The Hong Kong Polytechnic University

Department of Applied Biology and Chemical Technology

Proteomic Study of Harmful Algal Blooming Causative Agents: Nitrogen-Induced Growth and Identification of Dinoflagellates

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CERTIFICATE OF ORIGINALITY

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ABSTRACT

Harmful Algal Blooms (HABs) are an unavoidable worldwide problem and there is an apparent global increase in the occurrence of HABs. Consequently, there is an ever increasing risk of occurrence of HABs which represents expanding threats to human health, fishery resources and tourism industries. A huge and massive red tide occurred in the coastal waters of South China Sea, including Hong Kong from mid-March through mid-April in 1998. In Hong Kong alone, the blooms inflicted an estimated direct economic loss of around HK$ 250 million. There is an urgent need to understand the blooming mechanism. Although it is still not sure the extent to which the increase in red tides can be attributed to the increase of nutrient level, there is a strong relationship between algal blooming and the nitrogen load of coastal waters. Therefore, nitrogen is believed to be an important factor in the initiation and maintenance of phytoplankton blooms.

Dinoflagellates are the major HABs causative agents. Although there are many studies on the effects of nitrogen on the growth of dinoflagellates, very little is known about the changes in the cells at molecular level. In addition, dissection into the proteome of dinoflagellate was not reported. Proteomic studies using 2D gels with or without nitrogen supplements on dinoflagellates have the potential to uncover the cellular pathways and mechanisms involved in blooming at the molecular level.

Given that no effective method for controlling blooming is available yet, the best strategy for control is prevention. On top of it, a fore-warning system will be very useful. Therefore, rapid identification of HABs species is another important issue of the study of HABs. Traditional HABs species identification method is based on the morphological features. However, this type of taxonomic identification method not

only is time-consuming but also requires a high level of expertise. Because of the nature of the technique, taxonomic confusion and arguments on similar looking HAB species are common. To resolve the taxonomic debate, different types of identification methods are being introduced, including the molecular probes using lectins, antibody and oligonucleotide. However, none of them are entirely satisfactory. Therefore, a fast, simple, accurate and systematic identification method is required for field applications.

Studies described in this thesis were divided into two parts. The first part was aimed to study protein expression profiles in the model dinoflagellate *Alexandrium affine* under nitrogen stress. By investigating protein expressions in response to nitrogen availability, we may understand more about the mechanism of growth in this organism. Methodologies required to study protein expression profiles in dinoflagellates were derived and streamlined to allow production of high-quality two-dimensional gel electrophoretograms (2DE).When comparing cells grown under nitrogen depletion conditions with that under repletions, more than 15 differentially expressed proteins ($\geq$ 5-fold differences) were annotated by 2-DE. Two of them were found to be highly down-regulated ($\geq$ 16-fold) upon N-depletion; where one of them was 55 kDa with pI 5-6 and was successfully identified as ribulose-1,5 bisphosphate carboxylase/oxygenase form II (Rubisco II). Another one with 50 kDa, pI 5-6 and it was completely *de novel* and named as NAP50. Using a combination of N-terminal amino acid sequencing, tandem-mass spectrometry, PCR and molecular cloning technologies, the entire putative amino acid sequences of NAP50 were successfully obtained. When searched against the NCBI non-redundant database, no homology was found. Down-regulation of these two nitrogen-associated proteins (NAPs) was

further confined to occur upon 30 hours after N-depletion through the immunoblotting experiments. However, their mRNA expression levels remained unchanged during N-depletion and repletion. Therefore, the down-regulation of these two NAPs seems to be controlled at protein level rather than at transcriptional level. Down-regulation of these NAP proteins under N-depletion could be prevented either by the replenishment of N sources or the addition of protease inhibitors. Specifically addition of serine protease inhibitors (AEBSF and benzamidine) could prevent the proteins became down-regulated *in vivo* and *in vitro*. Therefore, it was speculated that specific serine protease(s) was involved in the degradation of both proteins under the N-depletion condition. This specific serine protease(s) was partially fractionated using benzamidine-bound affinity column chromatography and the active fractions further suggested that there could be a NAPs-specific serine protease(s) being activated in response to nitrogen stress. To the best of our knowledge, this is the first study of dinoflagellate proteome in response to nitrogen stress. The novel protein NAP50 and the involvement of a serine protease(s) in the NAPs degradation under nitrogen stress had never been reported as well. Results from the present study not only provide new insights on how dinoflagellates react with nitrogen (one of the main factors thought to trigger HABs) at molecular level, but also act as the first step to dissect proteomes of dinoflagellates under optimal growth conditions.

In the second part, it was aimed to design and validate a fast and accurate method for identification of dinoflagellates that is not dependent on morphological aspects. Different dinoflagellates were identified using their respective protein/peptide mass fingerprint profiles obtained with matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (MS). The peptide mass fingerprint

spectral patterns found for each species of dinoflagellates are unique and are easily distinguishable by visual inspection. In addition to the whole mass spectra, several specific biomarkers were identified from the mass spectrum of different species. These mass spectral patterns and the biomarker ions form an unambiguous basis for species discrimination. Results of our investigations clearly demonstrated the capability of this method in rapid identification of different species of dinoflagellates (namely *Alexandrium affine, Prorocentrum minimum, Scrippsiella rotunda, Karenia brevis and a yet to be identified species)*, In addition, identification and differentiation of closely related species such as *Alexandrium affine, Alexandrium catenella, Alexandrium tamarense, Alexandrium minutum,* can be accomplished easily by the MALDI-MS analysis. The method is simple, fast and reproducible. Lastly, I had demonstrated that it was possible to identify individual dinoflagellate species in a mixed population of different dinoflagellate species based on characteristic peak mass spectral patterns or species-specific signature biomarkers in the spectrum. Although the identification process would become very complicated when more than two species are presented in a mixed culture, it may still be possible to identify the species compositions with the aid of computer software based on the analysis of the specific signature biomarker peak ions. The workflow of HAB species protein/peptide peak mass fingerprint spectral profiling is a straightforward approach. It represents an excellent alternative to classical microscopic-based identification techniques. Furthermore, this approach shows great potentials to be used for the continuous monitoring of water samples for the possible occurrence of HABs.

PUBLICATIONS

Journal articles

Fred Wang-Fat Lee and Samuel Chun-Lap Lo, 2007. Proteomic Study of Micro-Algae: Sample Preparation for Two-Dimensional Gel Electrophoresis and *De Novo* Peptide Sequencing Using MALDI-TOF MS. Current Proteomics 4(2), 67-78. (see Appendix XIII).

Fred Wang-Fat Lee, Kin-Chung Ho and Samuel Chun-Lap Lo, 2008. Rapid Identification of Dinoflagellates Using Protein Profiling With Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry. Harmful Algae (Accepted, see Appendix XIV-XVI).

Fred Wang-Fat Lee and Samuel Chun-Lap Lo, 2008 The use of Trizol reagent (phenol/guanidine isothiocyanate) for producing high-quality two-dimensional gel electrophoretograms (2-DE) of dinoflagellates. Journal of Microbiological Methods. (Accepted, see Appendix XVII-XIX).

Conferences

Fred Wang-Fat Lee, John Hon-Kei Lum and Samuel Chun-Lap Lo, 2005. Protein identification using *de novo* sequencing of N-terminal sulfonated peptides from the toxic dinoflagellate (*Alexandrium spp.*) species with post-source decay on a matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI-TOF), in: Gordon Research Conference on Mycotoxins and Phycotoxins. (Abstract and poster presentation, see Appendix XII).

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Someone told me that PhD should stands for "Permanent head damage". Luckily, I didn't get any damage to my head after I have finished my PhD project. Although the project is very challenging, I am so enjoyed and got lots of funs during the past four years. I would like to take this opportunity to express my sincere gratitude to a number of people who assisted and contributed to my PhD project. Without their efforts, this work could never been made possible.

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LIST OF ABBREVIATIONS

AEBSF	4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride
ACN	Acetonitrile
AFCD	Agriculture, Fisheries and Conservation Department
APAF	Australian Proteome Analysis Facility
ASP	Amnesic shellfish poisoning
CAF	Chemically assisted fragmentation
CFP	Ciguatera fish poisoning
CID	Collision induced dissociation
COD	Chemical oxygen demand
DFAAs	Dissolved free amino acids
DON	Dissolved organic N
DSP	Diarrhoetic shellfish poisoning
ESI	Electrospray ionization
HABs	Harmful algal blooms
HCCA	α-cyano-4-hydroxycinnamic acid
IEF	Isoelectric focusing
ITS	Internal transcribed spacer
MALDI	Matrix-assisted laser desorption/ionization
MS	Mass spectrometry
Mud-PIT	Multidimensional protein identification technology
NAPs	Nitrogen-associated proteins
NaR	Nitrate reductase
NSP	Neurotoxic shellfish poisoning

PCPs	Peridinin–chlorophyll *a* proteins
PCR	Polymerase chain reaction
PMFs	Peptide mass fingerprints
PSD	Post-Source Decay
PSP	Paralytic shellfish poisoning
PTMs	Post-translational modifications
RACE	Rapid amplification of cDNA end
rRNA	ribosomal RNA
RUBISCO	Ribulose-1,5-bisphosphate carboxylase/oxygenase
SPITC	4-sulfophenyl isothiocyanate
2-DE	Two-dimensional gel electrophoresis
TCA	Trichloroacetic acid
TEM	Transmission electron microscopy
TFA	Trifluoroacetic acid
TOF	Time-of-flight
SA	Sinapinic acid

CHAPTER I

Literature Review

1.1 Red Tides and Harmful Algal Blooms (HABs)

1.1.1 The nature of Red Tide and HABs

Figure 1.1 Examples of red tide occurred in Hong Kong water [Adopted from Hong Kong red tide information network (www.hkredtide.org) dated September, 2007]

From time to time, especially near the coast, surface of the sea becomes bright red literally overnight (Figure 1.1). This phenomenon, called a red tide, has occurred for thousands of years. However, the term "red tide" is confusing as some red tides are not red! Further, red tides have nothing to do with the tide. They are massive

blooms of phytoplankton. Red tides may turn orange, brown, or bright green. To avoid further confusion, the scientific community is using the term “harmful algal bloom (HAB)” to describe all algal blooms, irrespective of whether they discolor the water or not.

Harmful algal blooms (HABs) result from the uncontrolled and sudden growth of a single, sometimes two, species of algae. Sudden occurrence of HABs has negative impacts (Dortch, 2001). They are both marine and freshwater algae and they are the foundation of all aquatic food chains. These microscopic planktonic algae are of critical importance as they serve as food for filter-feeding bivalve shellfish (oysters, mussels, scallops, clams) as well as the larvae of commercially important crustaceans and finfish (Hallegraeff, 2003). In any normal column of water, a mixture of species from many different taxonomic groups is usually present. Sometimes, one or more species can predominate. Occasionally, a particular combination of environmental conditions will result in high biomass with an unusual predominance of one or a few species. Proliferation of such plankton algae up to millions of cells per liter is termed as “algal blooms” (Hallegraeff, 2003). Sometimes, one of these species may be harmful as they contain toxins. With or without toxin, algal blooms may inflict harmful effects on the ecosystem and other living organisms (including human) (see below). Therefore, these blooms are named “harmful algal blooms (HABs)”. Some of the organisms considered HAB species do not fit all of the implied criteria (Dortch, 2001). For example, cyanobacteria which was no longer considered as algae, is still included. Some others are not photosynthetic at all. Finally, some algae can be harmful when they are present in very low abundance but can never form blooms.

A wide range of harmful effects are possible. Some HAB species produce

biotoxins that cause human illness or death after consumption of shellfishes or fishes that have accumulated the toxins from their diet. These toxins are particularly insidious because most are not destroyed by cooking (Dortch, 2001). One of the first recorded fatal cases of human poisoning after consuming shellfishes contaminated with dinoflagellate toxins was in 1793 (Hallegraeff, 2003). Such causative alkaloid toxins, like PSP, are so potent that even a very small quantity (about 500ug), which can be easily accumulated in just one 100g serving of shellfish, is fatal to human. On a global scale, nearly 2000 cases of human poisoning with nearly 15% mortality through fishes or shellfishes consumption are reported each year (Hallegraeff, 2003). There are other HAB species that specifically cause mortality in fishes and other animals. For example, whales and porpoises can become victims when they receive toxins through the food chain via contaminated zooplankton or fish (Geraci et al., 1989). These can result in economic losses and damages to the ecosystems.

Technically, HABs can be divided into three different types (Hallegraeff, 2003). Table 1.1 is a summary of these three types of HABs, together with representative examples of causative agents, ranging from dinoflagellates, diatoms, pymnesiophytes and raphidophytes and cyanobacteria. Type 1 is HAB species that grows so dense that they discolor the seawater, cause seawater to have a foul taste as well as odour and cause kills of fish and invertebrates through hypoxia or anoxia. Type 2 is toxin-producing species that enables accumulation of these toxins through the food chain from filter-feeders to humans. Type 3 is species that is non-toxic to humans but harmful to fishes and invertebrates as these HAB causative agents can damage and clog the gills of these animals. Collectively, HAB causative agents can kill fishes in different ways. Some can seriously damage fish gills, either mechanically or through

production of hemolytic substances. Some can kill fishes through oxygen depletion. In addition, other algae kill fishes through the production of extracellular neurotoxins. Whereas wild fish stocks have the freedom to swim away from problem areas, caged fishes are extremely vulnerable to such noxious algal blooms. Nevertheless, there is no definite correlation between algal concentrations and their potential harmful effects. For example, some dinoflagellate species such as *Dinophysis, Alexandrium* and *Pyrodinium* can contaminate shellfish with toxins, even at very low cell counts.

Table 1.1 Different types of harmful algal bloom. [Adopted and modified from (Hallegraeff, 2003)]

Type of HAB	Effect(s)	Example(s)
1. Species produce basically harmless water discolorations	Blooms can grow so dense that they cause indiscriminate kills of fish and invertebrates through oxygen depletion	Dinoflagellates: *Akashiwo sanguinea, Gonyaulax polygramma, Noctiluca scintillans, Scrippsiella trochoidea*; cyanobacteria: *Trichodesmium erythraeum*
2. Species produce potent toxins	Through the food chain to humans, causing a variety of gastrointestinal and neurological illnesses	PSP: *Alexandrium spp.* DSP: *Dinophysis spp.* ASP: *Pseudo-nitzschia spp.* (diatom) CFP: *Gambierdiscus toxicus* NSP: *Karenia spp.* Cyanobacterial toxin poisoning: *Anabaena circinalis* (freshwater)
3. Species that are non-toxic to human but harmful to fish and invertebrates	Damaging or clogging their gills	Diatoms: *Chaetoceros concavicorne*; dinoflagellates: *Karenia mikimotoi*; rephidophytes: *Heterosigma akashiwo*

Growth of HAB organisms are dependent on a species-specific optimal combination of environmental conditions, such as light, nutrient availability, temperature, salinity, water column stability, horizontal water movements and grazing by marine animals. Many HAB species have relatively low maximum growth rates. Consequently, researchers are trying to determine whether HABs have unique adaptation or requirements for growth that enable them to out compete other algae

under similar conditions. Some of the possibilities being considered include conditions that facilitate cyst formation and other life cycle strategies, vertical migration, development of grazer avoidance mechanisms or unique nutrient utilization capabilities. Since HAB causative agents are so diverse in nature, it is unlikely that a single explanation would be found.

1.1.2 Global increase of Algal Blooms

HABs are a global problem (Hallegraeff, 1993). It happens every where in the world ranging from Europe, America, Asia to Pacific including the western and eastern coast of The United States and Canada; Chile and Mexico; England, Spain, Finland, Italy, South Africa, China, Japan, Taiwan, India, New Zealand and Australia. Premazzi et al (1993) have summarized the global distribution of human intoxications by HABs toxins (Figure 1.2).

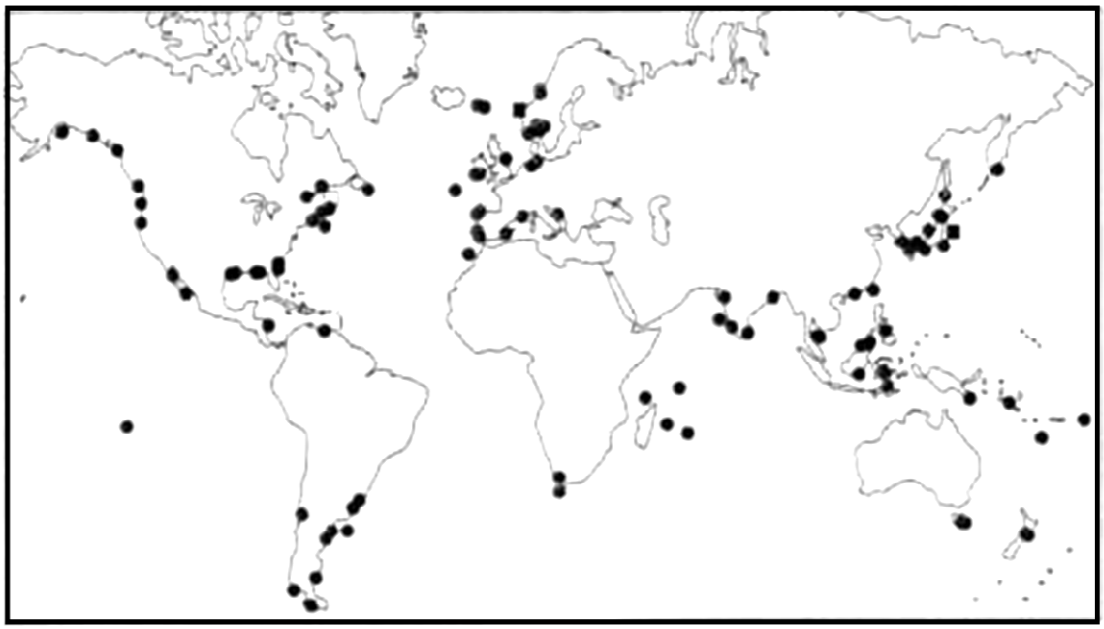


Figure 1.2 Global distributions of human intoxications by HABs toxins. Dots indicate cases of human intoxication.[Adopted from (Premazzi and Volterra, 1993)]

In fact, the public health and economic impacts of HABs appear to have increased in frequency, intensity and geographical distributions in the past three decades (Hallegraeff, 2003). One example, Figure 1.3 shows the increased global distribution of HAB toxins and toxicities from 1970 to 1999 (Sellner et al., 2003). Until 1970, toxic dinoflagellate blooms of *Alexandrium tamarense* and *Alexandrium catenella* were the only known from temperate waters of Europe, North America and Japan (Dale and Yentsch, 1978). However, blooms from these species were well documented throughout the Southern Hemisphere by 2000.

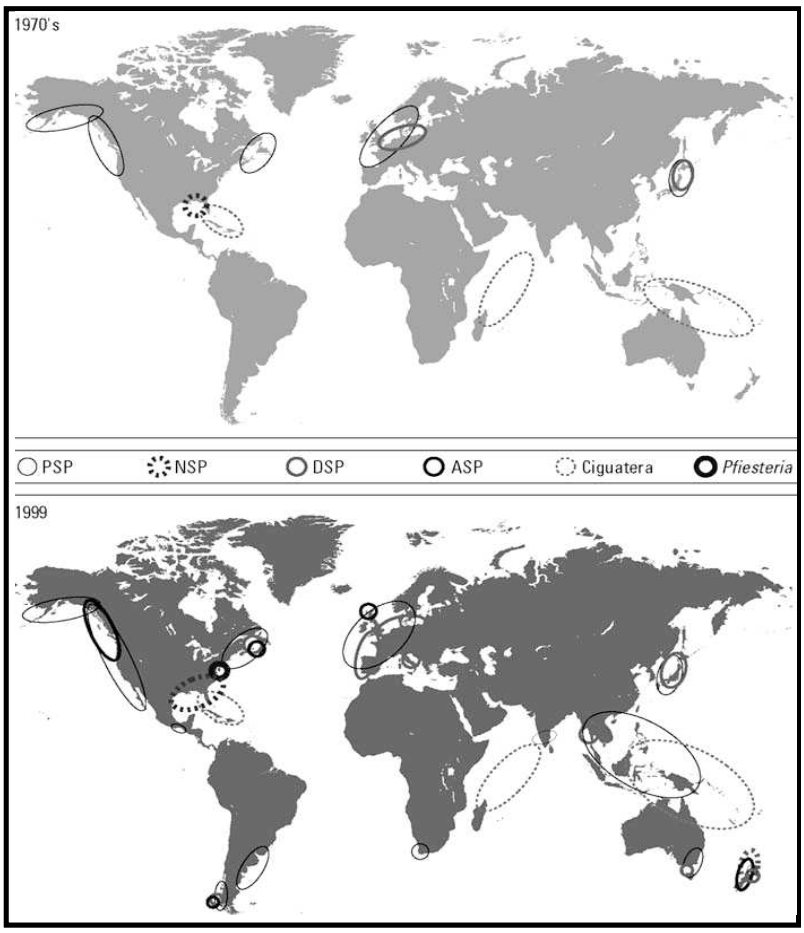


Figure 1.3 Known global distributions of HAB toxins from 1970 to 1999. Number of toxicities cases (dots) has increased from 1970 to 1999. PSP= paralytic shellfish poisoning, NSP= neurotoxic shellfish poisoning, DSP= diarrhoetic shellfish poisoning and ASP= amnesic shellfish poisoning. [Adopted from (Sellner et al., 2003)]

Several reasons has been proposed to explain the global increase in HABs observed (1) increased scientific awareness of toxic species; (2) increased utilization of coastal waters for aquaculture; (3) accelerated eutrophication; (4) unusual climatological conditions such as global warming and (5) transportation of dinoflagellate resting cysts in ship's ballast water or associated with translocation of shellfish stocks from one area to another (Anderson, 1989; Hallegraeff, 1993; Smayda, 1990; Tester et al., 1991).

1.1.3 Causes of red tides and HABs

1.1.3.1 Environmental conditions

Growth, abundance and seasonal distribution of HABs causative organisms are closely related to the temperature, salinity, light intensity, nutrient and current regimes in the sea. For example, different species of the genus *Alexandrium* show different optimal temperature and salinity for growth. A general increase in temperature, within the optimal range, would result in an increase in population growth and density. Undoubtedly various factors including nutrient upwelling; nutrient input; temperature; wind; rainfall; salinity and light have all been identified as contributory factors to occurrence of HABs.

1.1.3.2 Eutrophication

The rate at which the dinoflagellates, the causative agents of HAB, grow depends upon a wide range of factors including light, temperature, salinity, nutrient supply and grazing. Despite this apparent complexity, one of the major causes of HABs has been related to the input of nutrients. Nitrogen and phosphorus are the well known

macronutrient requirements important for growth and metabolic activities. In addition, it is known that phytoplankton also requires small amounts of micronutrients (found in trace amounts in the open oceanic waters) for optimal growth (Hudson and Morel, 1993). Some micronutrients like metals (Fe, Mn, Co, Ni, Cu and Zn); metalloids such as Se and non-metals such as iodine are required in metabolic processes for both marine and fresh water phytoplankton (Brand et al., 1983). The absence of one of these elements may limit growth (Brand et al., 1983), while an excess amount may also inhibit phytoplankton growth (Anderson and Morel, 1978; Brand et al., 1983). Several HAB incidences had been correlated to the increase in the discharge of nitrogen, phosphorus and trace metals to coastal waters (Hallegraeff, 2003). Therefore, the amount and types of nutrients provided for the microalgae are one of the important factors that may lead to "blooms" of such organism. However, the exact mechanisms of how such nutrients leading to extensive and sudden algal bloom are still unclear.

On the other hand, eutrophication is a condition in a water body where high concentrations of nutrients (mainly nitrogen and phosphorus) stimulate excessive algal (e.g dinoflagellates) growth and blooms. Growth and blooms of most of these algal species appear to be stimulated by "Cultural Eutrophication" from domestic, industrial and agricultural wastes (Hallegraeff, 2003). Nitrogen inputs to rivers, lakes and coastal areas have increased substantially from a variety of sources. This provides higher nutrient availability and a better environment for HAB species to growth (Dortch, 2001). Most marine systems are nitrogen-limited. Hence, when nitrogen is added, the phytoplanktons tend to grow or "bloom" until they run out of some other nutrients. In particular, diatoms might run out of silicate, which they need to make their shells (Smayda, 1989). This will shift the balance in favor of dinoflagellates.

While some diatoms produce toxins, dinoflagellates are more likely to do so. Moreover, some dinoflagellates produce more toxin when their supply of phosphorus runs low, which is likely to happen if we pump in nitrogen and not phosphorus (Guisande et al., 2002; John and Flynn, 2000; Touzet et al., 2007).

Several examples are listed below to show that HAB are directly related to the increase in the discharge of nutrients. Figure 1.4 illustrates an eight fold increase in number of red tides per year in Tolo harbour, Hong Kong, in the period 1976-1986 (Lam and Ho, 1989). During that period, there are 2.5-fold increases in nutrient loading, mainly contributed by untreated domestic and industrial waste and this is correlated with 6-fold increase in human population in Hong Kong.

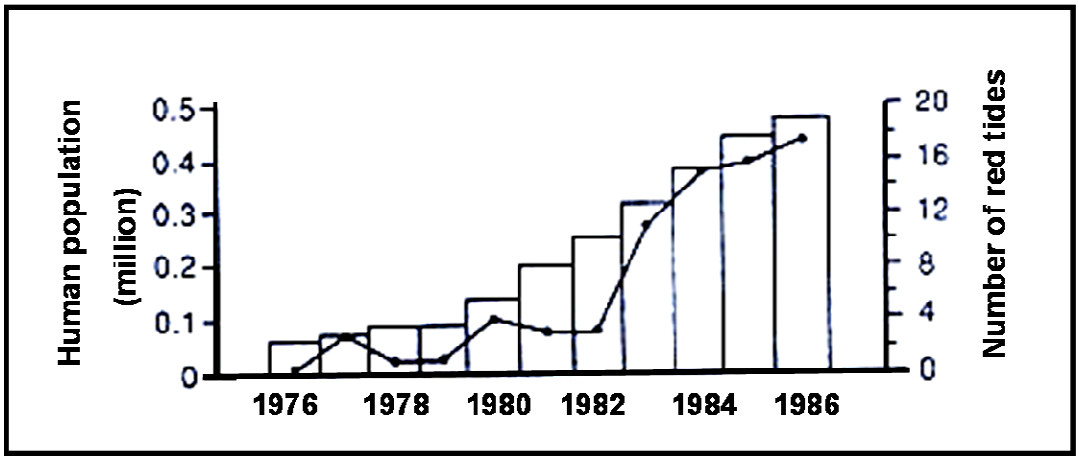


Figure 1.4 Correlation between the number of red-tide outbreaks per year in Tolo Harbour and the increase in the human population in Hong Kong (bar diagram), 1976-1986. [Adopted from (Lam and Ho, 1989)]

Similar outcomes can be seen in one of the major fish-farm areas in Japan, the Seto Inland Sea (Okaichi, 1989) (Figure 1.5). Between 1965 and 1976, the number of red-tide outbreaks progressively increased 7-folds, concurrent with a 2-folds increase in chemical oxygen demand (COD) loading, contributed mainly from untreated

sewage and industrial waste from pulp and paper factories. Afterwards, effluent controls were initiated to reduce the COD loading by about half (see arrow in Figure 1.5), secondary sewage treatment was introduced and phosphate was removed from household detergents. Following a time-lag of four years, the frequency of red tide events in the Seto Inland Sea had decreased by about 2-fold and maintained at a more stationary level.

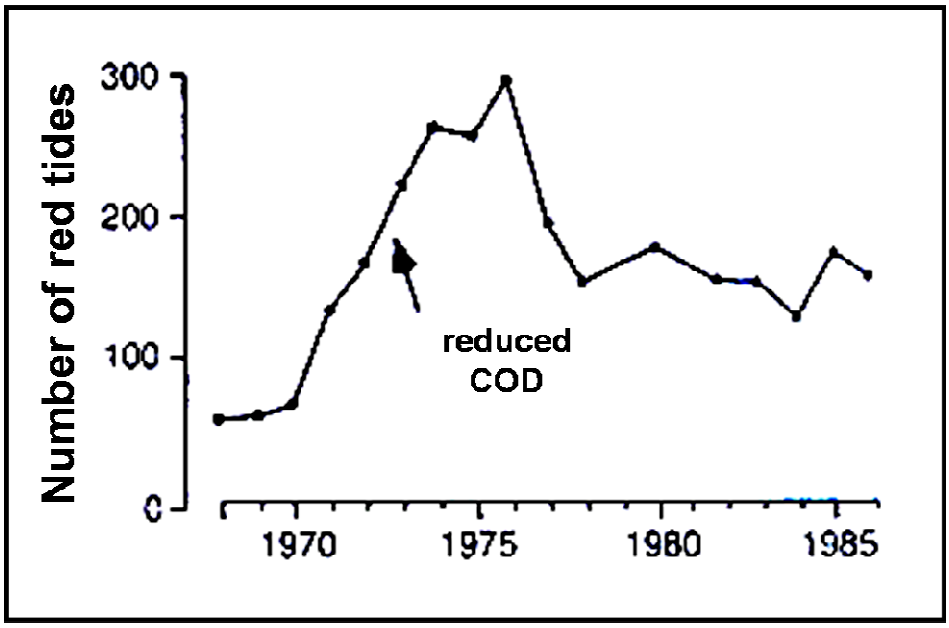


Figure 1.5 Long-term trends in the frequency of red tide outbreaks in the Seto Inland Sea, Japan, 1965-1986. COD: chemical oxygen demand. [Adopted from (Okaichi, 1989)]

Furthermore, Ho and Hodgkiss showed that HAB causative organisms were limited primarily by macro-nutrients and their growth was optimal at an atomic N: P (nitrogen to phosphate) ratio of about 4 to 22 (Hodgkiss and Ho, 1997). They further suggested that changes in such N to P ratio are highly relevant to incidence of HAB of the causative organisms.

1.1.3.3 Unusual climatological conditions

Some scientists believed that long term climatic shifts may be responsible for the apparent increase in both frequency and strength of HAB worldwide (Hallegraeff, 1993). Most of the studies proposed a link between both the greenhouse effect and EI Nino-Southern Oscillation (ENSO) event to algal blooming (Hallegraeff, 2003).

1.1.3.4 Transport of dinoflagellate cysts

It is still not clear on the mechanism of how a HAB is initiated. However, an inoculum must be present in order for one to begin, and yet the majority of HAB causative organisms do not appear in the water column throughout the year. Therefore, it comes to a question on how HABs spread to new places?

Some of the outbreaks of HABs in new areas have been shown to result from transport of HAB causative agents in ballast water in ships (Hallegraeff, 1998). It was estimated that a single ship can carry more than 300 million toxic dinoflagellates cysts (Hallegraeff, 1998; Hallegraeff, 2003)! Dinoflagellate cysts are the resting form which allow them to survive long period in dark ballast tanks (Hallegraeff and Bolch, 1992). Therefore, if ballast water was taken on during an HAB in one harbor and dumped in an area with similar environmental conditions, the chances of introducing a new species are increased. Moreover, live shellfish can contain cysts or vegetative cells in the water inside the shell and in the gut. Commercially activities on these cyst-containing shellfishes will allow spreading of cyst to new areas. In both cases high nutrient inputs in the new area are likely to increase the chance of survival for the introduced species.

According to Hallegraeff (2003), ballast water was first suggested as a vector in

the dispersal of marine planktons around 30 years ago (Hallegraeff, 2003). In the 1980s, there is a strong evidence that HAB organisms in Australia have been introduced through ballast water through commercial shellfish farm operations with disastrous consequences (Hallegraeff and Bolch, 1992). Paralytic shellfish poisoning (PSP) was unknown in the Australian region until the 1980s with the first outbreaks appeared in Melbourne which was caused by *Alexandrium catenella* and in Adelaide which was caused by *Alexandrium minutum.* In both places, the genetic affinities and fingerprinting obtained using ribosomal gene sequencing between Australian and Japanese strains of *A. catenella* as well as between Australian and European strains of *A. minutum* provided circumstantial evidence for the ballast water transportation (Scholin et al., 1995).

In addition, it was shown that the faeces and digestive tracts of bivalves were loaded with viable dinoflagellate cells and resistant resting cysts (Scarratt et al., 1993; Schwinghamer et al., 1994). Therefore, translocation of live shellfish stocks from one area to another would bring about the concomitant dispersal of algae (especially the resting cysts) into new areas.

1.1.4 Effects of HABs

1.1.4.1 Problems on human health

In some HAB causative species, for example dinoflagellates, at least 20 of them produce toxins which can affect human health via contaminated fishes and shellfishes. A recent estimate indicated that toxic algae caused 100,000 to 200,000 cases of serious poisoning annually. These poisoning cases lead to 10,000 to 20,000 deaths and a similar number of cases of paralysis as well as other serious consequences

(Hallegraeff, 2003). This is much higher than earlier estimates, which numbered in the hundreds of deaths. Therefore, the problem is worse than previously realized and it is deteriorating. Further, it should be reiterated that one does not have to eat the shellfishes to suffer. Swimming or boating in affected water, even breathing sea spray, can cause sore throats, eye irritation, and skin complaints.

There are several kinds of toxins produced by HAB causative agents, including paralytic shellfish poisoning (PSP), amnesic shellfish poisoning (ASP), neurotoxic shellfish poisoning (NSP), diarrhoetic shellfish poisoning (DSP) and ciguatera fish poisoning (CFP). These major algal toxins cause different problems to human health (Table 1.2).

Table 1.2 Major algal toxin groups causing human health problems. [Adopted from (Dortch, 2001)]

Syndrome	Toxin type	Toxins	Description of symptoms	Duration
Paralytic shellfish poisoning	Neurotoxins	Saxitoxin and derivatives	Tingling/burning of skin and extremities, especially mouth and fingers, loss of muscular coordination, giddiness/staggering, drowsiness, dry throat and skin, loss of speech and speech comprehension, rash, fever, gastrointestinal distress, respiratory paralysis, death in 2–24 h without immediate treatment	Starts in 30 min; lasts up to 3 days
Amnesic shellfish poisoning/ domoic acid poisoning	Neurotoxins	Domoic acid	Gastrointestinal distress, headache, disorientation, memory loss, seizures, respiratory difficulty, coma, death	Starts in 2–5 h; duration variable
Neurotoxic shellfish poisoning	Neurotoxins	Brevetoxins	Tingling and numbness of tongue, throat and lips, muscular aches, gastrointestinal distress, dizziness	Starts in 3–5 h; lasts several days
Ciguatera fish poisoning	Neurotoxins	Ciguatoxins, maitotoxin, gambieric acids, okadaic acid, prorocentrolid B, haemolysins	Gastrointestinal distress, headache, itching, temperature reversal and other abnormal sensations, joint and muscular pain, convulsions/visual hallucinations, dizziness, irregular pulse/loss blood pressure	Starts in 12–24 h; usually lasts 1 week, but occasionally months to years
Diarrhoetic shellfish poisoning	Neurotoxins	Okadaic acid, dinophysitoxins, pectenotoxins	Gastrointestinal distress, chills	Starts in 30 min to 12 h; lasts up to 3 days
None	Neurotoxins	Unknown toxins from *Pfiesteria piscicida*	Narcosis, sores, reddening of eyes, blurred vision, nausea/ vomiting, sustained difficulty breathing, kidney/liver dysfunction, memory loss, cognitive impairment	6 months to 6 years, depending on exposure
None	Neurotoxins	Anatoxin-A and homoanatoxin-A	Staggering, gasping, muscle fasciculations, cyanosis, respiratory arrest, death	Minutes to hours
None	Neurotoxins	Anatoxin-A(S)	Hypersalivation, lacrymation, diarrhoea, ataxia, death	Minutes to hours
None	Hepatotoxins	Microcystins and nodularins	Short-term response to acute exposure: weakness/recumbency, pallor, vomiting, diarrhoea, pooling of blood in liver, respiratory arrest, death Long-term response to acute exposure: liver failure, death	Hours to days
None	Cytotoxins	Cylindrospermopsin	Vomiting, headache, abdominal pain, enlarged and tender liver, diarrhoea, severe electrolyte imbalance	Days to weeks
None	Dermatoxins	Aplysiatoxins, debromoaplysiatoxins and oscillatoxin-A	Acute dermatitis, asthma-like symptoms	Immediate

1.1.4.2 Animal mortality

The devastating effects of HABs are not confined to human. HABs sometimes turn the sea into a dead trap because these blooms may either used up most of the dissolved oxygen or secrete toxins into the water. Most of the larger aquatic animals such as fishes will die and the sea became a sea of dead fishes (Figure 1.6), creating another health hazard. Besides fishes, HAB causative organisms affect other marine organisms. For example, whales and sea birds that ate intoxicated fishes were also killed. Naturally, nearly all animals in a food web will be affected and the effects of these toxins will be biomagnified when they were passed upward through the food chain (Work et al., 1993). Even herbivores have been affected, through they eat aquatic vegetation. For example, manatees in Florida (U.S.A) were killed by HABs on several occasions. They are exposed by just being in the water, or may be by breathing in toxic spray at the surface. In 1996, about 150 manatees were killed by HABs (Figure 1.7).

Figure 1.6 Devastating effects of HABs seen on the west coast of Florida where the proliferation of the toxic dinoflagellate *Gymnodinium breve* resulted in massive fish kills. [Adopted from The Harmful Algae Page (http://www.whoi.edu/science/B/redtide/) dated September, 2007]

Figure 1.7 More than 150 deaths of the Florida manatees occurred in 1996 due to harmful effects of algal toxins produced by *Gymnodinium breve.* [Adopted from The Harmful Algae Page (http://www.whoi.edu/science/B/redtide/) dated September, 2007]

As mentioned briefly before, not all harmful effects of HABs on marine animals are due to toxins. Fish kills can occur if the masses of phytoplankton suddenly die, even if they are not poisonous. Their decomposition depletes dissolved oxygen in water and fishes suffocate. In addition, thecate HAB causative agents can kill marine animals by their spines which are sharp and pointed. They can lacerate gills. Further, HAB causative agents can produce copious quantities of mucous and clog gills (Dortch, 2001).

Although massive animal mortality is the most obvious sign of a problem, much more subtle effects that are difficult to quantify are also possible (Dortch, 2001). Ecosystem structure can be altered if some organisms succumb to algal toxins and others do not. Fisheries recruitment can be severely and negatively affected if larval fishes of an ecosystem die preferentially as they are more susceptible to low levels of toxins. Moreover, the long-term survival of endangered species can be threatened, for example the manatees in Florida that was just mentioned. Figure 1.8 has summarized how HABs affect both human and marine animal.

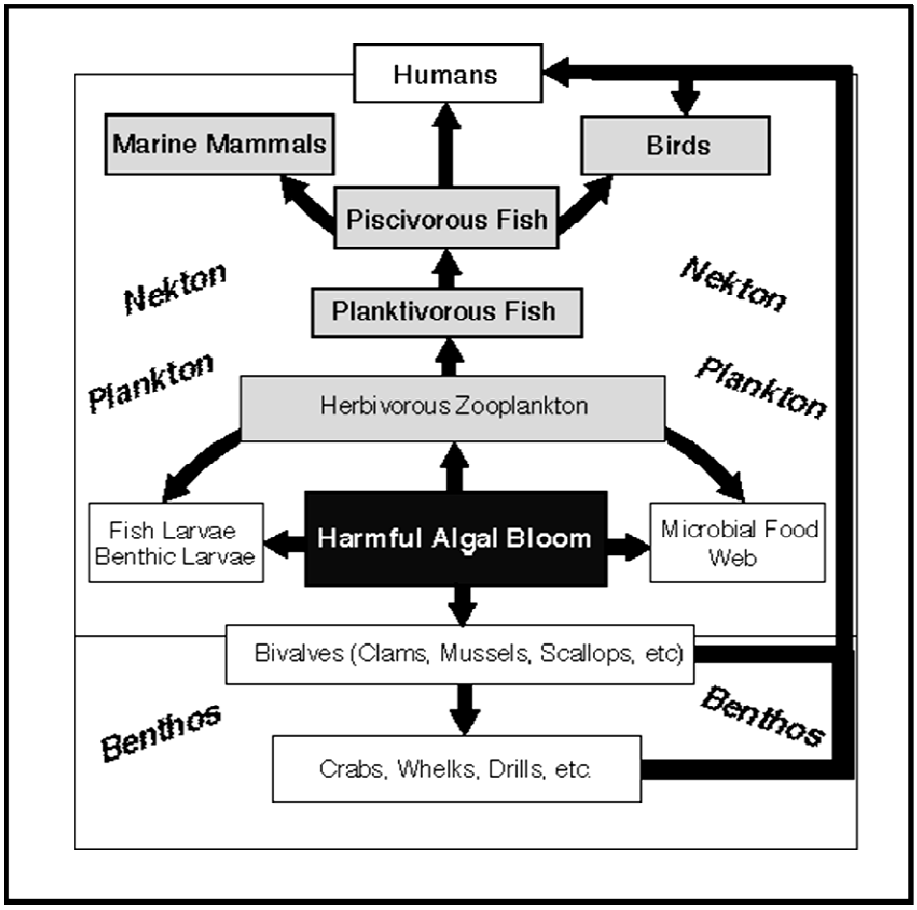


Figure 1.8 The conceptual model above illustrates routings through which algal toxins impact many different trophic compartments. Low oxygen levels in bottom water caused by non-toxic algae may also have adverse effects on the ecosystem. [Adopted from The Harmful Algae Page (http://www.whoi.edu/science/B/redtide/) dated September, 2007]

1.1.4.3 Economical problems

HABs cost hundreds of millions of dollars in losses to tourism and, especially, fisheries. Valuable shellfish fisheries are often closed as a result of HAB incidences or during seasons when HABs are mostly likely to occur. Even perfectly safe seafood may be rendered nearly worthless when the public perceives it as dangerous to eat. Fish farms and other aquaculture facilities are especially vulnerable because the caged fishes or other seafood cannot swim away from the contaminated area. In addition, these marine animals are usually held in high densities. Cost of HABs damage is

estimated at $49 million annually in the United States alone, and is many times higher globally (Hallegraeff, 2003).

Massive HABs occurred in mid-March through mid-April 1998 in the coastal waters of the South China Sea, including Hong Kong. It is the most serious HAB that had ever occurred in these waters (Lee and Qu, 2004; Yang and Hodgkiss, 2004). This HAB had invaded nearly all corners of the coastal waters of Hong Kong, including 22 of the 26 fish farms and five swimming beaches. In Hong Kong alone, 1260 fishery households were affected, 2500 tones of fishes were killed, and a direct economic loss of HK$ 250 million (about US$ 32 million) as estimated by the fish farmers (Yang and Hodgkiss, 2004). While in the Guangdong waters, it was reported that this HAB had killed more than 260 tones of fishes, causing a direct economic loss of about 40 million RMB (about US$ 4.8 million) (Yang and Hodgkiss, 2004).

1.1.4.4 Other harmful effects

Other than the effects mentioned above, HABs can also cause serious damages on tourism and recreational activities (Dortch, 2001). Most of the damages are the result from the accumulation of large quantities of biomass. Many species, including many toxic ones, form easily visible coloured (e.g reddish colours) (Figure 1.1) or bioluminescent (only some dinoflagellates) blooms. Moreover, algae produce volatile and water-soluble compounds. Therefore, a bloom can have a distinctive smell and impart unpleasant smell to the water. Finally, some species like *Phaeocystis spp*. blooms cause billows of foam to float on water surfaces and beaches. In Hong Kong, between 1980 and 2001, for example, about 15% of the HABs affected swimming beaches. The production of discolorations, repellent odour, foam, mucilage during

HAB induced negative effects on tourism and recreational activities. Figure 1.9 shows a huge HAB occurred in June, 2007 which spread through Hong Kong waters and affecting more than 14 beaches in Hong Kong. The HAB causative agent was identified by the authorities as *cochlodinium spp*. Although it was a non-toxic blooming, the massive cell growth (more than 10000 cells per milliliter), resulted in discoloration, repellent odour, foam and mucilage.

Figure 1.9 A huge HAB had spread through Hong Kong waters and affected more than 11 beaches. Brownish colours and foam were formed in the HAB caused by *cochlodinium spp.* in Gold Beach (Left). A total of 11 beaches were affected by the HAB and was suspended for swimming (Top right). Red tide samples were collected and monitored by officers from AFCD (Bottom right). [Adopted from South China Morning Post (SCMP) dated 05 June 2007]

1.1.5 Red tide and HABs in Hong Kong

There are several groups of phytoplankton that can cause algal blooms including diatoms (Class Bacillariophyceae, Phylum Heterokontae), dinoflagellates (Class Dinoflagellata, Dinophyceae, Phylum Dinophyta), haptophytes (Class Prymnesiophyceae or Haptophyceae, Division Haptophyta), and raphidophyceans (Class Raphidophyceae, Phylum Heterokontae). Among these groups, dinoflagellates and diatoms are the major groups that contribute to the HABs.

From the Agriculture, Fisheries and Conservation Department (AFCD) of the Hong Kong Special Administrative Region, there are around 330 aquatic organisms known to cause red tide globally and 70 of them had caused HABs in Hong Kong The most common one was the dinoflagellate *Noctiluca scintillans* which accounted for a third of the reported blooms (203 out of 634). Table 1.3 shows the occurrence and distribution of HAB species in different water control zones in Hong Kong from 1980 to 2001.

Hong Kong is one of the places that have most frequent occurrences of HABs throughout the world with an annual rate of 20-40 incidences (Smayda, 1990). (Figure 1.10 a). Spring is the peak season for the occurrence of HABs with more than 100 incidents from March to April (Figure 1.10 b). However, a majority of local algal blooms are non-toxic and seldom caused large scale damages.

Nevertheless, the first scientific report of a local HAB incident was on a *Noctiluca scintillans* bloom that occurred in the southern bays of Hong Kong in 1971. However, most HABs occur in the eastern waters including Tolo Habour and Channel, Mirs Bay and Port Shelter (Figure 1.10 c). From 1980 to 2001, 275 of 634 HABs (43%) occurred in the Tolo Habour. Therefore, this site was extensively studied.

Hodgkiss and Chan found that a total of 36 reports of blooming occurred in Tolo Habour during 1977-1984 (Hodgkiss and Chan, 1986). As a result of studies on the Tolo Harbour and Tai Tam Bay phytoplankton, the linkage between dinoflagellate blooms in Hong Kong waters and nutrient enrichment, particularly the atomic N: P ratios of 6 to 15 in seawater, has been clearly established (Hodgkiss and Ho, 1997) and they concluded that growth of most HAB causative organisms in Hong Kong coastal water is optimized at low N: P ratio of between 6 and 15.

As mentioned previously, the most extensive and damaging HAB in Hong Kong occurred from mid-March to mid-April, 1998. This bloom was caused by a new species- *Karenia digitata* (Yang and Hodgkiss, 2004; Yang et al., 2000). It inflicted seriously economic losses to fish farmers, associated workers and the Hong Kong economy.

Table 1.3 Occurrence and distribution of red tides species in Hong Kong from 1980 to 2001.

Species	Tolo Harbor	Mirs Bay	Eastern Buffer	Port Sheleter	Junk Bay	Victoria Harbour	Southern Waters	North Western	Western Buffer	Deep Bay	Total
Noctiluca scintillans		57		38		1	46	4	7		203
Skeletonema costatum	23	2		1	2	7	6	3	7		51
Gonyaulax polygramma	21	6		12			6	1	2		48
Mesodinium rubrum	8	7		7	1	1	10	4			38
Prorocentrum mininum	32	1									33
Prorocentrum triestinum	33										33
Ceratium furca	10	7		9							26
Scrippsiella trochoidea	14	3		2			1				20
Prorocentrum sigmoides	14	1		1							16
Heterosigma akashlwo	10	2						3			15
Heterocapsa circularisquama	12	2									14
Leptocylindrus minimus	10										10
Karenia mikimotoi	5	1		3							9
Prorocentrum dentatum	6	3									9
Cryptomonas sp.	8										8
Dactyiiosolen fragilissimus	6	1		1							8
Chaetoceros app.	6			1							7
Karenia digitata	1	3		2			1				7
Thalassiosira mala	6						1				7
Thalassiosira nordenskioldii	2	2				1	1		1		7
Akashiwo sanguinae	2	2						1		1	6
Thalassiosira proschkinae	5	1									6
Thalassiosira spp.	2				1		2				5
Dictyocha speculum		2		1			1				4
Eutreptiella spp.	4										4
Gymnodinium sp.		1		2			1				4
Gyrodinium instriatum						1		1	1	1	4
Leptocylindrus danicus	3	1									4
Prorocentrum micans	3	1									4
Pseudo-nitzschia	1						2		1		4
Gymnodinium simplex	3										3
Plagioseimis prolonga	3										3
Pseudo-nitzschia seriata	1					2					3
Trichodesmium sp.		1		1			1				3
Alexandrium tamarense				2							2
Cerataulina pelagica	2										2
Chaetoceros curvisetus			1				1				2
Chattonella ovata		1		1							2
Chattonella sp.		1					1				2
Cochlodinium polykrikoides							2				2
Cochlodinium sp.	2										2
Gymnodinium viridescens	1	1									2
Nitzschia longissima	1						1				2
Prorocentrum spp.	1	1									2
Teleaulax acuta	2										2
Trichodesmium erythraeum		2									2
Alexandrium catenella					1						1
Chaetoceros pseudocrinitus	1										1
Chaetoceros socialis							1				1
Chaetoceros sp.0105	1										1
Chaetoceros tenuissimus		1									1
Chattonella marina		1									1
Chlamydomonas sp.	1										1
cyclotella spp.	1										1
Cyttarocylis sp.				1							1
Eucampla zodiacus								1			1
Guinardla dellcatula	1										1
Guinardla striata	1										1
Gyrodinium spirale							1				1
Hermesinum adriaticum		1									1
Haematococcus pluvialls	1										1
Katcdinlum rotundatum								1			1
Leptocylindrus spp.							1				1
Odontella mobiliensis	1										1
Odontella sinensis	1										1
Pedinomonadaceae species	1										1
Prorocentrum baltlcum		1									1
Protoperidinium quinquecome	1										1
Pseudo-nitzschia spp.							1				1
Thalalssomonas sp.	1										1
Total: 70 species	275	117	1	85	5	13	88	19	19	2	674

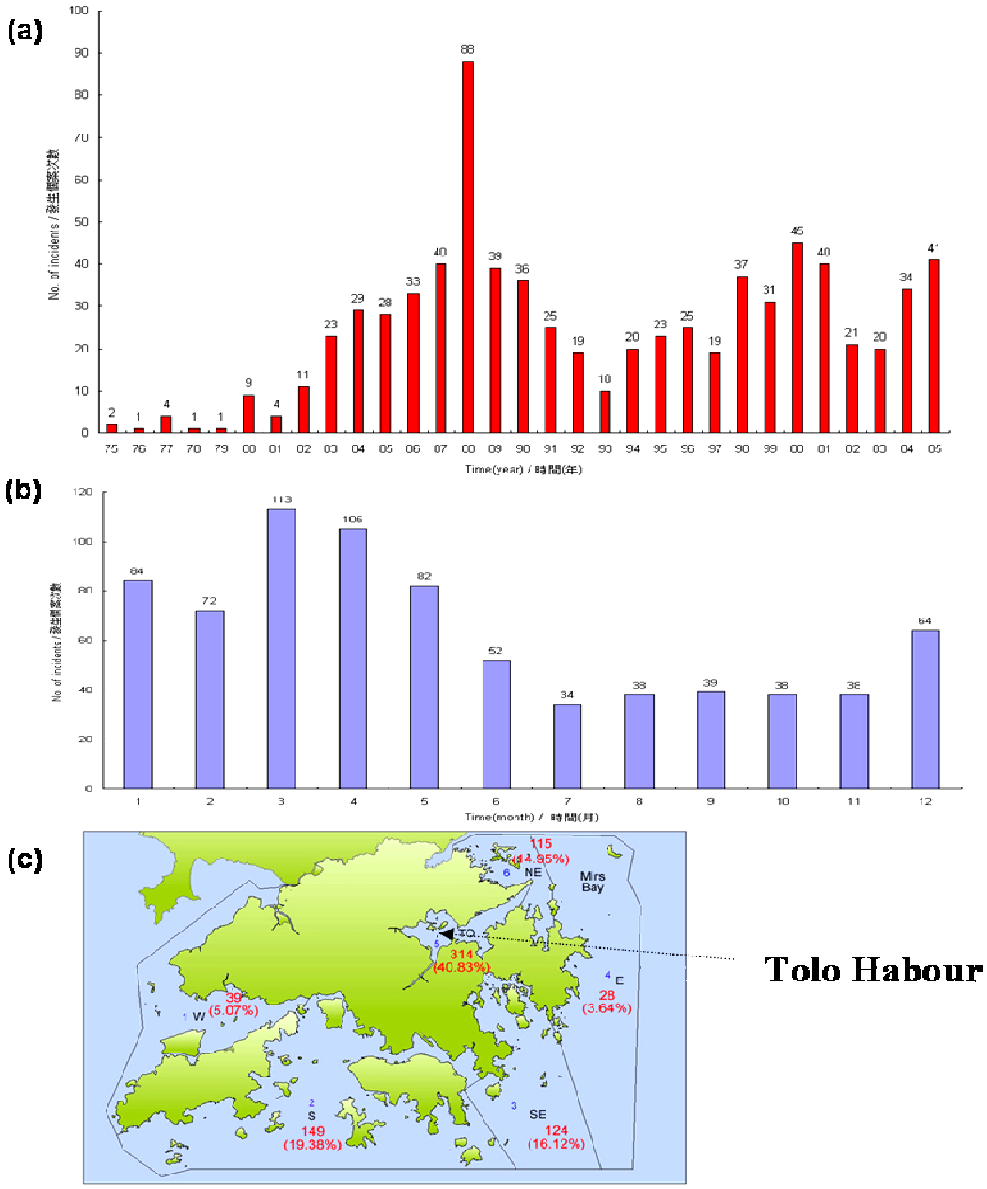


Figure 1.10 Number (a), Seasonal distribution (b) and Regional distribution (c) of red tide incident in Hong Kong from 1975-2005. Tolo habour (arrow) and its channel have the highest number of HAB incidences. [Adopted from Hong Kong red tide information network (www.hkredtide.org) dated September, 2007]

1.2 Toxins produced by HAB causative agents

1.2.1 Paralytic shellfish poisoning (PSP)

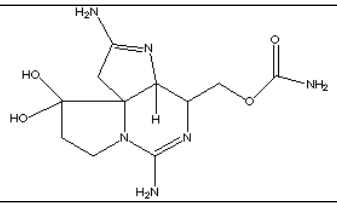
Paralytic shellfish poisoning (PSP) toxins are produced by some marine dinoflagellates including species of *Alexandrium, Pyrodinium bahamense var.*

compressum and Gymnodinium catenatum and a few freshwater cyanobacteria (Dortch, 2001). The occurrence of similar toxins in such widely divergent groups of organism is not readily explained. Organisms causing PSP are distributed globally. The wide distributions of the causative organisms and the severity of the illness induced make PSP one of the most serious HAB problems. Symptoms of PSP are listed in Table 1.2. Since HAB outbreaks often occur in areas with no monitoring and have significant local consumption of fishes and shellfishes, much human mortality results. The first serious epidemic ascribed with certainty to PSP occurred in San Francisco in 1927, with 102 cases of intoxication by shellfishes collected along the coast. This was also the first time when it was hypothesized that the diet of the shellfishes, mainly based on dinoflagellates, was the cause of poisoning

Saxitoxin (STX) is the main toxin group responsible for the syndrome of PSP (Table 1.4). It is a low molecular weight prehydropurine family of compounds (exemplary molecular weights around 300 Da) that act as potent Na^+ -channel blockers. It was firstly found in Alaska shellfish *Saxidomus giganteus* and later in several species of dinoflagellates. Some derivatives are more toxic than the others and additional changes in the structure and toxicity can occur as the toxins are passed up the food chain. The exact toxin profile can vary between different geographic isolates of the same species and is therefore useful as a marker. However, the amount and composition of toxins produced can be affected by environmental factors. Cysts are sometimes more toxic than vegetative cells and can lead to PSP outbreaks if they are consumed by shellfishes. On the other hand, the role of bacteria in toxin production is much debated. Some groups believed that STX is produced by marine bacteria that are isolated from dinoflagellates (Dantzer and Levin, 1997; Gallacher and Smith,

1999; Kodama et al., 1990). However, some suggested that there is no strong relationship on the PSP production and the dinoflagellate associated bacteria (Dantzer and Levin, 1997; Lu et al., 2000).

Table 1.4 The properties of saxitoxin [Adopted from the CBWInfo collection of factsheets on biological and chemical warfare agent (http: //www.cbwinfo.com /Biological /Toxins/ Saxitoxin.html)]

Structure	
CA Name	1H,10H-Pyrrolo[1,2-c]purine-10,10-diol,2,6-diamino-4-[[(aminocarbonyl)oxy]methyl]-3a,4,8,9-tetrahydro-, (3aS,4R,10aS)-
Trivial Names	●(+)-Saxitoxin ●Saxitoxin hydrate ●STX
Molecular Formula	$C_{10}H_{17}N_7O_4$
Molecular weight	299.3
Solubility	Freely soluble in water and methanol. Limited solubility in ethanol and acetic acid. Insoluble in lipid solvents
pKa in water	8.24

1.2.2 Diarrhoetic shellfish poisoning (DSP)

Diarrhoetic shellfish poisoning (DSP) was first documented in 1976 in Japan where it caused major problems for the scallop fishery (Yasumoto et al., 1980). It is the least serious of the human health problems caused by algal biotoxins. Unlike PSP, no human fatalities have ever been reported and patients usually recover within three days. However, the toxins are quite potent, less than 1000 cells per litre are enough to cause a symptom (Table 1.2). The linear polyethers okadaic acid (molecular weight =

805 Da) and dinophysitoxins (molecular weight = 2700 Da), the primary toxins responsible for DSP, inhibit protein phosphatases. They are produced by some members of the planktonic dinoflagellate genus, *Dinophysis*, and some benthic or epiphytic *Prorocentrum spp*. Most outbreaks of DSP were attributed to *Dinophysis spp*. because of their wider distributions. However, many planktonic *Prorocentrum spp*. have never been tested for toxin production and at least one, *P. minimum*, was associated with many human deaths in Japan (Hallegraeff, 2003).

1.2.3 Amnesic shellfish poisoning (ASP)

The illness is called Amnesic shellfish poisoning (ASP) because permanent loss of short-term memory is one of the distinguishing symptoms (Table 1.2). ASP was first described in 1987 when 107 people became sick after eating mussels from Prince Edward Island, Canada and three people died subsequently (Dortch, 2001; Hallegraeff, 2003). The causative toxin involved in ASP is domoic acid (molecular weight = 311.33). Domoic acid was originally isolated from a red alga called "doumoi" or "hanayanagi" (*Chondria armata*) in Japan. It is an analogue of the neurotransmitter glutamate and is thought to be produced by toxic diatoms of the genus *Pseudonitzschia.* However, it should be stressed that most *Pseudonitzschia* diatoms are not considered toxic. As long as light and nitrogen availability in the surrounding is adequate, domoic acid production is enhanced by factors that limit growth, such as limitation on phosphorus or silicate or low temperature, (Hallegraeff, 2003; Pan et al., 1998). The presence of bacteria can enhance toxin production, but is not required (Bates et al., 1995). However, the exact mechanism is unknown.

To date, reports of ASP in seafood products have been mainly confined to North

America and Canada. Although only in minute concentrations, they have also been detected in other regions of the world such as Europe, Australia, Japan and New Zealand (Hallegraeff, 2003). Numerous incidents occurred testified the seriousness of the ASP problem.

1.2.4 Ciguatera fish poisoning (CFP)

Ciguatera poisoning is a tropical fish-poisoning syndrome well known from coral reef area in the Caribbean, Australia, and especially in the French Polynesia. In the period of 1960-1984 in the French Polynesia, more than 24,000 patients were reported. This figure is more than six times the average for the whole of the Pacifics (Hallegraeff, 2003).

Ciguatera fish poisoning (CFP) occurred after the consumption of fishes harvested from tropical or subtropical reefs which were intoxicated with ciguatera. Herbivorous fishes became toxic from consuming dinoflagellates that contained ciguatera. This toxin can be biomagnified through the food chain, from small fishes grazing on the coral reefs into the organs of bigger fishes that feed on them (Hallegraeff, 2003).

The incidence rates and severity of CFP are highly variable. Although it is rarely fatal, CFP is an illness severe enough to prevent the use of fishes as food sources in many areas (Table 1.2). CFP causative species included the following: *Gambierdiscus toxicus, Ostreopsis lenticularis, O. siamensis, Coolia monotis, Prorocentrum lima, P. concavum, P. mexicanum, P. emarginatum, Amphidinium carterae* and *A. klebsii*. Different genera produce a variety of toxins, which have different effects. The toxicity can be influenced by environmental factors such as temperature and phosphate

availability.

1.2.5 Neurotoxic shellfish poisoning (NSP)

Neurotoxic shellfish poisoning (NSP) is caused by polyether brevetoxins produced by the unarmoured dinoflagellate *Karenia brevis,* which occurs primarily along the western coast of Florida, but also can occur throughout the Gulf of Mexico and as far north as North Carolina along the eastern US coast. Brevetoxins are a suite of polyether toxins, which activate voltage-sensitive Na+ channels and alter synaptic transmission. Brevetoxin-2 (molecular weight = 895.09) is the most abundant among the nine brevetoxins that have been characterized, whereas brevetoxin-1 (molecular weight = 867.1) is the most toxic. Initially *Karenia brevis* was though to be the only producer of brevetoxins, but several raphidophytes, known to cause fish kills, have recently been shown to produce brevetoxins as well. On the other hand, brevetoxins have chemical and biosynthetic affinities similar to that of CFP and DSP toxins. The amount and composition of different species of brevetoxins found within a particular species varies between isolates and is affected by environmental conditions. Although the toxins are not fatal, the blooms are a major threat to human health and cause significant ecological as well as economic damages An unusual feature of this organism is the formation of toxic aerosols that can lead to respiratory asthma-like symptoms in human (Table 1.2).

1.3 Dinoflagellates

Although diatoms can also produce toxic HAB, the aim of this study is to use dinoflagellates as the model. Therefore, literature that is relevant to dinoflagellates will be described.

1.3.1 Types of dinoflagellates

Dinoflagellates are important primary producers in coastal marine waters and are a single-celled microorganism adapted to a range of pelagic and benthic habitats throughout oceans of the world. Many species are cosmopolitan and they may be photosynthetic, heterotrophic or both. Over 2000 species have been documented worldwide and more than 50% are photosynthetic forms. There are also some parasitic and symbiotic forms. Dinoflagellates are important foundations of our food chain. Dinoflagellates are commonly between 5–2000 microns in length/diameter. Majority of dinoflagellate species is found in marine, but they can also be found in freshwater lakes, rivers and marshes. Most species are basically round in shape (Figure 1.11a) but some have particular shapes. For example, *Ceratium spp.* appeared as "folk" shape (Figure 1.11b). Dinoflagellates can be divided into armoured and naked. Armoured dinoflagellates contain a cell wall which is divided into cellulosic plates known as the theca (e.g. *Alexandrium spp*). Naked dinoflagellates contain only the cell membrane and without the theca (e.g. *Karenia spp*).

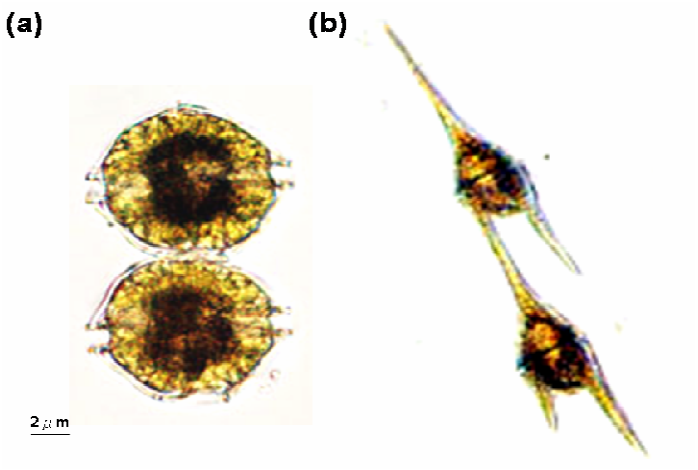


Figure 1.11 Some morphological shapes of dinoflagellates. (a) *Alexandrium spp.* with a typical "round" shape. (b) *Ceratium spp.*with a "folk" shape. [Adopted from Hong Kong red tide information network (www.hkredtide.org) dated September, 2007]

1.3.2 Cell biology of dinoflagellates

Most of the dinoflagellates are unicellular with two distinctive flagella that confer characteristic rotatory swimming motions. One flagellum is ribbon like and encircles the cell in a groove, and the other flagellum extends back. An equatorial groove or girdle divides a typical dinoflagellate cell into an epicone and a hypocone. The posterior flagellum extends through a depression called the sulcus (Figure 1.12) (Sze, 1998). In fact, the term "dinoflagellate" originates from the Greek word *dineo*, meaning "to whirl". Together with the cell membrane, the dinoflagellate cell covering material is a single layer of amphiesma that is consists of several to many closely adjacent, flattened amphiesmal (thecal) vesicles (Figure 1.13). Besides, the amphiesma consists of a continuous outermost plasma membrane, the outer plate membrane, and a single-membrane-bounded thecal vesicle. Inside this vesicle, there are a number of cellulosic thecal plates subtended by a pellicular layer. The structural component, cellulose, is mainly deposited in the thecal plates (Morrill and Loeblich, 1984). If the thecal vesicles of a species contain a thecal plate composed of cellulose;

such species are said to be armored (Figure 1.14 a). While if the amphiesmal vesicles of species are devoid or nearly devoid of contents, such species are referred to as unarmored (Figure 1.14 b).

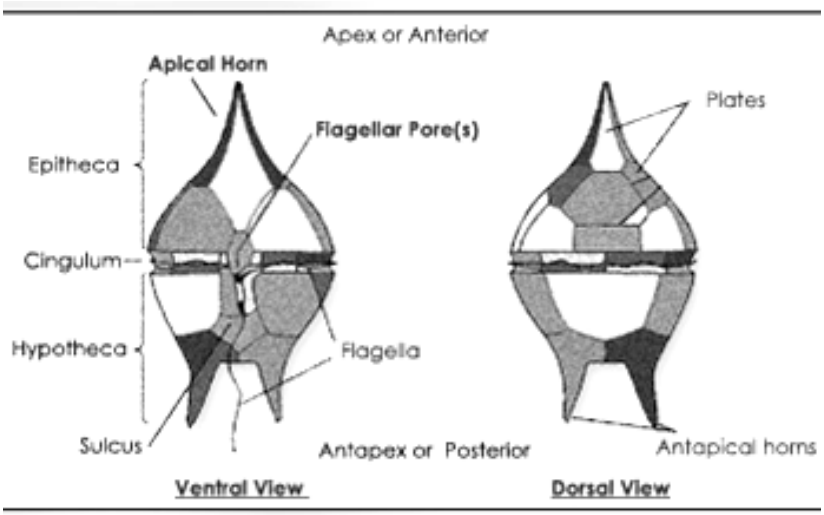


Figure 1.12 A typical dinoflagellate cell. [Adopted from Hong Kong red tide information network (www.hkredtide.org) dated September, 2007]

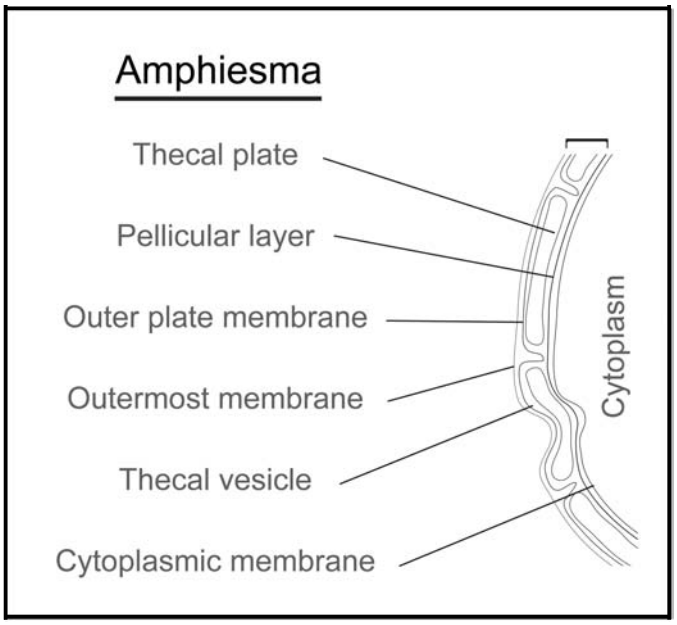


Figure 1.13 Schematic diagram of the amphiesma of a typical thecate dinoflagellate. [Adopted from (Morrill and Loeblich, 1984)]

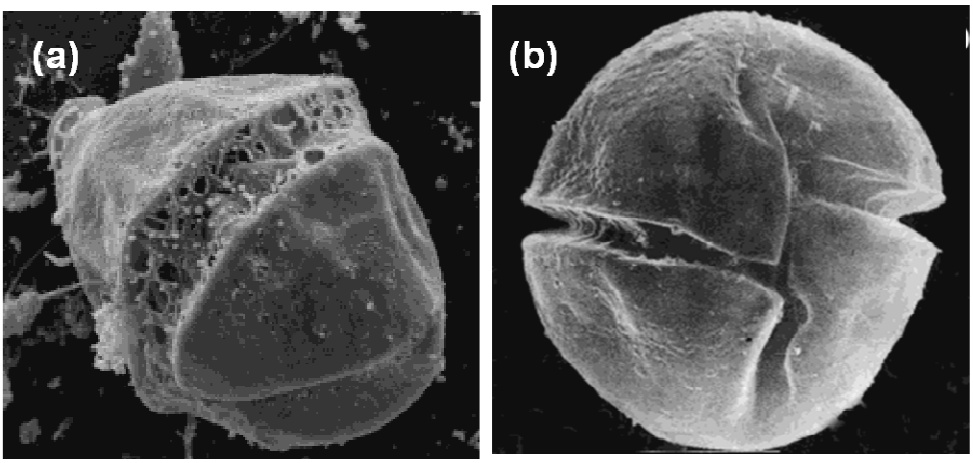


Figure 1.14 Scanning electron microscopy (SEM) of armored and naked dinoflagellates. (a) *Scrippsiella trochoidea* (Armored) and (b) *Karenia digitata* (unarmored or naked). [Adopted from (Chan et al., 2004a)]

A typical dinoflagellate cell has several features: (1) theca and associated structure (as mentioned above), (2) nucleus, (3) chloroplasts (for photosynthetic cells) and (4) different other organelles include golgi bodies near the nucleus (Figure 1.15) (Sze, 1998). The pusule is a distinctive dinoflagellate organelle consisting of a series of vesicles near the base of flagella. It functions in osmoregulation, macromolecule uptake, and secretion (Sze, 1998). Reserves are stored in the cytosol as starch or lipids (Sze, 1998).

Pigment composition for most of the plastid-containing dinoflagellates is similar, including chlorophyll a and c, xanthophylls peridinin, β-carotene and other carotenoids. Some of this pigment such as gyroxanthin diester, can be used as a pigment marker for the toxic HAB dinoflagellate, for example *Gymnodinium breve* (Millie et al., 1997; Richardson and Pinckney, 2004).

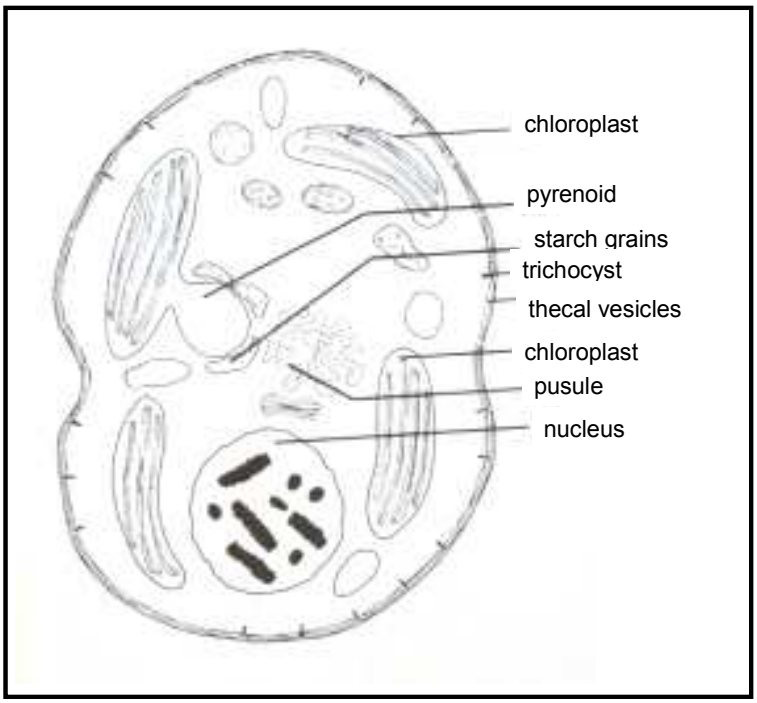


Figure 1.15 Sketch of a typical dinoflagellate cells, including nucleus with condensed chromosomes, chloroplast with protruding pyrenoid, thecal vesicles, trichocyst, pusule, and starch grains. [Adopted and modified from (Sze, 1998)]

1.3.3 Unusual features of dinoflagellates

Dinoflagellates are unique organisms with several unusual features (Graham and Lee, 2000; Sze, 1998). Firstly, the chromosomes of most dinoflagellates are permanently condensed (always visible) and lack the histone proteins that are characteristics of other eukaryotes (Figure 1.16). These chromosomes only, uncoil for a brief time to allow DNA replication to occur (de la Espina et al., 2005; Herzog and Soyer, 1981; Rizzo, 2003). Secondly, the dinoflagellate nuclear envelope remains intact throughout mitosis. There is no true mitotic spindle during nuclear division and it is entirely extra nuclear. Recently, Chan and Wong suggested that the liquid crystalline chromosomes of dinoflagellates are alternative to the nucleosome-based organization of chromosomes in this organism (Chan and Wong, 2007). Thirdly, DNA content of dinoflagellates is estimated to be about 10-100 times more than that of

human. That means they possess genomes many times greater than that of the human genome (approximately 3.0 pg·cell^{-1}). For example, *Prorocentrum micans* has one of the largest genomes known for photosynthetic phytoplantons, which is about 230 to 280 pg·cell^{-1} (Veldhuis et al., 1997). Fourthly, in photosynthetic dinoflagellate species, the plastid or chloroplast is surrounded by three membranes rather than the typical two or four (Gibbs, 1981). In addition, peridinin is a unique dinoflagellate chlorophyll protein that is responsible for light harvesting. Together with chlorophyll *a*, peridinin was bound to form peridinin–chlorophyll *a* proteins (PCPs) (Jeffrey et al., 1975; Peltier et al., 2000). Fifthly, probably the most unusual feature, it was found that the plastids contain form II instead of form I ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO). Rubisco II was found in some species of anaerobic proteobacteria only (Morse et al., 1995; Rowan et al., 1996). Form II Rubisco differs from the typical form I in that oxygen has a higher affinity than CO_2 for binding to the active site of the form II enzyme than to that of the form I enzyme (Whitney and Andrews, 1998). This observation suggests the presence of a specific mechanism for carbon-fixing, like inorganic carbon concentrating mechanism (Leggat et al., 1999). Lastly, molecular studies demonstrated that genome of dinoflagellate plastids are encoded on small single-gene or dual-gene minicircles (Barbrook and Howe, 2000; Barbrook et al., 2001; Hiller, 2001; Zhang et al., 2001; Zhang et al., 1999). Furthermore, of the plastid proteins found, they are all nuclear-encoded (Fagan et al., 1999; Hiller et al., 1995; Morse et al., 1995). Collectively, these and other unique cytological, ultrastructural, and genetic attributes present intriguing topics of research into the life of dinoflagellates.

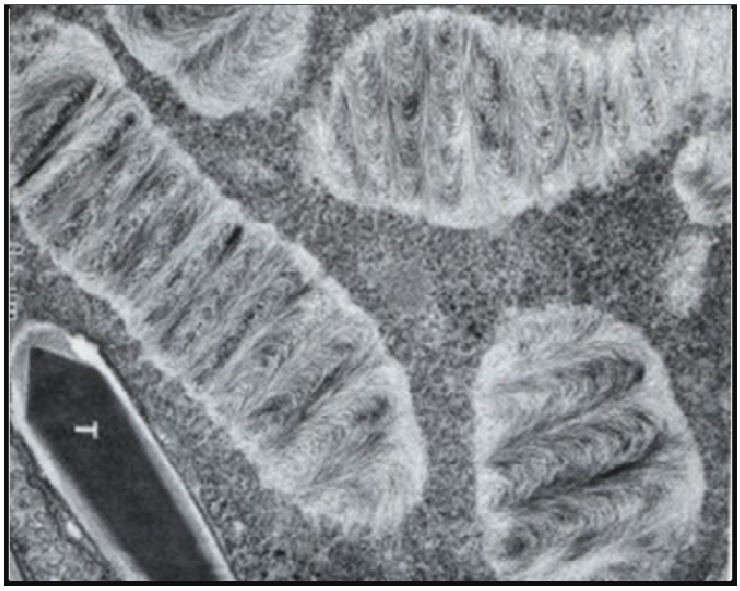


Figure 1.16 Permanently condensed chromosomes of dinoflagellate *Prorocentrum micans.* [Adopted from (Graham and Lee, 2000)]

1.3.4 Physiology and growth of dinoflagellates

1.3.4.1 Reproduction and life cycle

Dinoflagellates that spend most of their life cycle as unicellular flagellates undergo division by mitosis, and several have been documented to undergo sexual reproduction as well (Graham and Lee, 2000). Figure 1.17 shows the life cycle of a typical dinoflagellate. Dinoflagellate produces a resting cyst during periods of suboptimal growth conditions. The cyst sinks to the bottom of a column of water and they can excyst to release vegetative cells when there are favorable conditions. Vegetative cells swim to the surface to re-introduce the seed populations for blooming. Gametes will form again with the return of poor conditions (e.g nutrient depletion). Diploid, motile zygotes (planozygotes) will be present following gamete fusion. The zygotes will in turn become cyst again. In some species, temporary asexual cysts can be form. During asexual reproduction, cytokinesis in unarmored cells may involve a simple pinching in two along an oblique division line, with continuous synthesis of

new amphiesma parts and the cells may remain motile during the process. In contrast, armored cells may shed the cell covering prior to mitosis, and then each daughter cell resynthesizes a new theca. Sexual reproduction can be induced in the laboratory by reducing nitrogen concentration of the growth medium or by lowering the temperature. It is assumed that these factors are also related to induction of sexual reproduction in nature.

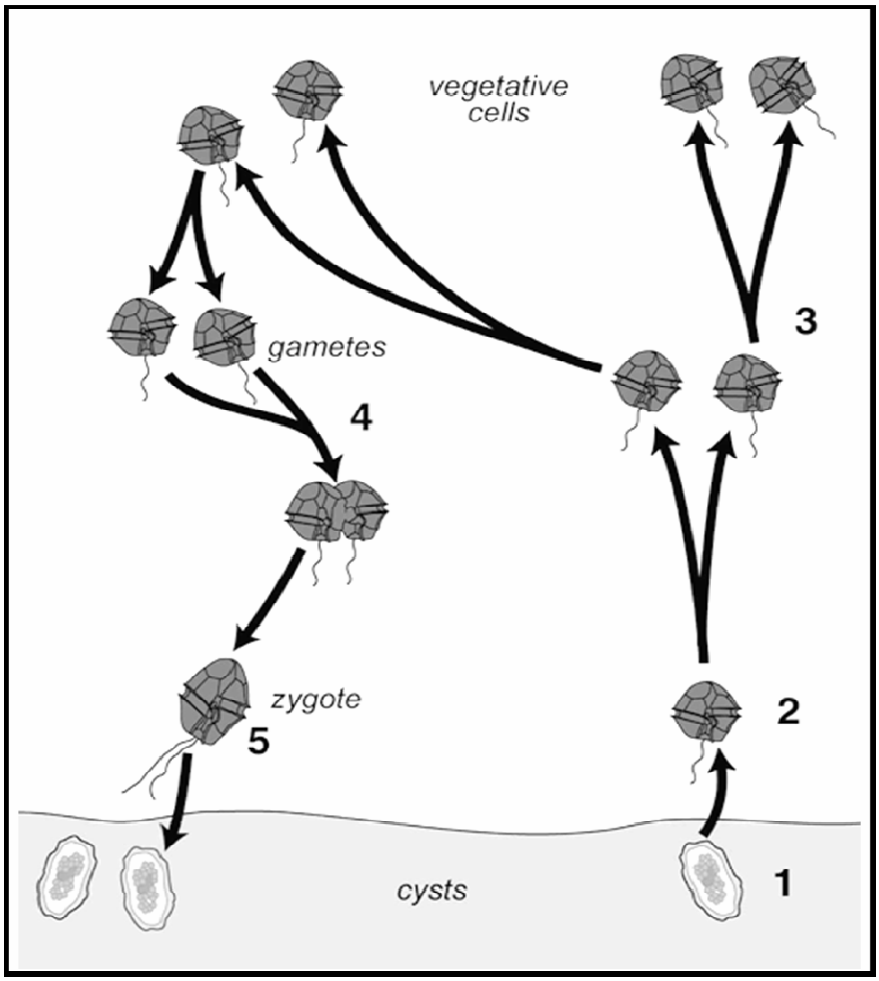


Figure 1.17 Life cycle of dinoflagellate. (1) Cyst resting stages that act as reservoirs for new population growth. (2) The resting cyst ruptures (excyst) to become swimming cells. (3) Division to produce a vegetative population. (4) Under certain conditions, like nutrients depletion, cell division is slowed down and gametes will be formed. (5) Gametes fused to form zygote and then cysts. [Adopted from (Sellner et al., 2003)]

1.3.4.2 Cysts

Cyst is a term used to describe a non-motile cell that lacks flagella and the ability to swim. Formation of cyst may arise from a variety of processes. It has been attributed to adverse changes in nutrients, irradiance, photoperiod, or temperature (Graham and Lee, 2000). Cyst development begins with formation of colorless peripheral region of cytoplasm. The planozygote stops swimming and sheds its flagella, after which the thecal plates separate and pull away from the cell surface. Cysts so formed will sink to the bottom. Cyst germination is inhibited by darkness and low oxygen. Excystment in the spring occurs through an aperture in the cyst wall, and then a normal theca is developed. Cyst formation ensure survival during adverse conditions, which may include temperatures that are too high or too low for growth or other adversities. For example, some *Pfiesteria* cysts can survive treatment with concentrated sulfuric acid or ammonium hydroxide (Graham and Lee, 2000). Usually, these cyst products are generated from sexual reproduction. Dormant resting cysts are often found in nature. For example, *Gonyaulax* cysts can survive in storage for as long as 12 years (Graham and Lee, 2000). The encystment process can also be induced by addition of indoleamines such as melatonin and 5-methoxytryptamine in laboratory cultures (Balzer and Hardeland, 1991; Balzer and Hardeland, 1992; Tsim et al., 1997). Like vegetative cells of toxic strains, cysts of these strains are toxic and may be ingested by shellfishes (Oshima et al., 1992). High number of cysts is usually found in sediments below coastal waters that had previously supported dinoflagellate blooms. As mentioned, cysts may be transported via water currents or ship ballast water to new locations and serve as inocula for bloom formation (see 1.1.3.4).

1.3.5 Detection and identification of dinoflagellates

Understanding of the HAB phenomena depends greatly on identification, detection and enumeration of specific species found in discrete water samples. Microscope-based cell identification methods have long been the standard methodology (Fensome et al., 1999). Usually, light-microscopic techniques; scanning electron microscopy (SEM) and transmission electron microscopy (TEM) are the commonly used tools. These type of identification methods rely greatly on the morphology of the target species. However, such traditional microscopic identification method is time consuming and always requires a high level of expertise to discriminate key morphological features indicative of HAB species. Moreover, it is impossible to discriminate between “toxic” and “non-toxic” species just by using morphological characteristics alone.

There are several techniques have been proposed for discriminating different and even very closely related HAB species. Molecular based methods for detecting HAB species are used routinely in many laboratories world-wide. Other methods which use lectins (Cho et al., 2001; Costas and Rodas, 1994), antibodies (Chang et al., 1999; Lopez-Rodas and Costas, 1999; Mendoza et al., 1995), ELISA (Xin et al., 2005) and oligonucleotide (Hosoi-Tanabe and Sako, 2005; Hosoi-Tanabe and Sako, 2006), were practiced. Recently, there are several other types of detection techniques had been proposed. These methods included the use of photopigments and absorption signatures (Millie et al., 1997) and lipid profiles (Mansour et al., 2005; Mansour et al., 1999) of HAB causative agents. However, these methods are still not commonly applied, and thus it would not be discussed in detail in this review. Instead, a brief discussion would be focused on the use of ribosomal RNA-targeted DNA probes as

this is a very common and useful method for HAB species identification.

PCR-based investigation on ribosomal gene is a very common molecular based technique used in identification of HAB causative agents. Ribosomal RNA (rRNA) genes are used as they usually include highly conserved regions common to most organisms (Figure 1.18). This is advantageous as primer are available that should be able to amplify any rRNA genes, allowing subsequent sequencing to locate unique sequences within variable regions which can then be targeted as unique molecular "signatures". For example, Adachi et al. have found an *Alexandrium tamarense*-specific primer TF1 (Adachi et al., 1996). On the other hand, rDNA cistron (ITS and NTS regions) allow specificity at even finer levels because they are not structural gene and have less conserved regions (Litaker et al., 2007).

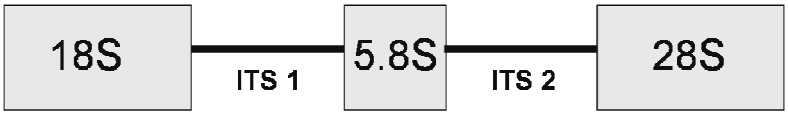


Figure 1.18 Schematic diagram of ribosomal RNA gene. ITS is the internal transcribed spacers.

1.4 Proteomics

Besides PCR-based methodologies, two-dimensional gel electrophoresis proteome reference maps had also been suggested for species-identification purposes (Chan et al., 2004a). On the other hand, with the provision of proteomic technologies, study on differential protein expression of dinoflagellates could provide insights into cellular responses upon nitrogen depletion and repletion (see below). Therefore, these methodologies were adopted in this study. A detailed review of proteomics and its application in research of HAB are in the following:

1.4.1 Proteomics and Two-dimensional gel electrophoresis (2-DE)

The term "proteome" was first coined in 1994 by an Australian postdoctoral fellow, Dr. Marc Wilkins in Macquarie University (Wasinger et al., 1995). In his definition, the proteome refers to the total set of proteins expressed in a given cell at a given time; and the study of which is termed as "proteomics."

Proteomics is the large-scale study of proteins, particularly their structures and functions, in cells. Proteomics has firmly been established as a powerful tool for understanding various biological problems (Reinders et al., 2004). Currently, for most proteomic investigations, proteins has to be resolved first before being separated and identified by mass spectrometry. Two-dimensional gel electrophoresis (2-DE) or some emerging technologies like multidimensional protein identification technology (Mud-PIT) and shotgun peptide sequencing (Hu et al., 2007), are now the most commonly used techniques in the quantitative expression studies of large sets of complex protein mixtures. Very expensive setup is required for Mud-PIT and shotgun peptide sequencing and they are not commonly available. In this investigation, 2-DE was used.

2-DE is an important protein resolving procedure in proteomics. It was invented more than 30 years ago (O'Farrell, 1975). The use and set up of 2-DE had recently been extensively reviewed (Gorg et al., 2000; Gorg et al., 2004). It is a powerful and widely used method for the analysis of complex protein mixtures extracted from cells, tissues, or other biological samples. This technique sorts protein according to two independent properties in two discrete steps. In the first-dimension step, isoelectric focusing (IEF) (Figure 1.19a) was applied in which proteins are separated by their isoelectric points (*pI*). In the second-dimension step, SDS-polyacrylamide gel

electrophoresis (SDS-PAGE) (Figure 1.19b) which separated proteins according to their molecular weights were applied. After protein visualization procedures, each spot on the resulting 2D gel corresponds to a single protein in the sample. Hundreds of different proteins can thus be separated. Information such as the protein pI, the apparent molecular weight, and the amount of each protein can also be obtained. Moreover, each protein spot can be identified by mass spectrometric techniques (see below).

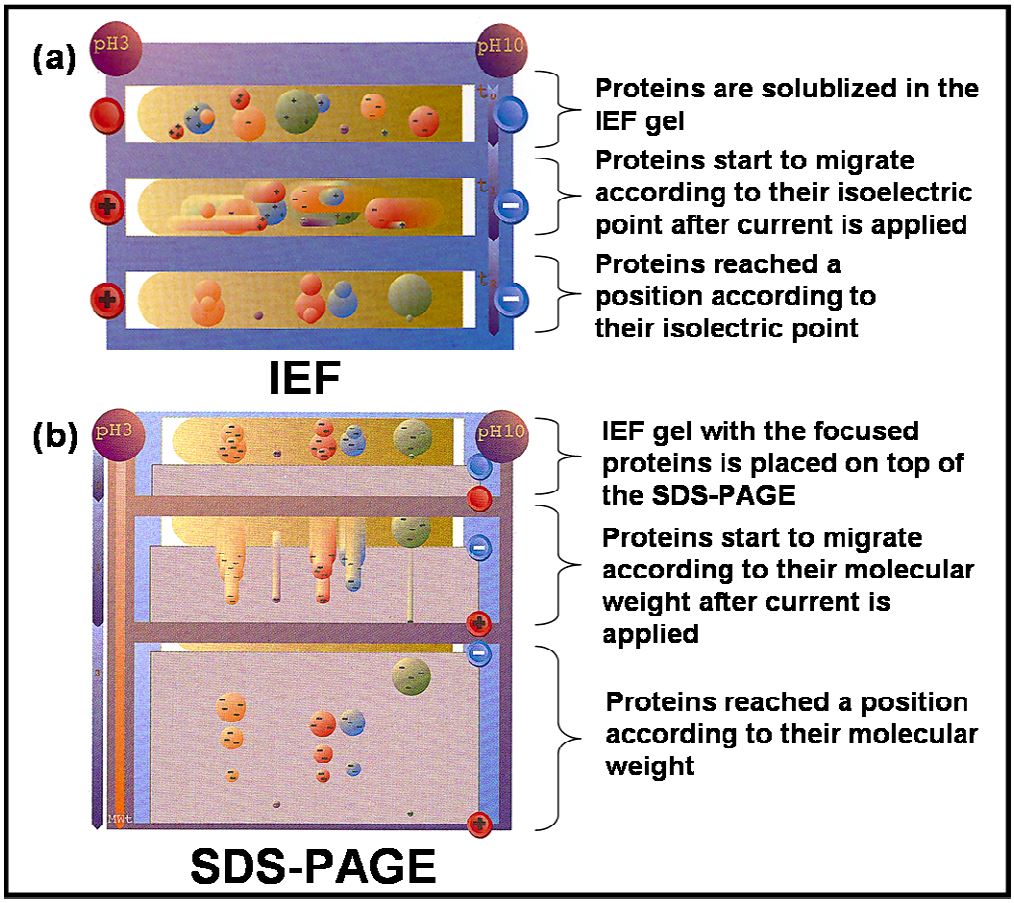


Figure 1.19 Schematic diagram illustrating the principle of (a) IEF (first dimension) and (b) SDS-PAGE (second dimension). In the IEF, protein molecules with a net positive charge will migrate toward the cathode and becoming progressively less positively charged as it moves through the pH gradient. When it reach a pH level that it has no net charge (pI), it will stop. Proteins with a net negative charge will move in exactly the opposite direction. While in the SDS-PAGE, Protein molecules are separated according to their molecular weights.

Despite of the emerging alternative proteomic technologies such as Mud-PIT and shotgun peptide sequencing (Hu et al., 2007), 2-DE is still one of the most commonly used techniques in proteomics research. It is because 2-DE is relatively cheap to set up and it provides a fast overview of the proteome of interest. It is still the only technique that can be routinely conducted in parallel for the quantitative expression profiling of large sets of complex protein mixtures (Gorg et al., 2004). In addition, the primary strength of 2-DE is the detection of proteins with post-translational modifications (PTMs) and it is easy observed in 2-DE gels. In many instances, post-translationally modified proteins appear as distinct spot trains in the horizontal/vertical axis of the 2-DE gels. In comparison with 2-DE protein expression map approach, liquid-chromatography-linked-tandem-mass-spectrometry (LC-MS/ MS) based analytical methods cannot provide information on isoelectric points and molecular masses of intact proteins. Further, it should be reiterated that 2-DE analysis not only can provide information on protein expression levels and their PTM, it can also allows isolation of proteins in significant amounts (even up to mg levels if required) for downstream structural analysis or *de novo* sequencing by MS/MS or Edman degradation (Celis and Gromov, 1999; Graves and Haystead, 2002; Ong and Pandey, 2001). Figure 1.20 showing the workflow of the 2-DE/MS approach as a proteomic analysis platform.

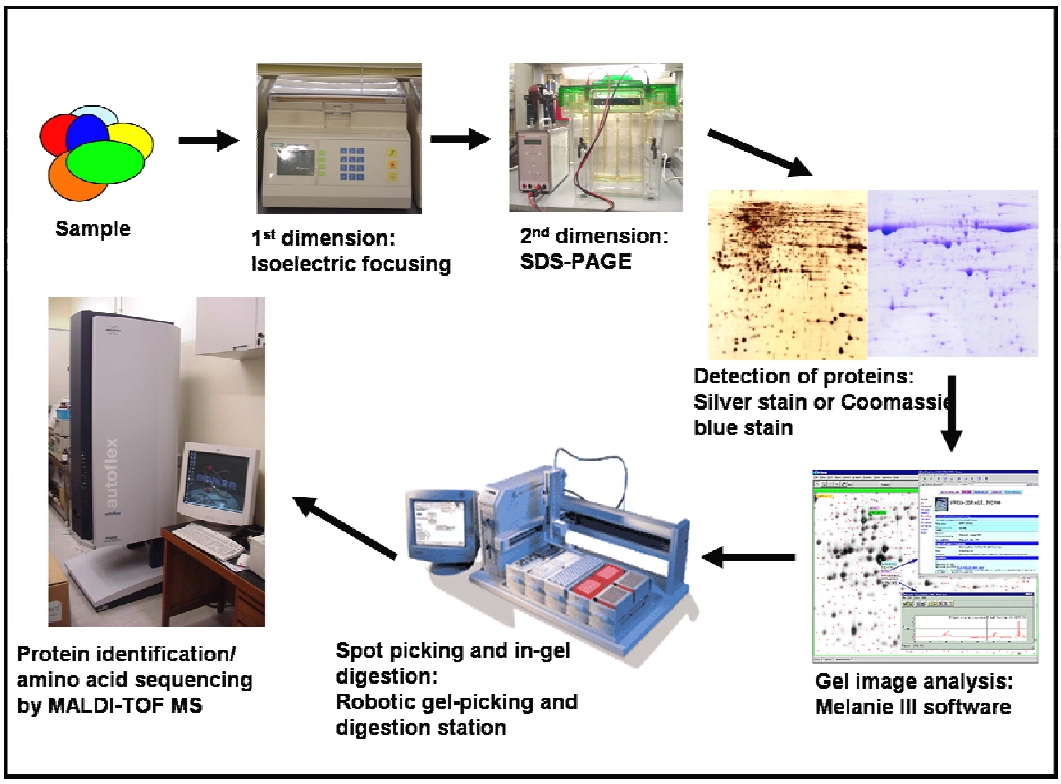


Figure 1.20 Schematic diagram showing the workflow of 2-DE/MS approach as a proteomic analysis platform.

1.4.2 Proteomics of dinoflagellates

Although the ever increasing risk of occurrence of HABs represents expanding threats to human health and economy, many questions about these dinoflagellates are unknown. For examples, it is currently unknown why these autotrophs would need to produce toxins. Mechanisms of blooming and pathways by which these toxins are synthesized are unknown. Consequently, proteomic studies on these dinoflagellates have the potential to uncover cellular pathways and mechanisms involved in toxin production and/or blooming at the molecular level.

Although rare, there were several proteomic studies on dinoflagellates with the uses of 2-DE and MS approach. These studies can be divided into three broad categories: (1) the study of blooming and toxin- producing biomarker of

dinoflagellates (Chan et al., 2005; Chan et al., 2004a; Chan et al., 2004b; Chan et al., 2002; Chan et al., 2006); (2) the study of cell surface proteins of dinoflagellates (Bertomeu et al., 2003) and (3) the study of circadian expressed proteins in dinoflagellates (Akimoto et al., 2004; Markovic et al., 1996; Milos et al., 1990).

Chan and co-authors reported that *Prorocentrum triestinum* grown under different growth phases and growth conditions showed different differentially expressed protein profiles. They found several preblooming (PB1, PB2, and PB3) and blooming proteins (BP1 and BP2) which were differentially expressed before and after blooming respectively (Chan et al., 2004b). In addition, by comparing protein expression on 2DE gels from samples of toxic and non-toxic dinoflagellates, a differentially expressed toxin biomarker (T1) was found. N-terminal sequencing of the blooming proteins and internal amino acid sequencing of T1 were successfully obtained (Chan et al., 2006). Complete DNA sequence of T1 was obtained subsequently. That series of proteomic studies were the first set of investigations on blooming as well as toxin-releasing mechanisms on dinoflagellates and some preliminary understanding on these aspects in molecular terms were achieved.

On the other hand, the first report on surface proteins of dinoflagellates was from Bertomeu and co-authors (2003) using I^{125} labeling approach. They had successfully obtained several peptide sequences of p43, a cell surface protein that was isolated from *Lingulodinium polyedrum* (a dinoflagellate) through 2-DE. Amino acid sequences of p43 was obtained by microsequencing performed with an unspecified mass spectrometer (Bertomeu et al., 2003). Degenerate primers derived from these amino acid sequences were designed and subsequent PCR amplifications aiming to obtain the full cDNA sequence were performed. The complete cDNA sequence

encoding p43 was obtained eventually.

Proteomic technologies had also been applied to study the circadian processes of dinoflagellates. Several cellular processes in the marine dinoflagellate *Lingulodinium polyedra* (formerly called *Gonyaulax polyedra*) were found to exhibit circadian rhythms. Some proteins involved in these processes were found to be regulated at the translational level only (Mittag, 2001). Because of that, characteristics of these proteins have to be studied at the protein level. On the other hand, Milos and coworkers labeled proteins *in vivo* using cultures from the day- and night- phases before being resolved by 2-DE (Milos et al., 1990) Several proteins were found to be differentially expressed at different time. This approach was taken further by amino acid microsequencing of the labeled proteins and four proteins were successfully identified from the peptide sequences obtained (Markovic et al., 1996). These proteins are ribulose 1,5 bisphosphate carboxylase/oxygenase (RUBISCO); the nuclear encoded glyceraldehydes-3-phosphate dehydrogenase (GAPDH), a protein component of the oxygen evolving complex; and the peridinin-chlorophyll a-binding protein (PCP). All these proteins are related to photosynthesis and under circadian regulation. In 2004, with the same *L. polyedra* as Bertomeu *et al.* (2003), Akimoto and his collaborators (2004) had study biological rhythmicity in terms of expressed proteins in these algae using proteomic technologies. In their studies, cells were harvested at different time points of the light-dark regime and protein extracts from these cells were resolved by 2-DE. Out of about 900 protein spots detected, 28 were found to display differential expression in a diurnal pattern. 20 out of these 28 proteins were identified by LC-ESI-MS/MS. The remaining 8 proteins, which were also found to exhibit diurnal changes, were not identified at that time due to insufficient genome

information of the dinoflagellates. With provision of more advanced technologies currently available, *de novo* protein sequencing and bioinformatic search for protein annotation that relied on homologous or highly conserved protein sequences can be performed for identification of these proteins. Nevertheless, Akimoto and colleagues had demonstrated the feasibility of applying proteomic approach in studying differential protein expression in dinoflagellates. The combination of 2-DE, MALDI-TOF MS and amino acid sequencing by Edman degradation or other types of MS, has provided a useful approach for the identification and annotation of differentially expressed proteins in dinoflagellates.

Although protein identification utilizing peptide mass fingerprints (PMFs) obtained from MALDI-TOF MS analysis and bioinformatic searches has greatly improved efficiency of the identification process over classical Edman sequencing procedures, successful protein identification using PMFs required a concomitant provision of genome information of the organisms of interest. Nonetheless, genomic sequences of many subclasses of *Dinophyceae* (dinoflagellates) are completely unknown. It should be reiterated that incomplete genome information on dinoflagellates has limited applications of proteomic techniques in the studies on those organisms. Thus, *de novo* protein/peptide sequencing is important for acquiring protein sequences on samples of interest from these organisms.

1.4.3 De novo proteins/peptides sequencing

1.4.3.1 Edman sequencing

In the 1960s, *de novo* protein sequencing was performed by the Edman degradation chemistry (Edman and Begg, 1967). It is a stepwise procedure to degrade

proteins from their N-terminal end. Although details of Edman sequencing is outside the scope of this review, various technical developments and advances of microsequencing had enable fabrication of highly sensitive and fully automated protein sequencers (Shively, 2000; Tsugita, 1987; Walker, 1994). Although Edman sequencing continues to contribute to the field of protein biochemistry and proteomic research, it has a number of serious limitations. The most significant limitation is the slow speed of the sequencing reaction. One typical degradation cycle is around 45 minutes with a narrow-bore HPLC. Therefore, only a limited number of peptides can be sequenced every day. Furthermore, eukaryotic proteins are often blocked at their N-termini, i.e. without an α-carbon (Gevaert and Vandekerckhove, 2000). De-blocking procedures including enzymatic digestion to open up the N-terminus have to be performed before amino acid sequencing. However, it should be noted that after a protein was blotted onto a PVDF membrane, each sample can only be used once in N-terminal sequencing. There is no chance for repeating the experiment using the same blotted sample except when it was N-terminal blocked. Inadequacy of Edman sequencing in these respects makes it less popular than other sequencing techniques (see below). Nonetheless, Edman sequencing still remains as a fallback peptide sequencing method when mass spectrometric methods failed to yield useful sequencing information.

1.4.3.2 Peptide sequencing by MALDI-TOF mass spectrometry (MS)

Provision of MS has significantly reduced the need for Edman sequencing because it is more sensitive and provides higher sample throughput (Bienvenut et al., 2002). MS itself can only give considerable information on the peptide mass as well

as the amino acid compositions of peptides. MS does not determine the order of the amino acids (Standing, 2003). Therefore, *de novo* peptide sequencing usually requires tandem mass spectrometry or MS/MS. Generally, for MS/MS peptide sequencing, a particular precursor ion is selected in the first masses scan before being fragmented either by collision induced dissociation (CID) or metastable decay. Subsequently, the fragmented ions can be separated by their mass/charge during the second MS scan. This procedure may yield a series of ions with ladder like appearance which may contain sufficient information to determine the amino acids sequences. Further, it was found that the presence of matrix greatly facilitated the ionization process.

Matrix-assisted laser desorption ionization (MALDI) in MS was developed by Karas and Hillenkamp in the late 1980s (Karas and Hillenkamp, 1988). MALDI *de novo* sequencing has been carried out using CID and metastable decay (Medzihradszky et al., 2000; Schilling et al., 1999; Spengler et al., 1992; Yergey et al., 2002). There are several attractive properties for the analysis of peptides by using MALDI MS. Firstly; it has a relatively high tolerance to salts and impurities when compared to that of electrospray ionization (ESI). Therefore, requirement for sample purity for MALDI analysis is relatively low and extensive sample cleaning steps can be avoided. Secondly, the sample intended for MALDI analysis is co-crystallized with the matrix and dried on a target plate. This allows the sample to be used until it is depleted; so data can be obtained for different ion species produced from a single peptide mixture. Because of these advantages, *de novo* peptide sequencing using MALDI MS is becoming very popular (Spengler et al., 1992; Yergey et al., 2002); particularly after pretreatment of the sample by derivatization such as sulfonation (Keough et al., 2001; Keough et al., 2000; Keough et al., 2002; Keough et al., 1999),

as described in section 1.4.3.2.2.

1.4.3.2.1 MALDI-TOF MS-PSD

There are two different fragmentation reactions in MALDI-MS. Firstly, fragmentation of ions occurred in-source following laser impact and it can occur within a few hundred nano-seconds (Lennon and Walsh, 1997). Another one is the Post-Source Decay (PSD) (Kaufmann et al., 1993). It takes a longer time scale (μs) following extraction of MALDI-ions out of the source region into the field-free region (in the flight tube) and the peptide ions decayed because of the energy departed in the desorption process. Fragment ions maintain the same velocity as their intact precursor ions in the field-free region. However, the fragmented ions could not travel as far as intact ions into the reflectron field due to their lesser kinetic energy. Therefore, the lower the mass of the ions, the sooner the ions will be ejected out of the reflectron field. It will then appear as a lower apparent mass in the spectra. Because the dynamic separation range is limited for most reflectron fields at a defined voltage, therefore, a regime of gradually stepped down voltages is required to collect the complete set of fragment masses in the range of interest (usually 10-15 segments are obtained). The final sequences could be deduced after stitching together the spectra from different voltage ranges. Alternatively, a “curved-field reflectron” in the TOF mass detector will enable simultaneous focusing of a wide mass range of fragment ions generated by metastable decay. The entire PSD fragment ions spectrum can be obtained in a single experiment (scan) without changing voltages. Provision of the ‘curved-field reflectron’ has therefore circumvented the time-consuming reflector voltage stepping procedures (Cornish and Cotter, 1994).

In addition, when delayed extraction is applied in the ion source of a MALDI MS, precursor ions are energetically cooled down which brought about a concomitant reduction of the rate of PSD fragmentation by at least an order of magnitude (Kaufmann et al., 1996). Part of this loss is balanced by a better signal/noise ratio which results from a significantly improved mass resolution of the PSD fragment ions ($M/\Delta M$ up to 1800 compared with $M/\Delta M$ = 200-500 under prompt extraction). While this compensatory effect is true for the middle to high mass range of PSD fragment ions, it gradually vanishes towards the low mass end of the PSD mass scale where, in the case of linear peptides some important immonium ions are lost. Taken overall for most practical work with PSD, delayed extraction improves quality of the PSD spectra and that high energy collisional post-source activation can compensate for the occasional loss of analytical information (Kaufmann et al., 1996).

It should be stressed that there are many types of fragment ions were produced during the fragmentation processes. Both the N-terminal fragment ions (a-, b-, c- and d- type ions) and C-terminal fragment ions (x-, y-, and z-type ions) as well as the internal fragment ions can be formed from double chains cleavages and they can be observed in the PSD spectra (Chaurand et al., 1999) . [For the nomenclature of fragment ions, please refer to (Biemann, 1988)]. These ions are complementary to each other and can be used to validate accuracy of *de-novo* sequences obtained. However, interpretation of these spectra and deduce a "*de-novo*" peptide sequence is a highly skillful task. In fact, the major drawback of *de-novo* peptide sequencing using PSD is that the output of fragment ions spectra is too complex. As mentioned, interpretation of the results is complicated, labor-intensive and certainly required a high level of proficiency. Therefore, it was argued against the use of

MALDI-TOF-PSD as a standard peptide sequencing method (Samyn et al., 2004). However, it should be noted that publications in reputable journals in the proteomic field nowadays required validation of at least one amino acid sequence per protein identified. As validation experiments with antibodies-based are not always possible because of lack of suitable antibodies, MALDI-TOF-PSD or MALDI-TOF-TOF operation for sequence confirmation is fast becoming a necessity in any proteomic laboratory.

1.4.3.2.2 Derivatization and N-terminal sulfonation

As mentioned above, in order to produce a simple but informative mass spectrum, chemical modification or derivatization of the peptides is highly desirable. Several derivatization techniques had been developed (Hellman and Bhikhabhai, 2002; Keough et al., 2001; Keough et al., 2000; Keough et al., 2002; Keough et al., 1999; Keough et al., 2003; Lindh et al., 2000; Shen et al., 1999; Sonsmann et al., 2002). Because of its relative simplicity, a notable derivatization procedure called the "sulfonation of the peptide N terminus" that was originated by Keough and his collaborators (Keough et al., 2001; Keough et al., 2000; Keough et al., 2002; Keough et al., 1999; Keough et al., 2003) is one of the most popular methods. (One version of this sulfonation procedure" was adopted in my studies (see below)). The sulfonation technology facilitated *de novo* peptide sequencing by using MALDI-TOF-PSD. In their method, a sulfonic acid group was introduced at the N-terminus of a peptide. This chemical modification facilitates protonation of amide bonds of the protein backbone. Protonation destabilizes amide bonds which lead to only b- and y- type ions being produced during PSD fragmentation. However, the negative charge at the

N-terminus neutralizes the positive charge normally located at the C-terminus; thereby suppressing the formation of b-ions in positive mode. Hence, a series of y-type ions dominated the PSD spectrum will be formed (Figure 1.21). Interpretation of this spectrum for *de-novo* sequence will be a much simpler operation. The three most commonly used sulfonation reagents and their general advantages and disadvantages are listed in Table 1.5 below.

Table 1.5 Comparison of the three most commonly used N-terminal sulfonation reagents for *de novo* peptide sequencing.

sulfonation reagents	advantages	disadvantages	useful references
chlorosulfonylacetyl chloride (CSAC)	● commercially available ● quite reliable for the derivatization of low-level Arginine-terminated tryptic peptides ● allows sulfonation of subpicomole quantities of tryptic peptides	● too reactive to used in water, it must be used in non-aqueous conditions ● poor stability ● more purification and cleaning steps are required	Keough *et al.*, 1999
3-sulfopropinic acid NHS-ester	● Developed into a commercial available sequencing kit named chemically assisted fragmentation (CAF) (Amersham Biosciences, USA), which are more friendly to be used. ● water compatible ● unwanted sulfonation side products can be selectively reversed by using hydroxlamine	● relatively expensive ● have to be freshly prepared	Keough *et al.*, 2002; Hellman and Bhikhabhai, 2002
4-sulfophenyl isothiocyanate (SPITC)	● commerically available ● derivatization reaction is efficient in aqueous solution ● stable, can be prepared into stock solution ● much less expensive	● may need larger amounts of protein materials (nanomoles)	(Gevaert et al., 2001; Marekov and Steinert, 2003; Wang et al., 2004)

Sulfonation was first accomplished by acylation with 2-sulfobenzoic acid cyclic anhydride and chlorosulfonylacetyl chloride (Keough et al., 1999). This procedure was then refined by using a water soluble reagent, 3-sulfopropinic acid NHS-ester (Keough et al., 2002) which had been commercialized as the CAF (chemically assisted fragmentation) sequencing kit by Amersham Biosciences (Hellman and Bhikhabhai, 2002). The procedures were further refined by guanidination of the ε-amino group of lysine-terminated tryptic peptides with O-methylisourea hydrogen sulfate (Keough et al., 2000; Keough et al., 2002). This step converts lysine residues into more basic homoarginine residues. This modification protects the ε-amino groups against unwanted reaction with sulfonation and therefore allows it to become suitable for *de novo* sequencing reaction by MALDI-TOF-PSD.

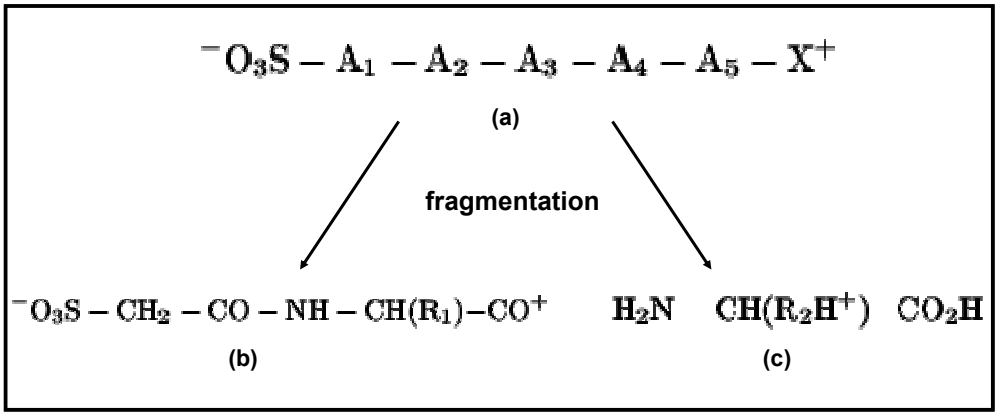


Figure 1.21 Schematic diagram illustrating sulfonation facilitates protonation of amide bonds of the protein backbone in PSD MALDI of tryptic peptides. (a) Tryptic peptides containing an N-terminal sulfonic acid after sulfonation, where A_i are amino acid residues and X are Lys or Arg residue. The strong acid group at the N terminus will be deprotonated, exactly counterbalancing the C-terminal positive charge. The major products after fragmentation reactions have structures like **(**b) and (**c)**, which would be produced by fragmentation of a derivatized tryptic dipeptide (R_2 is the basic side chain of a Lys or Arg residue). Fragments containing the N terminus will be suppressed in the positive ion mode because the negative charge on the sulfonate group neutralizes the C-terminal positive charge in **(**b). A single series of y-type ions (**c**) should be enhanced because they contain the protonated C terminus. [Adopted and modified from (Keough et al., 1999)]

Recently, another effective sulfonation procedure was reported using 4-sulfophenyl isothiocyanate (SPITC) as the derivatization reagent (Gevaert et al., 2001; Marekov and Steinert, 2003; Wang et al., 2004). Wang and his collaborators (2004) have improved the operation procedures and increased sensitivity on the derivatized peptide. The advantage of this method is that SPITC is much less expensive (about $16 per gram with less than 1 mg required for one reaction) and it is relatively stable. Our laboratory found this method is simple and fast in obtaining *de novo* peptide sequences of dinoflagellate proteins isolated by 2-DE gels. Figure 1.22 summarized the procedures used to obtain *de novo* amino acid sequences for novel peptides/proteins isolated from dinoflagellates. Briefly, after gel plugs containing the spots of interest were excised (visualized by protein stains such as Coomassie Brilliant blue), it was washed and dried. Trypsin digestion was performed on the protein inside the gel plug before dividing the tryptic digested mixture into two portions. One fraction was analyzed by MALDI-TOF MS to yield a peptide mass fingerprint (PMF) spectrum. This PMF spectrum can be used for identification purposes through bioinformatic searches in the relevant genome databases. If the PMF spectrum is not enough for identification of the novel protein, the other portion of the tryptic digest will be subjected to SPITC sulfonation as described (Wang et al., 2004). After cleaning-up with zip-tips, the sulfonated tryptic peptide mixtures were subjected to another MALDI-TOF MS analysis. A PMF spectrum of these sulfonated peptides was obtained (from the analysis of this second fraction). When compared to the PMF spectrum obtained with the non-sulfonated portion (from the first fraction), the sulfonated peptide was identified as the same peptide will have a mass difference of 215 Da apart in between the two spectra. This 215 Da represents the mass of SPITC.

Subsequently, a MALDI-TOF PSD will be performed on this particular SPITC tagged peptide. The *de novo* amino acids sequences would be easily deduced by the mass distance between adjacent y ions in the spectrum. Therefore, this sulfonation technique performed with MALDI-TOF PSD analysis provides a fast and simple way to obtain *de novo* peptide/protein sequences of dinoflagellate proteins.

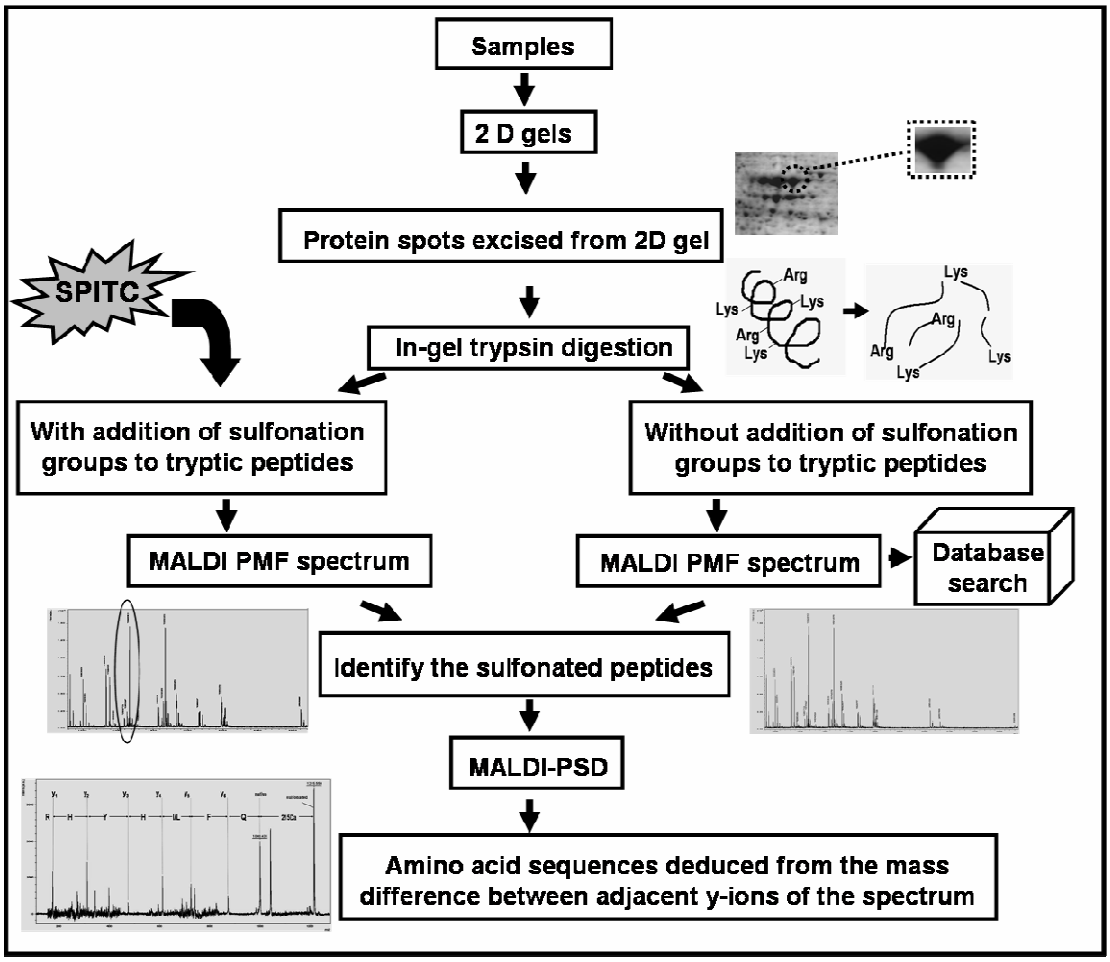


Figure 1.22 Schematic diagram illustrating the procedures used to obtain *de novo* peptide sequences for novel proteins by sulfonation.

After some peptide amino acid sequences were obtained, the next question to ask is how to get the entire amino acid sequences of the protein. Peptide sequences obtained earlier could provide the raw information required for determining the overall protein sequence. Further, a protein can be digested with different specific proteases to yield different set of peptide fragments. Sequence coverage of this protein will be increased when different types of proteases are used to generate different sets of peptides. By getting sequences of these peptide fragments, these sequences can be stitched together and extended. However, a complete protein sequence can only be obtained by this method if the peptides coverage is very close to 100%. Completeness of the coverage can be checked by a MALDI measurement of the overall protein mass. On the other hand, we can determine the complete protein sequence even if only limited or partial peptide sequence information is available. It is because a single peptide sequence (if it is long enough with a minimum of 7 amino acids) allows the construction of a short oligonucleotide probe. This probe can be used for isolation of the gene that encodes this target protein. However, success of this approach is limited when the peptide sequence at hand is not long enough. A long peptide sequence will allow us to have a higher chance of success as we can design primers that have low degeneracy (i.e amino acids encoded by one or two base triplets).

1.5 Aims and objectives

The study was divided into two main parts. The first aim was to find and investigate differentially expressed proteins in the model dinoflagellate *Alexandrium affine* under nitrogen stress. By investigating protein expression profiles in response to nitrogen availability, we may understand more about the mechanism of the unusual

growth behavior of this organism. This part of study was further subdivided into several sections. Firstly, background information including growth curve, generation time, identities, of a local species of *Alexandrium affine* was determined. The effects of different nitrogen sources and different concentrations on the cell growth were also measured. Data generated was used to establish nitrogen stress model for subsequent proteomic analysis. Secondary, different sample preparation methodologies were investigated in order to establish a fast and effective method for producing a high-quality 2-DE for subsequent protein expression profiling study. Thirdly, cells under nitrogen- depleted and repleted conditions were subjected to 2-DE analysis in order to find the differentially expressed proteins. The differentially expressed proteins would be identified by MALDI-TOF MS. Lastly, proteins related to nitrogen stress and with drastically changes in the protein expression (more than 10-fold) were chosen for further analysis. Expression changes of the proteins in relation to nitrogen stress were further validated by using immunoblotting analysis in different nitrogen conditions and in different time points.

For the second part of this thesis, it was aimed to identify and differentiate different dinoflagellates with their proteins profiles by MALDI-TOF MS. Different dinoflagellate species were first identified by analysis on their ribosomal gene sequences before analysis with MALDI-TOF MS. In order to yield a good mass spectrum for subsequent protein profile analysis, various sample preparation methodologies were tested and optimized. In addition, to test the validity of the protein-profiling based identification method, the sensitivity, specificity and reproducibility of the method were investigated. Finally, the capability of the method for differentiating different species in mixed cultures was determined.

CHAPTER II

Effects of different nitrogen sources on the growth of dinoflagellates

2.1 Introduction

There is an increasing evidence that a global increase in nutrient levels of coastal waters through riverine and sewage inputs (Hodgkiss and Ho, 1997; Middelburg and Nieuwenhuize, 2000); atmospheric deposition (Howarth et al., 1996) and bottom layer inputs (Mccarthy and Kamykows.D, 1972) are the major causes of algal blooming. Although it is still not sure the extent to which the increase in HABs can be attributed to the increase of nutrient levels, there is a strong relationship exists between algal blooming and the nitrogen load of coastal waters. For example, a 5- fold increased in dissolved nitrate levels between 1978 and 1985 coincided with the increased in HAB incidences from 2 cases in 1978 to 17 cases in 1984 (Hodgkiss and Chan, 1983; Hodgkiss and Chan, 1986; Hodgkiss and Ho, 1997).

Nitrogen is believed to be an important factor in the initiation and maintenance of phytoplankton blooms (Paerl, 1997; Smayda, 1990). For example, the outbreaks of high density *cochlodinium polykrikoides* blooms in the coastal seawaters in Korea were found to be resulted from rainfall – initiated inflows of high levels of nitrate (Lee, 2006). Nitrogen in both organic and inorganic forms is generally available for phytoplankton from either endogenous or exogenous N sources. Recent studies had shown that the blooms from dinoflagellatess tend to associate with high N concentrations and particularly the reduced forms such as ammonium and urea (Glibert et al., 2001; Lomas et al., 2001). Smayda (1989) pointed out that there was a general consensus that phytoplankton growth in the sea is often nutrient limited (Smayda, 1989). Thus, nitrogen addition tends to relax nutrient limitation and causing

"blooms" of the cells. High concentrations of N are usually found in discharge areas such as bays and estuaries (Matsuoka, 1999). When dinoflagellates exposed to this high concentration of N, some physiological changes such as growth and cellular metabolism can be expected to accelerate in these cells. Taken together, the amount and form of N supply are regarded as an important factor for regulating the growth and cellular metabolism in dinoflagellate cells.

Undoubtedly, there were many physiological studies that offered valuable insights into the potential of dinoflagellates for utilizing various nitrogen sources and their corresponding physiological effects. However, information on the proteome of HAB organisms in response to nitrogen depletion/repletion is very limited. To the best of our knowledge, there is no report at the proteome level upon the effects of nitrogen availability in HAB causative organisms. As mentioned, one of the main goals of this Ph.D. project here is try to identify and investigate the proteins that are related to the changes of nitrogen availability status of dinoflagellates. However, it is important to understand the effects of nitrogen sources on the growth physiology of *Alexandrium affine*, the model of study, prior to subsequent proteome studies. It should be stressed that this *Alexandrium spp*. was selected as a studying model based simply because of its fast growth rate. Identity of the selected strain was confirmed through analysis of the ribosomal genes. Growth phases and generation time of the species were also determined. After establishing background information of the selected strain, the effects of different nitrogen sources and nitrogen availability on the growth of these cells were investigated. All these data gathered are important as they served as a foundation for the establishment of a nitrogen-depletion /repletion platform for subsequent proteomic analysis.

2.2 Materials and methods

2.2.1 Origin of the strains available

Unialgal cultures of *Alexandrium spp.* were kindly donated by Prof. John I. Hodgkiss previously of The University of Hong Kong. Totally 9 local strains of dinoflagellate *Alexandrium spp.* were obtained and they were all isolated in Junk Bay, Hong Kong. Time of collection of these 9 strains are listed in Table 2.1..

Table 2.1 Harvesting time of 9 *Alexandrium* strains that were isolated from the Hong Kong seawaters.

Strains of *Alexandrium spp.* used	Sample day
1	1997 November
2	1997 December
3	1998 March
4	1998 March
5	1998 August
6	1998 August
7	1998 October
8	1998 August
9	1998 August

2.2.2 Seawater and medium

Synthetic seawater based K medium was used for culturing the dinoflagellates (Keller et al., 1987). Synthetic seawater (Table 2.2) (Instant Ocean, USA) was prepared with ddH_2O at a salinity of 25 parts per thousand (ppt) (salinity was checked routinely with refractrometer) and stored at 4 ℃ before used. All synthetic seawater was filtered with 0.45 μm nylon membrane (Millipore, USA) and autoclaved in Teflon culture bottle, prior to subculture. Nutrients required to make up K medium (Keller et al., 1987) (Table 2.3) nutrients were added to the autoclaved synthetic seawater aseptically. Unless stated or otherwise, all chemicals were obtained from Sigma (USA).

2.2.3 Cultivation and maintenance of *Alexandrium spp.*

Stock cultures of all dinoflagellates were kept at exponential growth phase by transferring to new medium every five or six days in a ratio of 1:10 v/v. Vegetative cells from cultures in mid- or late-exponential phase of growth were inoculated into freshly prepared culture medium. Possible contamination of algal culture was monitored by regular microscopic examination. The cultures were grown at 22℃ under a 16:8 hours light:dark cycle and at a light intensity of 120 μE Lux $m^{-1}s^{-1}$ provided by cool white fluorescent tubes in a Conviron growth chamber (Model EF7, USA).

2.2.4 Cell counts

Cell density was counted at the same time daily. Briefly, 1 ml of each culture was taken and fixed with 10 μl Lugol's solution and counted under light microscope with a Sedgwick-Rafter cell counter.

Table 2.2 Composition of Instant Ocean Sea salts

Ion	Instant Ocean^ (ppm)	Seawater* (ppm)
Chloride	19,290	19,353
Sodium	10,780	10,781
Sulfate	2,660	2,712
Magnesium	1,320	1,284
Potassium	420	399
Calcium	400	412
Carbonate/bicarbonate	200	126
Bromide	56	67
Strontium	8.8	7.9
Boron	5.6	4.5
Fluoride	1.0	1.28
Lithium	0.3	0.173
Iodide	0.24	0.06
Barium	less than 0.04	0.014
Iron	less than 0.04	less than 0.001
Manganese	less than 0.025	less than 0.001
Chromium	less than 0.015	less than 0.001
Cobalt	less than 0.015	less than 0.001
Copper	less than 0.015	less than 0.001
Nickel	less than 0.015	less than 0.001
Selenium	less than 0.015	less than 0.001
Vanadium	less than 0.015	less than 0.002
Zinc	less than 0.015	less than 0.001
Molybdenum	less than 0.01	0.01
Aluminum	less than 0.006	less than 0.001
Lead	less than 0.005	less than 0.001
Arsenic	less than 0.004	0.002
Cadmium	less than 0.002	less than 0.001
Nitrate	None	1.8
Phosphate	None	0.2

^ Data for synthetic seawater provided from Instant Ocean (USA)
* Data for seawater values were adopted from (Pilson, 1998)

Table 2.3 Compositions of K- medium [Adopted from (Keller et al., 1987)]

Nutrients	Final Concentration (M)
Tris (pH7.2)	$1x10^{-3}$
$NaNO_3$	$8.83x10^{-4}$
Na-glycerophosphate	$1x10^{-5}$
NH_4Cl	$5x10^{-5}$
H_2SeO_3	$1x10^{-8}$
NaEDTA	$1x10^{-4}$
$FeCl_3.6H_2O$	$1.17x10^{-5}$
$CuSO_4.5H_2O$	$4x10^{-8}$
$ZnSO_4.7H_2O$	$8x10^{-8}$
$CoCl_2.6H_2O$	$5x10^{-8}$
$MnCl_2.4H_2O$	$9x10^{-7}$
$NaMoO_4.2H_2O$	$3x10^{-8}$
Biotin	$2.1x10^{-9}$
Vitamin B_{12}	$3.7x10^{-10}$
Thiamine	$3x10^{-7}$

2.2.5 Selection of dinoflagellate *Alexandrium* strain as a study model

All 9 strains of *Alexandrium* were grown to 8000 cell ml^{-1} and 10 ml culture was inoculated to 250 ml culture flask in 90 ml medium. All cultures were grown in conditions mentioned above (see section 2.2.3). Cell density was counted at the same time at 2-3 days intervals; 1 ml of each culture was taken and fixed with 10 μl Lugol's solution and counted under light microscope with a Sedgwick-Rafter counter. The fastest growing strain was selected by comparing the growth rate of all 9 strains.

2.2.6 PCR-based identification

Selected model strain was identified by the analysis of the ribosomal gene sequences. Fifty ml cells were collected by centrifugation (1500 x g for 10 mins at room temperature) from mid-exponential culture and frozen in liquid nitrogen prior to mechanical cell disruption and DNA extraction with a kit (Roche, Switzerland). ITS regions containing the 5.8S rDNA were amplified from the extracted genomic DNA

using ITSA and ITSB primers (Adachi et al., 1994; Adachi et al., 1996). PCR was performed under conditions: 95℃ 5 min; 35 cycles of 94℃ 45 s, 50℃ 45 s and 72℃ 1 min; 72℃ 10 min. PCR products were cloned into pGEM-T easy vectors (Promega, USA) prior to DNA sequencing. DNA sequencing of all cloned plasmids were performed by commercial facilities using traditional dideoxy-methodolgy.

2.2.7 Monitoring of growth phases and generation time

Growth phases and generation time can be determined and calculated from the growth curve of the model strain. 10 ml cells in late–log phase (with 8000 cells ml^{-1}) were inoculated to 90 ml of medium in triplicate. Cell density was counted at the same time everyday. Growth rate can be calculated from the exponential growth phase of the corresponding growth curve. The experiment was repeated 3 times, with triplicate in each batch of experiment. The growth curve was plotted by cell density against days and each data point is the mean of the results from 3 batches of experiments.

In general,

Growth rate constant (K) = $(\log N_1 - \log N_0)/(t_1 - t_0)$ $(\log 2)$

Where, N_0 and N_1 indicate the cell numbers at time t_0 and t_1 respectively.

And,

Generation time= 1/(growth rate constant)

2.2.8 Effects of different nitrogen sources on the growth of selected strain

Approximately 10^6 cells were harvested from mid-log phase culture by centrifugation (1500 x g for 10 mins at room temperature). Harvested cells were washed twice with sterile synthetic seawater. Cells were equally inoculated into six lots of 100 ml K-medium with different nitrogen sources (Urea, L-aspartate, glycine, ammonium or nitrate) added at different concentrations (5 μM-N, 10 μM-N, 20 μM-N, 40 μM-N, 100 μM-N, 200 μM-N and 400 μM-N), and each concentration was performed in triplicates. The initial cell density of each 100 ml cells was in 500 cell ml^{-1} and it was checked by cell counting. The whole experiment was repeated for three times. All cultures were grown for 20 days and cells from each culture was count under microscope at the same time everyday. Each data point on the curve represents the mean of triplicate results in one single run. Representative results from the 3 batches of experiments were shown in the results section below.

2.2.9 Effects of nitrogen stress on the growth of selected strain

2.2.9.1 Nitrogen depletion

Around 10^5 cells were harvested from mid-log phase culture by centrifugation (1500 x g for 10 mins at room temperature). Harvested cells were washed twice with sterile synthetic seawater and inoculated into 100 ml K-medium (without any nitrogen sources). The initial cell density of each 100 ml cells was in 500 cell ml^{-1} and it was checked by cell counting. The growth was monitored by cell count at the same time every day. The whole experiment was repeated three times, with triplicates in each run. Each data point on the curve represents the mean of triplicate results in one single run. Representative results from the 3 batches of experiments were shown in the results

section below.

2.2.9.2 Nitrogen repletion

The procedures in 2.2.9.1 were repeated, but different nitrogen sources including Urea (200 μM-N), L-aspartate (10 μM-N), glycine (10 μM-N), ammonium (40 μM-N) and nitrate (200 μM-N) (in the optimal concentration) were added at day 3, day 8 and day 13 after the cells were inoculated into 100 ml K-medium that had no nitrogen sources added. The growth was monitored by cell counts at the same time every day. The whole experiment was repeated three times, with triplicate in each run. Each data point on the curve represents the mean of triplicate results in one single run. Representative results from the 3 batches of experiments were shown in the Results section below.

2.2.9.3 Nitrate measurement

Around 10^6 cells were harvested from mid-log phase culture by centrifugation (1500 x g for 10min at room temperature). Harvested cells were washed twice with sterile synthetic seawater and inoculated into 100 ml K-medium with or without nitrogen sources added. All cultures (with and without nitrogen) were grown and harvested at various time points (0 hour, 24 hour, 48 hour, 72 hour and 96 hour). The cell-free medium was subjected to nitrate measurement. Nitrate was measured by Nitrate Test Kit (NECi, USA) and all procedures were performed according to instruction manual of the kit. Briefly, supernatant from the cell suspensions were removed. Fifty μl of medium samples were added to 950 μl assay buffer (25 mM KH_2PO_4, 0.025 mM EDTA; pH7.5). Fifty of 1mM NADH and 20 μl of 1 unit nitrate

reductase were added to the mixture. The mixture was mixed and allowed to stand for 20 minutes at room temperature. Five hundred μl of color reagent 1 (1% sulfanilamide in 3N HCl) and color reagent 2 (0.02% N-Naphthylethylenediamine) were added. Samples were allowed to stand at room temperature for 10 mins and the absorbance was measured at 540 nm.

Nitrite (NO_2) concentration was measured. All steps were repeated by omitting nitrate reductase and NADH from the samples. The amount of nitrate was expressed as mg per 10^6 cells in the culture. The whole experiment was repeated three times, with triplicate in each run. Each data point on the curve represents the mean of triplicate results in one single run. Representative results from the 3 batches of experiments were shown in the result section below.

2.2.9.4 Activity assay of nitrate reductase (NaR)

Around 10^6 cells were harvested from mid-log phase culture by centrifugation (1500 x g for 10 mins at room temperature). Harvested cells were washed twice with sterile synthetic seawater and inoculated into 100 ml K-medium (with or without nitrogen sources). All cultures (with and without nitrogen) were grown and harvested at various time points (0 hour, 48 hour and 96 hour). NaR activity was measured by NaR Activity Assay Kit (NECi, USA) and all procedures were performed according to instruction manual of the kit. Briefly, cell pellets were resuspended in 200 μl extraction buffer (0.1 M potassium phosphate, 1 mM EDTA, pH 7.5) with 1% PVPP (g/v), 0.08% L-Cysteine (g/v) and 10 μl/ml protease inhibitor cocktails (Sigma, USA) were added. Cells were lysed by sonication (a total of 3 mins with short pulses of 5-10 seconds each) on ice. Lysis of cells was confirmed using light microscope.

Supernatant from these cell suspensions were removed and subjected to the assay. One hundred μl of samples were added to 1.8 ml assay buffer (25 mM KH_2PO_4, 0.025 mM EDTA; pH 7.5) containing 10 mM nitrate. One hundred μl of 2 mM NADH was added to the mixture. The mixture was mixed and time was recorded at 30℃. Reaction was stopped by adding 100 μl 1 M zinc acetate at time 20 mins, 40 mins and 60 mins. Reaction mixtures with the same ingredients as above but omitting NADH was used as blank. Five hundred μl of color reagent 1 (1% sulfanilamide in 3N HCl) and color reagent 2 (0.02% N-Naphthylethylenediamine) were added. Samples were allowed to stand at room temperature for 10 mins and the absorbance was measured at 540 nm. Background signals were also measured. One hundred μl samples were added to 3 ml assay buffer and measured at 540 nm. The net absorbance of cell samples at 540 nm equals to the absorbance from NaR measurement minus the background absorbance from the cellular materials per se. The final absorbance equals to the average of (net A540 nm from 60 mins – net A540 nm from 40 mins) and (net A540 nm from 40 mins – net A540 nm from 20 mins). Activity of NaR was expressed as nmole of nitrite produced per hour per gram of cell (wet weight). The whole experiment was repeated three times, with duplicate in each run. Each data point on bar chat represents the mean of results from the 3 batches of experiments.

2.3 Results and discussion

2.3.1 Selection of dinoflagellate *Alexandrium* strain as a study model

The *Alexandrium* genus has been known to produce potent neurotoxins which cause paralytic shellfish poisoning (PSP). It is a very common HAB causative agent in many coastal regions around the world (Hallegraeff, 1993; Hallegraeff, 2003). Therefore, this dinoflagellate genus was chosen as the model to study in the project. Nine local *Alexandrium* strains were provided by Prof. John I. Hodgkiss previously of The University of Hong Kong and one of them would be selected as the studying model. The selection was based on two criteria, the selected strain should be fast-growing and with at least 10000 cell ml^{-1} cell density in their maximum cell capacity. Table 2.4 shows the growth rate and maximum cell capacity of all nine strains. Strain no.9 was the fastest-growing strain among all the strains. The growth rate of this strain was 0.92 divisions per day, while others were mostly in between 0.6-0.8 divisions per day. Although the growth rate of strain no.7 (about 0.9 divisions per day) was comparable to that of strain no.9, the maximum cell density of strain no.9 (12000 cell ml^{-1}) was much greatly than that of strain no.7 (7500 cell ml^{-1}). Thus, strain no.9 was chosen as the model to study for subsequent investigations. The high growth rate and cell density of this strain attained would greatly facilitate success of the project. For example, more cells for harvest from a shorter period of grow would allow sufficient amount of samples for performing 2-DE.

Table 2.4 Growth rate and generation time of different *Alexandrium spp.* Strain no.9 was chosen as the model to study (bold)

Strains	Growth rate constant (K) (generation day^{-1})	Generation time (g) (day)	Maximum Capacity (cell ml^{-1})
1	0.80	1.25	10000
2	0.62	1.61	9500
3	0.85	1.17	8500
4	0.76	1.31	7000
5	0.76	1.32	9000
6	0.84	1.19	8500
7	0.90	1.11	7500
8	0.63	1.60	8000
9	**0.92**	**1.07**	**12000**

2.3.2 PCR-based identification of selected *Alexandrium* strain

From the information provided by Prof. John I. Hodgkiss, the selected strain was previously identified as *Alexandrium tamarense* based on morphological characteristics. The genus *Alexandrium*, which consists more than 20 species (Yuki and Fukuyo, 1992) and they share highly similar morphological details. Figure 2.1 shows the light microscopic picture of the selected *Alexandrium* strain and this is a typical outlook of *Alexandrium spp.* However, biochemical identifications had never been done on these strains before!

Figure 2.1 Light microscopic picture of the selected strain-*Alexandrium affine*

In order to clarify the identity of the selected strain, sequences of the internal transcribed spacer (ITS) rDNA of the strain were analyzed. The ITS regions were selected because the regions diverge rapidly during speciation and it was believed to serve as a unique species-specific "DNA barcode" for species identification purposes (Litaker et al., 2007). This region has been successfully used to identify a number of HAB species (Adachi et al., 1996; Hudson and Adlard, 1996). The ribosomal gene sequence of the region ITS1-5.8S-ITS2 of the selected strain was found matched exactly with two *Alexandrium affine* species (AC1 and AS1) and highly similar to other four *Alexandrium affine* species (H1, IEO-PA4V, IEO-PA8V and CCMP112) from the NCBI database (Figure 2.2). The entire ITS region of the selected strain contains 525 base pairs and the sequence was submitted to GenBank of NCBI with accession number of EF579793 (Appendix V).

These results strongly suggested that this selected strain is *Alexandrium affine* rather than *Alexandrium tamarense* as we were told! Therefore, it reaffirmed the common notion that closely-related and looking-alike species like *Alexandrium* are difficult to be identified solely by their morphological features. Therefore, other identification methods are needed for an accurate and fast HABs species identification purposes. A novel method for HAB species identification by protein profiling using MALDI-TOF MS would be discussed in more detail in the second part of the project, Chapter VI.

It should also be stressed that the ITS sequence of the selected strain matched exactly as the two *Alexandrium affine* strains, AC-1 (accession no: DQ176665) and AS-1 (accession no: DQ176664). This suggests that these dinoflagellates may actually be the same strain. AC-1 and AS-1 were both isolated from China by the

same group of authors (Tang X, Yu R, Yan T, Wang Y and Zhou M), while the selected strain was isolated locally in Hong Kong water. It is often not surprising that same strain is found from different locations of the world. As mentioned, HAB organisms can be carried from one place to another though ballast water and live shellfish stocks translocation (Hallegraeff, 2003; Hallegraeff and Bolch, 1992). As in the case of HAB occurred in Australia in 1980s, the causative agent *Alexandrium catenella* was found to be the same strain as another one found in Japan. In both places, genetic fingerprinting using ribosomal gene sequencing provided circumstantial evidences for the identities between these strains (Scholin et al., 1995).

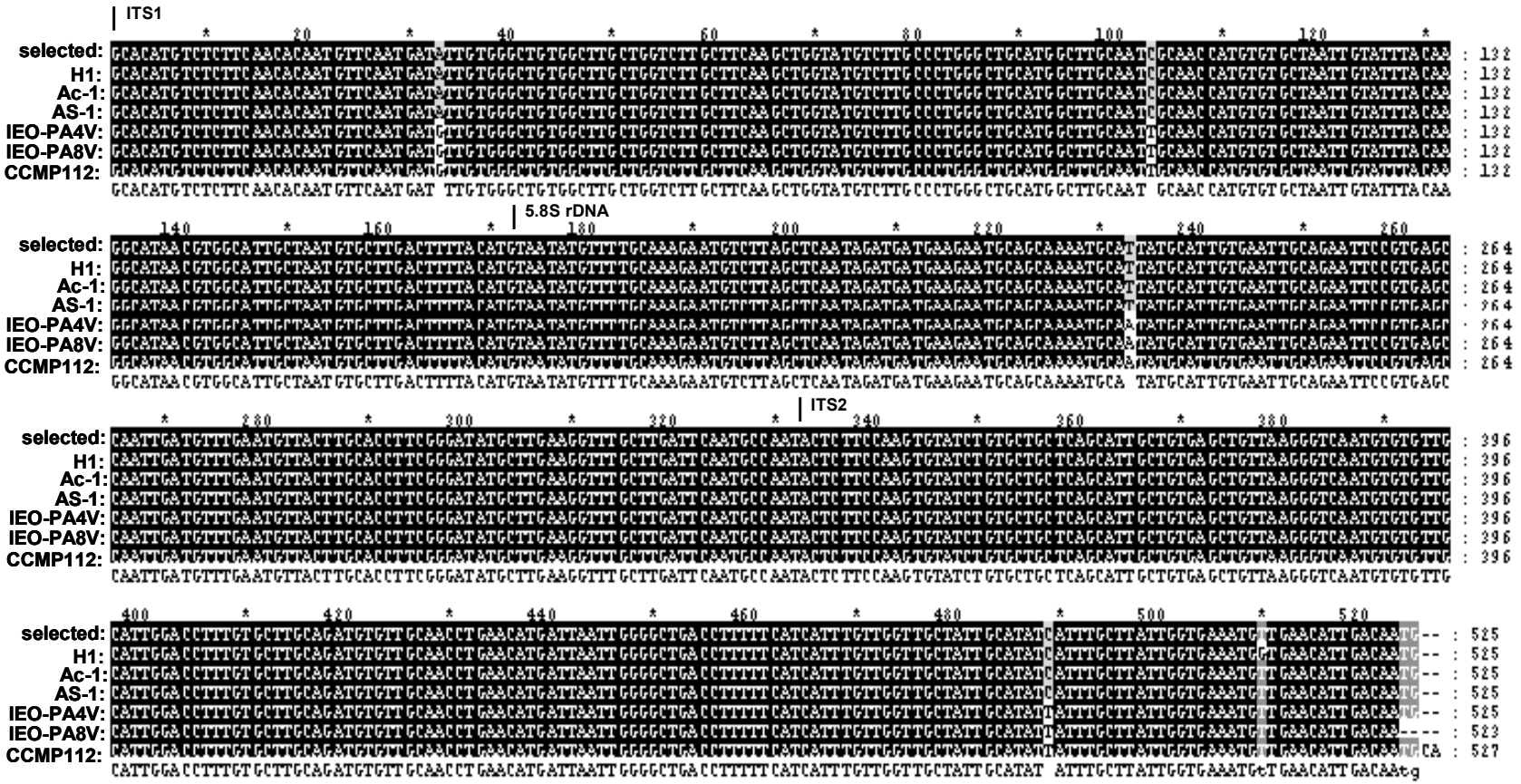


Figure 2.2 Complete sequence of ITS 1, 5.8S and ITS2 of selected strain and aligned with other stains of *Alexandrium affine*. The sequences region of ITS1 (1-173bp), 5.8s (174-333bp) and ITS 2 (334-525bp) are shown. Entire ITS sequences of the selected strain (EF579793) is aligned with other *A.affine* strains including H1(AB006995), AC-1(DQ176665), AS-1(DQ176664), IEO-PA4V (AJ632094), IEO-PA8V (AJ632095) and CCMP112(AY831409). Sequence of selected strain matched exactly as that of AC-1 and AS-1; and only with one to few nucleotide differences to the others.

2.3.3 Growth cycle

Growth rates shown in Table 2.1 were measured in every 2-3 days for model strain selection purposes. However, in order to determine different distinctive growth phases of the selected *A.affine* strain, a comprehensive growth curve of the selected strain was measured by cell counting every day (Figure 2.3). The growth pattern of cells grown in batch cultures gave a typical growth curve with three distinct phases. As indicated in Figure 2.3, inoculated cells require 1 day for adaptation (lag phase) prior entering the log phase for exponential growth from day 2 to day 6. Cells reached a maximum cell capacity of around 12000 cell ml^{-1} on day 6 or 7. Since nutrients were becoming limiting, the cells would entered to stationary phase (where rate of division = rate of death) beyond day 7. The cells would finally in death phase from day 10 onwards (where rate of death > rate of division) because of the decline of nutrients.

In this part of the experiment, the specific growth rates derived during culturing was around 0.94 day^{-1} which was similar to that calculated (0.92 day^{-1}) in Table 2.1. In the literature, there was only a few studies that had been conducted on *Alexandrium affine*. Flynn et al. (1996) determined the effects of low nitrogen and low phosphate medium on its growth and physiology and they found that the growth rate of cells grown in low nitrogen is around 0.5 day^{-1} (Flynn et al., 1996). Another study reported the growth rates of *A. affine* as 0.25-0.34 day^{-1} at 20-30℃ (Band-Schmidt et al., 2003). These results indicated that growth rates, in response to different environment, vary among strains and isolates. Variability in growth had been shown to exist in *Alexandrium* species under different environmental factors (Hwang and Lu, 2000; John and Flynn, 2000). The large differences in the rates observed could be attributed to the variations of growing conditions. In addition, the effects of flask swirling might

have biased these results, as turbulence was known to affect the growth rate of some dinoflagellates (Sullivan and Swift, 2003). Overall, by reference to the literature, growth rates of *Alexandrium* species normally range from 0.1 to 0.9 day^{-1}. Therefore, the selected *A .affine* stain of the present study exhibited a relatively higher growth rate (0.9 day^{-1}) when compared to most of the other strains in the genus.

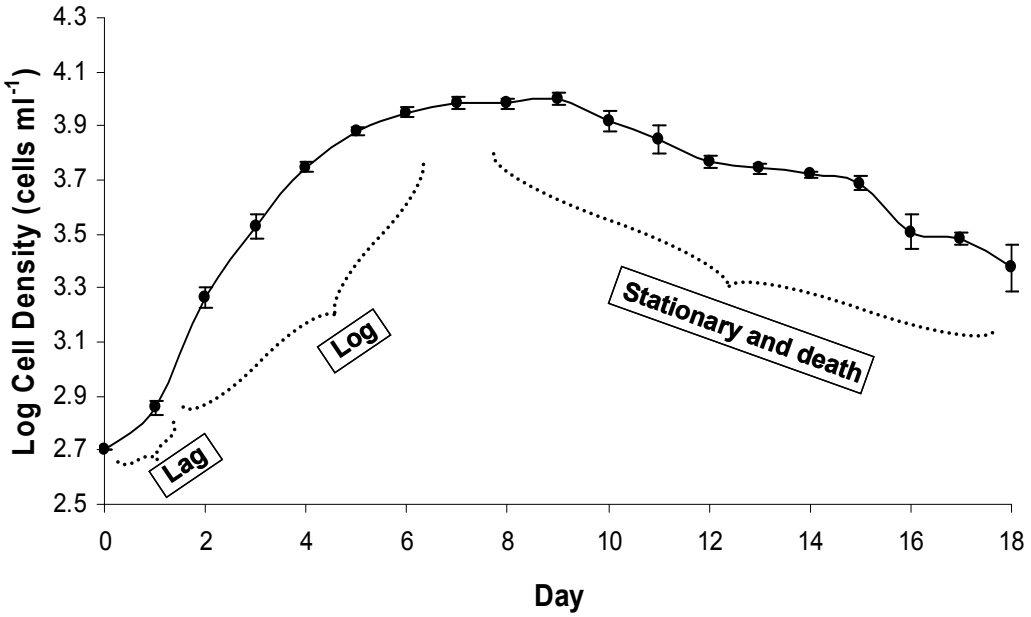


Figure 2.3 Growth curve of the selected strain (*Alexandrium affine*). Different growth phases are indicated. Lag phase (day 0-day 1), Log phase (day 2-day 6) and stationary/death phase (beyond day 7). (Bars indicates $\pm$ S.D)

2.3.4 Effects of different nitrogen sources on the growth of *Alexandrium affine*

Recent studies showed that dinoflagellates blooms tend to associate with high N concentration particularly with the reduced forms of N sources such as ammonium and urea. High concentrations of N are increasingly found in discharge areas such as bays, coastal estuaries and at the bottom layer of water column (Glibert et al., 2001; Lomas et al., 2001). In addition, atmospheric N input is also considered as an important source for rapid growth of phytoplankton (Peierls and Paerl, 1997). Recently, there are considerable evidences that show various forms of nitrogen such

as organic nitrogen (e.g amino acid), reduced nitrogen (e.g urea), in addition to nitrate and ammonium, can support fair to good growth of dinoflagellates (John and Flynn, 1999; Leong and Taguchi, 2004). Undoubtedly, these physiological studies offer a valuable insight into the potentials of HABs causative organism in utilization of various nitrogen sources. However, a comprehensive comparison of the data is difficult due to methodological differences among experiments, the influence of growth stages, and clone-specific responses of various *Alexandrium* strains in responses to different environmental conditions. Given that the selected *A. affine* strain has a relatively higher growth rate when compared to most of others reported in the literature and hence indicative of variations in physiological machineries, the responses to different nitrogen sources and concentrations on its growth were studied.

Different nitrogen sources including nitrate, ammonium, urea, glycine and aspartate at various concentrations were used in batch culture experiments as the sole N source for investigating the growth of *A. affine* (Figure 2.4). Results indicated that *A. affine* has the ability to assimilate different kind of nitrogen sources for cell growth. However, when these cells were exposed to different N sources with various concentrations, some physiological alterations such as growth rate and maximum cell capacity could be seen.

Growth rates observed in the three independent batch culture experiments were highly similar for all N sources with all concentrations tested (Figure 2.4) (data for experimental runs 2 and 3 not shown). With nitrate as the sole N source (Figure 2.4a), cell abundance and growth rates of *A. affine* increased with increasing concentrations and the maximum growth rate of 0.9 day^{-1} was found at concentration of 200 μM-N (Figure 2.5), at which a maximum cell density of 12000 cells ml^{-1} was attained

(Figure 2.4a). However, both cell density and growth rate were greatly reduced to 9500 cells ml^{-1} and 0.5 day^{-1} respectively, with nitrate concentration 400 μM-N as the sole N source. In ammonium-enriched cultures (Figure 2.4b), cells did not grow at concentrations 5 to 10 μM-N. The cell density and growth rate were maintained at around 2000-3000 cell ml^{-1} and 0.1-0.2 day^{-1} respectively. Cells were found to increase in cell numbers when ammonium concentration reaches 20 μM as the sole N source. The maximum cell density of 7000 cell ml^{-1} and growth rate of 0.69 day^{-1} were found at N concentration 40 μM (Figure 2.5). Nevertheless, cell death occurred when N concentration $\geq$ 100 μM. Cells exposed to increasing urea concentrations showed an increase in both cell abundances and growth rates similar to those observed in nitrate-enriched cultures (Figure 2.4c), with cell abundance of 11000 cell ml^{-1} and growth rate of 0.7 day^{-1} at N concentration of 200 μM (Figure 2.5). However, it should be noted that nitrate-enriched cultures survived better while all urea-enriched cells died with urea concentration of 400 μM. Further, responses of *A. affine* to both inorganic and organic N (organic N-enriched cultures (i.e glycine and aspartate)) were similar (Figure 2.4d and e). Cells showed the best growth rate at N concentration of 10 μM while the maximum growth rate and cell density are around 0.73-0.75 day^{-1} and almost 8000 cell ml^{-1} respectively. Both growth rates and cell abundances were greatly reduced to 0.2 day^{-1} and 2000-3000 cell ml^{-1} respectively at N concentrations at 20 μM and above while and cells death occurred when N concentration was raised beyond 40 μM (Figure 2.5). Table 2.5 summarized the optimal concentrations of various N sources and the corresponding maximum growth rates / cell densities of the growth of *A. affine*.

In this study, the highest maximum growth rate and cell abundance were

obtained in nitrate-enriched cultures, showing that nitrate is the preferred N source for this strain. Dinoflagellates can normally grow well with increasing nitrate concentrations as observed in the present study, even at very high concentrations (i.e 400 μM-N equivalent to ~1800 μM nitrate). For example, some have found *Alexandrium tamarense* grows very well even at nitrate concentrations of more than 800 μM (Parkhill and Cembella, 1999). However, reduced N sources such as ammonium and urea can lead to inhibition of growth when at high concentrations. For example, a study found that growth of *Alexandrium minutum* was inhibited at concentrations > 25 μM-N of ammonium and urea (Chang and McClean, 1997). While the present study showed that ammonium > 40 μM-N inhibited the growth of *A. affine*, and there was no inhibition of growth by urea. Instead, *A.affine* can utilize a wide range of urea concentrations, just similar to that of nitrate (Figure 2.5). Our results are similar to that described by (Leong and Taguchi, 2004). They observed that the growth of *Alexandrium tamarense* was inhibited with ammonium concentrations > 50 μM-N and there was also no urea-inhibitory effects on growth. In the present study, although ammonium becomes toxic when levels were higher than 40 μM-N, cells showed the highest growth rate when concentration of ammonium was at 40 μM-N (Figure 2.5). It indicates that these cells are more likely to grow with ammonium among the N sources at concentrations that are below the toxicity level.

Dissolved free amino acids (DFAAs) are one of the most labile forms of dissolved organic N (DON). Two DFAAs, glycine and aspartate, were used to evaluate effect on the growth of *A.affine*. Similar to that of ammonium-enriched cultures, amino acid-enriched cultures have a relative low tolerance to the amino acid levels (Figure 2.5). A growth inhibition of the cells was observed when amino acid

concentration > 10 μM-N. The optimal growth of *A.affine* was at concentration of 10 μM-N (equivalent to around 50 μM glycine and 80 μM aspartate); at which both the growth rates and maximum cell densities were the highest among the N sources (Figure 2.4). The optimal DFAA concentration found in this study was consistent with other studies with the concentration was generally around 50 μM (John and Flynn, 1999). The use of amino-N as a sole N source for algal growth has long been debated and there are only a few related studies on this issue (Dixon and Syrett, 1988; Flynn and Fielder, 1989; John and Flynn, 1999). It is because the difficulties in ensuring the amino-N are being used directly by the dinoflagellates rather than via bacterial regeneration. DFAAs form a significant proportion of DON in marine waters, ranges from 0 to 5 μM. Therefore, they could provide an additional source of nitrogen for the growth of marine microalgae.

Differences in the results obtained from the present study and that of the literature illustrated that the mechanism of N utilization and tolerance level for each N substrate maybe different between species, even if they belong to the same genus of *Alexandrium*.

On the other hand, it was emphasized that occurrence of algal blooms are highly related to nutrient ratios (i.e N:P ratio) in addition to nutrient loading. Hodgkiss and Ho (1997) estimated that the optimal N:P ratio for growth of most HABs organisms in coastal waters of Hong Kong was between 6 to 15. However, I am unable to determine the equivalent N:P ratios for comparison in the present study as the results were obtained differently. The ratios reported by Hodgkiss and Ho were estimates using data of seawater obtained during the blooms while my data are on nitrogen artificially supplied to the cultures. Dynamic changes of nutrients in the sea are

completely different from that in laboratory cultures.

The ability to take up, adapt and change in growth rates depending on the N sources suggests that N sources may affect the dynamics of dinoflagellate bloom. *A.affine* is able to utilize different forms of N sources supplied (nitrate, ammonium, urea, glycine and aspartate), showing that these N sources may serve as direct N source for growth of *A. affine* in the coastal environment. Therefore, the form of N supply can be regarded as an important factor in regulating growth of *A. affine*. The ability to adapt and acclimate to different N environments of *Alexandrium* is a beneficial strategy for ensuring uninterrupted growth and proliferation. This advantage allows dinoflagellate cells to maintain high growth in the competitive coastal environment.

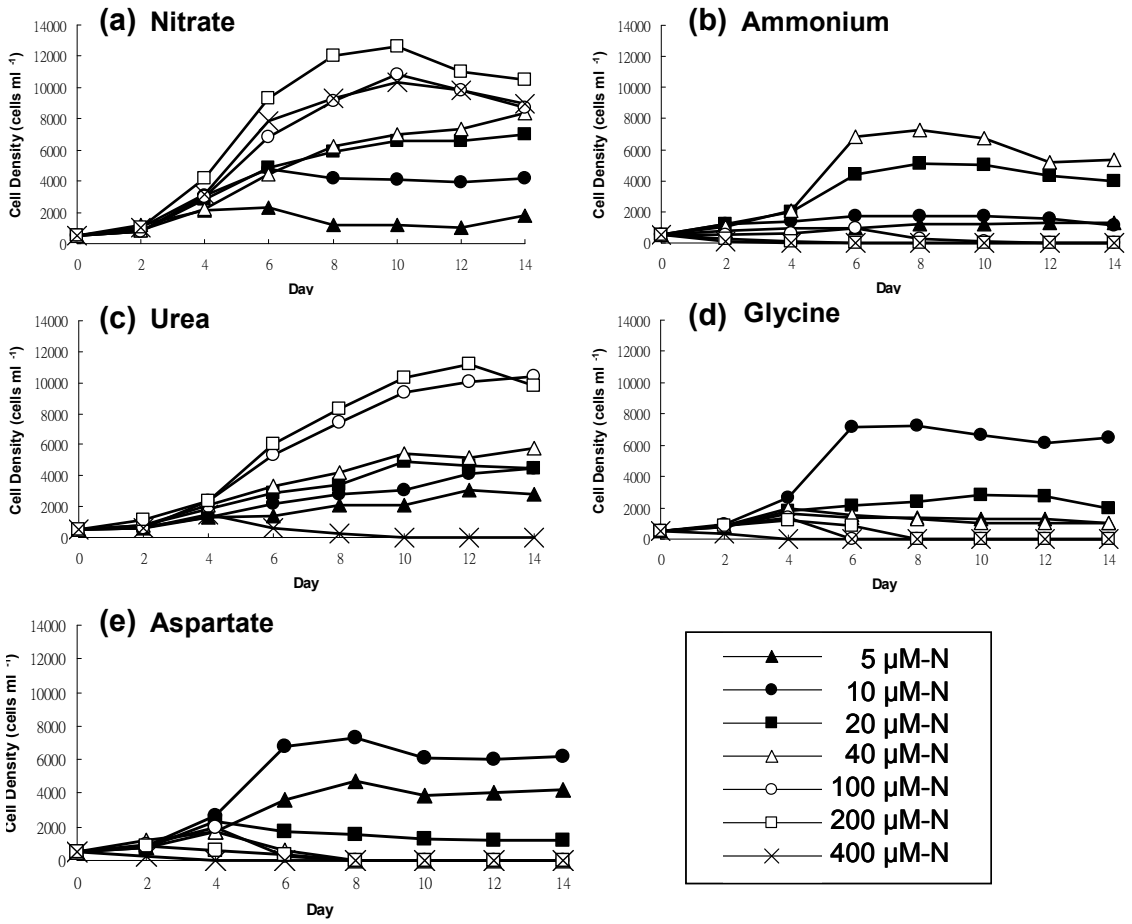


Figure 2.4 Cell growth of *Alexandrium affine* with different nitrogen sources. Cell density is determined during experimental run for (a) nitrate, (b) ammonium, (c) urea, (d) glycine and (e) aspartate; at 5, 10, 20, 40, 100, 200 and 400 µM-N for 14 days. Closed triangle indicates 5 µM-N; closed circle indicates 10 µM-N; closed square indicates 20 µM-N; open triangle indicates 40 µM-N; open circle indicates 100 µM-N; open square indicates 200 µM-N and cross indicates 400µM-N.

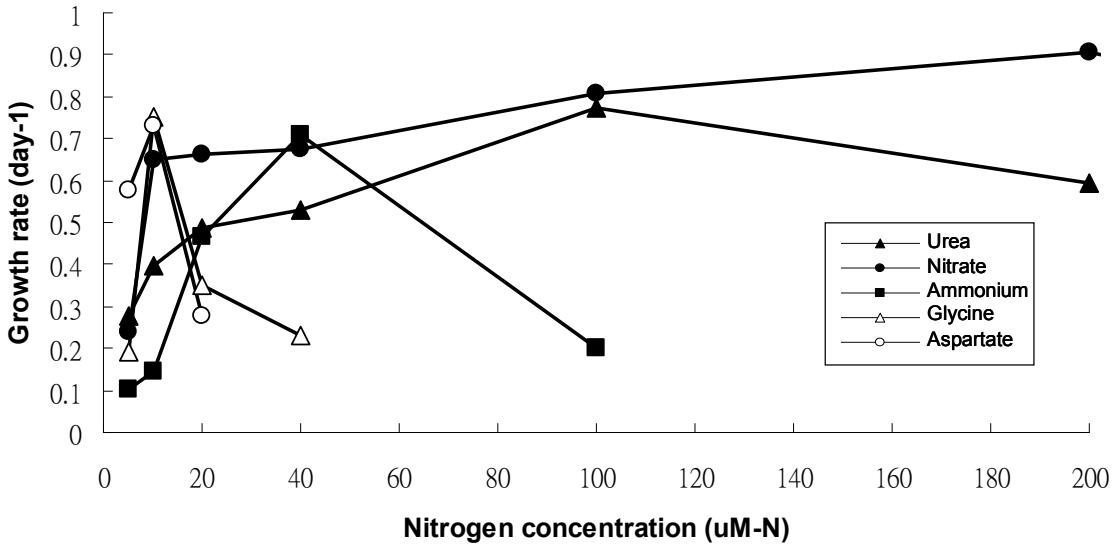


Figure 2.5 Growth rates of *Alexandrium affine* as a function of nitrogen concentrations of various nitrogen sources. Closed circle indicates nitrate; closed square indicates ammonium; closed triangle indicates urea; open triangle indicates glycine and open circle indicates aspartate. Growth rate of 400 μM-N as sole N source is 0.5 day^{-1} and it is not shown in the figure.

Table 2.5 Summary of optimal concentration of different nitrogen sources and the corresponding growth rate and maximum cell density of the growth of *Alexandrium affine*

Nitrogen source	Optimal concentration		Growth rate (day-1)	Maximum cell density (cell ml^{-1})
	μM-N	μM		
Nitrate	200	880	0.9	12000
Ammonium	40	57	0.69	7000
Urea	100	210	0.7	11000
Glycine	10	54	0.75	8000
Aspartate	10	82	0.73	7000

2.3.5 Effects of nitrogen stress on the growth of *A. affine*

2.3.5.1 Nitrogen depletion and repletion

Most marine systems are nitrogen-limited. When nitrogen is added, the phytoplanktons tend to grow or "bloom" until they run out of something else. Therefore, understanding the cellular responses to nitrogen stress and nitrogen replenishment is important to understand the "blooming" mechanism. The present study is aimed to investigate the growth physiology of *A..affine* under nitrogen depletion and repletion conditions.

Growth rates in the three independent batch culture experiments were highly similar for all N sources studied (Figure 2.6) (data for experimental runs 2 and 3 not shown). The nitrogen-dependent growth experiments showed that when nitrogen was depleted, the growth of the dinoflagellate cells was stopped. These cells did maintain a small amount of growth in the first two days even when no nitrogen was added. This was most probably because there were intracellular pools or other N reserves in the organism and these commodities would provide some residual substrates for growth (Boyer et al., 1987). After the first two days, cell growth were started to slow down and maintained at a basal cell density at around 500-700 cell ml^{-1} throughout the entire period of 14 to 18 days. Upon the replenishment of nitrogen to the N-depleted cultures, the growth of cells was resumed. After N was re-added, N-repleted cells would undergo a lag phase of 1 day. The growth patterns, growth rates and the maximum cell density of the N-repleted cultures were highly similar to that of normal cultures. Moreover, cells would resume growth when N was re-added at different time (i.e day 3, day 8 and even day 13) to the N-depleted cultures. Their growth curves were highly similar. These results indicated that N-depleted cells were kept in a

"ready-to-growth" status for long period and growth would be resumed upon N addition independent of the time when N was added back to the system. Furthermore, as expected, the results of the replenishment of various nitrogen sources were similar. The present study not only helps to understand the growth physiology of *A.affine* under nitrogen depletion and repletion but also provide information for choosing particular time points and N-status for subsequent proteomic analysis.

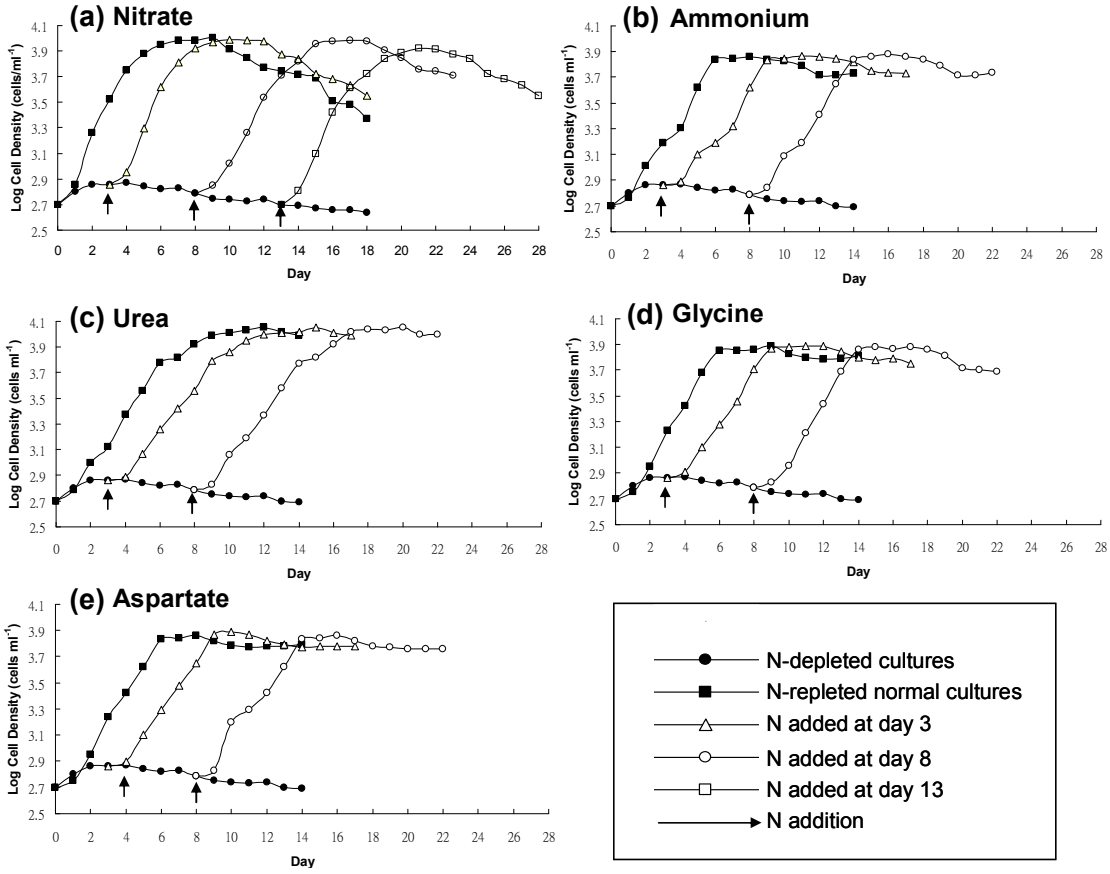


Figure 2.6 The effect of N-depletion and repletion on the growth of *Alexandrium affine*. Different nitrogen source (a) nitrate, (b) ammonium, (c) urea, (d) glycine and (e) aspartate was used or re-added to the N-depleted culture. Closed circle indicates the cell growth under N-depleted condition. Closed square indicates the normal cell growth with nitrogen provided according to the optimal concentration listed in Table 2.2. Open triangle indicates nitrogen was re-added at day 3 to the N-depleted culture. Open circle indicates nitrogen was re-added at day 8 to the N-depleted culture. Open square indicates nitrogen was re-added at day 13 to the N-depleted culture. Arrow indicates the time of N re-addition.

2.3.5.2 Amount of nitrate and the NaR activity

Although growth of *A.affine* under the N-depleted and repleted conditions was measured in this study, nothing is known about the amount of nitrogen uptake in both with or without N cultures. Hence, the following study was trying to understand the amount of N present extracellularly in the with/without N cultures by measuring the amount of nitrate and nitrate reductase (NaR) activity.

Figure 2.7 has shown the amount of nitrate of the culturing medium of the growing *A.affine* cultures with or without nitrogen. Nitrate measured in the three independent batch cultures was highly similar (data for experimental runs 2 and 3 not shown). In the nitrogen-depleted cultures, there was no detectable nitrate in the culturing medium throughout the entire period (i.e 96 hours). For the nitrate-enriched cultures, amount of nitrate in the culturing medium have dropped around 60% after 96 hours. While amount of nitrate in the culturing medium of the nitrate/ ammonium-enriched cultures have only dropped around 40% after 96 hours. The detection limited of the nitrate test is 0.05 ppm nitrate-N (equivalent to 3.6 μM nitrate). The dropped of the amounts of nitrate in the N-enriched cultures were probably due to the N uptake by the cells. However, the amount and rate of nitrate uptake by the cells was much higher in nitrate-enriched cultures than that of nitrate/ ammonium-enriched cultures. This is because when both nitrogen sources are present, the ammonium is preferentially assimilated due to lesser energy is required for assimilating the reduced N.

Other than measuring amount of nitrate of the N-depleted and N-enriched cultures, assay on the nitrate reductase (NaR) activity were also measured to provide circumstantial evidence to support cellular nitrogen uptake activities. NaR is the first

enzyme involved in the nitrogen reduction process and it is believed to be the rate-determining step. Since NaR is generally induced by nitrate and repressed during ammonium assimilation, it has been proposed that its assay might be a valuable tool for indicating the nitrogen source for phytoplankton (Eppley and Coatswor.Jl, 1968; Harrison, 1973). High NaR activity indicates that nitrate was the primary nitrogen source. On the other hand, low or non-existent NaR activity indicates that another form of nitrogen was being utilized or that the nitrogen source was exhausted. Figure 2.8 shows the NaR activity measured in N-depleted and N-enriched cells for 96 hours. NaR activity of N-depleted cells was maintained for the first 24 hours and rapidly dropped to undetectable level at and beyond 48 hours. This indicates that there was no or very low N available for cell growth in the N-depleted cultures. On the other hand, NaR activity of both N-enriched cells increased with time, with a sharply increased upon 48 hours. However, NaR activity of nitrate-enriched cells was much higher than that of nitrate/ammonium-enriched cells. These results are consistent to the nitrate measurement study. The difference in the NaR activity of both N-enriched cells (with and without ammonium) was most likely due to the NaR repression occurred in the presence of ammonium.

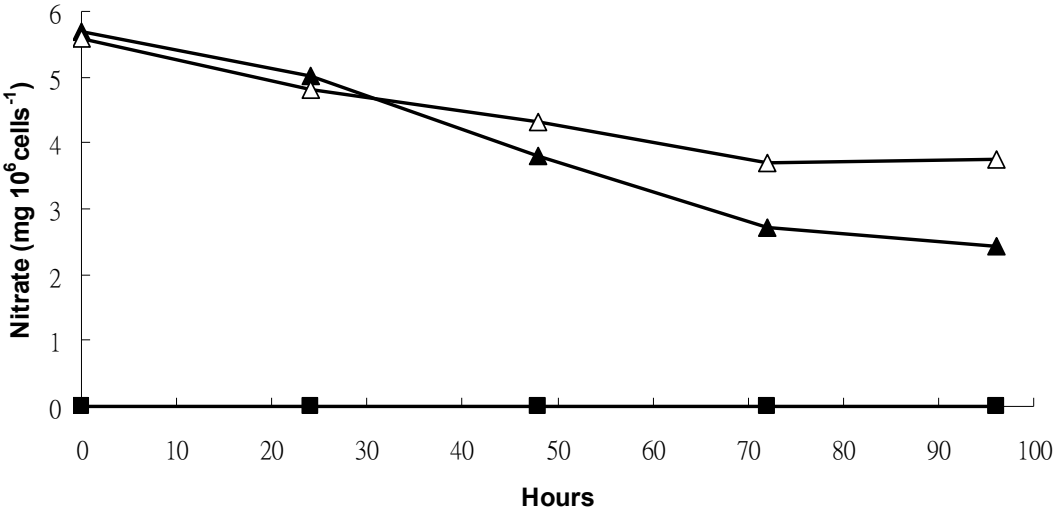


Figure 2.7 Amount of nitrate in the culturing medium of the growing *A.affine* with or without nitrogen supplement. Closed square indicates amount of nitrate in N-depleted culture. Closed triangle indicates amount of nitrate in nitrate-enriched culture. Open triangle indicates amount of nitrate in nitrate/ammonium-enriched culture. Amount of nitrite of N-depleted was not detectable through out the entire period (data not shown).

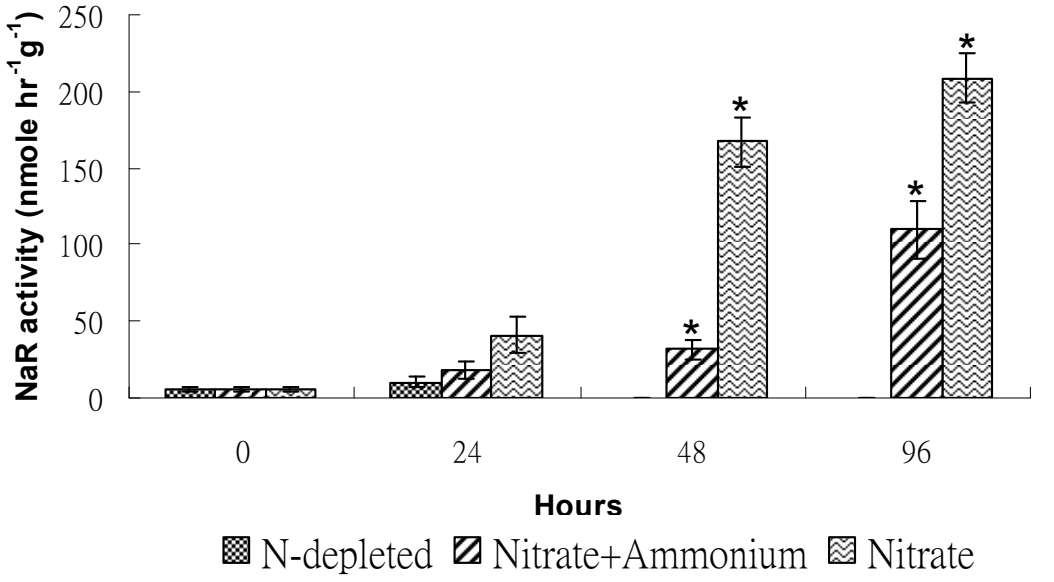


Figure 2.8 NaR activity of the growing *A.affine* with or without nitrogen supplement. NaR activity assay performed on cells grown under N-depleted, nitrate-enriched and nitrate/ammonium-enriched culture. (Bars represents $\pm$ S.D). Asterisk represents significant differences (p<0.01) when compared to NaR activity of N-depleted cells.

2.4 Conclusions

A fast-growing *Alexandrium species* was selected as the model strain to study and it was positively identified as *Alexandrium affine* through ribosomal gene analysis. Morphological identification in this case is misleading. Growth rate and different growth phases were well characterized. The selected strain was shown to be able to utilize different type of N sources (i.e nitrate, ammonium, urea, and amino acids) and with different growth responses to each individual nitrogen source. Optimized level to support growth for each individual N source was found which would be very useful for subsequent analysis. Cells would not growth under N-depleted condition. However, cell growth could be resumed by N replenishment independent of the time of N provision. N measurement and NaR activity assay provided circumstantial evidences that nitrate was not available in the N-depleted cultures and a rapid uptake was occurred in the N-enriched cultures. Although the present study could not clearly evaluate the metabolic pathways of N utilized and the assimilation of each individual N substrate, it showed that the physiological growth response of *A.affine* to various N and its nutritional status. More than that, results from the present study are important as they serve to build a foundation for the establishment of a nitrogen-depletion /repletion platform for subsequent proteomic analysis in this project.

CHAPTER III

The production of high-quality two-dimensional gel electrophoretograms (2-DE) of dinoflagellates

3.1 Introduction

As mentioned earlier, many questions relating to blooming/toxin-producing is unknown. For example, questions such as the exact mechanism of how HABs occurred, how these dinoflagellates sensed the changing nutrient and ambient environment and mechanisms of toxin-secretion are currently unknown. Nevertheless, there is an ever increasing risk of occurrence of HABs and that represents expanding threats to human health, the fishery resources and tourism industries. With the advance of proteomic technologies including 2-DE gels, researchers can attempt to uncover differential protein expression at different stages of laboratory induced blooming. Effects of different nutritional stages and ambient temperature etc. on protein expression can be studied at the proteome level. Researchers in this field can collectively aim to decipher cellular pathways, mechanisms of blooming and environmental effects at the whole proteome level. Study of the proteome or protein expression in HAB species is therefore important in understanding their blooming mechanisms.

Of the limited number of publications on studies on proteomes of dinoflagellates, most rely on the high power of resolution of 2-DE (Akimoto et al., 2004; Chan et al., 2005; Chan et al., 2004a; Chan et al., 2004b; Chan et al., 2002; Chan et al., 2006). Since its description more than 30 years ago (O'Farrell, 1975), 2DE has remained one of the most commonly used techniques in proteomics research today. It allows a fast and relatively inexpensive overview of protein changes in cellular processes. Further,

2-DE allows the isolation of proteins in at least several tens of nanogram amounts and that fit very nicely into subsequent protein identification methods such as MALDI-TOF MS, ESI-MS, MS/MS and Edman microsequencing (Celis and Gromov, 1999; Graves and Haystead, 2002; Ong and Pandey, 2001).

A high quality 2-DE gel with well resolved protein spots is the pre-requisite for finding differentially expressed proteins and successful MALDI-TOF MS identification subsequently. For example, a protein spot could not be identified if it co-migrates together with another protein in a 2-DE gel. Presence of interfering substances in significant quantities also renders successful 2-DE of dinoflagellates more difficult. However, to obtain a high quality protein samples of dinoflagellates subsequent to 2-DE requires tedious sample preparatory steps (Chan et al., 2002). The lengthy preparatory steps are necessary to remove high endogenous levels of salts, nucleic acids (estimated at least 10-100 times more than human), polysaccharides, phenolic compounds, pigments, and other interfering compounds. All these substances will interfere with the IEF-focusing process, the first step of 2-DE. The classical method includes sample cleaning up steps which take a few days and the amount of proteins loaded cannot excess 100 μg. Therefore, it is important to develop a simple and effective sample preparation methodology in order to produce high-quality 2-DE for subsequent comparative protein expression study. In the study, two dinoflagellate species, *Alexandrium affine* and *Scrippsiella spp.* were used. Five 2-DE sample preparation methods including two lysis-buffers, acetone precipitation, TCA/acetone precipitation and Trizol reagent; were compared and evaluated. Furthermore, methods of cell lysis including homogenization and sonication, effects of the addition of protease inhibitors and IEF loading methods including rehydration loading and

cup-loading were also evaluated. A method that could produce 2-DE gels of higher quality in terms of spot numbers, spot intensity and resolution would be used in the subsequent studies.

3.2 Materials and Methods

3.2.1 Dinoflagellate species and Culture conditions

Unialgal cultures of *Alexandrium affine* and *Scrippsiella species* were kindly donated by Prof John I. Hodgkiss previously of the University of Hong Kong. Seawater based K or f/2 media were used for culturing the dinoflagellates (Keller et al., 1987). Stock cultures of all dinoflagellates were kept at exponential growth phase by transferring to new medium every five or six days in a ratio of 1:10 v/v. Vegetative cells from cultures in mid- or late-exponential phase of growth were inoculated into freshly prepared culture medium. Possible contamination of algal culture was monitored by regular microscopic examination. The cultures were grown at 22℃ under a 16:8 hours light:dark cycle at a light intensity of 120 μE Lux $m^{-1}s^{-1}$ provided by cool white fluorescent tubes in a Conviron growth chamber (Model EF7, USA).

3.2.2 Cell counts

One ml of each culture was taken and fixed with 10 μl Lugol's solution and counted under light microscope with a Sedgwick-Rafter cell counter.

3.2.3 Preparation of protein extracts

Figure 3.1 is a flow chart summarizing workflow of the five methods evaluated.

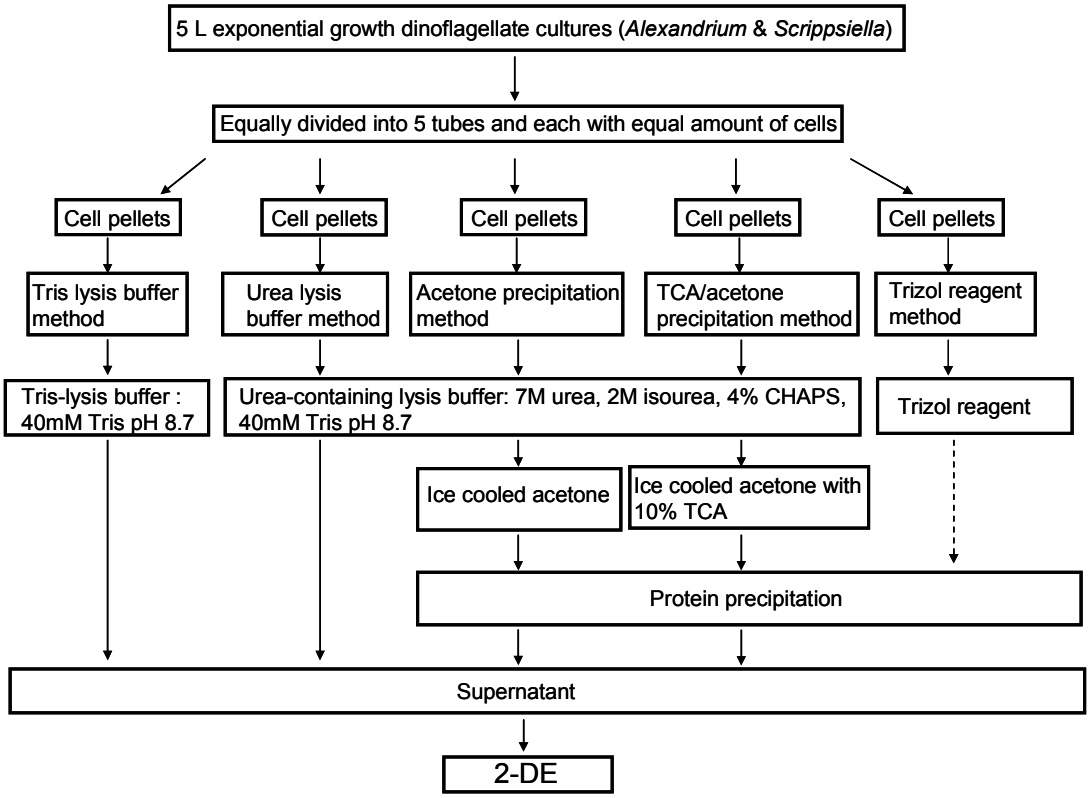


Figure 3.1 Schematic diagram showing the five sample preparatory procedures for 2-DE. (Dotted line indicates Trizol protein extraction procedures according to user manual)

Briefly on the procedures, 5L of mid-log phase cells of both dinoflagellate species were harvested by centrifugation at 1000 x g for 15 mins at 4℃. The cell pellets were then equally divided into five 2ml micro-centrifuge tubes (for the 5 different types of extraction procedures, see below) and equal amount of cells in each tube were checked by cell counts under light microscope. Pellets were rinsed with sterile seawater and centrifuged at 1000 x g again for 15 mins at 4℃. Cell pellets were stored at -80℃ until used. Proteins were extracted according to the 5 different

extraction methods listed, including two lysis-buffers (urea-containing buffer: 7 M urea, 2 M thiourea, 4% CHAPS, 20 mM Tris pH 8.7 and Tris-buffer: 40 mM Tris pH 8.7), acetone precipitation, TCA/acetone precipitation and Trizol extractions.

For protein extraction using lysis buffer, 500 µl of lysis buffer (urea-containing buffer or Tris buffer) was added to the cell pellets. Cells were disrupted by sonication (a total of 3 mins with short pulses of 5-10 seconds each) on ice. Lysis of cells was confirmed using light microscope. Cell lysates were then centrifuged at 14000 x g for 15 mins at 4℃ to collect the supernatant. Subsequently, the supernatant collected was placed into a new eppendorf tube and store at -80℃ until use.

For sample preparation relying on acetone precipitation or TCA/acetone precipitation, 2.5 ml of ice-cold acetone or acetone with 10% TCA was added to a 500 µl protein extracts in urea-containing lysis buffer and kept at -20℃ overnight to allow precipitation of proteins. Proteins precipitated were washed twice with ice-cold acetone before allowing to dry in the air. Subsequently, 500 µl of fresh urea-containing lysis buffer was added to re-solubilize the protein pellet. The protein solution was ready for 2-DE.

In protein extraction using Trizol (Roche, Switzerland), preparation was performed according to that of the manufacturer with some modifications. Briefly, 1 ml Trizol reagent was added to the cell pellet and subjected to sonication (a total of 3 mins with short pulses of 5-10 seconds) on ice. Lyses of cells were confirmed using light microscope. Subsequently, 200 µl of chloroform was added to the cell lysate before shaking vigorously for 15 seconds. The mixture was allowed to stand for 5 mins in room temperature before being centrifuged at 12000 x g for 15 mins at 4℃. The top pale-yellow or colorless layer was removed. 300 µl of ethanol was added to

resuspend the reddish bottom layer and the mixture centrifuged at 2000 x g for 5 mins at 4℃. Supernatant was transferred to a new tube and 1.5 ml of isopropanol was added. The mixture was allowed to stand for at least 20 mins for precipitation of proteins to complete. It was then centrifuged at 14000 x g for 10 mins at 4℃. Pellet obtained was briefly washed with 95% ethanol before allowed to air dried. Five hundred µl of lysis buffer was added to solubilize the protein pellet before loading onto the first dimension IEF. To the evaluate on the cell lysis methods and the effects of protease inhibitors, the above procedures were repeated but homogenization was used instead of sonication and with/without the addition of protease inhibitor cocktails (containing a mixture of AEBSF, E-64, pepstatin, bestatin and 1,10 phenanthroline) (Sigma, USA).

3.2.4 Protein determination

Protein quantification in the urea-containing protein samples was performed using a modified Bradford protein assay (Bio-Rad, USA) (Ramagli and Rodriguez, 1985).

3.2.5 Two dimensional gel electrophoresis (2-DE) and imaging analysis

Typically, 340 µl sample containing 60-80 µg of sample proteins (for silver staining gel) in rehydration buffer (containing 7 M urea, 2 M thiourea, 4% CHAPS, 0.2% DTT and 3.4 µl of IPG buffer pH 4-7) was used to rehydrate the IPG strip (18 cm) pH 4-7 (Bio-Rad, USA) for 16 hours. For samples to be cup-loaded (for comparative studies), protein samples were added in the sample cups after rehydration of the IEF strips. IEF was performed using a Protean-IEF cell (Bio-Rad, USA).

Voltage was applied according to the following: 1 hour at 100V, 2 hour at 300V, 2 hours 1000V, 2 hours 4000V and 5 hours 8000V. Following IEF, the gel strip was equilibrated with equilibration buffer (50 mM Tris pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 1% DTT and trace amount of bromophenol blue) for 15 mins. The gel strip was then placed in fresh equilibration buffer containing 1% iodoacetamine (instead of DTT) for another 15 mins. The second dimension SDS-PAGE was performed using 10% or 12% polyarylamide gel running at a constant current of 15 mA/gel until the bromophenol blue dye reached the end of the gel. After electrophoresis, the gel was silver stained. Stained gels were scanned and the images were analyzed by Melanie III (GeneBio, Switzerland) as described in the user manual.

3.3 Results and discussion

3.3.1 Comparison of 2-DE gels obtained by different extraction methods

The success of obtaining high-quality 2-DE electrophoretogram is greatly dependent on sample preparations before the IEF run. Typically, IEF is tolerant of salt concentrations up to 50 mM (Kirkland et al., 2006). Therefore, in order to have good quality IEF for 2-DE, a thorough desalting of the sample is required. In marine dinoflagellates, there are quite a large amount of interfering substances including salts, nucleic acids, polysaccharides, phenolic compounds and pigments. If untreated, these interfering substances will cause aberrant patterns in the 2-DE electrophoretograms. Thus, sample preparation should include steps to get rid of these substances. Application of Trizol-extraction methodology to obtain proteins for 2-DE had been reported in halophilic proteins with satisfactory results (Kirkland et al., 2006). In comparison with 2-DE electrophoretograms obtained using protein samples prepared with the two lysis-buffer methods or acetone or TCA/acetone precipitation methods, those obtained using Trizol extraction method showed better results. They have a higher resolution of protein spots than those prepared by using the other four methods mentioned. The results were consistently observed in the two dinoflagellates species (*Alexandrium affine* and *Scrippsiella spp*) used in this study (Figure 3.2).

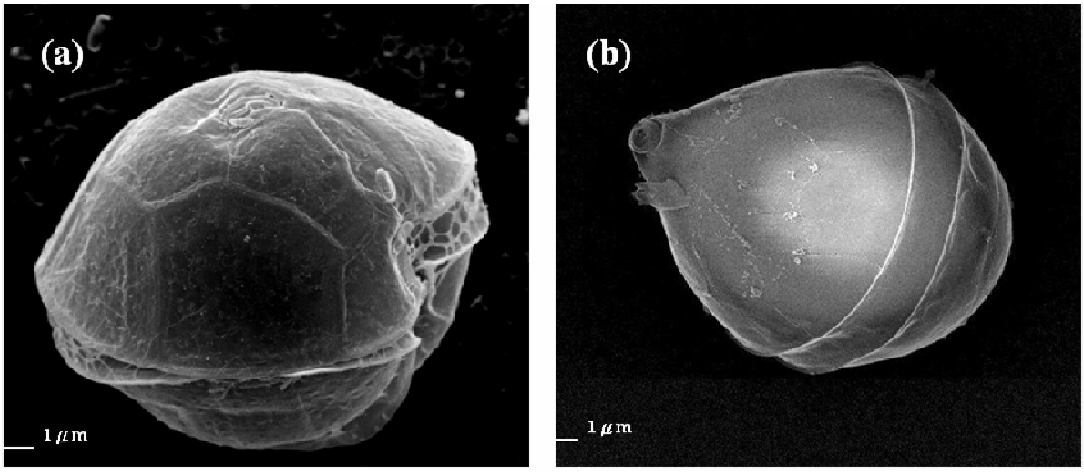


Figure 3.2 Scanning electron micrographs (SEM) of *Alexandrium spp* and *Scrippsiella spp* isolated from Hong Kong waters. SEM was taken at x 2120 (scale bar = 1μm) of (a) *Alexandrium spp* and (b) *Scrippsiella spp.*

Proteins extracted by direct sonication of the dinoflagellate cells in both lysis buffers produced 2-DE electrophoretograms that had excessive horizontal and vertical streakings (Figure 3.3 and Figure 3.4). Obviously, 2-DE from Tris-lysis buffer was much worse than that of urea containing-lysis buffer. None of the protein spots in the 2-DE from Tris-lysis buffer were focused. There were a lot of smearing and/or aberrant patterns. For example, there was a massive of dark area appeared in the anode side of the gel using samples from *Alexandrium spp.* (Figure 3.3a) and a big "cross" appeared in the gel using samples from *Scrippsiella spp.* (Figure 3.4a). Although 2-DE from urea containing-lysis buffer was better than that of Tris-lysis buffer, most of the proteins were still not being focused properly and appeared either as smearing or in aberrant patterns (Figure 3.3b and 3.4b). In addition, high backgrounds with relatively fewer spots were seen. The better results of the 2-DE from urea containing-lysis buffer were attributed to the better protein solubilizing power of the buffer (which containing urea, thiourea and detergents). However, the "unremoved" endogenous interfering substances in these dinoflagellate cells have

greatly affected focusing of the IEF step in the 2-DE. Therefore, these methods are not the best choice for 2-DE of dinoflagellates.

As for the acetone (Figure 3.3c and 3.4c) and TCA/acetone (Figure 3.3d and 3.4d) precipitation methods, the precipitation step was used both to concentrate proteins in the samples and also to separate the proteins from potentially interfering substances. Therefore, resolution and intensity of the protein spots were better than those obtained with the two lysis-buffer extraction methods. However, phenolic and some other contaminants were coextracted together with the proteins, resulting in several horizontal and vertical streakings with a high background. This is easily observable in enlarged areas of the gel (Figure 3.7). Although TCA/acetone precipitation method had further improved the 2-DE from that of acetone precipitation alone, when compared to Trizol extraction method for 2-DE, relatively high background in some areas were still presented in the gel (Figure 3.3d and 3.4d). In addition, some spots were still not well focused and appeared as indistinct image. Therefore, further cleaning-up procedures are required to remove the substantial interfering substances that remained in the acetone or TCA/acetone precipitated samples.

Of the five protein extraction procedures, Trizol-extraction resulted in the best resolution of stained spots with a clear background (Figure 3.3e and 3.4e). The Trizol-sample preparation strategy is simple and fast. There are only a few steps to perform and the preparation time just requires a few hours. When compared to the lengthy and complicated extraction procedures described previously (Chan et al., 2005; Chan et al., 2004a; Chan et al., 2004b; Chan et al., 2002), the shorter time of sample preparation of the Trizol-extraction method decreased the risk of protein degradation and losses. The beneficial effects of Trizol extraction method included

improvements on spot resolution, spot numbers and intensity in 2-DE. The improvements can be attributed to the great efficiency of Trizol in the removing nearly all of the interfering substances. Figure 3.5 shows the steps involved in Trizol protein extractions. DNA/RNA, pigments, salts and other interfering substances like phenolic compounds were removed sequentially. Because a relatively clean sample was prepared, therefore proteins could be well separated and focused in the IEF. The excellent 2-DE patterns obtained by this Trizol-extraction method would allow comprehensive protein expression studies on dinoflagellates with 2-DE and thus greatly contributes to the research field of proteomics.

In the literature, there is only one comprehensive study on optimizating protocols for sample preparations of dinoflagellates for 2-DE studies (Chan et al., 2002). The authors had compared different procedures and methods on cell disruption; extractions buffers; and pre-electrophoretic treatments (such as addition of protease inhibitor and RNase/DNase/endonuclease, ultrafiltration, desalting and protein precipitation etc.). They found that Tris-extraction buffer and TCA/acetone precipitation enabled good protein resolution on subsequent 2-DE. Between the Tris buffer extraction and TCA/acetone precipitation procedures, Tris-buffer extraction procedure was found to yield a greater number of spots (407 spots) than that of TCA/acetone precipitation (387 spots). However, the optimized protocol involving many desalting and concentrating steps. The additional desalting and concentrating steps could improve quality of the final results, but each additional step would result in selective losses of some proteins. Samples prepared by methodologies described by Chan and coworkers (2002) yield only around 400 spots. Therefore, it seems that there was a significant loss of proteins with that sample preparatory protocol. Proteins

lost may be a consequence of the lengthy desalting and concentrating steps. Although there is no one-step procedure for sample preparation for 2-DE, one should always aim for a simpler sample preparatory protocol in order to minimize protein losses and ensure reproducibility.

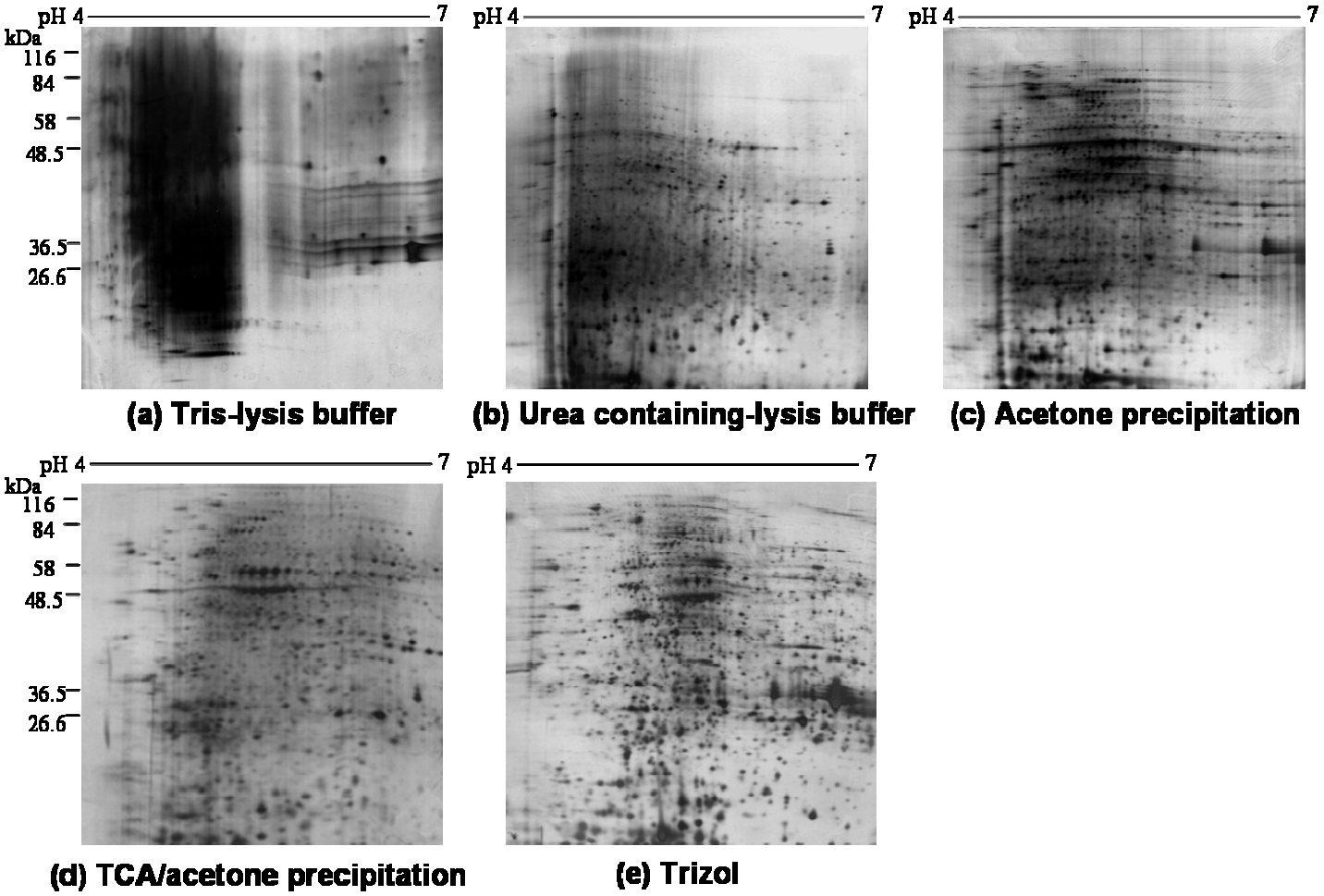


Figure 3.3 2-DE of total protein extracts of *Alexandrium affine* prepared using five different sample preparation methods. The methods including (a) Tris-lysis buffer method, (b) Urea containing-lysis buffer method, (c) Acetone precipitation method, (d) TCA/acetone precipitation method and (e) Trizol protein extraction method.

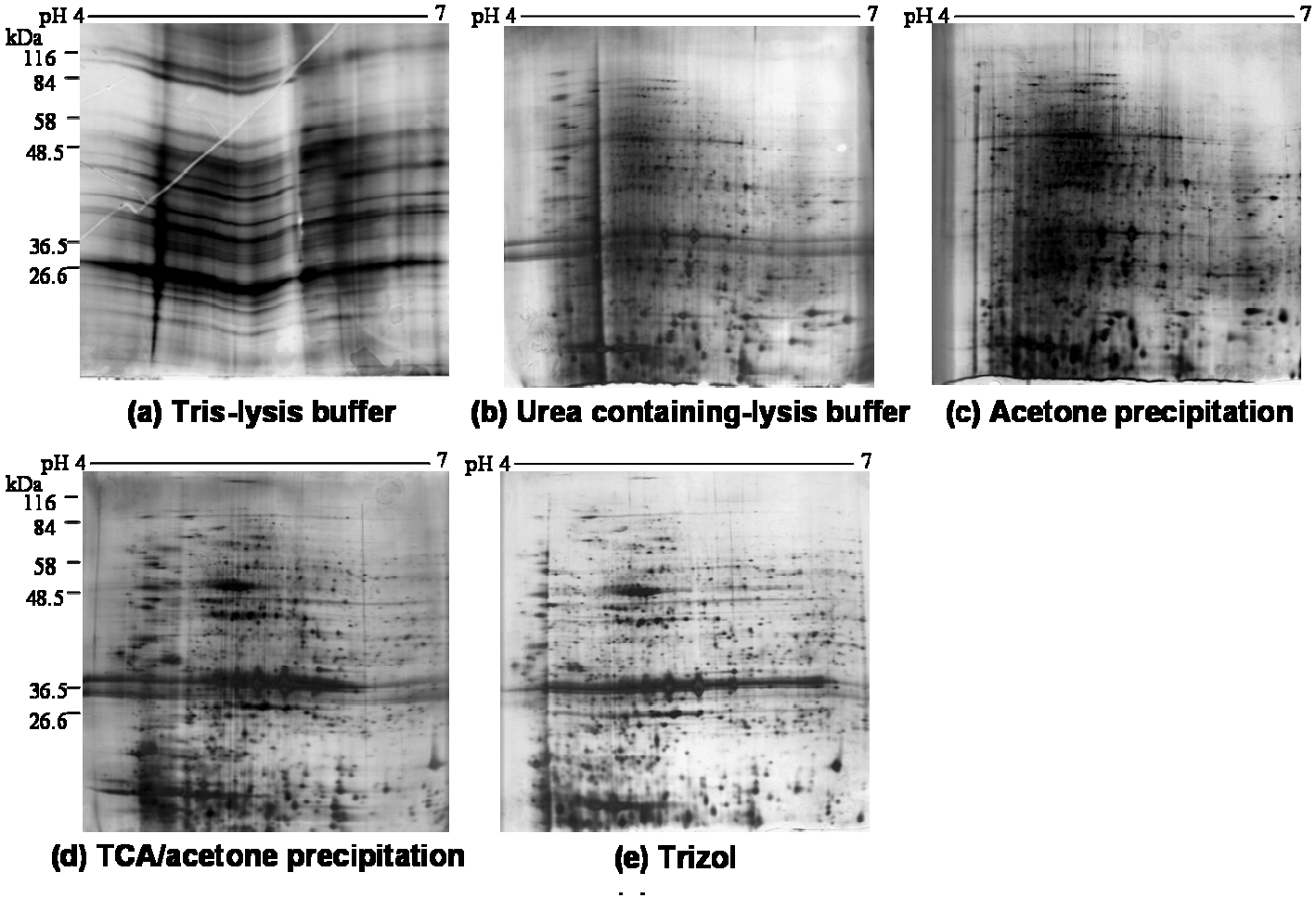


Figure 3.4 2-DE of total protein extracts of *Scrippsiella spp* prepared using five different sample preparation methods. The methods including (a) Tris-lysis buffer method, (b) Urea containing-lysis buffer method, (c) Acetone precipitation method, (d) TCA/acetone precipitation method and (e) Trizol protein extraction method.

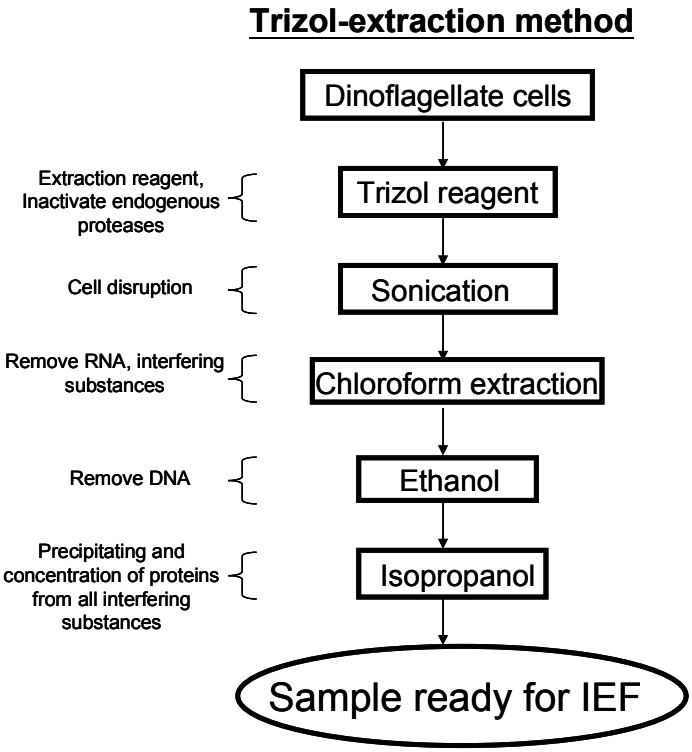
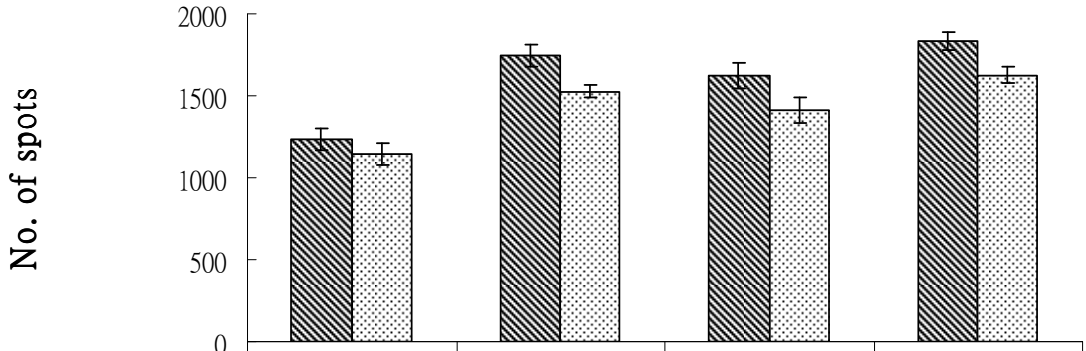


Figure 3.5 Workflow of Trizol protein extractions. Different interfering substances were sequentially removed during the various steps of extraction.

3.3.2 Total numbers and intensity of spots

Since the spots in the 2-DE from Tris-lysis buffer extraction were not detectable, 2-DE of other four extraction methods were compared. With the help of the Melanie III software, it was found that 2-DE gels generated using proteins extracted from the *Alexandrium affine* with the urea containing-lysis buffer extraction method, the acetone precipitation, TCA/acetone precipitation and Trizol–extraction methods yielded an average of 1233, 1741, 1622 and 1835 protein spots respectively (Figure 3.6). On the other hand, those 2-DE using *Scrippsiella spp.* sample yield an average of 1147, 1527, 1410 and 1627 spots for the four extraction methods respectively (Figure 3.6).



	Lysis buffer	Acetone precipitation	TCA/acetone precipitation	Trizol
Alexandrium	1233±65	1741±65	1622±83	1835±55
Scrippsiella	1147±67	1527±38	1410±80	1627±47

Figure 3.6 Comparison of total number of spots found on 2-DE gels ran using samples obtained from the two dinoflagellates species, *Alexandrium affine* and *Scrippsiella spp*. with the five different extraction methods. Results presented are the mean values from three independent experiments. Numbers indicate the spot numbers ± S.D. Lysis buffer presented was the urea containing-lysis buffer. Spots from Tris-lysis buffer prepared 2-DE was not counted due to the high background and aberrant patterns of the gels.

Excessive streaking, smearing and weakening appearance of the 2-DE would make it difficult to discern any appreciable number of spots in some regions in the 2-DE developed either with the lysis buffer or the acetone precipitation methods (Figure 3.3 and 3.4). Although the corresponding region in the gel developed using TCA/acetone precipitation shows spots that with better focusing, the Trizol method remains the best that all spots could be resolved individually (as indicated in the circle and rectangle regions shown in Figure 3.7). There were not only an obvious increase in spot numbers in these regions, but also many faint protein spots could now be seen clearly with the use of Trizol extraction method, presumably due to a clearer background. One point should also be highlighted is that some spots in the TCA/acetone prepared 2-DE (Figure 3.7d and 3.7i) were in different pattern to the

other gels. Changes of the protein locations may be attributed to the protein modifications in the extremely acidic environment with TCA. Therefore, Trizol prepared 2-DE gels showed the highest of spot numbers, intensity and resolution.

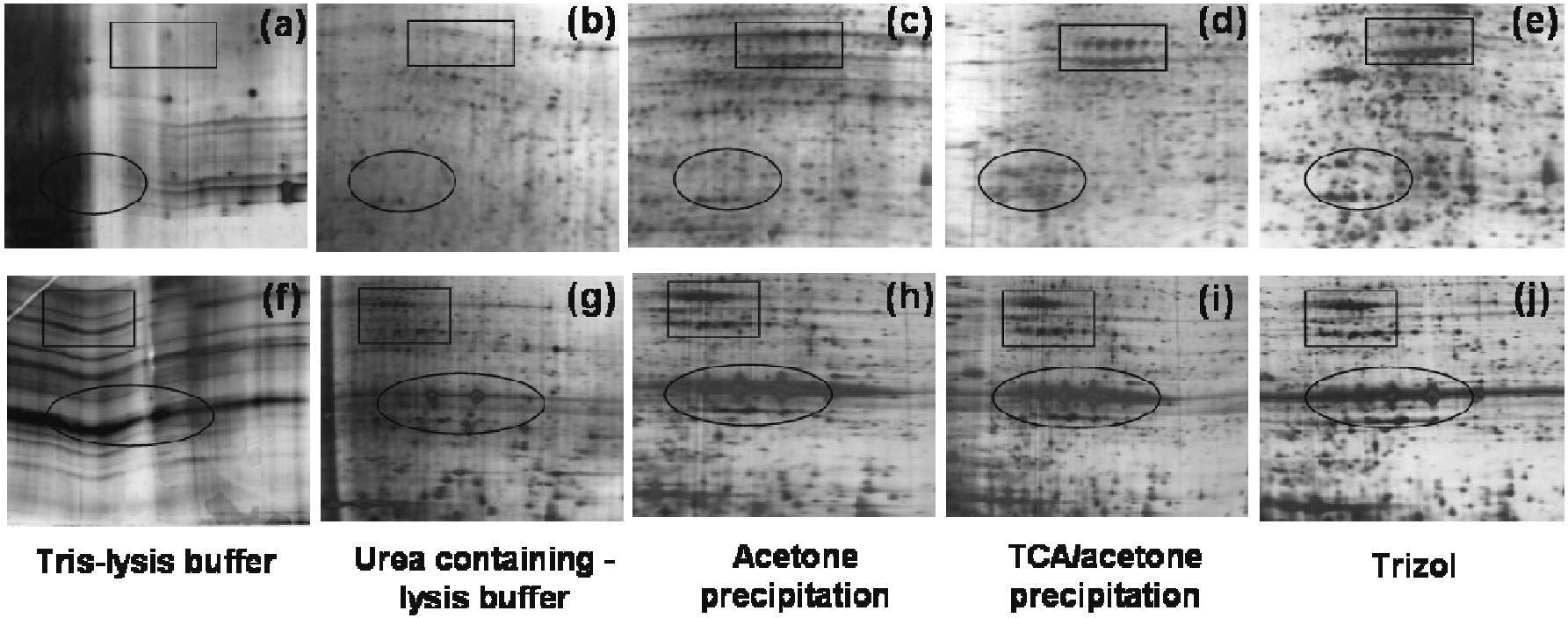


Figure 3.7 Enlargement of 2-DE images from representative regions among the five protein extraction methods: Tris-lysis buffer; Urea containing-lysis buffer; Acetone precipitation; TCA/acetone precipitation and Trizol-extraction method. Image (a)-(e) and (f)-(j) are gels from *Alexandrium affine* and *Scrippsiella spp.* samples respectively. Circle and rectangle regions indicate some representative spots that have progressively improved from lysis buffer extractions to Trizol extractions in term of both resolution and intensity.

3.3.3 Homogenization and sonication

After comparing five different extraction procedures, Trizol protein extraction method was found to be the best. However, lyses of dinoflagellate cells were not easy due to their surrounding thecal plates. Thus, the efficiency of cell lysis protocol was also one of the important factors for getting more protein spots in the 2-DE gels subsequently. Therefore, cell lysed by either sonication or homogenization was compared. Protein yield of around 900 μg per 10^7 cells was found from samples that were sonicated. On the other hand, protein yield of only around 500 μg per 10^7 cells was found from samples that were homogenized. Furthermore, on 2-DE analysis, samples obtained with sonication showed more protein spots than that with homogenization if the same amount of proteins were loaded (Figure 3.8). Therefore, the cell disruption efficiency for sonication seems to be much higher than that of homogenization. Moreover, sonication was easier to perform and can handle smaller amount of samples than homogenization. Inference of the results from the present study was the same as that of Chan et al (2002). Therefore, based on the higher cell disruption efficiency and ease of handling, sonication was the method of choice.

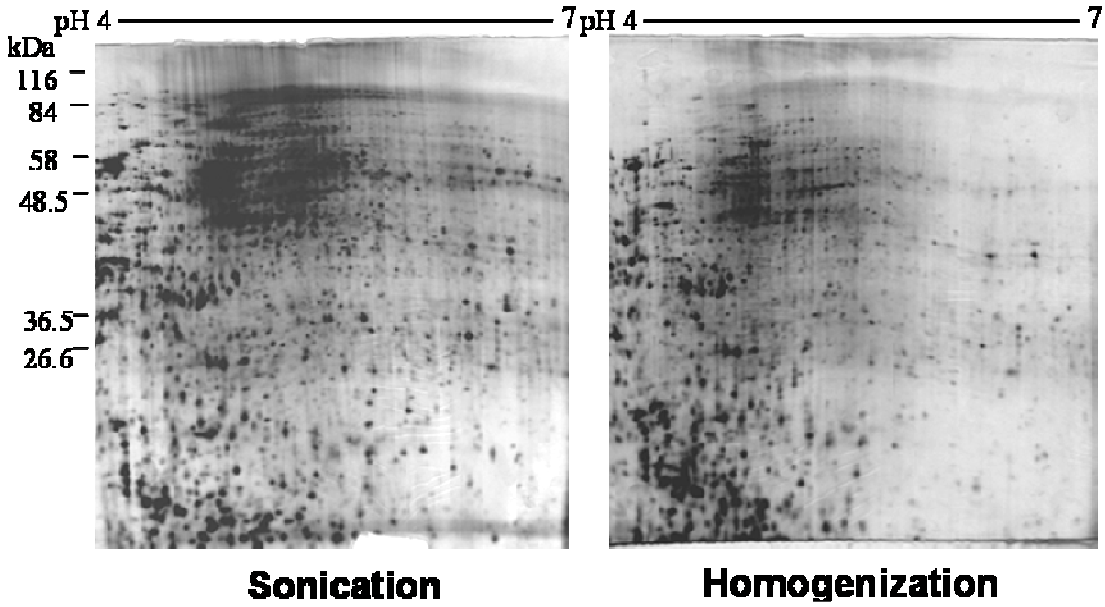


Figure 3.8 Comparison of the 2-DE gels obtained using proteins extracted from *Alexandrium affine* by the Trizol extraction method with two different cell lysis protocols, homogenization (Right) and sonication (Left).

3.3.4 Effects of protease inhibitors

As elaborated earlier, there are three fundamental steps in sample preparation for 2-DE: (1) cell disruption, (2) inactivation of proteases, and (3) removal of interfering substances (Gorg et al., 2000; Gorg et al., 2004; Herbert, 1999; Shaw and Riederer, 2003). Cell disruption and removal of interfering substances were already discussed previously. On the other hand, presence of proteases activities is also an important factors that have to be considered in the sample preparation of 2-DE. Although Chan et al (2002) claimed that there were only minor differences when the extraction were done with or without the presence of protease inhibitors, nearly 40 spots were missing when protease inhibitors were not added. Given that their method did not resolve as many protein spots as we did, we would like to evaluate whether the addition of protease inhibitors to Trizol extraction buffer were necessary for minimizing endogenous proteolytic degradation of proteins in samples before 2-DE. Figure 3.9

shows the 2-DE electrophoretograms from samples that either with or without the addition of protease inhibitors during the Trizol protein extraction. The number of spots in both gels were very similar (with protease inhibitors: 1783 spots; without protease inhibitors: 1779 spots). The result indicates that most of the endogenous proteases were inhibited in the Trizol reagent. Therefore, Trizol extraction procedures could be carried out in the absence of protease inhibitors.

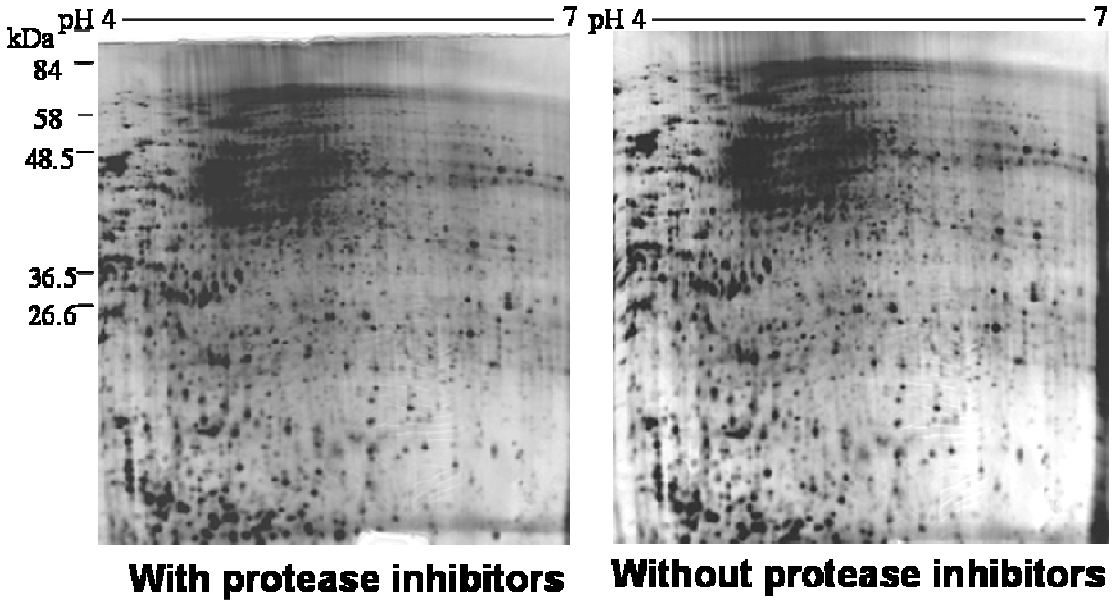


Figure 3.9 Comparison of the number of spots obtained in 2-DE gels using proteins extracted from *Alexandrium affine* by Trizol extraction method with (Left) and without (Right) the addition of protease inhibitors.

3.3.5 Rehydration versus Cup-loading method

Cup loading was reported to improve resolution of 2-DE electrophoretograms, especially when the sample was applied in the anode side (Gorg et al., 2000). However, only upto 100 µl could be loaded. Larger sample loads inevitably lead to protein precipitation in the sample cup. Rehydration loading instead of cup-loading could accommodate larger sample volumes (greater than 100 µl) and without the problems with precipitation. In this study we found that the 2-DE patterns and spot resolution obtained with the Trizol-extracted samples in rehydration loading mode was similar to that obtained with the cup loading mode in the pH 4-7 range (Figure 3.10). In addition, for the other protein extraction methods, there were significant differences in the 2-DE gel patterns obtained from samples prepared with the two sample loading methods. Nevertheless, a high-quality 2-DE gel can be obtained relatively straight-forward with proteins in the sample extracted by the Trizol method. Samples could be added directly onto the IEF tray, i.e. without cup-load. The 2-DE pattern as well as resolution obtained upon rehydration loading was highly similar to that of cup loading (Figure 3.10). The enablement of using rehydration loading could avoid problem of the sample cups leak and protein precipitation. It allowed larger amount of proteins to be loaded onto the strips without limitation on the sample volumes. These would also greatly facilitate the *de novo* protein/peptide sequencing for dinoflagellate proteins.

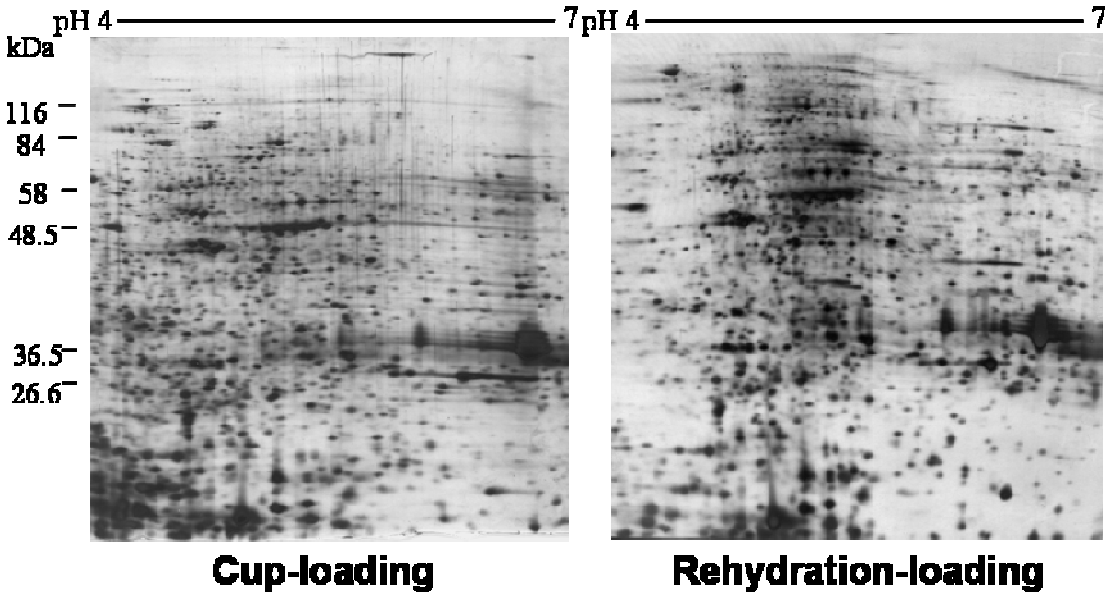


Figure 3.10 Comparison of the 2-DE gels of proteins extracted from *Alexandrium affine* by Trizol extraction method with two different IEF sample loading methods (Right) rehydration loading and (Left) cup loading at anode.

3.4 Conclusions

Trizol, a monophasic mixture of phenol and guanidine isothiocyanate that is commercially available, is typically used to isolate RNA from cell and tissue samples. In this study, it is shown that using Trizol is a simple, fast and effective method for preparing proteins samples for 2-DE analysis of dinoflagellates. Of the five protein extractions evaluated, Trizol extraction method was found to be the best to produce high-quality 2-DE gels using dinoflagellate samples. In addition, sonication was better than homogenization for cell disruption. Most of the endogenous proteases were inhibited in the Trizol reagent. Therefore, Trizol extraction procedures could be carried out in the absence of protease inhibitors. It was also found that 2-DE gel patterns and spot resolution obtained with the Trizol-extracted samples in rehydration loading mode were similar to those obtained with the cup loading mode. Thus, a larger amount of proteins can be loaded and this would greatly facilitate the production of high-quality and high protein-loaded 2-DE gels (mg of proteins loaded) for some downstream processing such as MALDI-TOF-PSD or MALDI-TOF/TOF which requires a larger amount of proteins. This method is being used for subsequent investigations in this thesis.

CHAPTER IV

2-DE analysis of protein expression profiles of dinoflagellates under nitrogen stress

4.1 Introduction

As mentioned, dinoflagellates are the major HAB causative agents and the mechanism of algal blooms is still poorly understood. However, it is generally accepted that the causes of algal blooms are governed by many factors (Hallegraeff, 1993; Hallegraeff, 2003). Nevertheless, nitrogen is believed to be one of the most important factors in the initiation and maintenance of algal blooms (Paerl, 1997; Smayda, 1990). It is believed that nitrogen is limited in the open sea. Therefore, the impact of increased influx of nitrogen will be drastic in the initiation of algal blooms. Many studies were carried out to understand the effects of nitrogen sources on the growth rate, cell density, cell size, pigment composition and toxin production on dinoflagellates (Boyer et al., 1987; Flynn et al., 1996; John and Flynn, 2000; Lee, 2006; Leong et al., 2004; Leong and Taguchi, 2004; Parkhill and Cembella, 1999). However, very little is known about the biochemical and molecular responses of the cells to the effects of nitrogen. It is important to understand what is happening inside the cells and what biochemical machinery that are operating in response to the effects of nitrogen. However, there were no any protein expression studies on how dinoflagellates response to the loading of nitrogen.

From results presented in Chapter II, cell density of *A. affine* was found to maintain at a low level (~ 500 – 1000 cell ml^{-1}) in nitrogen-depleted conditions and cells were initiated to growth rapidly after the replenishment of nitrogen sources. Therefore, in order to found out what and how the expression of proteins would be

changed in the response to nitrogen depletion and subsequent repletion, 2-DE of cellular homogenates of *A. affine* under both nitrogen-depletion and repletion conditions were examined. Through the identification and expression patterns of differential expressed proteins in responses to different N status, insights into the molecular mechanism involved in the initiation of algal blooms may be formulated.

4.2 Materials and methods

4.2.1 Cultivation of cells

A. affine was cultivated as described previously in preceding chapters.

4.2.2 Proteomic analysis of dinoflagellate under nitrogen stress

A. affine cells under nitrogen depletion and subsequent repletion conditions were subjected to 2-DE analysis aiming to find differentially expressed proteins. To prepare the nitrogen depletion samples, *A. affine* cells were grown to late-log phase (day 6) (at around 10000 -13000 cell ml^{-1}) and collected by centrifugation (1500 x g 15 mins) at room temperature. The pellets were washed twice with sterile synthetic seawater (without any N) to avoid any carry-over of nitrogen from the previous medium. The pellets were then inoculated into a new medium that had no nitrogen source. The N depleted cultures were subjected to growth condition as mentioned in 4.2.1., and the initial cell concentrations were determined by cell counting under microscope. From the results in Chapter II (Figure 2.6), cell would maintain a small amount of growth in the first day and stopped in day 2. This growth was believed to be supported by the N reserves inside these cells. Therefore, cells harvested at 48 hours from the depleted cultures were used as "N-depleted" samples. The cells were counted under the microscope and harvested by centrifugation (1500 x g 15 mins) at room temperature. The harvested cell pellets were kept at -80℃ until use.

For the preparation of nitrogen-repletion samples, nitrate (200 μM-N, refer to Table 2.1) was added to the 2-day nitrogen depletion cultures. From the results in Chapter II (Figure 2.6), cells were in lag phase in the first day and start to growth from day 2. Therefore, cells of the nitrogen repleted cultures were harvested after 24

and 48 hours and these samples were named as “N- repletion” samples. The cells were counted under the microscope and harvested by centrifugation (1500 x g 15 mins) at room temperature. The harvested cell pellets were kept at -80℃ until use.

4.2.3 Preparation of protein extracts

Proteins were extracted according to the optimized method developed as described in Chapter III. Briefly, cell pellets of either N depletion or repletion were added with 1 ml Trizol reagent (Roche, Switzerland). The cell mixtures were subjected to short pulses of sonication for 3 minutes on ice. Cell lysis was confirmed by light microscopy. Subsequently, 200 µl of chloroform was added to the cell lysate before shaking vigorously for 15 seconds. The mixture was allowed to stand for 5 minutes in room temperature before being centrifuged at 12000 x g for 15 minutes at 4℃. The top pale-yellow or colorless layer was removed. Three hundred µl of ethanol was added to resuspend the reddish bottom layer and the mixture centrifuged at 2000 x g for 5 minutes at 4℃. Supernatant was transferred to a new tube and 1.5 ml of isopropanol was added. The mixture was allowed to stand for at least 20 minutes for precipitation of proteins to complete. It was then centrifuged at 14000 x g for 10 minutes at 4℃. Pellet obtained was briefly washed with 95% ethanol before allowed to air dried. Five hundred µl of lysis buffer was added to solubilize the protein pellet before loading onto the first dimension IEF.

4.2.4 Protein determination

Protein quantification in the urea-containing protein samples was performed using a modified Bradford protein assay (Bio-Rad, USA) (Ramagli and Rodriguez,

1985).

4.2.5 Two dimensional gel electrophoresis (2-DE) and imaging analysis

Typically, 340 µl sample containing 80 µg of sample proteins (for silver staining gel while 700 µg proteins are needed for coomassie staining gel) in rehydration buffer (containing 7M urea, 2M thiourea, 4% CHAPS, 0.2% DTT and 3.4 µl of IPG buffer pH 4-7 or pH 3-10) was used to rehydrate the IPG strip (18 cm) pH 4-7 or pH 3-10 (Bio-Rad, USA) for 16 hours. 2DE was performed according to methodology described in Chapter III.. After electrophoresis, the gel was either silver stained or coomassie blue (R250) stained before being scanned by an Epson scanner (Perfection 1200U). The images were analyzed by Melanie III (GeneBio, Switzerland) as described in the user manual. Each 2-DE sample was repeated in triplicates and the differential protein expression was confirmed only when it was consistently present in all three gels. Moreover, difference in spot optical density must be at least by 5-fold.

4.2.6 MALDI-TOF MS analysis

Differentially expressed proteins were excised from the coomassie blue stained gel and transferred to a microcentrifuge tube. The gel plugs were washed twice in 25 mM NH_4HCO_3 in 50% Acetonitrile (ACN). Subsequently, the gel plugs were washed with 100% ACN and dried under vacuum for 10-15 minutes. In-gel trypsin digestion was performed by adding 20 ng/ml of trypsin in 25 mM NH_4HCO_3 overnight at 37℃. For MALDI-TOF mass spectrometry analysis, 1µl peptide mixture was mixed with 1 µl matrix solution (HCCA, saturated solution in ACN: 0.1% TFA (1:1)) on the target plate before being dried and analyzed with a MALDI-TOF mass spectrometer

(Autoflex; Bruker, Germany) in reflector mode over a mass range of 1000-3000 Da and using external mass calibration with the calibration standards from the manufacturer. Spectra from 150 shots at several different positions on the target plate were combined to generate a peptide mass fingerprint (PMF) for bioinformatic database searches. PMF obtained was searched against the NCBI non-redundant database using the search engine MASCOT.

4.2.7 *De novo* amino acid sequencing

4.2.7.1 N-terminal sulfonation and post-source decay (PSD) analysis

De-novo peptides/proteins sequencing was performed with the aid of N-terminal sulfonation by methodologies previously described in (Wang et al., 2004). Briefly, 10 mg/ml 4-sulfophenyl isothiocyanate (SPITC) was added into the tryptic peptides and incubated at 55°C for 30 min for the sulfonation reaction to occur. Sulfonated peptides were cleaned by absorption onto and subsequently eluted from zip-tips before being analyzed with MALDI-TOF mass spectrometer using post-source decay (PSD) as described in the user manual (Autoflex, Bruker, Germany). The acquired PSD spectrum was analyzed by the de novo sequencing function in Biotools 3.0 (Bruker, Germany). Amino acid sequences were deduced from the ladder sequences (spectrum) obtained and were searched against the NCBI non-redundant database.

4.2.7.2 Liquid-chromatography linked tandem mass spectrometry (LC-MS/MS) analysis

Proteins excised from a coomassie stained 2-DE were digested by trypsin as described previously. Dried peptide extracts were re-dissolved in 20-60 µl of water, depending on the staining abundance of the protein spots, and 10 µl was loaded using

an autosampler on a nano-LC-MS/MS Ultimate 3000 system (Dionex, Netherlands) interfaced on-line to an ion-trap mass spectrometer HCTultra (Bruker, Germany). The mobile phase was composed of 100% H2O (solvent A) and 20:80 H_2O: ACN v/v (solvent B). Peptides were first loaded onto a trapping microcolumn C18 PepMAP100 at a flow rate of 20 ml min^{-1}. After 4 minutes, they were back-flush eluted and separated on a nanocolumn C18 PepMAP100 at a flow rate of 300 nl min^{-1} in the mobile phase gradient: from 0 to 50% of solvent B in 60 min, 50–90% B in 10 min and 100% B in 10 min; % B refers to the solvent B content in an A and B mixture. Peptides were infused into the ion-trap mass spectrometer *via* a dynamic nanospray probe and analyzed in positive mode. Three most abundant precursor ions detected in the full MS survey scan (*m/z* range of 350–1500) were isolated within a 4.0 amu window and fragmented. MS/MS fragmentation was triggered by a minimum signal threshold of 500 counts and carried out at the normalized collision energy of 35%. MS/MS spectra were analyzed by Data Analysis Software from Bruker (Bruker, Germany) and *de novo* sequences were predicted with Biotools 3.0 (Bruker, Germany).

4.2.7.3 Edman microsequencing

Part of the N-terminal peptides/proteins sequencing were done in the Australian Proteome Analysis Facility (APAF) in Macquarie University. Proteins excised from a coomassie stained 2-DE were washed twice with milli-Q water and send to APAF for subsequent N-terminal sequencing. Briefly, the samples were first reduced and alkylated in-gel with DTT and acrylamide. They were then in-gel digested with trypsin at 37ºC for 16 hr. The peptides were extracted from the gel and fractionated by

RP-HPLC using a 1.0mm diameter column with a 100 μl min^{-1} flow rate. Two minute fractions were collected into 96-well plates. Eluting fraction that appeared to contain a single peptide was selected for N-terminal sequencing. Ninety μl of the fraction was loaded onto a biobrene-treated, precycled glass fibre filter and subjected to 13 cycles of Edman N-terminal sequencing using an Applied Biosystems 494 Procise Protein Sequencing System on a Pulsed Liquid sequencing method. Performance of the sequencer is assessed routinely with 10 pmol β-Lactoglobulin standard.

4.2.8 cDNA sequence of NAP50

A.affine cells were grown as mentioned previously. During exponential growth, 200 ml culture were collected and centrifuged at 4℃ with 1500 x g for 15 minutes. The cell pellets were resuspended in Trizol reagent (Roche, Switzerland) for RNA isolation according to the manual instructions. First-strand cDNA was synthesized with approximately 2 μg of total RNA extracted. Reverse transcribing reaction was performed by SuperScript™ III RT (Invitrogen, USA) with random primers according to the instructions of the manufacturer. Synthesized cDNA was used as template for PCR with degenerate primers designed from the peptide sequences of NAP50 (Table 4.1). PCR were performed under conditions: 95℃ 5min; 35 cycles of 94℃ 45s, 55℃ 45s and 72℃ 2min; 72℃ 10min. PCR products were cloned into pGEM-T easy vectors (Promega, USA) prior to DNA sequencing. DNA sequencing of all cloned plasmids were performed by commercial facilities using traditional dideoxy-methodolgy.

The 3’ ends of NAP50 cDNA were obtained using the RACE method (GeneRacer kit, Invitrogen, USA). This method was originally designed to synthesize

and amplify cDNA selectively from mRNA molecules that have a poly (A) tail on the 3' end. For the subsequent nested PCR, precisely matched primers were designed based on the partial cDNA sequences obtained. Table 4.2 has shown all the PCR primers used in RACE. PCR was carried out for 95℃ 5min; 35 cycles of 94℃ 45s, 50℃ 45s and 72℃ 1min; 72℃ 10min. PCR products were cloned into pGEM-T easy vectors (Promega, USA) prior to DNA sequencing. DNA sequencing of all cloned plasmids were performed by commercial facilities using traditional dideoxy-methodolgy.

The N-terminal of NAP50 was found by N-terminal Edman protein sequencing. The sequencing was done in The Protein Structure Core Facility (PSCF) of university of Nebraska, US. Briefly, NAP50 from the 2-DE was transferred onto PVDF membrane as mentioned previously. The PVDF membrane-bound proteins were visualized by 0.1% coomassie blue R-250 in 40% methanol for 1 min, and then destained in 40% methanol and 10% acetic acid. NAP50 was excised and subjected to N-terminal sequencing.

Table 4.1 Degenerate primers designed for PCR of NAP50 cDNA

Primer	Degenerate primer sequence (5'→3')	Peptide sequences	Sources
NAP1F	GGNTTYAARGAYGAYTTYGAYGCN	GFKDDFDAWR	Edman microsequencing
NAP1R	NGCRTCRAARTCRTCYTTRAANCC		
NAP2F	GGNATHTGGGARGARYTNCCNAAR	GIWEELPK	Edman microsequencing
NAP2R	YTTNGGNARYTCYTCCCADATNCC		
NAP3F	TTYGCNGAYTGGAAYGCNGARTA	AFADWNAEYE	LC MS/MS
NAP3R	RTAYTCNGCRTTCCARTCNGC		

F: forward primer
R: reverse primer

Table 4.2 Primers used in RACE of NAP50

Primer	Sequences (5'→3')
3'Race_f	GCTGGGGCACTGCTGACGTGCTTTAC
3'Race_f_nested	CGTGCTTTACAAGTCTGAGGCCATTG
Generacer 3'	GCTGTCAACGATACGCTACGTAACG
Generacer 3'_nested	CGCTACGTAACGGCATGACAGTG

4.3 Results and discussion

4.3.1 Differential protein expressions of *A.affine* under nitrogen stress

Vegetative cells of *A.affine* grown in N-depleted and repleted conditions were harvested and analyzed by 2-DE. In the beginning of the experiment, 2-D gels with pH range 3-10 were used to analyze the protein expression (Figure 4.1). Such broad pH range allow a large overview of the protein expressions in the 2-DE. However, most of the proteins were located at the acidic side of the gels. It was also compatible to the results of Chan et al. (Chan et al., 2005; Chan et al., 2004a; Chan et al., 2004b; Chan et al., 2002; Chan et al., 2006). The 2-D gels from their studies also showed that most of the proteins of *P. triestinum* were appeared in the acidic side of the gels. In general, resolution of the 2-DE electropherogram using pH range 3–10 was unsatisfactory because most of the protein spots were crowded at the middle part of the gel. Therefore, it was difficult to find out the differential expressed proteins from the gels. To better resolve the 2-DE electropherogram, we decided to perform all the other 2-DE in the pH range 4–7 (Figure 4.2). Given that equal amounts of proteins were loaded onto each gel and the intensities of silver staining were normalized with standard markers, several groups of differentially expressed proteins were found (circled in Figure 4.2). Moreover, the differentially expressed proteins in 2-DE of N-repleted 24 hr were almost the same as that of N-repleted 48hr. It indicates that most of the protein expressions have already changed in the lag phase (first 24 hours) after N replenishment (refer to chapter II, Figure 2.6). Since amount of cells in N-repleted 48hr were much higher than that of N-repleted 24hr and the higher amount of cells would allow more proteins to be extracted, 2-DE from N-repleted 48hr would be used to compared that with N-depleted for subsequent 2-DE analysis.

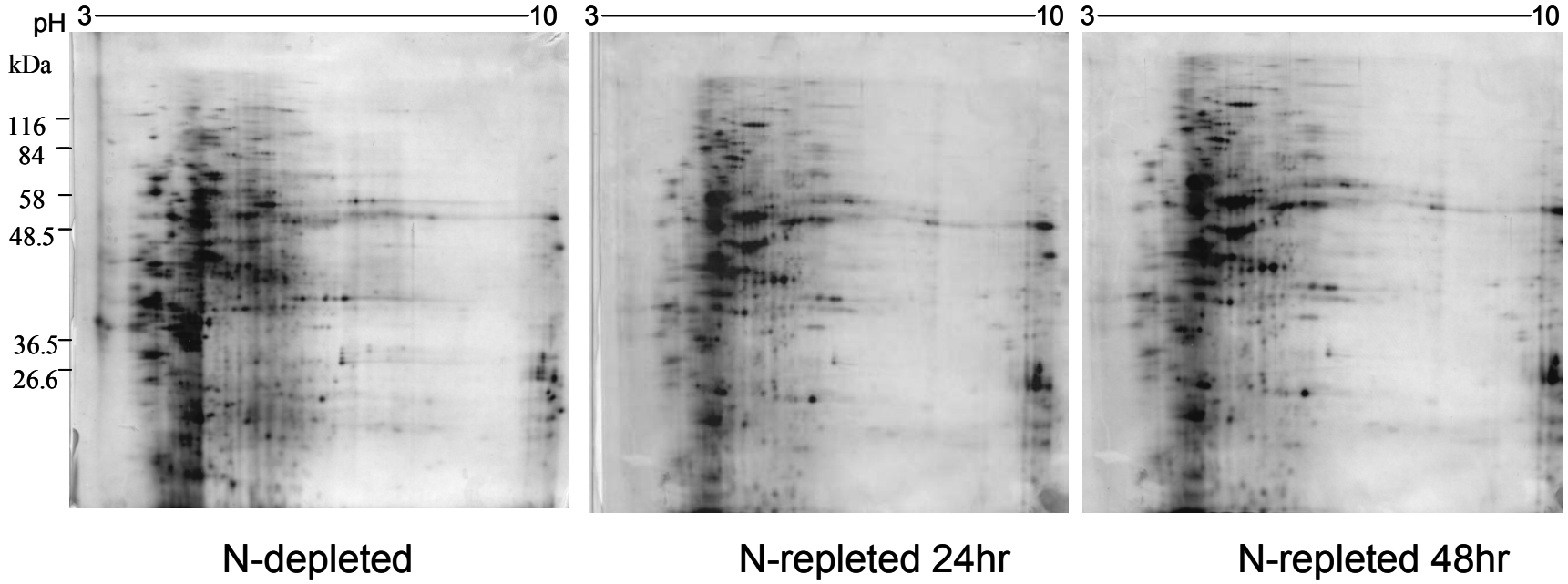


Figure 4.1 2-DE protein expression profiles of 40 µg proteins extracts of *A. affine* over a pH range of 3 to 10. Three conditions: N-depleted; N-repleted 24hr and N-repleted 48hr are shown as indicated. The second dimension was a SDS-PAGE with 10% polyacrylamide gel. Protein visualization was performed with silver stained.

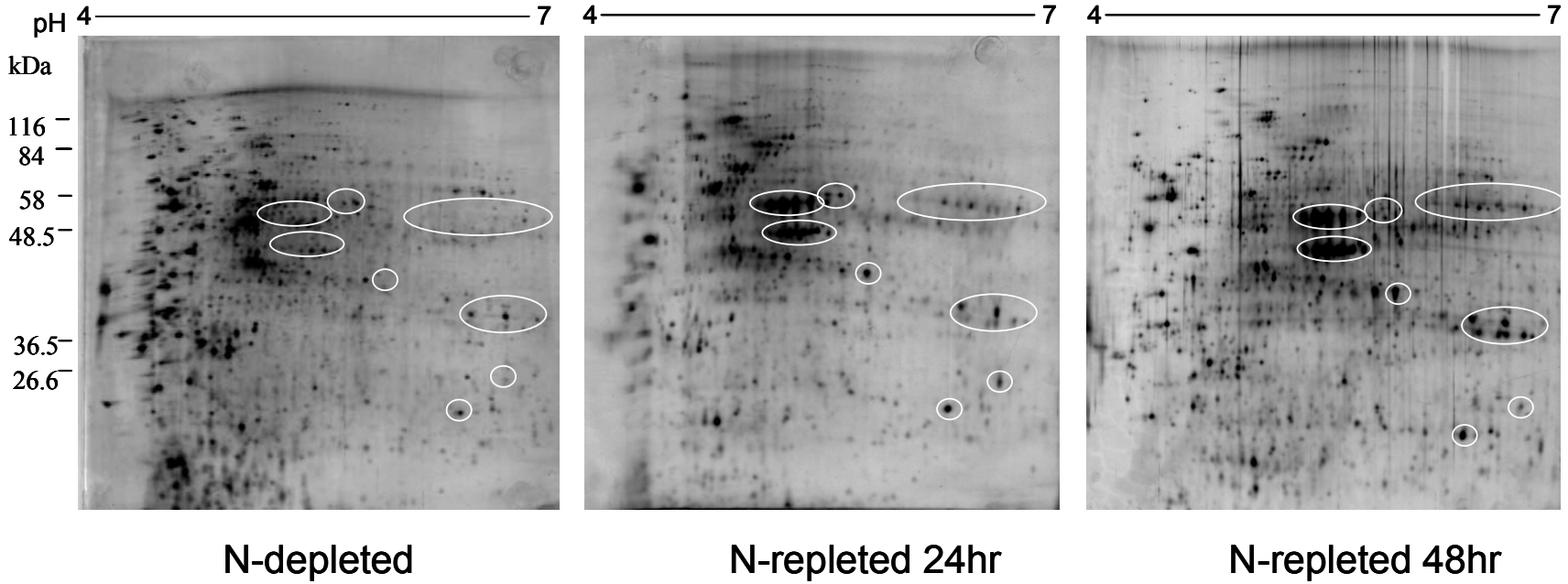


Figure 4.2 2-DE protein expression profiles of 60 µg protein extracts of *A. affine* over a pH range of 4 to 7. Three conditions: N-depleted; N-repleted 24hr and N-repleted 48hr are shown as indicated. The second dimension was a SDS-PAGE with 10% polyacrylamide. Protein visualization was performed with silver stain. Differential expressed proteins are circled.

Amounts of proteins available in silver staining gel (~ pg to ng) were generally enough to obtain a PMFs with MALDI-TOF MS. However, proteins stained with coomassie brilliant blue (~μg) were usually required for the *de novo* protein sequencing, especially when performing MALDI-PSD. The higher the amount of protein provided, the higher the success for amino acid sequencing. Given that most of the genome of *A. affine* is unknown, it is important to obtain coomassie blue stained 2-DE gels with higher protein loading for subsequent *de-novo* sequencing. Seven hundred μg of total proteins of *A. affine* from both N-depleted and repleted conditions were subjected to 2-DE analysis (Figure 4.3). The proteins were clearly resolved and the differentially expressed proteins found (circled) were same as that in the 2-DE stained with silver stained (Figure 4.2). The differentially expressed proteins were numbered (arrow) according to Table 4.3. As detected by 2-DE gel analysis software Melanie 3, the total protein spots of both 2-DE gels from N-depleted and N-repleted were 1736 and 1853 respectively. However, only proteins with high expression fold changes (at least by 5 folds) were selected (Table 4.3).A total of 22 differentially expressed proteins (spot1- 22) were found from 2-DE with pH 4-7 (Figure 4.3). Although the proteins were better resolved in the 2-DE over the pH range of 4-7 when compared to pH 3-10, some differentially expressed proteins may still be too close to others. Since most proteins were located around pH 4-6, 2-DE gels were repeated using IEF strips with pH range 4-5 (Figure 4.5) and pH range 5-6 (Figure 4.4). From the results of these gels, in addition to the 22 differentially expressed proteins found previously (from Figure 4.3), a further 11 differentially expressed proteins were found. These proteins were also numbered according to Table 4.3 and they were selected based on the 5-folds changes cut off point as mentioned

previously. Among these 11 proteins, 5 were found from 2-DE gel with pH range 5-6 (spot 23- 27) (Figure 4.4) and another 6 were found from 2-DE with pH range 4-5 (spot 28- 33) (Figure 4.5). Therefore, by comparing the 2-DE of N-depleted and N-repleted, a total of 33 differentially expressed proteins were found (Figure 4.6 and Table 4.3). These proteins were selected and focused for further investigations. It should be stressed that these proteins exhibited more than 5 folds in their protein expression. This significantly reduces the possibility of finding false positive differentially expressed proteins. Out of these 33 differentially expressed proteins, 12 of them showed at least 15-fold of differential expression (Table 4.3). Moreover, most of them were up-regulated in N-repletion and only 2 (spot 22 and 30) were down-regulated. These nitrogen responsive proteins were believed to be involved in a wide range of cellular processes that may facilitates the cells to growth rapidly upon resumption of nitrogen supply. Because of their tight association with the availability of nitrogen, these differentially expressed proteins were named as nitrogen-associated proteins (NAPs).

On the other hands, we found that the 2-DE profile of N-repleted 48hr was highly similar to the 2-DE profile of mid-log phase (Figure 4.7). It strongly suggested that protein expressions were resumed to their exponential growth status after nitrogen was replenished to nitrogen-depleted cultures. Although studying the protein expressions in different growth phases were not the scope of the present study, it can easily be observed that there were some groups of proteins which decreased their protein expressions when the cells went from mid-log phase of growth to stationary phase (Figure 4.7). Therefore, these results further implied that the N-repleted cultures were growing exponentially rather than keeping at stationary growth.

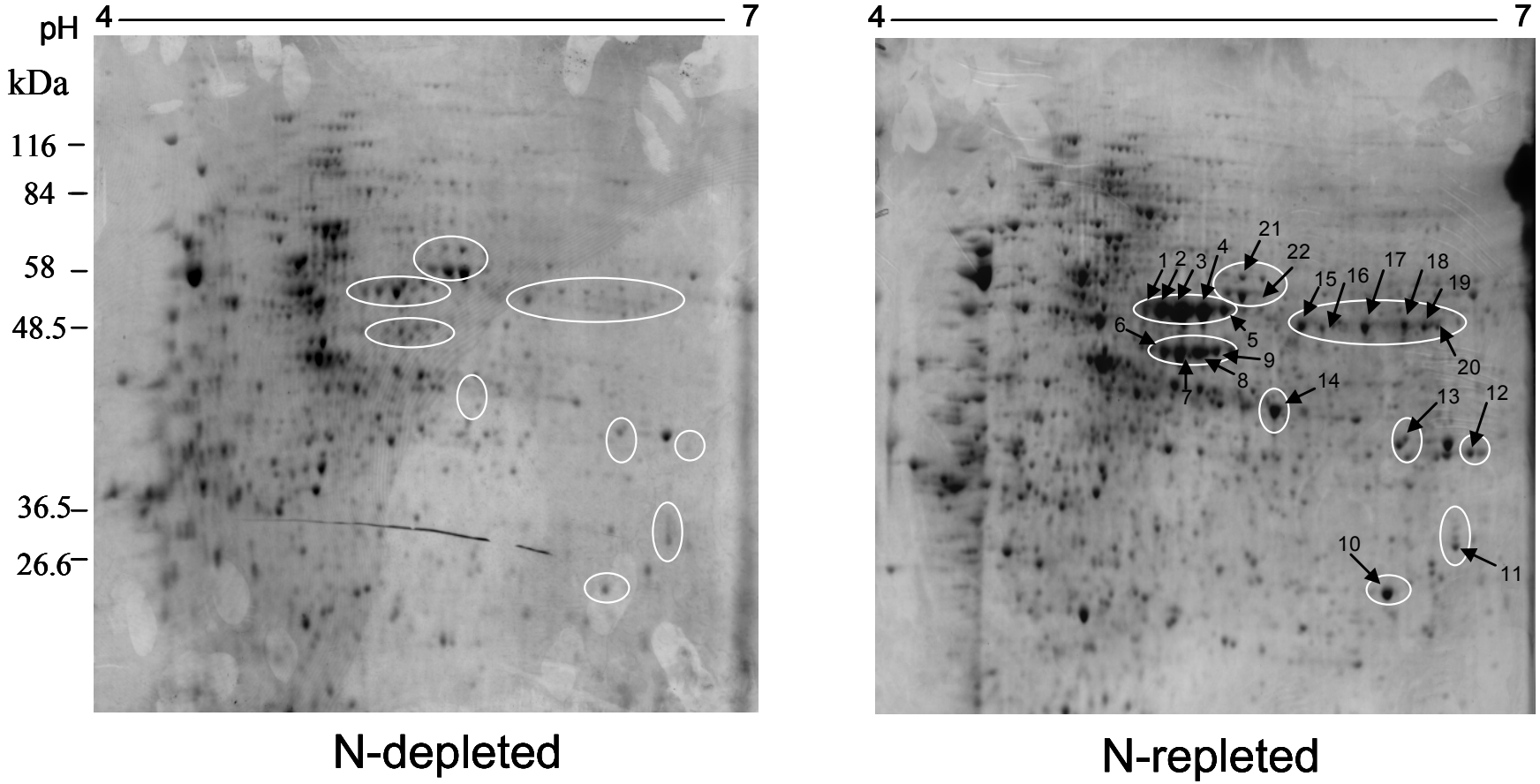


Figure 4.3 2-DE protein expression profiles of 700 µg **of protein extracts of *A. affine* over a pH range of 4 to 7.** Two conditions: N-depleted and N-repleted 48hr are indicated. The second dimension was a SDS-PAGE in a 10% polyacrylamide gel. Proteins visualization was performed with coomassie blue staining. Differential expressed proteins are circled and numbered according to Table 4.3.

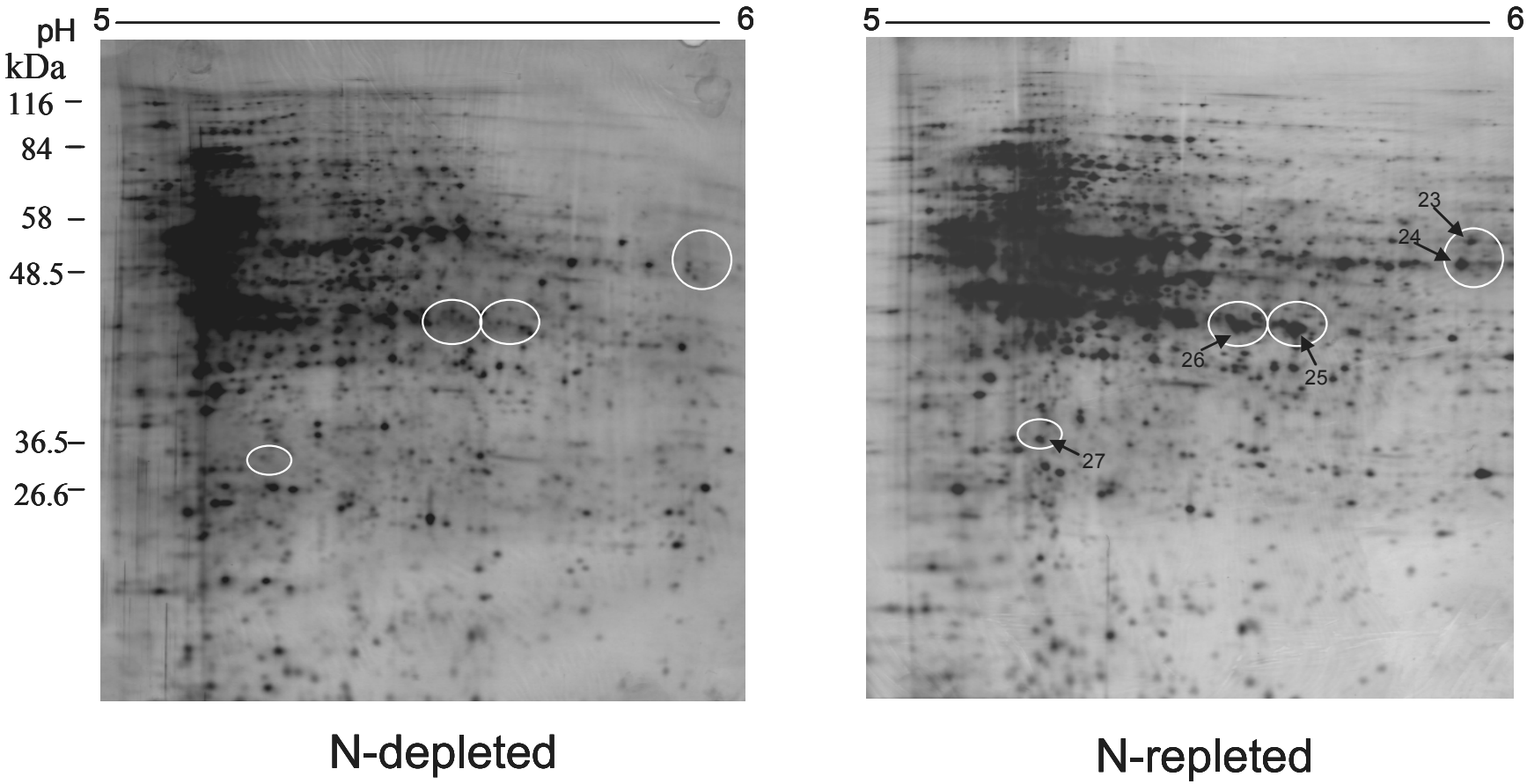


Figure 4.4 2-DE protein expression profiles of 80 µg protein extracts of *A. affine* over a pH range of 5 to 6. Two conditions: N-depleted and N-repleted 48hr are indicated. The second dimension was a SDS-PAGE in a 10% polyacrylamide gel. Protein visualization was performed with silver staining. Differential expressed proteins are circled and numbered according to Table 4.3.

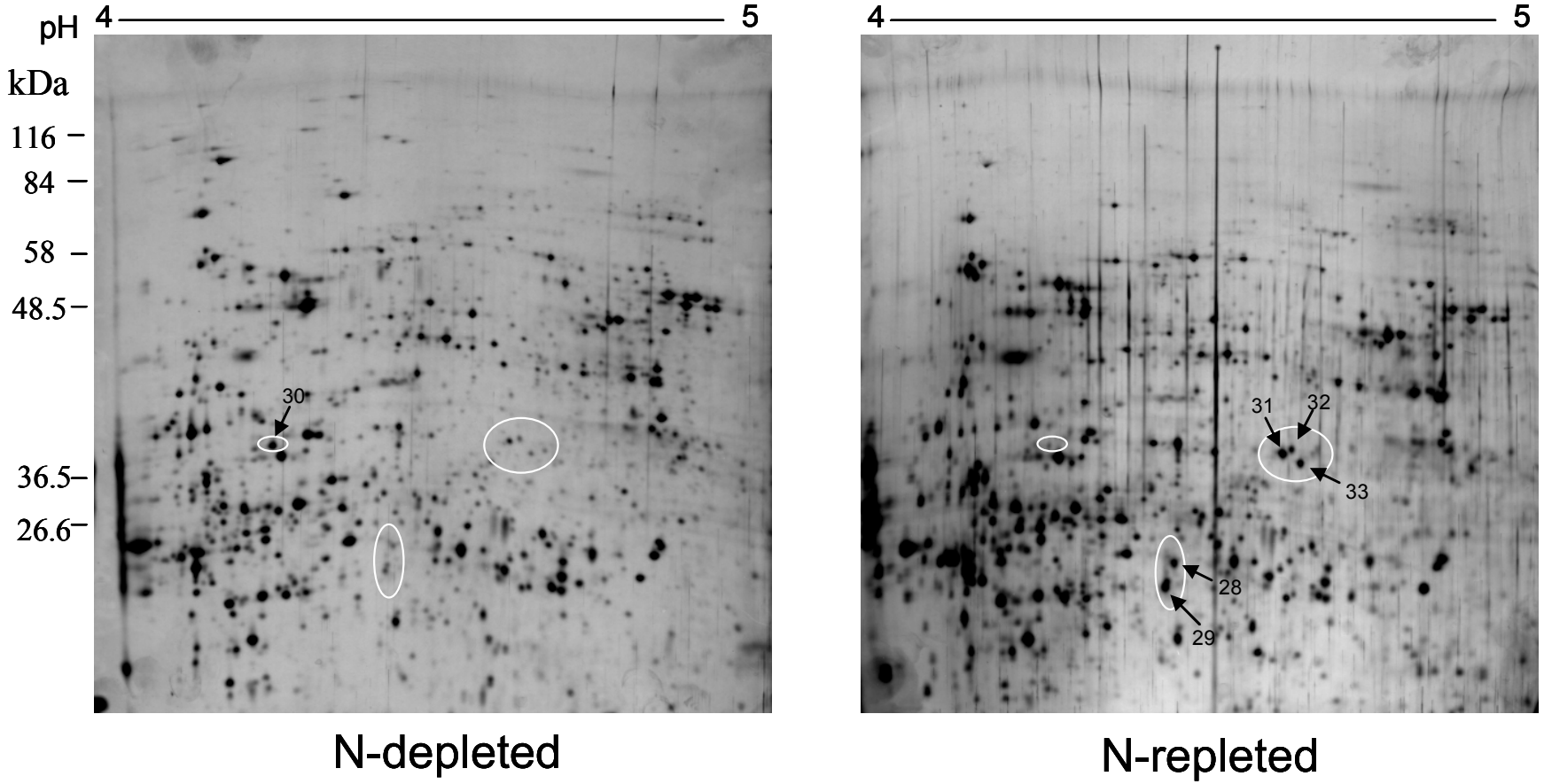


Figure 4.5 2-DE protein expression profiles of 80 µg protein extracts of *A. affine* over a pH range of 4 to 5. Two conditions: N-depleted and N-repleted 48hr are indicated. The second dimension was a SDS-PAGE in a 10% polyacrylamide gel. Protein visualization was performed with silver staining. Differential expressed proteins are circled and numbered according to Table 4.3.

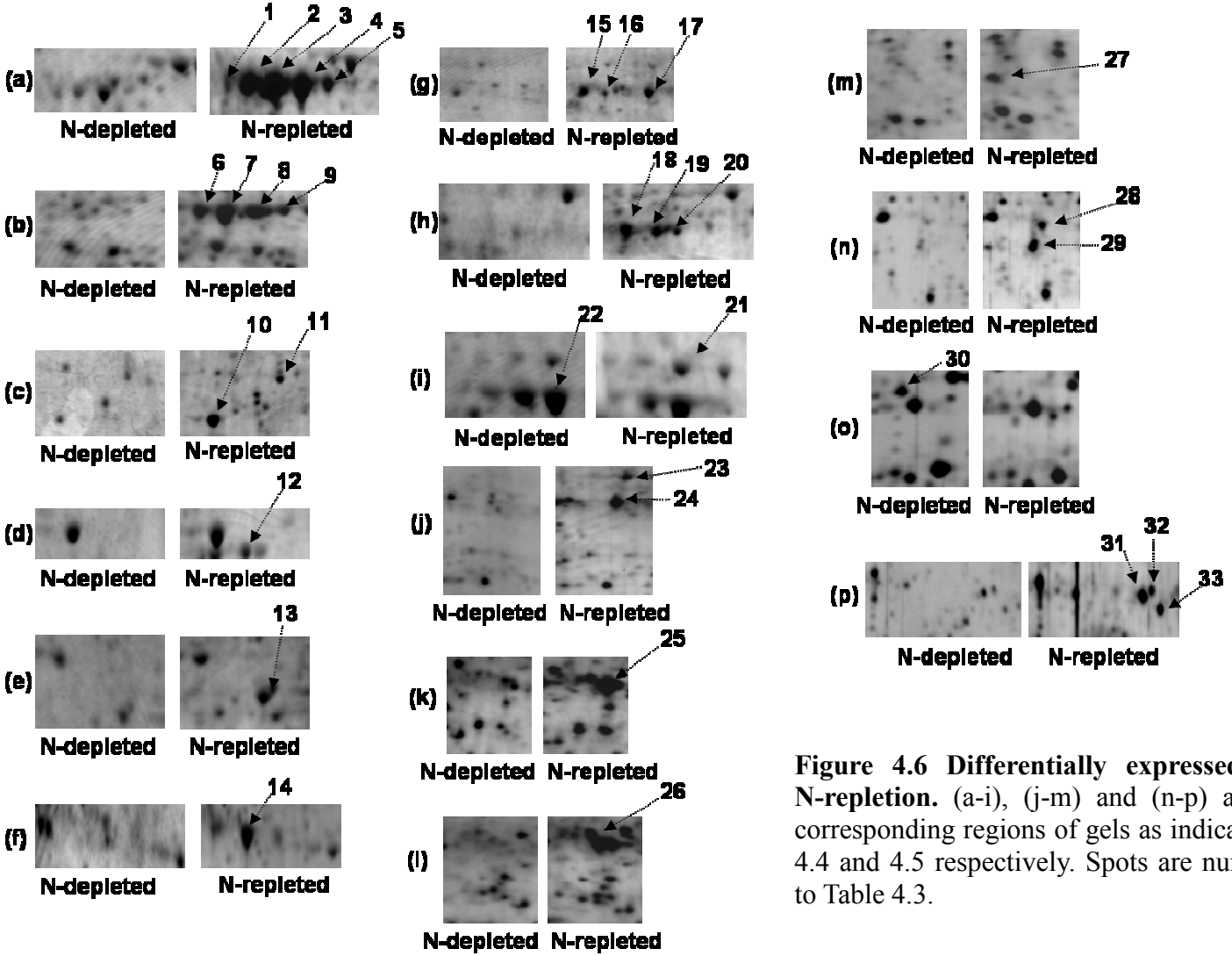


Figure 4.6 Differentially expressed proteins after N-repletion. (a-i), (j-m) and (n-p) are the magnified corresponding regions of gels as indicated in Figure 4.3, 4.4 and 4.5 respectively. Spots are numbered according to Table 4.3.

Table 4.3 Differentially expressed proteins as analyzed by 2-DE (with expression changes by at least 5-folds)

Spot	Isoelectric point *(pI)*	Molecular mass (kDa)	Protein expression [a]	Folds of change [b]
1	5.3	55	up-regulated	18*
2	5.4	55	up-regulated	17*
3	5.5	55	up-regulated	17.5*
4	5.6	55	up-regulated	18*
5	5.7	55	up-regulated	18*
6	5.4	50	up-regulated	17*
7	5.5	50	up-regulated	18.6*
8	5.6	50	up-regulated	18.6*
9	5.7	50	up-regulated	17.3*
10	6.4	25	up-regulated	15.5*
11	6.7	30	up-regulated	6.4
12	6.8	38	up-regulated	10.9
13	6.5	38	up-regulated	10.1
14	5.9	40	up-regulated	16.2*
15	6.0	53	up-regulated	10.1
16	6.2	53	up-regulated	10.8
17	6.3	53	up-regulated	11.3
18	6.5	53	up-regulated	9.8
19	6.6	53	up-regulated	10.5
20	6.7	53	up-regulated	9.7
21	5.7	59	up-regulated	7.6
22	5.8	58	down-regulated	16.2*
23	5.9	57	up-regulated	13.2
24	5.9	55	up-regulated	13.8
25	5.7	40	up-regulated	14.8
26	5.6	40	up-regulated	14
27	5.3	36	up-regulated	8.2
28	4.5	26	up-regulated	10.1
29	4.5	20	up-regulated	10.4
30	4.3	38	down-regulated	11
31	4.7	38	up-regulated	7.1
32	4.7	38	up-regulated	7.8
33	4.7	37	up-regulated	7.3

[a] Protein expression according to spots in N-repleted 2-DE compared to that of N-depleted.

[b] Folds of changes of protein expressions were detected in terms of optical intensity of spots in N-repletion to that of N-depletion by 2-DE analysis software Melanie 3.

* Protein spot with differential expression over 15 -fold and selected for amino acid sequencing

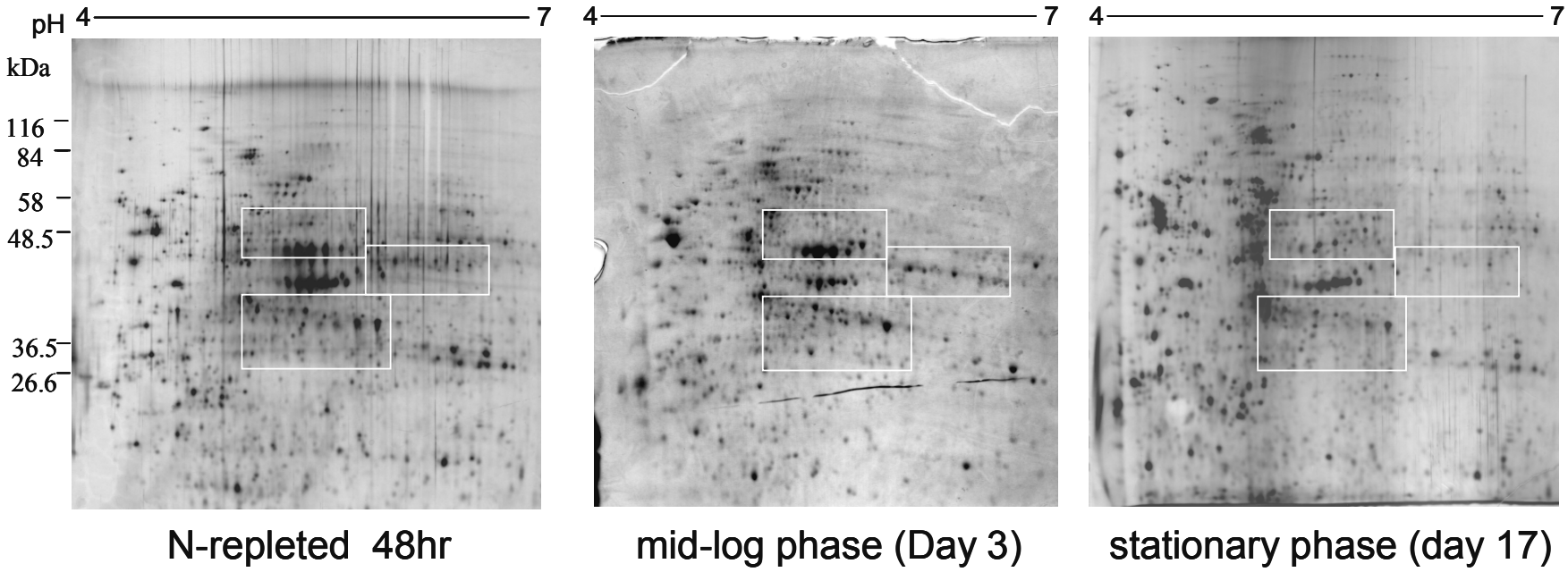


Figure 4.7 2-DE protein expression profiles of 60 µg of protein extracts of *A. affine* over a pH range of 4 to 7. Three conditions: N-repleted 48hr, mid-log phase and stationary phase are indicated. The second dimension was a SDS-PAGE in a 10% polyacrylamide gel. Protein visualization was by silver staining. Squares indicate differentially expressed proteins in the three conditions.

4.3.2 Protein identification by MALDI-TOF mass spectrometry

Attempts to identify all the differentially expressed proteins (as indicated in Table 4.3) were performed using in-gel tryptic digestion and MALDI-TOF MS. After tryptic digestion, peptide mass fingerprints (PMFs) of each protein were obtained (spot 1-5 in Figure 4.8; spot 6-9 in Figure 4.12; spot 10 in Figure 4.17; spot 14 in Figure 4.18; spot 22 in Figure 4.19 and the rest are in Appendix I). Out of all 33 differentially expressed proteins, only 5 protein spots were successfully identified. By bioinformatics searches from the NCBInr databases using the Mascot search engine with the PMFs obtained from spot 1-5 (Figure 4.8), it showed that spot 1-5 were the same protein. It is Ribulose 1,5-bisphosphate carboxylase/oxygenase II (Rubisco II). PMFs of these 5 protein spots were nearly identical (Figure 4.8). With the slightly different *pI* points in the 2-DE gels (*pI* ranged from 5.4-5.7, and the same molecular weights of 55kDa (Table 4.3)), it strongly suggested that these 5 proteins were isoforms of the same protein (Rubisco II). Figure 4.9 shows results of a MASCOT search using PMF obtained from spot 2. It shows that the protein is Rubisco II with a MOWSE score of 98. The MOWSE score represents the probability of the query protein matched with the identified protein from the database. According to the search algorithm of MASCOT, any protein with score higher than 64 is considered as significant (not by chance) and $p<0.05$. Besides the protein scores, sequence coverage of the peptide masses of spot 2 to the protein sequence of the matched protein was 15.2 % (Figure 4.10). To confirm if the proteins are indeed Rubisco II, two peptides (1000.431 and 1537.916 m/z) from the digested proteins were sulfonated and the amino acid sequences were analyzed by MALDI-PSD (Figure 4.11). As mentioned previously in section 1.4, sulfonation would greatly facilitate the amino acid

sequencing with MALDI-PSD by simply generates y-type ions in the PSD spectrum. It was clearly observed in the PSD spectrum of both sulfonated peptides (1215.55 and 1753.05 m/z) that the dominating peaks were mainly y-ions. Thus, amino acid sequences were easily deduced from the mass difference between adjacent y-ions with the mass tolerance limited to $\pm$ 0.5 Da. Sequence tag: QF[I/L]HYHR and YW[I/L]S[I/L]TEED[I/L][I/L]R were obtained. (Isoleucine and leucine have the same molecular mass and hence that amino acid was shown as I/L.) These sequence tags were also used to search against the NCBInr database. Sequence QF[I/L]HYHR matched with sequence QFLHYHR of Rubisco II of the *Symbiodinium spp.* (accession no. AAY51977). In addition, sequence YW[I/L]S[I/L]TEED[I/L][I/L]R matched with sequence YADLSLTEEDLIK of Rubisco II of the *Prorocentrum minimum* (accession no. AAO13049.1). Since the monoisotopic mass of tryptophan (W= 186.07) in YWI/LSI/LTEEDI/LI/LR is same as that of alanine (A= 71.04) + aspartic acid (D= 115.03) in YADLSLTEEDLIK, it suggested that both sequences were actually same as each other. Therefore, from the results of both the PMFs and peptide sequences data of spot 1-5 confirmed that they are Rubisco II. The significance of Rubisco II will be discussed in details in next chapter.

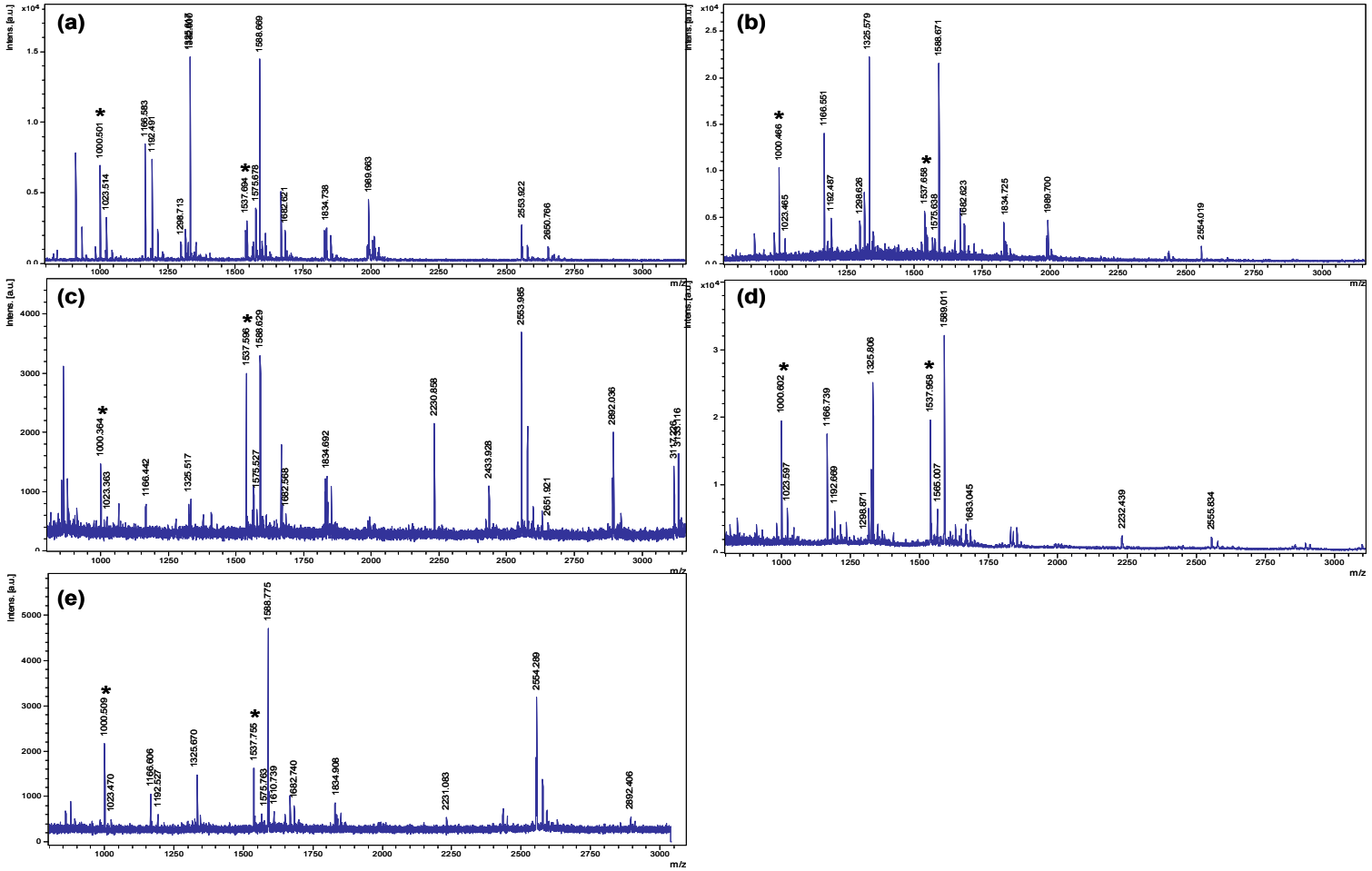


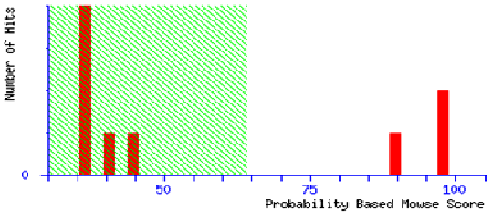
Figure 4.8 Peptide mass fingerprints (PMFs) of (a) spot 1; (b) spot 2; (c) spot 3; (d) spot 4 and (e) spot 5 obtained using MALDI-TOF mass spectrometry. Asterisk indicates peptides (1000.4 and 1537.9 m/z) that are found to be sulfonated and chosen for subsequent Post-source decay (PSD) analysis.

Mascot Search Results

```
User              : Fred lee
Email             : fredeflee@hotmail.com
Search title      :
Database          : NCBInr 20071006 (5535125 sequences; 1915185439 residues)
Taxonomy          : Other Alveolata (135036 sequences)
Timestamp         : 11 Oct 2007 at 09:07:21 GMT
Top Score         : 98 for gi|11545461, ribulose 1,5-bisphosphate carboxylase oxygenase large subunit precursor
```

Probability Based Mowse Score

Protein score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 64 are significant (p<0.05).



1. gi|11545461 **Mass:** 63469 **Score:** 98 **Expect:** 2.3e-05 **Queries matched:** 7
   ribulose 1,5-bisphosphate carboxylase oxygenase large subunit precursor [Symbiodinium sp.]

2. gi|75289311 **Mass:** 79611 **Score:** 97 **Expect:** 2.9e-05 **Queries matched:** 8
   Ribulose bisphosphate carboxylase, chloroplast precursor (RuBisCO)

3. gi|37727268 **Mass:** 43966 **Score:** 89 **Expect:** 0.00017 **Queries matched:** 6
   ribulose 1,5-bisphosphate carboxylase oxygenase form II [Prorocentrum minimum]
   gi|33324785 **Mass:** 34491 **Score:** 76 **Expect:** 0.0032 **Queries matched:** 5
   ribulose 1,5-bisphosphate carboxylase/oxygenase large subunit [Prorocentrum minimum]
   gi|37727246 **Mass:** 33177 **Score:** 76 **Expect:** 0.0035 **Queries matched:** 5
   ribulose 1,5-bisphosphate carboxylase oxygenase form II [Prorocentrum minimum]
   gi|37727256 **Mass:** 33178 **Score:** 76 **Expect:** 0.0035 **Queries matched:** 5
   ribulose 1,5-bisphosphate carboxylase oxygenase form II [Prorocentrum minimum]
   gi|37727272 **Mass:** 33205 **Score:** 75 **Expect:** 0.0043 **Queries matched:** 5
   ribulose 1,5-bisphosphate carboxylase oxygenase form II [Prorocentrum minimum]
   gi|37727260 **Mass:** 43978 **Score:** 71 **Expect:** 0.011 **Queries matched:** 5
   ribulose 1,5-bisphosphate carboxylase oxygenase form II [Prorocentrum minimum]
   gi|37727244 **Mass:** 43978 **Score:** 71 **Expect:** 0.011 **Queries matched:** 5
   ribulose 1,5-bisphosphate carboxylase oxygenase form II [Prorocentrum minimum]
   gi|33317821 **Mass:** 50468 **Score:** 68 **Expect:** 0.02 **Queries matched:** 5
   ribulose 1,5-bisphosphate carboxylase oxygenase large subunit [Prorocentrum minimum]

Figure 4.9 Representative MASCOT search results from NCBInr database search using the PMFs obtained in Figure 4.8. Result gave an identification of Ribulose 1,5-bisphosphate carboxylase oxygenase II (Rubisco II) of accession no as indicated and the highest MOWSE score was 98 (scores greater than 64 are considered as significant ($p<0.05$)).

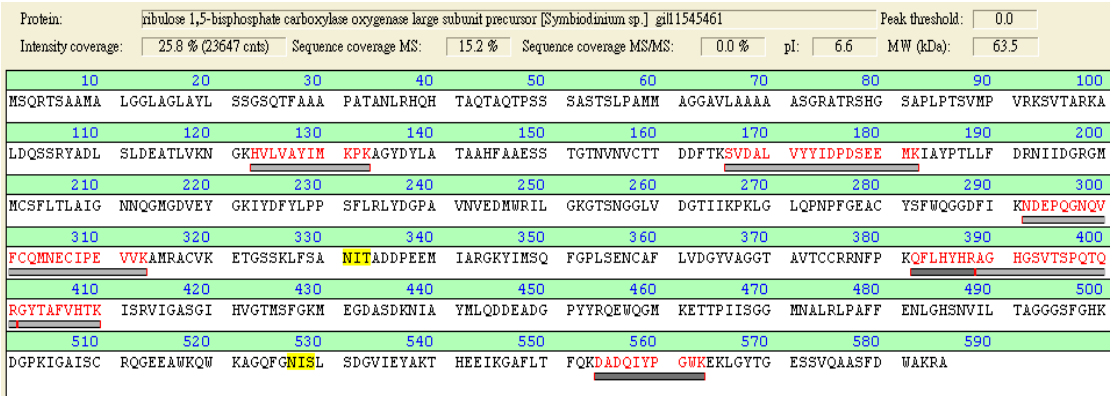


Figure 4.10 Sequence coverage of peptide masses in Figure 4.8 against the matched protein in the database.

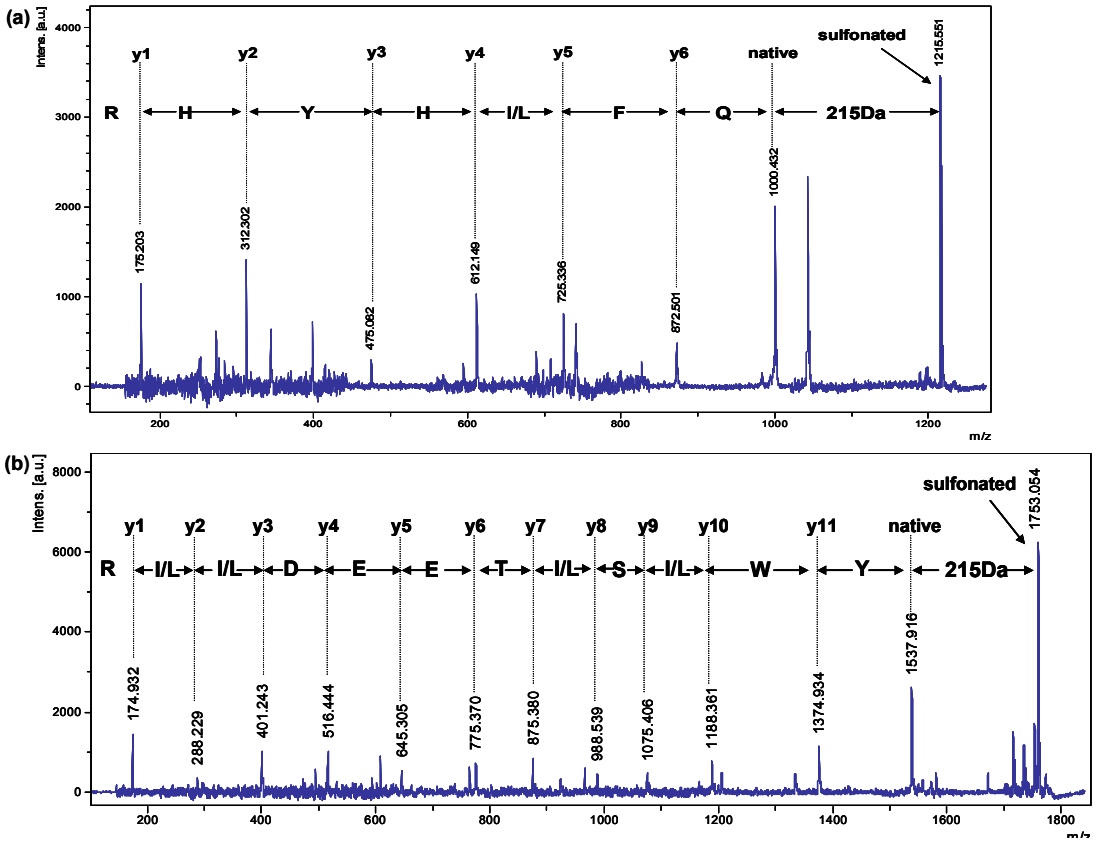


Figure 4.11 PSD spectrums of sulfonated peptides with (a) 1215.55 and (b) 1753.05 m/z. The sulfonated peptides1215.55 and 1753.05 m/z were derived from sulfonation of the native peptides 1000.431and 1537.916 m/z with SPITC (215 Da) respectively. Amino acid sequences are deduced from the mass difference between adjacent y-ions and 2 sequence tags were obtained: QF[I/L]HYHR and YW[I/L]S[I/L]TEED[I/L][I/L]R The second amino acid W may be actually be A and D as shown from the database, because molecular mass of Tryptophan (W) 186.07 = Alanine (A) 71.04 + Aspartic acid (D) 115.03.

4.3.3 *De novo* protein/peptide sequencing

Because of the limited DNA and protein sequences information of dinoflagellates in the database, it is not surprising that bioinformatic searches using PMFs obtained from the 28 proteins were not all successful. In our establishment, to take the identification further, the next level was *de novo* sequencing with sulfonation using MALDI-PSD. However, a significant amount of protein is required, i.e. they have to be reasonably strongly stained with Coomassie brilliant blue. Therefore, proteins that were of very high differential protein expressions (> 15-fold) and highly stained with coomassie blue were selected (spot 6-9, 10, 14 and 22) for amino acid sequencing with MALDI-PSD (Table 4.3). For other proteins, attempts are currently in progress either to obtain a sufficient enough amount for MALDI-PSD analysis, or to perform other amino acid sequencing methods such as N-terminal sequencing and LC MS/MS.

PMFs of spots 6-9 were highly similar with almost identical peptide masses (Figure 4.12). Results revealed that these multiple protein spots were isoforms of the same protein. Their differences in *pI* could be accounted for the post-translational modification (PTM) of the protein. Spots 6-9 were located just slightly below that of Rubisco II (spot 1-5) (Figure 4.3) and with around 50kDa, *pI* ranged from 5.4-5.7 (Table 4.3). A peptide (1152.5 m/z) was found to be sulfonated and therefore chosen for subsequent Post-source decay (PSD) analysis. Amino acid sequences (YHFATMN[I/L]R) are deduced subsequently (Figure 4.12). Beside Rubisco II (Spots 1-5), their differential protein expressions were the highest among all the differentially expressed proteins found, with more than 17-folds differences (Table 4.3). Thus, further investigations on this group of proteins were taken. In order to obtain more amino acid sequences of this group of proteins, LC-MS/MS and Edman

microsequencing were also performed. For the LC-MS/MS, digested peptides from the proteins were separated by loading onto a reverse-phase C-18 column. This fractionation was aimed to restrict the amount of peptides to one or two in each fractions prior entering to the ion-trap mass spectrometry. The elution profile was shown in Appendix II. Peptide with the highest amount in each fraction was selected and further fragmented by collision induced dissociation (CID) to produce a ladder sequence. However, one of the difficulties for performing amino acid sequencing with LC-MS/MS is the analysis of the MS/MS spectrum. Unlike sulfonation-MALDI-PSD, more than one type of ions would be generated from the ion-trap MS/MS. Although ions generated would mainly be y-, a- and b- type ions, the interpretation of the *de novo* amino acid sequences is complicated. Nevertheless, four relatively more reliable sequences from four peptides were found: 626.2 (2+) *m/z* (Figure 4.13); 542.6 (2+) *m/z* (Figure 4.14); 672.2 (2+) *m/z* (Figure 4.15) and 654.2 (2+) *m/z* (Figure 4.16). The sequences were deduced with the aid of softwares (Data analysis and Biotools 3.0; Bruker, Germany). Both the MS and MS/MS spectrum, together with the deduced amino acid sequences were shown below: QVETEASNMK (Figure 4.13); ALDAPLA (Figure 4.14); AFADWNAEYE (Figure 4.15) and FFEAESADY (Figure 4.16). For the Edman microsequencing, the digested peptides after separated by the reverse-phase column, instead of LC MS/MS, they were subjected to Edman sequencing. Three peptides (P1-F-05, P1-F-06 and P1-G06) from the eluted fractions were selected for N-terminal sequencing (Appendix III). Amino acid sequences of the three peptides obtained were: AFA[G/D/I][T/W/D]AA[V/E/G][L/Y/M]YK (P1-F-05 fragment); GFKDDFDAWR (P1-F-06 fragment) and GIWEELPK (P1-G06 fragment) (Appendix IV). The uncertain amino acid sequence of P1-F-05 was attributed to the

likely presence of more than one peptide in the same fraction.

For spot 10, peptide (1813.9 m/z) was found to be sulfonated and chosen for subsequent Post-source decay (PSD) analysis. Amino acid sequences (NXYSQ[I/L]TYNQVR) were deduced from the mass difference between adjacent y-ions of the spectrum (Figure 4.17). "X" represents gap in the peptide sequence which contain one or more amino acids. This protein has a relatively lower molecular mass with around 25kDa, and *pI* was around 6.4 (Table 4.3). For spot 14, two peptides (1076.4 and 1819.2 m/z) were found to be sulfonated and chosen for subsequent Post-source decay (PSD) analysis. Amino acid sequences (AXA[I/L]NGFGR and NXYDNEWGYSXR) were deduced from the mass difference between adjacent y-ions of the spectrum (Figure 4.18). The molecular mass and *pI* of this protein were around 40kDa and 5.9 respectively (Table 4.3). Unlike proteins mentioned above, protein spot 22 was found to be highly down-regulated upon the N-repletion (Figure 4.3).Two peptides (886.4 and 1682.8 m/z) were found to be sulfonated and chosen for subsequent PSD analysis. Amino acid sequences (YFAGX[I/L]R and EXAD[I/L]XAADHFR) were deduced from the mass difference between adjacent y-ions of the spectrum (Figure 4.19). The molecular mass and *pI* of this protein were around 58kDa and 5.8 (Table 4.3).

Table 4.4 has summarized the *de novo* amino acid sequences obtained. Searching using these partial amino acid sequences against the NCBInr database, Protein DataBank and SWISS-PROT by BLAST (except that for Rubisco II, spot 1-5) revealed no any homology in the database. Nevertheless, using these amino acid sequences data, degenerate primers could be designed for PCR amplification of the corresponding genes. This may allow further investigation such as DNA sequencing

to be performed. Since these differential expressed proteins were all novel and highly associated with nitrogen, these proteins were named as nitrogen-associated proteins (NAPs). In addition, to be more specific, spot 6-9, 10, 14 and 22 were named as NAP50, NAP25, NAP40 and NAP58 respectively. The number indicates the molecular mass of the protein.

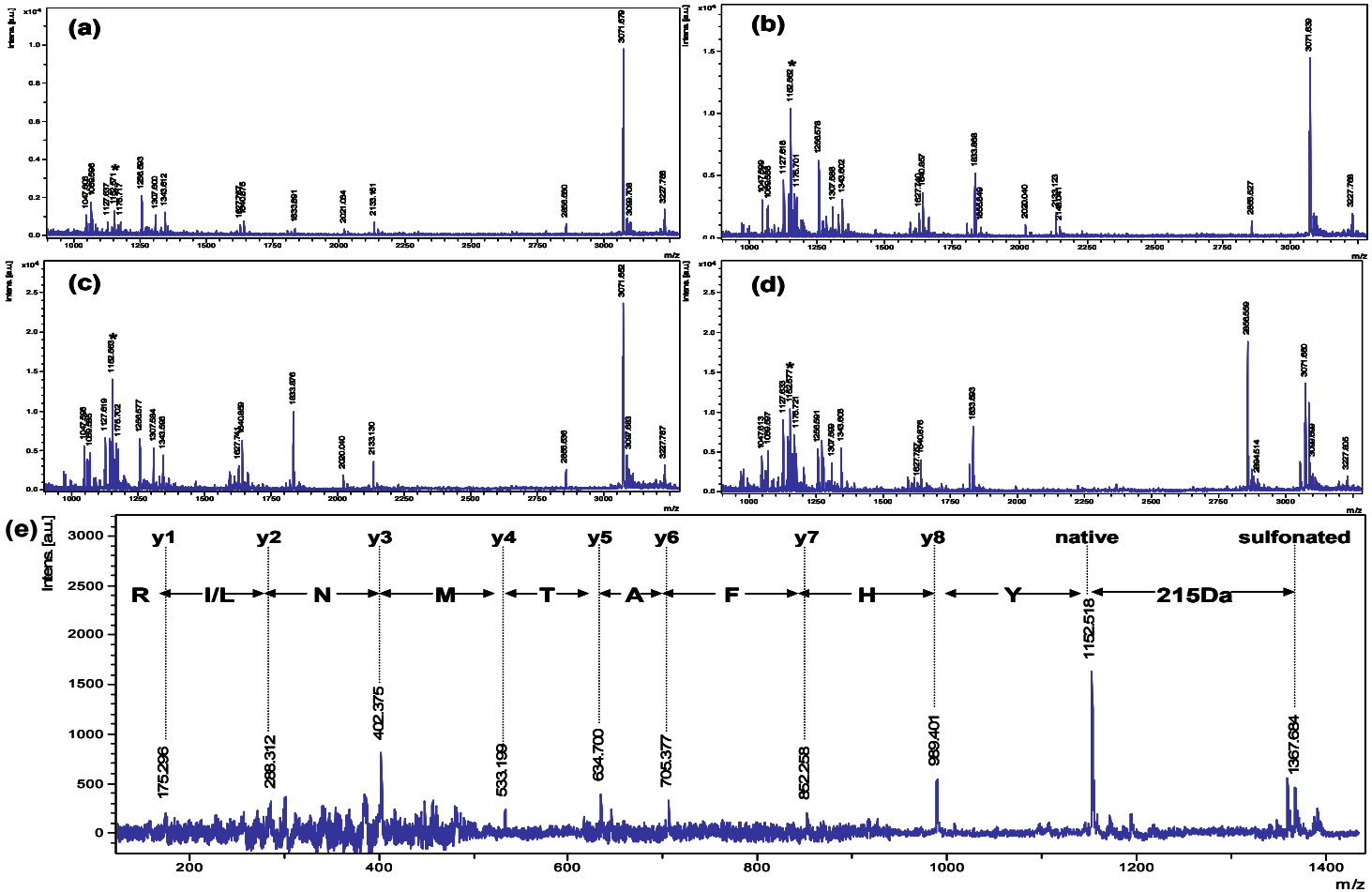


Figure 4.12 Peptide mass fingerprints (PMFs) of (a) spot 6; (b) spot 7; (c) spot 8; (d) spot 9 and (e) PSD spectrum obtained using MALDI-TOF mass spectrometry with sulfonated peptide ion 1367.684 (m/z). Asterisk indicates peptide 1152.5 (m/z), was sulfonated to form 1367.684 and hence chosen for subsequent PSD analysis. Amino acid sequence (YHFATMN[I/L]R) was deduced from the mass difference between adjacent y-ions of the spectrum.

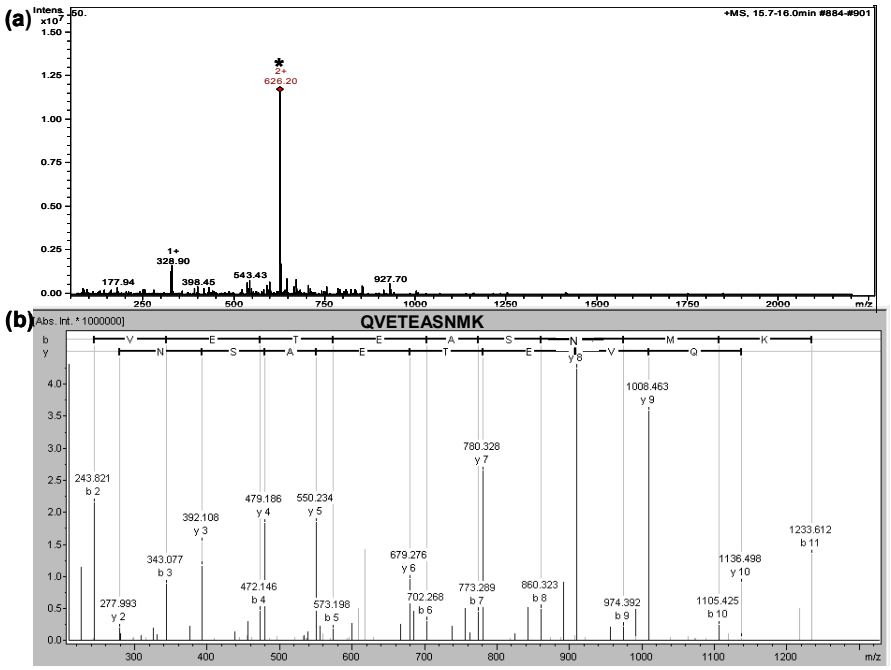


Figure 4.13 MS and MS/MS spectrum obtained from LC-MS/MS ion trap mass spectrometry. (a) MS spectrum of peptides eluted in fraction 50 from the LC (C-18 RP column). Asterisk indicates peptide was selected for MS/MS analysis. (b) MS/MS spectrum of the selected peptide [626.2 (2+) *m/z*]. Amino acid sequences (QVETEASNMK) derived from the y and b ions was indicated.

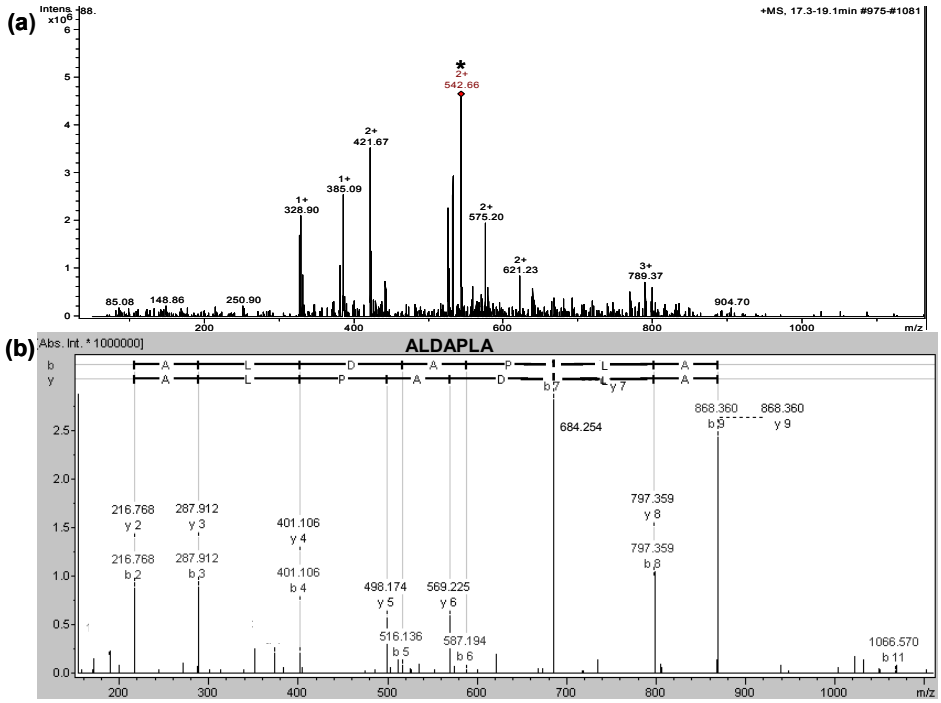


Figure 4.14 MS and MS/MS spectrum obtained from LC-MS/MS ion trap mass spectrometry. (a) MS spectrum of peptides eluted in fraction 88 from the LC (C-18 RP column). Asterisk indicates peptide was selected for MS/MS analysis. (b) MS/MS spectrum of the selected peptide [542.6 (2+) *m/z*]. Amino acid sequences (ALDAPLA) derived from the y and b ions was indicated.

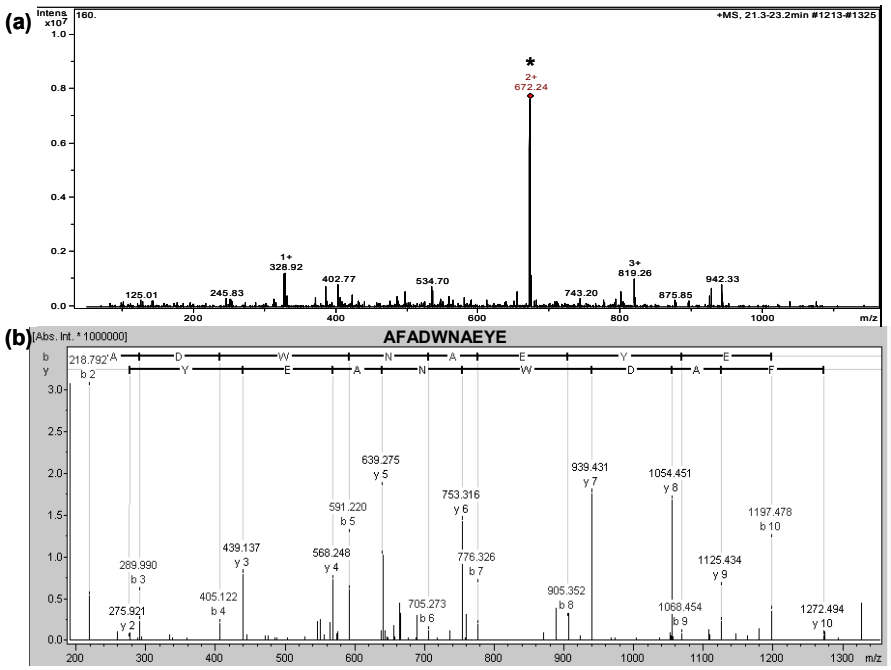


Figure 4.15 MS and MS/MS spectrum obtained from LC-MS/MS ion trap mass spectrometry. (a) MS spectrum of peptides eluted in fraction 160 from the LC (C-18 RP column). Asterisk indicates peptide was selected for MS/MS analysis. (b) MS/MS spectrum of the selected peptide [672.2 (2+) *m/z*]. Amino acid sequences (AFADWNAEYE) derived from the y and b ions was indicated.

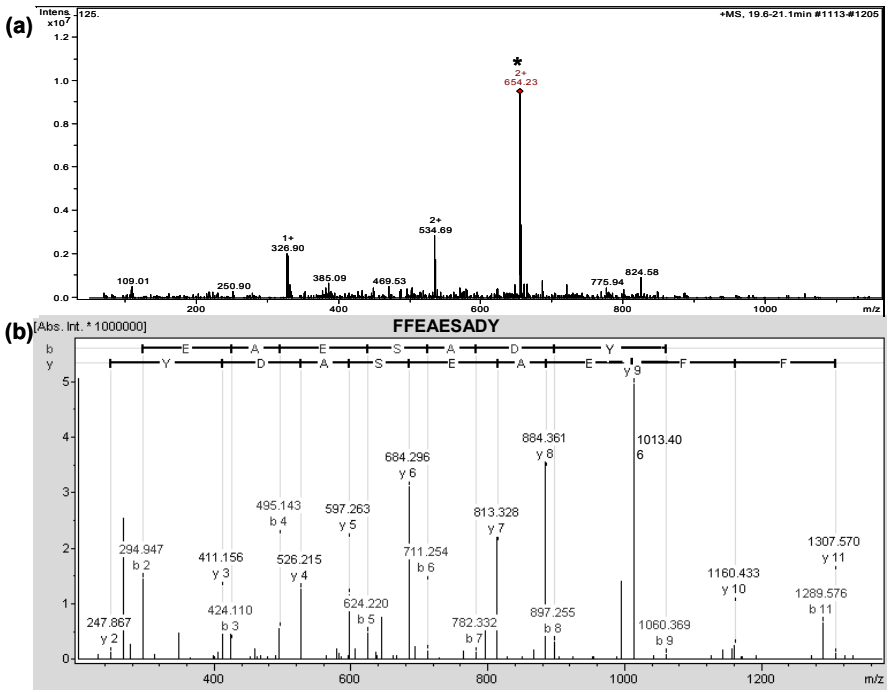


Figure 4.16 MS and MS/MS spectrum obtained from LC-MS/MS ion trap mass spectrometry. (a) MS spectrum of peptides eluted in fraction 125 from the LC (C-18 RP column). Asterisk indicates peptide was selected for MS/MS analysis. (b) MS/MS spectrum of the selected peptide [654.2 (2+) *m/z*]. Amino acid sequences (FFEAESADY) derived from the y and b ions was indicated.

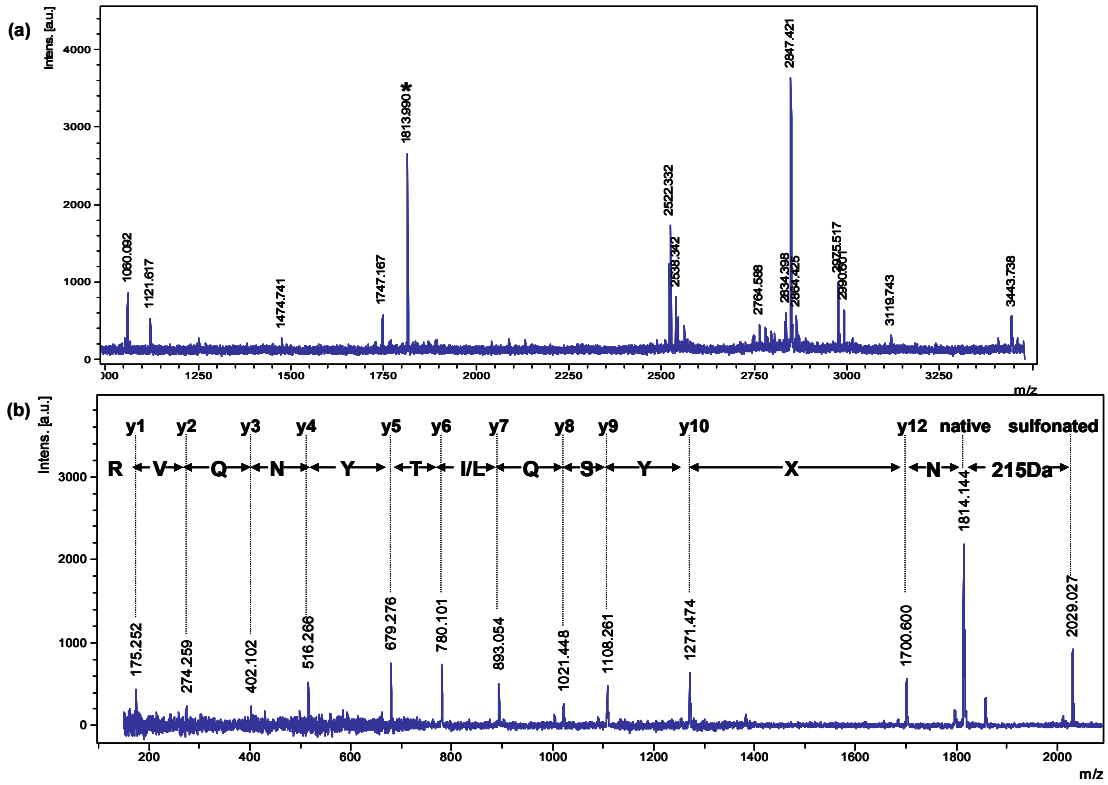


Figure 4.17 Peptide mass fingerprints (PMF) of (a) spot 10 and (b) PSD spectrum obtained using MALDI-TOF mass spectrometry. Asterisk indicates peptide (1813.9 m/z) that was found to be sulfonated and chosen for subsequent PSD analysis. Amino acid sequences (NXYSQ[I/L]TYNQVR) was deduced from the mass difference between adjacent y-ions of the spectrum. X represents gap in the peptide sequence which contain one or more amino acids

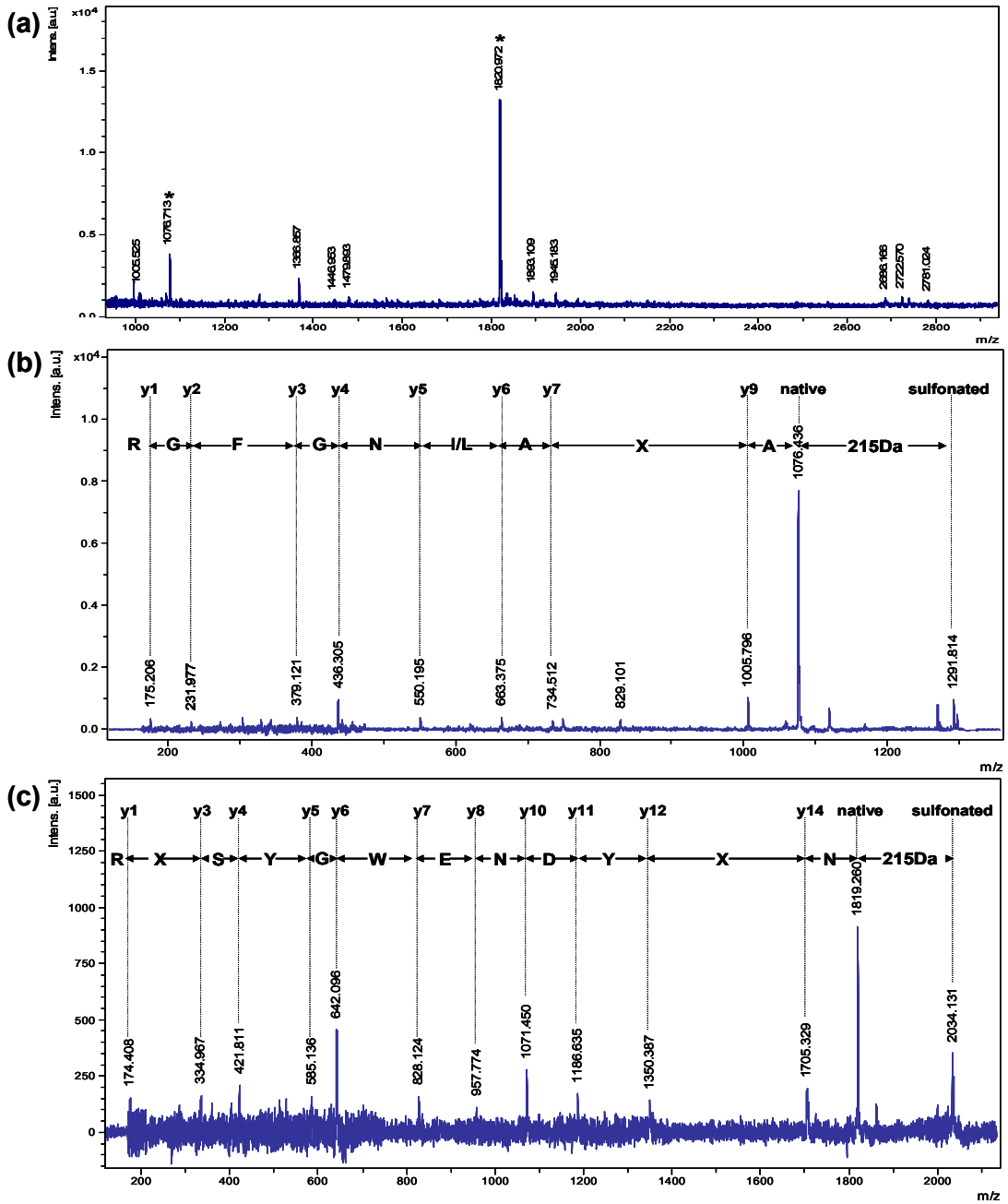


Figure 4.18 Peptide mass fingerprints (PMFs) of (a) spot 14 and PSD spectrum of peptide (b) 1076.4 and (c) 1819.2 *m/z* obtained using MALDI-TOF mass spectrometry. Asterisk indicates peptides (1076.4 and 1819.2 m/z) that were found to be sulfonated and chosen for subsequent PSD analysis. Amino acid sequences (AXA[I/L]NGFGR and NXYDNEWGYSXR) were deduced from the mass difference between adjacent y-ions of the spectrum. X represents gap in the peptide sequence which contain one or more amino acids

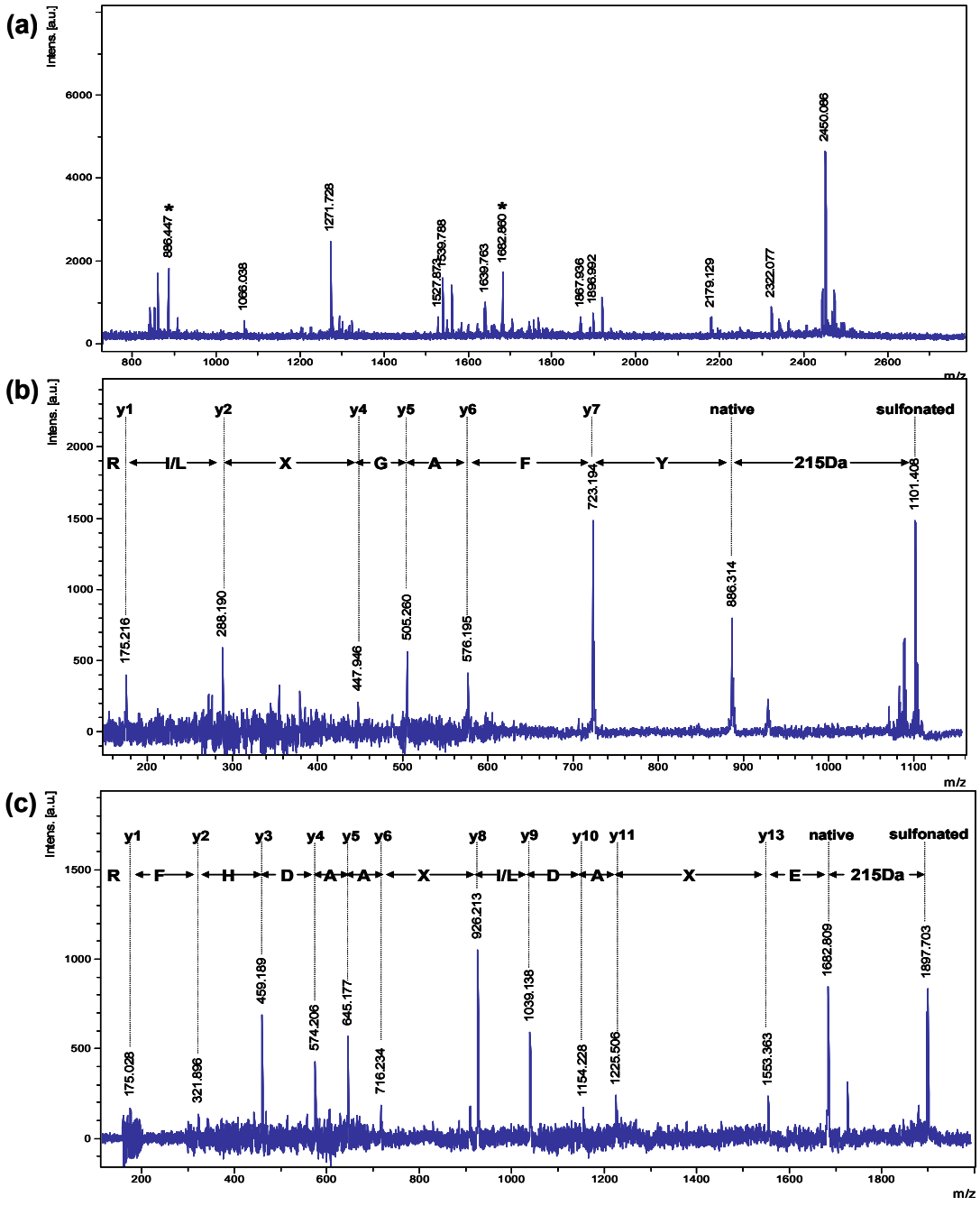


Figure 4.19 Peptide mass fingerprints (PMFs) of (a) spot 22 and PSD spectrum of peptide (b) 886.4 and (c) 1682.8 *m/z* obtained using MALDI-TOF mass spectrometry. Asterisk indicates peptides 886.4 and 1682.8 (m/z) that were found to be sulfonated and chosen for subsequent PSD analysis. Amino acid sequences (YFAGX[I/L]R and EXAD[I/L]XAADHFR) were deduced from the mass difference between adjacent y-ions of the spectrum. X represents gap in the peptide sequence which contain one or more amino acids

Table 4.4 Amino acid sequences of protein spots 1-5, 6-9, 10, 14 and 22.

Spot	Protein	Peptide mass (m/z)	Amino acid sequences	Sources
1-5 [b]	Rubisco II	1000.4 (1+) 1537.9 (1+)	QFI/LHYHR YWI/LSI/LTEEDI/LI/LR	Sulfonated-MALDI-PSD
6-9 [b]	NAP 50	1152.5 (1+)	YHFATMNI/LR	
		626.2 (2+) 542.6 (2+) 672.2 (2+) 654.2 (2+)	QVETEASNMK ALDAPLA AFADWNAEYE [a] FFEAESADY	LC MS/MS
		1237.5 (1+) 952.4 (1+) NA	GFKDDFDAWR [a] GIWEELPK [a] AFA(G/D/I)(T/W/D)AA(V/E/G)(L/Y/M)YK	Edman micro-sequencing
10	NAP 25	1814.1 (1+)	NXYSQI/LTYNQVR	Sulfonated-MALDI-PSD
14	NAP 40	1076.4 (1+) 1819.2 (1+)	AXAI/LNGFGR NXYDNEWGYSXR	
22	NAP 58	886.4 (1+) 1682.8 (1+)	YFAGXI/LR EXADI/LXAADHFR	

[a] Amino acid sequences were selected for designing degenerate primers for NAP50
[b] Spots believed to be isoforms of the same protein
X represents gap in the peptide sequence which contain one or more amino acids

4.3.4 Putative cDNA sequence of NAP50

At the outset of this work, a putative amino acid sequences of NAP50 (spot 6-9) from *A. affine* had been determined. Degenerate primers were designed from the peptide sequences obtained (Table 4.1). Three PCR products with around 1100 bp (with primers NAP1F + NAP3R); 800 bp (with primers NAP1F + NAP3R) and 300 bp (with primers NAP2F + NAP3R) were successfully obtained (Figure 4.20). DNA sequence of the 1100 bp product including that of the 800 bp and 300 bp products, suggesting that the three PCR products obtained were coming from the same gene. Sequences of 3' ends of the cDNA were found by RACE method subsequently. Gene-specific primers were designed from the location closed to the 3' ends (Table 4.2). Final PCR products of around 400 bp were obtained from the 3' RACE (Figure 4.20). For the N-terminal of the protein, it was found by the N-terminal Edman sequencing.

The length of the cDNA sequence obtained was around 1.5 kb. Sequence of the longest clone is given in Figure 4.21. This open reading frame contains 6 out of 7 peptide sequences obtained from the *de novo* sequencing (Table 4.4) and they were shown in underlining in Figure 4.21. The sequence ALDAPLA was not found in the derived amino acid sequence of NAP50, presumably correspond to contaminating proteins that were close and probably co-migrate with NAP50 on the 2-DE gels. Furthermore, the derived protein sequence with 400 amino acids with a predicted molecular weight of 47 kDa which is very close to the apparent molecular weight 50 kDa obtained from our 2-DE analyses. Therefore, it indicates that the sequence obtained does indeed encode NAP50. Further, the possibility that sequence of NAP50 might be derived from a bacterial contaminant in our unialgal (but not axenic) cultures

are highly unlikely, because the cDNA (3' RACE) was prepared from polyadenylated RNA. Hence to summarize, full amino acid sequence of NAP50 was obtained.

The entire derived protein sequence was also used in BLAST protein searches, but no positive identification could be found. Although it was very exciting to obtain the entire sequence of a novel protein, it's near zero degree of sequence identity is insufficient to predict the function of the NAP50. Results of cellular localization studies as well as changes in expression of NAP50 in relation to nitrogen loading are to be presented in Chapter V.

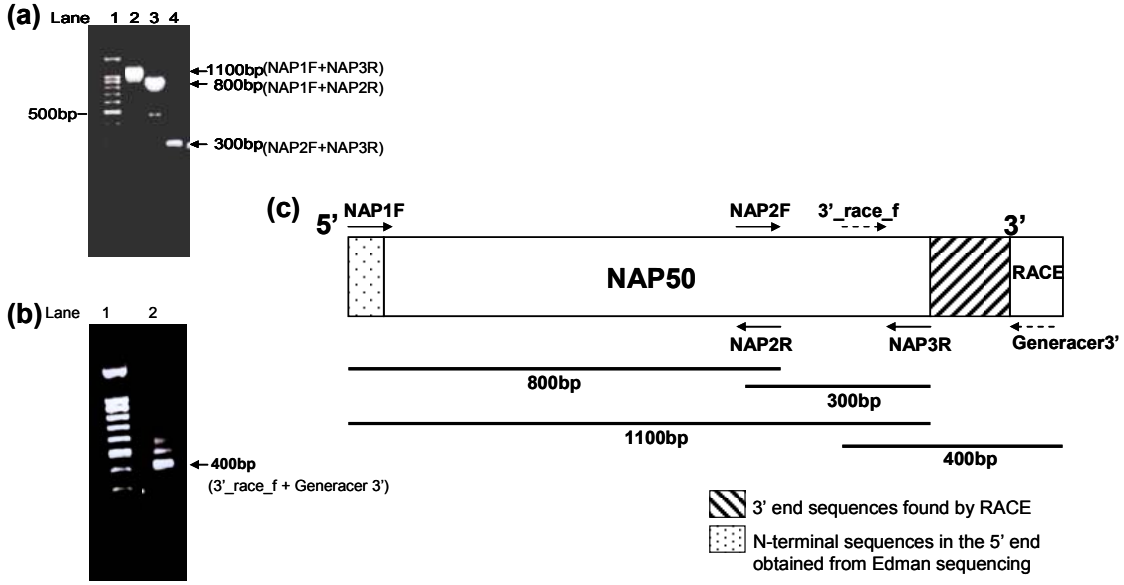


Figure 4.20 Determination of NAP50 sequences by the degenerate primers. (a) Three PCR products with around 1100bp (with primers NAP1F + NAP3R); 800bp (with primers NAP1F + NAP3R) and 300bp (with primers NAP2F + NAP3R) were successfully obtained from the PCR with the corresponding primers. (b) PCR products of around 400 bp were obtained from the 3' RACE. (c) schematic diagram showing NAP50.

ggattcaaggacgacttcgacgcctggcgctcctccttgagctctgaggagaaggcaatg
G F K D D F D A W R S S L S S E E K A M
ctccagaagcaggcgcggaacgagtatgacaagaagttccgcaagtctgacgaattcaaa
L Q K Q A R N E Y D K K F R K S D E F K
aagaacctgccagaggagaaggtgcagtctttcgcgaagatcttaggcaagtttttgaa
K N L P E E K V Q S F A K I L G K F F E
gcggagtccgcggactacacgaaggaggtcgctgcccggtcgcctgactacgatggcctc
A E S A D Y T K E V A A R S P D Y D G L
gccaagcgtgcgggggagaagaagatggacttcagcctcaagaacaagattgtcgaggtg
A K R A G E K K M D F S L K N K I V E V
aaccgcgacgccgaccgccgctaccactttgcgacgatgaacatccgcgaagcgcagcgc
N R D A D R R Y H F A T M N I R E A Q R
aaaggtgagttgttcccgcagagctcaccgctcactgagaagtgggtcgtccagaatgat
K G E L F P Q S S P L T E K W V V Q N D
gacgaggagtcgcaccagaaggccctggatgtcctggagttcatcaagagggccaaggac
D E E S H Q K A L D V L E F I K R A K D
gaccccagctgcccaccggacgccaagccctacatggaggaatgggtgcagaagggcatc
D P S C P P D A K P Y M E E W V Q K G I
ccacccatgggcgagaacatcgagctcccgatcccccaggtcctggcgaaccagctgcat
P P M G E N I E L P I P Q V L A N Q L H
accatgtctatgatgatcaagaacggcttcgagggagctgcgaaggacaagactgaggct
T M S M M I K N G F E G A A K D K T E A
gaggtggcggcagaggagaagaagctgccggagattgctgcgggcaccatcaagcagctg
E V A A E E K K L P E I A A G T I K Q L
gtggacaagtacgtatctgcgcgcgatcaggtggagaccgaagcgagcaacatgaaggcc
V D K Y V S A R D Q V E T E A S N M K A
ttcttccgtgcgcaagaggacatgccaggcaagaccaaggcagatgtgctcaaggggatc
F F R A Q E D M P G K T K A D V L K G I
tgggaagagctgccaaaaaggacgaagaagcccttgccccccactggacgaggaggttctt
W E E L P K R T K K P L P P L D E E V L
gcagagctcgcggagatcccggcaaacattgaaggccaggacaagcacagctggggcact
A E L A E I P A N I E G Q D K H S W G T
gctgacgtgctttacaagtctgaggccattgatgccttcggcatgaagtacctccttggt
A D V L Y K S E A I D A F G M K Y L L G
gtctttgaaaccaaagatgaggctcaaaaggcatttgccgactggaacgcagaatacgag
V F E T K D E A Q K A F A D W N A E Y E
aaggcccgcgtggaaatgaaagctgagatggagcagtggggcaagcaggagcaggcgcgc
K A R V E M K A E M E Q W G K Q E Q A R
ttggaccgtgacacttccggcccagagcgcatcaagaaggtcttggaagaatcaaggcgt
L D R D T S G P E R I K K V L E E S R R
tgagctgcatgcccgcactgcctagcccattgtattagccagtctttgcagatgcagctt
gcaacaagcaagcaggttccggtggttccatgagtgcatttttgatcgaaaaaaaaaaaa
aaaaaaaa

Figure 4.21 Putative amino acid sequences of NAP50. Peptide sequences obtained from *de novo* sequencing (Table 4.4) (underlined). Stop codon (TGA) (Box with solid line). N-terminal sequence obtained from edman sequencing (Box with dotted line).

4.4 Conclusions

Proteomic study with 2-DE analysis of studying model *A.affine* under N-depletion and repletion was performed in the present study. A total of 33 differentially expressed proteins were found. All of them showed at least 5-fold differential expression differences upon N-depletion and subsequent repletion. Thirty-one of them were found to be up-regulated and 2 of them were down-regulated after the repletion of nitrogen. Of these differentially expressed proteins, 5 protein spots were successfully identified as Ribulose 1,5-bisphosphate carboxylase/oxygenase II (Rubisco II) and its isoforms. Further, full sequence of NAP50 was obtained. However, no sequence homology could be found through bioinformatic searches through the various known databases. Partial amino acid sequences of several other NAPs were also obtained. However, cloning experiments to obtain their full sequences were not performed due to time constraints.

CHAPTER V

Drastically changes in the protein expression of nitrogen-associated proteins (NAPs) under nitrogen stress

5.1 Introduction

Nitrogen is one of the most important nutrients for the growth of dinoflagellates and it is also believed to be associated with the causes of HABs. Since the exact mechanism of HAB is uncertain, knowing the molecular responses of dinoflagellates to the re-loading of nitrogen is very important. In the literature, it is generally believed that proteins in living organism can exist in various forms and each protein has its own life time, but generally does not exist beyond lifetime of the organism. Therefore, turnover and quality control of proteins are physiologically important for sustaining life of the organism (Gottesman et al., 1997). Studies in recent years have revealed the regulatory roles that subsets of rapidly degraded proteins play in metabolic and developmental processes in both prokaryotes and eukaryotes. Several lines of evidence support that protein degradation occurs in plants during senescence (Hortensteiner and Feller, 2002). It is envisioned that leaf senescence is a complex highly regulated developmental phase characterized by protein degradation. It is generally believed that the breakdown of some specific proteins like Rubisco is of great physiological significance as these proteins provide additional amino acids for mobilization to other reproductive organs of the plant. Beyond senescence, protein degradation often occurred in responses to stress. For example, presence of proteases have been implicated in degradation of light-harvesting phycobiliproteins in nutrient-deprived cyanobacteria (Collier and Grossman, 1994). In addition, induction of proteases in response to nitrogen has also been described in some diatom and

chlorophyte species (Berges and Falkowski, 1998). It should be noted that, in contrast to higher plants, very little is known about proteolytic protein degradation in microalgae and especially dinoflagellates.

Sequel to the 33 differentially expressed proteins found from the 2-DE analysis on *A. affine* grown under N-depletion (Chapter IV), most of the NAPs were found to have minimal expression levels during N-depletion. However, their expression levels increased dramatically upon N-repletion. On the other hand, Rubisco II and NAP50 were found to be down-regulated drastically for more than 16-fold during N-depletion. Why are these proteins down-regulated? What are the molecular mechanisms leading to the degradation of these proteins? Is there any protease involved in the protein degradation under the N-depletion condition? In an attempt to answer these questions, a series of immunoblotting experiments were performed.

5.2 Materials and methods

5.2.1 Production of antibodies against NAP50

Antibody was raised in female Sprague Dawley rats. Coomassie blue stained spots of NAP50 were excised from the 2-DE gels for preparation as immunogen. Before injection into rats, identities of NAP50 spots excised were validated by comparing their PMFs to that of NAP50. In order to have enough antigens for immunization, eight to ten protein spots of the same protein were pooled. After excision from the 2DE gels, these protein spots were destained by destaining solution (40% methanol, 10% acetic acid) before being washed extensively with PBS (pH7.4) until pH of the washing buffer is near 7.4. Subsequently, gel spots were homogenized into a slurry-like suspension. This suspension was mixed 1:1 with Complete / Incomplete Freund's Adjuvant (Sigma, USA) depending whether it is the initial injection or subsequent booster shoots. One ml of the emulsion was injected into each rat subcutaneously near the hind limbs. The process was summarized in Table 5.1.

Table 5.1 Antibody production of NAP50

	Duration [a]	**Number of Spots injected**	**Serum collection**
Primary Immunization [b]	3 weeks	10	nil
1st booster	2 weeks	8	nil
2nd booster	2 weeks	9	nil
Final booster	2 weeks	8	From eye [c]
Killing and final serum collection	nil	nil	From whole animals

[a] Time duration before next injection
[b] Complete Freund's Adjuvant was used in the primary immunization, and Incomplete Freund's Adjuvant was used in subsequent booster.
[c] Serum was taken from the rats before final booster

In order to check the antibody production progress, periodical bleeding was performed to obtain 1ml of blood from each immunized rat. The blood was allowed to clot in room temperature for 30 minutes before being stored overnight at 4^{o}C. Subsequently, the blood was centrifuged at 1500 x g at 4^{o}C for 20 minutes. Clear supernatant obtained was the anti-serum (containing polyclonal antibodies) produced and was aliquoted before being stored in -20^{o}C.. Care of these rats was performed according to the Code of Practice for Care of the Animals of The Hong Kong SAR Government. Ethnics approval for this part of the experiment was obtained from the Animal Subjects Research Ethnics Subcommittee.

5.2.2 Western blotting

5.2.2.1 Sensitivity and specificity of polyclonal antibodies generated against NAP

To test the sensitivity and specificity of the anti-NAP50 antiserum, total protein extracts of *A. affine* were subjected to one- and two-dimensional gel electrophoresis. Twenty μg and 700 μg of proteins were loaded to the 1D and 2DE gels respectively. Subsequent to 1D or 2D electrophoresis, resolved proteins were electro- blotted onto a nitrocellulose membrane at 100V for 2 hour at 4℃. Subsequently, the protein-blotted membrane was rinsed with TBS buffer (20mM Tris-HCl and 137 mM NaCl, pH 7.6) before being blocked with 5% BSA in TBS buffer for 2 hours at room temperature. Subsequently, the membrane was probed with anti-NAP50 antiserum diluted 1:1000; 1:2000; 1:3000; 1:4000 and 1:5000 with 1% BSA in TBS for 1 hour at room temperature. (Only 1:4000 was used for 2-DE western blot.) For probing experiments with Rubisco II, the membrane was probed with rabbit antibodies to Rubisco II diluted 1:4000 with 1% BSA in TBS for 1 hour at room temperature. (The polyclonal

antibody to Rubisco II was a kind gift from Professor David Morse, Montreal University, Canada.) After washing six times with TBS buffer containing 1% Tween 20 (TBST buffer), the membrane was probed either with anti-NAP50 or anti-Rubisco II. After incubation for 1 hour at room temperature, the membrane was washed again before being incubated with either peroxidase conjugated anti-rat IgG antibodies (diluted 1:2000 with 1% BSA in TBS; Santa Cruz Biotechnology, USA) or with peroxidase conjugated anti-rabbit IgG antibodies (diluted 1:100000 with 1% BSA in TBS; Santa Cruz Biotechnology, USA) for 1 hour respectively. After washing six times with TBST buffer, expression levels of Rubisco II and NAP50 were assessed by the amount of chemiluminescent signals generated using Supersignal Chemiluminescent Substrate kit (Pierce Chemical, USA) with the procedures performed according to that described by the manufacturer.

5.2.2.2 Analysis of protein expression level of NAPs under different conditions

Unless stated otherwise, all western blotting experiments were performed with the same conditions as detailed above. Proteins extracted from equal cell amounts were loaded onto the SDS-PAGE.

5.2.2.2.1 Nitrogen-depletion

A. affine cells at mid-log phase were harvested by centrifugation at 1500 x g at room temperature. Cells were washed twice with sterile synthetic seawater. Cells were inoculated into a 100 ml fresh K-medium with or without any nitrogen sources. Unless stated otherwise, nitrate was used as the nitrogen source for the entire experiment. The initial cell density of each 100 ml cells was set at 2000 cell ml^{-1} and

it was checked by cell counting. Growth of these cells was monitored by cell count at the same time every day. Cells were harvested at various time points (from time 0 – 48 hours) and analysed by western blotting as described above. On the other hand, proteins were loaded either according to equal protein amounts or equal cell amounts. For equal protein loading, 20 µg of total proteins were loaded and alpha-tubulin (Santa Cruz Biotechnology, USA) was used as loading control for protein load. For equal cells loading, 20 µl of proteins extracted from the same amount of cells ($1x\ 10^5$) from various treatment were loaded. All the western blotting experiments were repeated at least twice. Representative results were shown.

5.2.2.2.2 Normal growth

A. affine cells at mid-log phase were harvested by centrifugation at 1500 x g at room temperature. Cells were washed twice with sterile synthetic seawater. Cells were inoculated into a 100 ml fresh K-medium. The initial cell density of each 100 ml cells was set at 500 cell ml^{-1} and it was checked by cell counting. The cell density was monitored by cell count at the same time every day or every 5 hours for 35 hours. Cells were harvested at various time points (from time 0 – 35 hours or day 1-16) and analysis by western blotting as described above.

5.2.2.2.3 Repletion of nitrogen

The procedures described in 5.2.2.2.1 were repeated. However, nitrogen sources were added back to the N-depleted cultures at 24 and 36 hours. Cells replenished with N at 24 and 36 hours were harvested at 36 and 48 hours respectively. These cells were analyzed by western blotting as described above.

5.2.2.2.4 Phosphate-depletion

The procedures described in 5.2.2.2.1 were repeated. However, instead of nitrogen, cells were inoculated into a 100 ml fresh K-medium with or without any phosphates. Cells were harvested at various time points (from time 0 – 72 hours) and analysis by western blotting as described above.

5.2.2.2.5 Protease(s) activation

The procedures described in 5.2.2.2.1 were used. However, protease inhibitors were added to the N-depleted cultures at 24 and 36 hours instead of nitrogen. 1.5 µl /ml protease inhibitor cocktails (sigma, USA) or individual protease inhibitor including 200 µM AEBSF (serine proteases inhibitor); 2 µM E64 (cysteines protease inhibitor); 1 µM PepA: Pepstatin A (acid proteases inhibitor); 1 µM Best: Bestatin (aminopeptidases inhibitor) and 100 µM Phen: 1,10 Phenanthroline (metalloproteases inhibitor) were used. Allowing 12 hours to react, cells added with protease inhibitors at 24 and 36 hours were harvested at 36 and 48 hours respectively. Cells were harvested were analyzed by western blotting as described above.

5.2.3 Immunohistochemistry

A. affine cells at the exponential grow phase were fixed with 3% glutaraldehyde in 0.3M phosphate buffer pH7.4, for 35 min. The cell sectioning and transmission electron microscopy was performed kindly by Prof. David Morse (Montreal University, Canada). Anti-NAP antibody was used as the primary antibody. A 20 nm gold-labeled goat anti-rat antibody (Ted Pella, USA) was used as the detection antibody.

5.2.4 Analysis of mRNA expression level of NAPs by real-time PCR

A. affine total RNA was isolated from cells using the Trizol reagent (Roche, Switzerland) according to the manufacturer's instructions. The RNA preparation was then subjected to on-column digestion with RNase-free DNase I from the RNeasy Mini kit (Qiagen, Germany) to remove contaminating genomic DNA. Samples were checked for residual genomic DNA by standard PCR or by real-time PCR using the corresponding primers (Table 5.2).

Table 5.2 Primers used in real-time PCR

Gene	Primers	Sequences 5'→ 3'	Product size
NAP	NAP_rt_f	GACTACGATGGCCTCGCCAAGCGTGC	200 bp
	NAP_rt_r	CGTCCTTGGCCCTCTTGATGAACTCCAG	
Rubisco II	RBC_rt_f	GGMAACAAYCAGGGTATGGG	300 bp
	RBC_rt_r	ATGCACTCGTTCATCTGGCA	
Actin	Actin_rt_f	CCDGAYGGSAACATCATCAC	300 bp
	Actin_rt_r	CTTVGAGATCCACATYTGCTGGAA	

RNA samples were deemed to be free of genomic DNA if no amplification product was detected by agarose gel electrophoresis (or by real-time PCR) after at least 30 cycles of amplification. Reverse transcriptase PCR (RT-PCR) reactions were performed with 100 ng of total RNA and iQ^{TM} SYBR Green Supermix (Bio-rad, USA) according to the manufacturer's instructions. PCRs were performed with the following conditions: denaturation at 94°C for 45 s, annealing at 55°C (60 °C for NAP50) for 45 sec., and elongation at 72°C for 1 min for 35 cycles. For real-time RT-PCR, first-strand cDNA was prepared by using Superscript III Plus RNase H$^-$ Reverse Transcriptase (Invitrogen, USA) with random hexamer primers as described in the protocol provided by the manufacturer. The resulting cDNA was amplified by using

the Smart Cycler system (Cepheid, USA) in a final reaction volume of 25 µl which contained 3 µl of first-strand cDNA, 0.3 µM concentrations of each primer and 12.5 µl SYBR-Green supermix (Bio-rad, USA). The amplification conditions for real-time PCR were as follows: denaturation at 95°C for 45 s, annealing at 55°C (60 °C for NAP50) for 45 s, and elongation at 72°C for 45 s for 45 cycles. The gene-specific primers used in RT-PCR and in the real-time PCRs are listed in Table 5.2. The threshold cycle for each real-time PCR was determined from a second derivative plot of total fluorescence as a function of cycle number by using the software package supplied with the Smart Cycler system. Real-time PCRs were carried out for three batches of experiments with duplicates in each batch. After some real-time PCRs, the end-point amplification products were visualized by electrophoresis in 1% agarose gels. Determination of real-time PCR efficiencies of reference gene (actin), target genes (NAP50 and Rubisco II) with their corresponding primers and the relative mRNA expressions were calculated according to procedures described previously (Livak and Schmittgen, 2001; Pfaffl, 2001).

5.2.5 Partial purification of rubisco-degrading serine protease(s) by benzamidine column

To test whether the target protease(s) would bind to benzamidine, the procedures described in 5.2.2.2.5 were repeated. However, 4 mM benzamidine was added to the N-depleted cultures instead. Cells added with benzamidine at 24 and 36 hours were harvested at 36 and 48 hours respectively. Harvested cells were analyzed by western blotting as described previously.

In order to purify the protease, a functional assay for the protease has to be developed. When performing this in vitro assay of the target protease(s), testing cells

were first prepared. Equal number of log-phase cells (around 1 x 10^5 cells) was harvested and aliquoted into eppendorf tubes. These cell pellets were used as testing cells and were kept at -80 ℃ until use. Cell extracts of N-depleted or repleted cultures were added to testing cells. When performing the control, buffer (0.05M Tris pH8.2 with 0.02M $CaCl_2$) was used to replace cell extracts. In the purification process, cell extracts, fractions or buffer containing the testing cells were disrupted by sonication (a total of 3 minutes with short pulses of 5-10 seconds each) on ice. Lysis of cells was confirmed using light microscope. Cell lysate was then centrifuged at 14000 x g for 15 minutes at 4℃ to collect the supernatant. Subsequently, the supernatant collected was analyzed by western blot with the anti-rubisco II antiserum as mentioned previously.

To partially purify the rubisco-degrading protease(s), two methods were applied and the entire purification process was performed at 4℃. Method 1: Proteins in the total cell extracts were loaded onto a benzamidine column. After washing with buffer, proteins that remained bound were first eluted with glycine-HCl pH2.7 followed by binding buffer containing 20mM benzamidine. Method 2: Proteins bound to benzamidine column were eluted with stepwise concentrations of benzamidine (5mM, 10mM, 20mM and 40mM) before being chased by elution with glycine-HCl pH2.7. Seventy to 150 L of N-depleted cells were harvested and kept at -80 ℃ until use. The presence of the target protease(s) was checked by the degradation of rubisco II with the western blotting analysis. Ten ml of binding buffer (0.05 M Tris-HCl, 0.5 M NaCl, pH 7.4) was added to the cell pellets. Cells were disrupted by sonication (a total of 3 minutes with short pulses of 5-10 seconds each) on ice. Lysis of cells was confirmed using light microscope. Cell lysate was then centrifuged at 14000 x g for 15 minutes

at 4℃ to collect the supernatant. Subsequently, the supernatant collected was loaded onto a benzamidine column (HiTrap 1ml) (Amersham Biosciences, USA) equilibrated with binding buffer. A flow rate of 1 ml/min was used. The column was washed with washing buffer (1 M Tris-HCl, 0.5 M NaCl, pH 7.4) until the OD280nm was zero. Glycine-HCl pH 2.7 or binding buffer with benzamidine (5mM-40mM) was used to elute the bound proteases and 0.5 ml fractions were collected. Protein fractions were pooled before concentrated and buffer exchanged to (0.05M Tris pH8.2 with 0.02M $CaCl_2$) by an Amicon YM-10 (Millipore, USA). One μg of proteins from the original protein extracts, flow-through and eluted fractions were analyzed by adding to the testing cells and assay the present of the rubisco-degrading protease(s) before being analysed by SDS-PAGE and western blot.

5.3 Results and discussion

5.3.1 Sensitivity and specificity of anti-serum raised against NAP50

Antibodies of a specific dinoflagellate protein were successfully generated in rats by using a 2-D gel spot as the antigen (Chan et al., 2006). As mentioned, anti-rubisco II antibody was kindly provided by Prof. David Morse (Montreal University, Canada) to allow the protein expression studies of rubisco II. However, to investigate the changes in protein expression of NAP50 under different N conditions, measurement of NAP50 levels were required. I therefore raised rat polyclonal antibodies to *A.affine* NAP50 and determined its titer and specificity with the immunoblotting analysis. Control experiments either in the absence of primary antibody or using preimmune serum showed no reaction with *A.affine* protein extracts (data not shown).

Figure 5.1 shows total proteins of *A.affine* probed with various dilutions of anti-NAP antibodies. In the protein blot, there was only one single band observed with an apparent molecular weight of 50 kDa (Figure 5.1). The signal observed was increased when the more of the antibodies used. However, the signal from the antibodies with dilution 1:5000 was relatively weak. Therefore, dilution with 1:4000 was used for the subsequent western blotting analysis. Although there was only one band observed from the protein blot shown in Figure 5.1, I cannot rule out the possibility that non-specific proteins with same molecular weight of NAP50 may be presented.

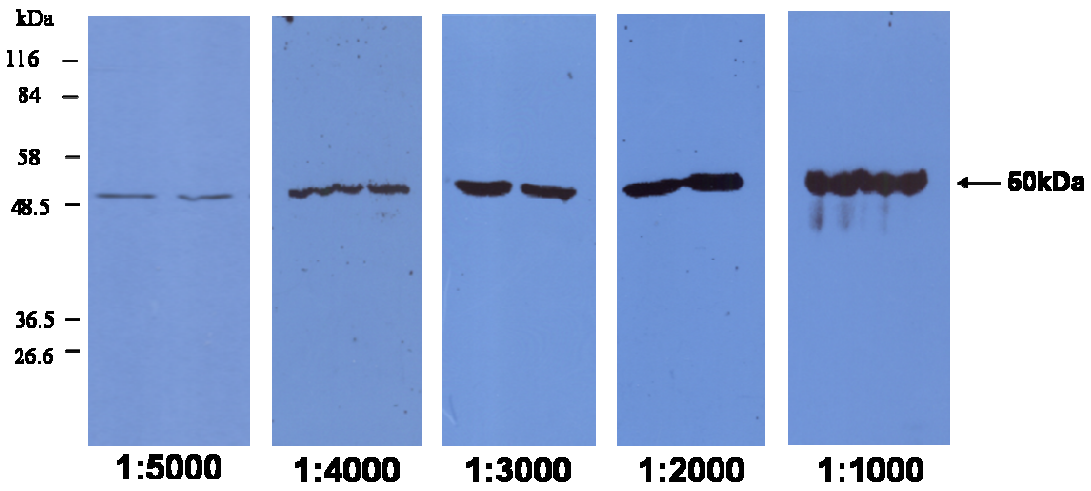


Figure 5.1 Western blotting analysis of total protein extracts of *A.affine* with antibodies raised against NAP50. Different titers from 1:1000 to 1:5000 (from right to left) of anti-NAP50 anti-serum were evaluated.

To further confirm the specificity of the anti-NAP50 antibodies, total proteins from *A. affine* were analyzed by 2D SDS-PAGE followed by western blot (Figure 5.2). Four protein spots were detected from the immunoblot analysis with anti-NAP50 antibodies (Figure 5.2b). These spots have a molecular weight of 50 kDa with *pI* ranged from 5.4-5.7, and they correspond to protein spot 6-9 (Figure 4.3). To further confirm these four spots detected were NAP50, rubisco II was probed together with NAP50 in the western blot analysis (Figure 5.2c). Five spots of rubisco II were observed and located just at the top of NAP50. Their appearance and relative location bear remarkable resemblance to that of NAP50 and rubisco II. Further, these four protein spots were positively detected when probed by anti-NAP50 antibodies. Therefore, the four spots detected by the anti-NAP50 antibodies were highly likely to be NAP50. These spots are isoforms of NAP50 either occurred naturally or being formed by laboratory manipulations.

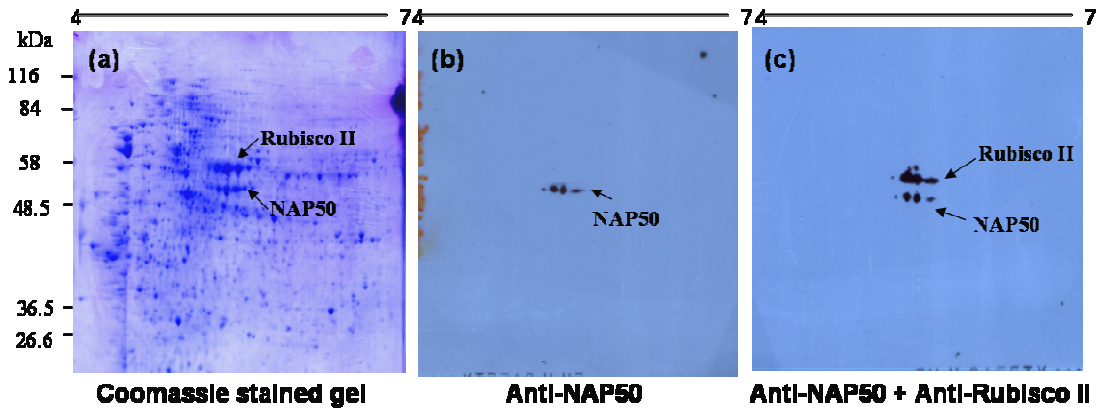


Figure 5.2 Western blotting analysis of 2-DE of *A.affine* with antibodies raised against NAP50. (a) 2-DE of *A. affine* total protein extracts stained with coomassie brilliant blue, (b) western blot of the 2-DE with anti-NAP50 antiserum and (c) western blot of the 2-DE with both anti-NAP50 antiserum and anti-rubisco II antiserum. Arrows indicate the corresponding spots of NAP50 and rubisco II.

5.3.2 NAP50 is specific in *Alexandrium* species

In order to test whether NAP50 was also presented in other dinoflagellate species, protein extracts from different dinoflagellate species were analyzed by western blotting (Figure 5.3). Six different dinoflagellates were tested. They included *Scrippsiella rotunda* (accession no: EF579794), *Prorocentrum minimum* (accession no: EF579797), *Karenia brevis* (CCMP 2281); *Alexandrium tamarense* (CCMP 1598); *Alexandrium minutum* (CCMP 113) and *Alexandrium affine* (accession no: EF579793). Interestingly, positive signals were only observed in *Alexandrium* species. Although I cannot rule out the possibility that NAP50 may be presented in other dinoflagellate species that were not tested in this study, it seems likely that NAP50 is specific to the genus- *Alexandrium*. Therefore, if this protein is present in *Alexandrium* only and tightly regulated in accordance to different nitrogen conditions, it may indicates that the nitrogen metabolism of *Alexandrium* is different from that of other dinoflagellates. Another possibility is that protein with same function but with less homology to NAP50 is presented in other dinoflagellates. More research has to be performed using

more dinoflagellate cultures.

Figure 5.3 Western blotting analysis of protein extracts of different dinoflagellate species. 20 µg of total proteins of various species were loaded for the SDS-PAGE. *Sc: Scrippsiella rotunda* (accession no: EF579794)*; Pro: Prorocentrum minimum* (accession no: EF579797)*; Ka: Karenia brevis* (CCMP 2281)*; At: Alexandrium tamarense* (CCMP 1598)*; Ac: Alexandrium minutum* (CCMP 113)*; and Af: Alexandrium affine* (accession no: EF579793).

5.3.3 Subcellular localization of NAP50

One of the important questions after a novel protein is found is the cellular localization of this protein. Given that NAP50 is also a novel protein, its subcellular localization will be very interesting. The most commonly used method is immunohistochemical localization experiments using specific antibodies. Therefore, *A. affine* cells were fixed, sectioned and incubated with anti-NAP50 IgG (purified from protein-G affinity column) before visualized using gold labeled secondary antibodies and examination under transmission electron microscopy (TEM) (Figure 5.4). As shown in the figure, most of the gold labeled particles were observed within the chloroplast and there was only a few of them observed in the other regions of the cells.

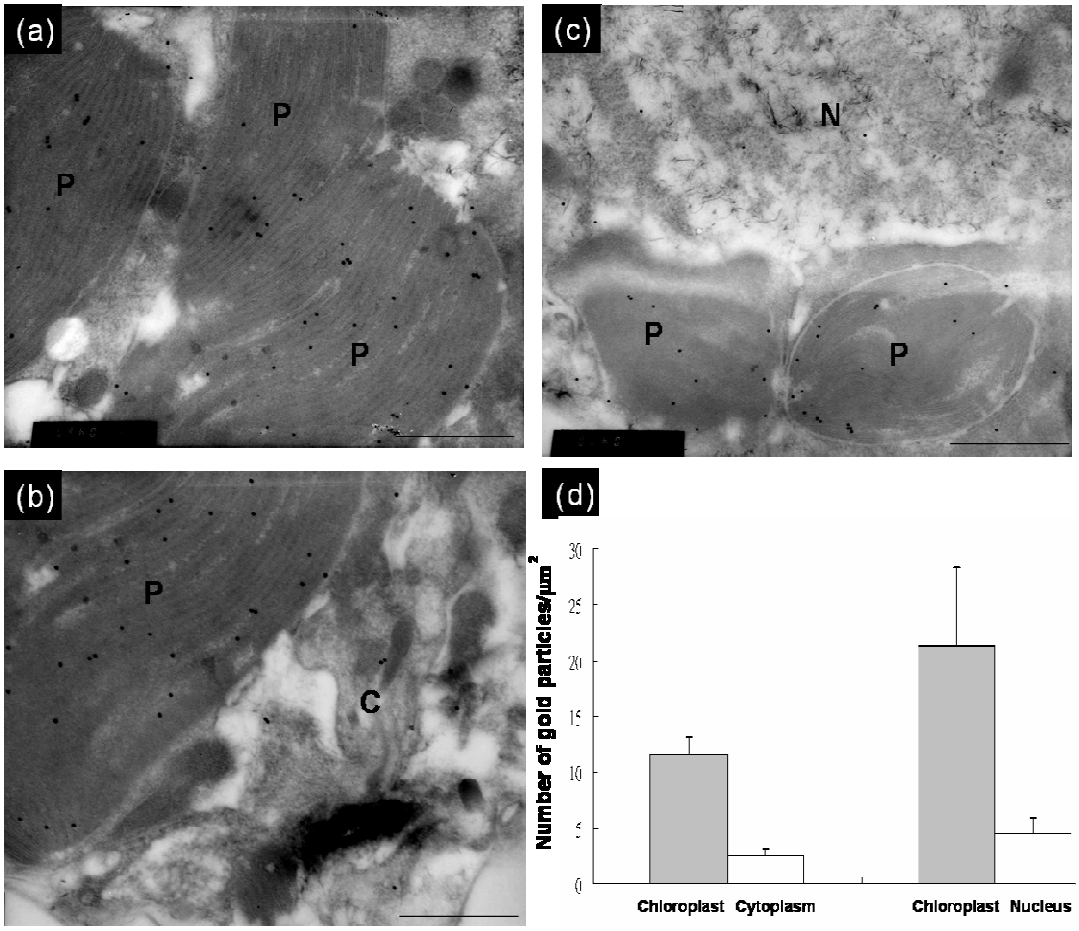


Figure 5.4 Immunohistochemical localization of NAP50 in *A. affine* cell sections. Cell sections of *A.affine* incubated with the polyclonal anti-NAP50 antibody, followed by a gold-conjugated (20nm) rabbit anti-rat secondary antibody. Representative electron micrographs with 25000 x magnification of (a) P: chloroplast; (b) P: chloroplast and N: nucleus and (c) P: chloroplast and C: cytoplasm, were shown. Gold labeling is concentrated on chloroplasts with little labeling apparent on other regions of the cell. (d) the number of gold particles over micron square regions were counted, and the SEM for each were calculated (n=5). Scale bar indicates 1 μm.

Given that our previous results suggested that anti-NAP50 antibody is reasonably specific in the concentration that was used (Figures 5.1 to 5.3), these results presented in Figure 5.4 strongly supported that NAP50 is localized in the chloroplasts of *A. affine*.

5.3.4 Analysis of protein expression of NAPs (rubisco II & NAP50) in nitrogen depletion and repletion conditions

5.3.4.1 Degradation of NAPs under nitrogen-depletion

In both prokaryotes and eukaryotes, most cellular proteins are stable in vivo (Mosteller et al., 1980). However, normally stable proteins would become unstable when under stressed or abnormal environments (Thiel, 1990). Studies in recent years have revealed important regulatory roles of some rapidly degraded proteins in metabolic and developmental processes. To understand the degradation mechanism of proteins under N-depletion, comprehensive studies on possible degradation of rubisco II and NAP50 were performed. It was found that expressions of these proteins were relatively stable during N-repletion. Results from equal protein loading (Figure 5.5a) were similar to that loading with equal numbers of cells (Figure 5.5b). However, both rubisco II and NAP50 were drastically degraded at 48 hour during N-depletion (Figure 5.5, -48 hours). Results of the present study further confirmed the down-regulation of these proteins found in 2-DE analysis in previous studies (Chapter IV). Image analysis of the intensities of the degraded proteins during N-depletion and N-repletion were performed. It was found that both rubisco II and NAP50 have degraded at least more than 16-folds when compared to their original expression levels in the N-repleted condition (Figure 5.6). In addition, when the intensities were measured by software (Melanie 3.0), both proteins were around 40-folds less than that of their original levels. On the other hand, from the western blotting analysis of serial samples from N-depleted cells at every two hours, both proteins were found to be degraded at the 30^{th} hour (Figure 5.7). Further, both proteins were found to be degraded at a dramatic rate within two hours (from the 28^{th} hour to the 30^{th} hour). Thus, the results indicate that both proteins were rapidly degraded into small peptides

or amino acids. Interestingly, levels of rubisco II were also found to be degraded during N-depletion in other species including *Scrippsiella rotunda* (different genus) and *Alexandrium tamarense* (same genus) (Figure 5.8). These result indicated that the degradation of rubisco II under N-depletion may be a universal molecular response to nitrogen depletion in dinoflagellates.

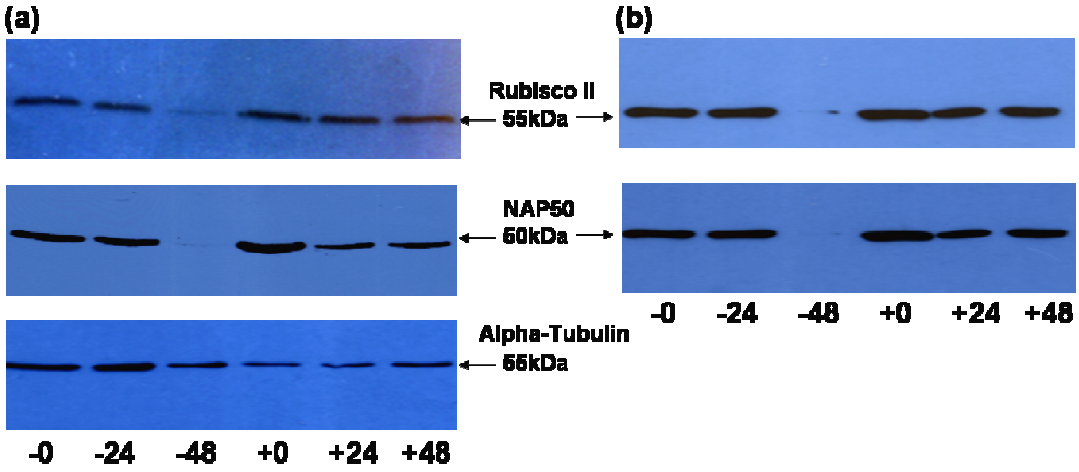


Figure 5.5 Western blotting analysis of NAP50 and rubisco II of *A. affine* under nitrogen depletion. *A.affine* cells grown under N-depletion (-0, -24, -48) and N –repletion (+0, +24, +48) for 48 hours; were analyzed with anti-NAP50 or anti-Rubisco II antiserum. Proteins were loaded according to (a) equal protein amounts and alpha-tubulin was used as control for protein load; (b) equal number of cells.

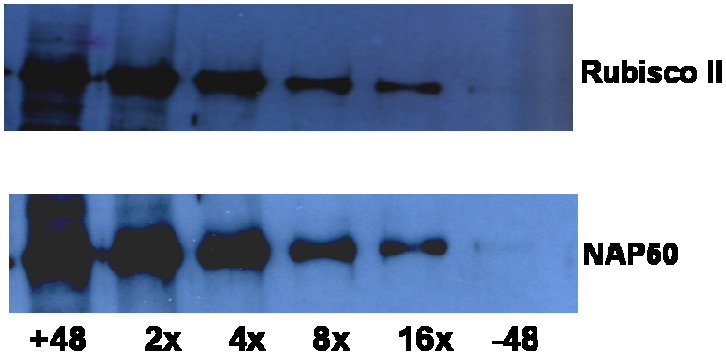


Figure 5.6 Levels of rubisco and NAP50 under N-depletion. Serial dilutions of N-repleted sample (+48) and their protein levels were shown. Protein levels of N-depleted sample (-48) (last lane) were 16-fold less than that of +48.

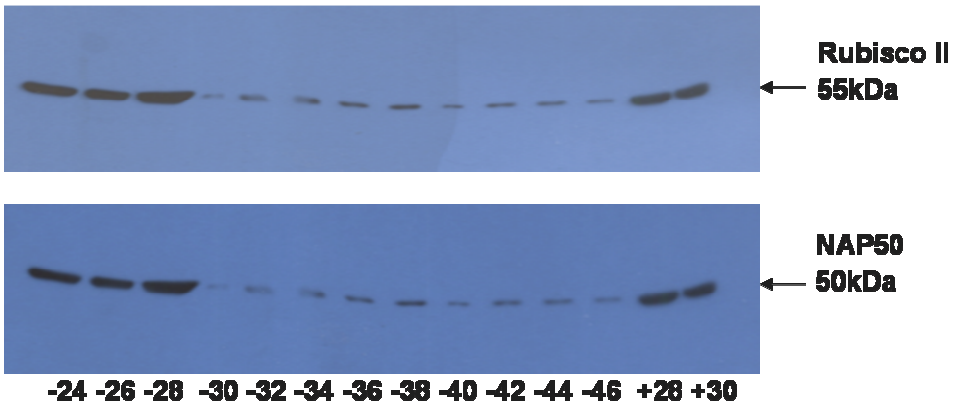


Figure 5.7 Western blotting analysis of NAP50 and rubisco II of *A. affine* under nitrogen depletion for every 2 hours time points. *A.affine* cells grown under N-depletion (-24 to -46) and N –repletion (+28 and +30) for 48 hours; were analyzed with anti-NAP50 or anti-rubisco II antiserum. Both proteins were found to be degraded upon 30 hours under N-depletion.

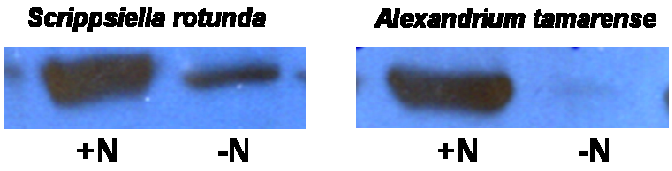


Figure 5.8 The degradation of rubisco II under N-depletion in other dinoflagellate species. Levels of rubisco II was found also be degraded in other species including *Scrippsiella rotunda* (different genus) and *Alexandrium tamarense* (same genus).

5.3.4.2 Unchanged expression level of NAPs in normal growth cycles and phosphate-depletion

Both rubisco II and NAP50 were found to be rapidly degraded under nitrogen depletion. However, it is also important to know the protein levels of both proteins under their normal growth and daily cycles (Figure 5.9).

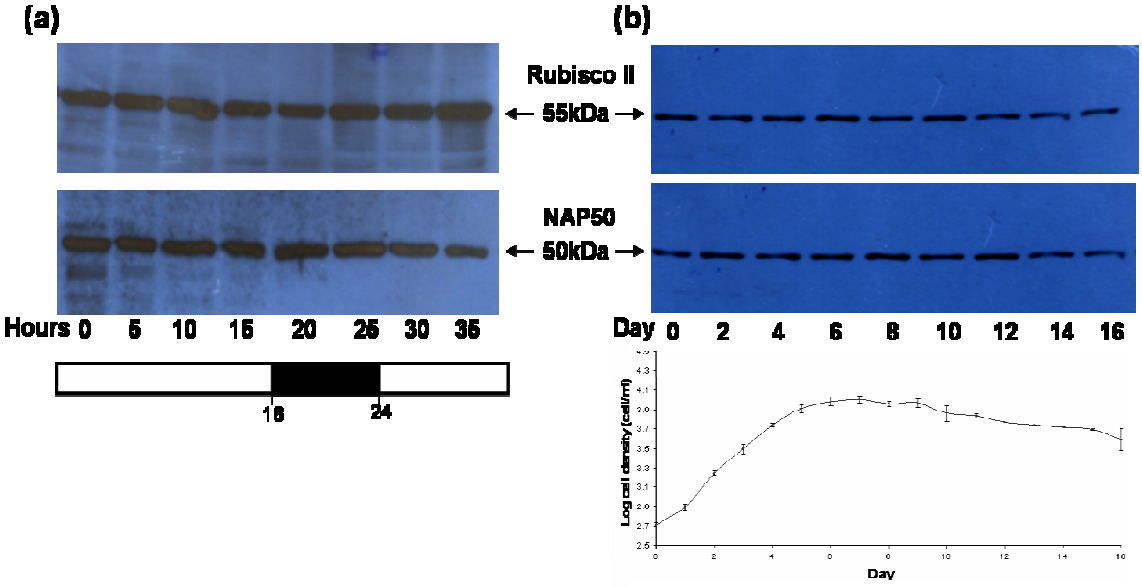


Figure 5.9 Levels of rubisco II and NAP50 were found to be constant during the (a) daily and (b) growth cycle. Closed bar and open bar represents dark and light period respectively. Numbers correspond to the time at which protein samples were taken. Growth curve was shown corresponding to the protein expression during day 0-16.

Image analysis of the intensities of bands in the immunoblots revealed that rubisco II was expressed at a constant level during its daily and growth cycles. Constant level of rubisco II expression during the daily cycle had been reported in Gonyaulax (a dinoflagellate) (Nassoury et al., 2001). Other than nitrogen, phosphate is another nutrient that is very important to the growth of dinoflagellates. Therefore, the protein expressions of both proteins were also measured in phosphate-depleted condition. No change in expression levels of both proteins were found in either

phosphate- depleted or repleted conditions (Figure 5.10). As the amounts of proteins are constant for the daily- and growth cycle, and because the protein expressions do not vary in either phosphate- depleted or repleted conditions, it is highly likely that the drastic protein degradation of rubisco II and NAP50 are due to the effect of nitrogen depletion.

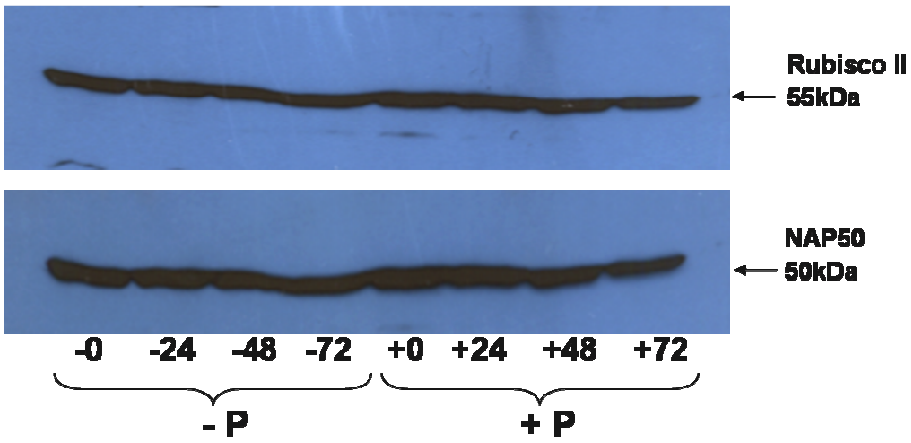


Figure 5.10 Levels of rubisco II and NAP50 were found to be constant under the phosphate –depletion and repletion conditions. Expression levels of these proteins in *A. affine* cells grown under P-depletion (-0 to -72) and P-repletion (+0 to +72) conditions for 72 hours were analyzed using anti-NAP50 or anti-rubisco II antibodies. Expression levels of both proteins were found to be unchanged under both conditions

5.3.4.3 Protection of NAPs from degradation by the replenishment of nitrogen

Interestingly, it was found that the degradation of rubisco II and NAP50 can be prevented by the replenishment of nitrogen sources (Figure 5.11). When different nitrogen sources (such as nitrate, ammonium, urea, and glycine) were supplied to the growth medium of N-depleted cells, both proteins were not degraded subsequently. In addition, nitrogen sources added to the N-depleted cells even after the proteins have degraded (36+N) also shows the same result. Although detail mechanisms behind the phenomena are not yet understood, it indicates the protein degradation processes are reversible and highly regulated by nitrogen.

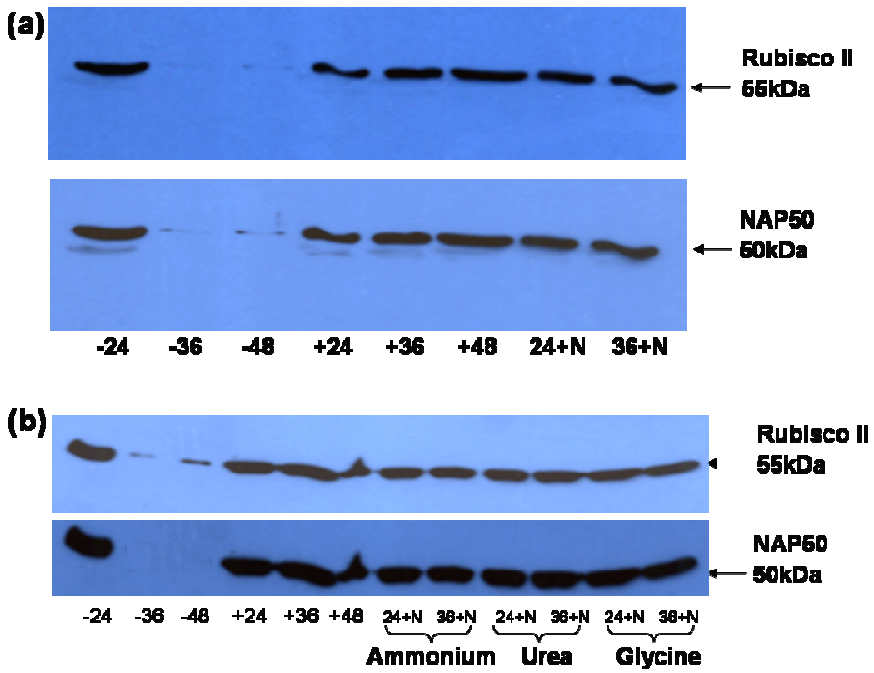


Figure 5.11 Resumption of rubisco II and NAP50 levels after replenish of nitrogen sources. *A.affine* cells grown under N-depletion (-24, -36 and -48); N –repletion (+24, +36 and +48) and N-replenishment at specific time during N-depletion (N added at 24 hour: 24+N; at 36 hour: 36+N) for 48 hours; were analyzed with anti-NAP50 or anti-rubisco II antibodies. Samples with N added at 24 hour (24+N) and at 36 hour (36+N) were analyzed at 36 and 48 hour respectively. Different N sources including (a) nitrate and (b) ammonium, urea and glycine were used to replenish the N-depleted cells.

5.3.5 Analysis of mRNA expression of NAPs

To investigate whether the protein degradation process is also controlled at the transcriptional level, real time PCR was used to determine the transcripts levels of rubisco II and NAP50 during different nitrogen-depletion and repletion conditions (Figure 5.12). Interestingly, the results suggested that mRNA levels for both rubisco II and NAP50 are constant in both nitrogen-depletion and repletion conditions. Further, their levels are independent of the addition of nitrogen or protease inhibitor (AEBSF). There does not seem to have a correlation between mRNA abundance of rubisco II and NAP50 with the availability of nitrogen. Thus, these observation excludes transcriptional control as a possible mechanism for regulating both rubisco II and NAP50 degradation in this alga.

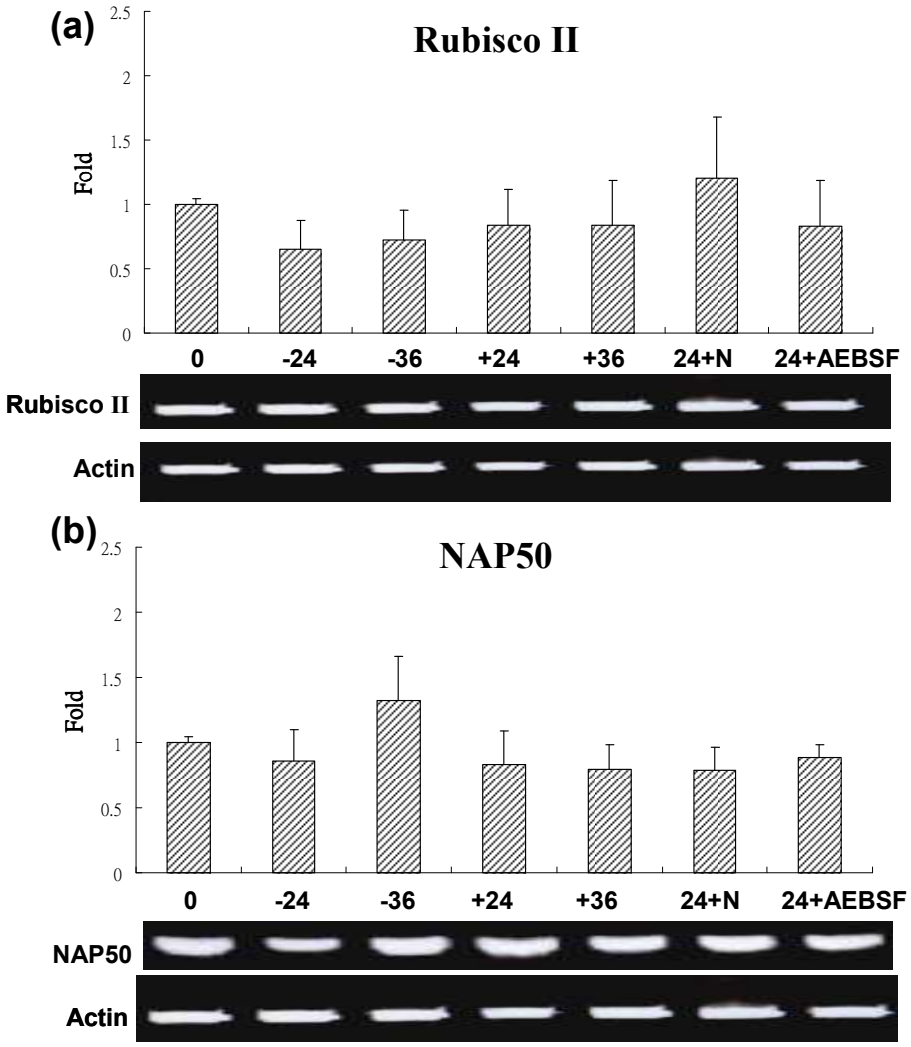


Figure 5.12 Real time PCR analysis on the mRNA expression levels of rubisco II and NAP50. Various time points and treatments including time 0 (0); N-depletion (-24 and -36); N-repletion (+24 and +36); N-replenishment at 24 hour (24+N) and serine protease inhibitor AEBSF added at 24 hour (24+AEBSF), were evaluated. The RT-PCR was performed with specific primers designed for Rubisco II and NAP50, and β-Actin was used as internal control for each treatment (see materials and methods). Time 0 was used as control and the fold changes of different treatments were relative to that of control. Gel images show the end-point amplification products visualized by electrophoresis in 1% agarose gels. The experiment was repeated three times and with duplicate in each experiment. Bar indicates $\pm$ S.D. among data from three batches of experiments. The result indicates there were no significant differences between the treatments and the control (p>0.05).

5.3.6 Activation of a protease(s) under nitrogen-depletion conditions for the degradation of NAPs

5.3.6.1 The degradation of NAPs by the actions of a protease(s)

Although the degradation of both rubisco II and NAP50 during nitrogen-depletion was found to be controlled at protein level rather than at transcriptional level, the next question is what are the active species that degrade these two proteins? In plants, rubisco is known to be degraded during leaf senescence and under various stressful conditions (Hortensteiner and Feller, 2002). There is very limited information on the triggering mechanisms that cause rubisco degradation in vivo. However, it is generally believed that the degradation may be triggered by a rubisco specific protease(s). Furthermore, induction of proteases in response to nitrogen-deprived conditions had also been described in some diatoms (Berges and Falkowski, 1998) and cyanobacteria (Collier and Grossman, 1994). Thus, it is interesting to investigate if the degradation of rubisco II and NAP50 was triggered by the actions of a protease(s). A protease inhibitor cocktail including AEBSF (serine proteases inhibitor), E64 (cysteines protease inhibitor), pepstatin A (acid proteases inhibitor), bestatin (aminopeptidases inhibitor) and 1,10 phenanthroline (metalloproteases inhibitor) was added to the N-depleted cells. As seen in Figure 5.13, both proteins were prevented from degradation in the present of protease inhibitors. It indicates that specific protease(s) are induced or activated during N-depletion and involved for the degradation of rubisco II and NAP50.

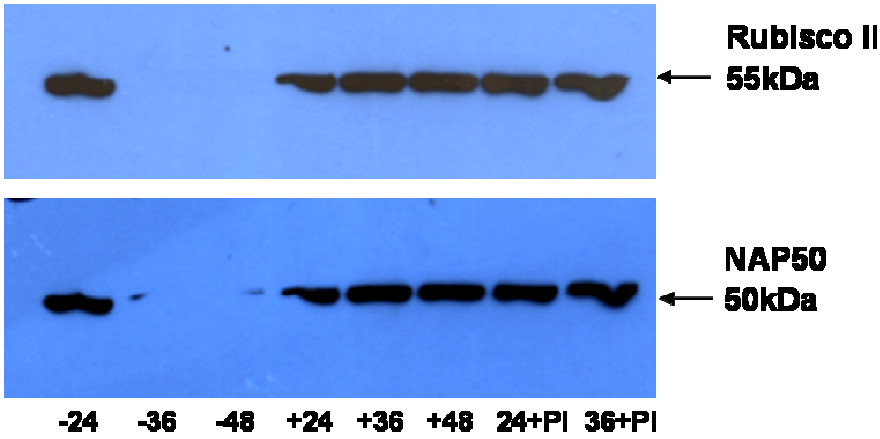


Figure 5.13 The degradation of rubisco II and NAP50 can be inhibited by the addition of protease inhibitors under N-depletion. *A.affine* cells grown under N-depletion (-0 ,-36 and -48); N –repletion (+0, +24 and +48) and N-depletion with protease inhibitor cocktails (PI) added at 24 hour (24+PI) and 36 hour (36+PI); were analyzed with anti-NAP50 or anti-Rubisco II antibodies. Samples with PI added at 24 hour (24+PI) and at 36 hour (36+PI) were analyzed at 36 and 48 hour respectively. Both proteins were found to be protected by the presence of protease inhibitors under N-depletion.

To further identify which type of protease(s) is actually responsible for the degradation of both proteins, individual protease inhibitor of the cocktail was added to the N-depleted cells. From the results shown in Figure 5.14, AEBSF appeared to be the single most effective inhibitor for protecting the degradation of rubisco II. It therefore suggests that serine protease(s) is the dominant enzyme responsible for the rubisco II degradation. In contrast to AEBSF, 1,10 phenanthroline was also effective against NAP50 degradation, suggesting that NAP50 degradation was performed by the actions of serine- and metallo-protease(s). Interestingly, it should be noticed that the degradation of both proteins were effectively prevented by the protease inhibitors either adding before (24+PI) or after (36+PI) the activation of protease(s) (Figure 5.13). Thus, it is likely that the degradation of both proteins was mainly performed at post-translational level. That is, there are constitutive synthesis of both rubisco II and NAP50. Then, one or a group of specific protease(s) are being activated in the

response to nitrogen-depletion and degrade both proteins for some yet to be determined functions.

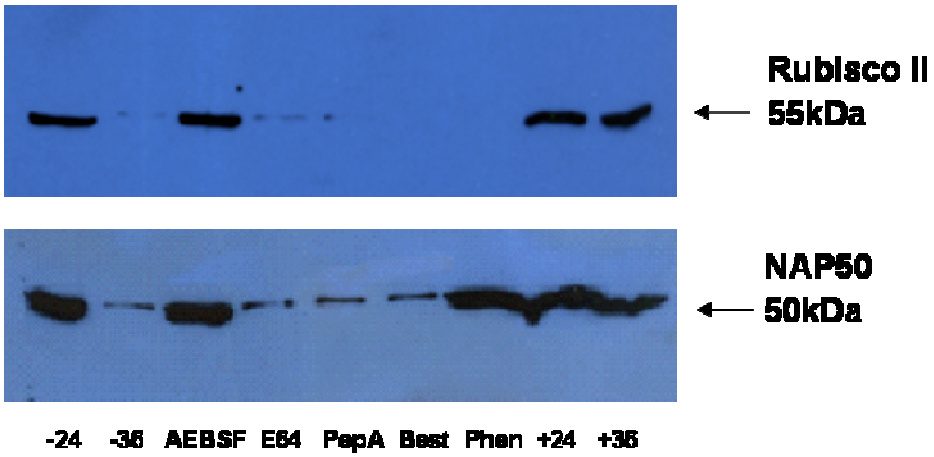


Figure 5.14 Serine or metallo- protease(s) were involved in the degradation of rubisco II and NAP50 under N-depletion. *A.affine* cells grown under N-depletion (-24 and -36); N –repletion (+24 and +36) and N-depletion with specific protease inhibitor added at 24 hour were analyzed with anti-NAP50 or anti-rubisco II antibodies. Protease inhibitors used includes: AEBSF (serine proteases inhibitor); E64 (cysteines protease inhibitor); PepA: Pepstatin A (acid proteases inhibitor); Best: Bestatin (aminopeptidases inhibitor) and Phen: 1,10 Phenanthroline (metalloproteases inhibitor). Rubisco II was found to be degraded by serine protease(s) and NAP50 was degraded by serine and metalloproteases(s) under N-depletion.

Protein degradation by specific proteolytic proteases during nitrogen stress in dinoflagellates has never been reported. The precise functions and mechanisms of the protein degradation during nitrogen depletion remain unknown. In addition, it cannot be ruled out that the proteins degradation were also triggered by other means, such as to combat the production of reactive oxygen species (ROS) as mentioned in some recent studies (Ishida et al., 1997; Ishida et al., 1998). However, results from the present study suggested that nitrogen deprivation leads to the possible induction or activation of serine/metalloproteases(s) which are the main factor that mediate degradation of rubisco II and NAP50. Since no rudiment of semi-breakdown

intermediates could be detected in the immuno-blots, it indicated the final products of degradation should be amino acids. It is not sure about the purposes of the protein degradation and possible fates of the amino acids produced. Nevertheless, the degradation of some chloroplast proteins like rubisco is known to be closely related to nitrogen economy in plants. Rubisco is the major protein in plant and it represents the major fraction of chloroplast nitrogen. It is believed that the breakdown of this protein to amino acids is to regenerate the nitrogen sources (Hortensteiner and Feller, 2002). However, it is difficult to predict the roles of protein degradation in N-deprived dinoflagellates. There are several reasons. Firstly, it should be stressed that plant is a multicellular organism while dinoflagellate is a single-celled organism. Nitrogen remobilization from one organ to another in plant is not directly applicable in dinoflagellates. Secondary, rubisco in dinoflagellates is of the form II enzyme which is totally different from that (form I) existed in plants. This form II enzyme differs from typical form I enzyme in that it is composed only of large subunits that share limited sequence homology with that of form I Rubisco (Morse et al., 1995; Rowan et al., 1996). Importantly, unlike form I enzyme, form II enzyme has a higher affinity for oxygen than carbon dioxide, suggesting that there may be special mechanisms to allow the effective carbon-fixation in this photosynthetic organism (Nassoury et al., 2001). Therefore, the hypothesis that these chloroplast proteins, rubisco and NAP50, act as N storage proteins and the actual functions of the degraded products remained an open question in the case of dinoflagellates.

5.3.6.2 Degrading mechanisms of rubisco is different from that of NAP50

There is a report which showed that protein synthesis is required for proteolysis under nitrogen starvation in cyanobacteria and that proteolysis is inhibited by incubating the nitrogen-deprived cells in the dark (Thiel, 1990). To address the possibility that the proteolytic protein degradation in N-deprived dinoflagellate was also inhibited by stopping protein synthesis and with darkness incubation, a complementary series of experiments was performed. For rubisco II, proteolysis was inhibited by either the addition of cycloheximide or incubating the nitrogen-deprived cells in the dark (Figure 5.15). This indicates that protein synthesis from the cytoplasm is required for the early activation of protease(s) or that protease(s) required for proteolysis are synthesized in response to N-depletion. In addition, the rubisco II degradation was abolished by darkness, suggesting that either the activation or the actions of the protease(s) is an energy- or light-dependent process. However, it should be noticed that proteolysis of rubisco II was only inhibited by incubating the N-deprived cells in dark before the presence of the protease(s) (0+dark and 24+dark), but not after the presence of the protease(s) (36+dark). These results indicated that light or energy is required for the initiations or activations of the protease(s) rather than the proteolytic actions. The case of NAP50 is completely different from that of rubisco II. Addition of both cycloheximide and chloramphenicol did not show any inhibitions of the proteolysis of NAP50, suggesting that protease synthesis or activations of protease(s), in either the chloroplast or cytoplasm, was not required in the process. Furthermore, incubating N-deprived cells in the dark did not completely inhibit the NAP50 degradation. The partially inhibitions showed that the activation of NAP50-degrading protease(s) was not totally light- or energy- dependent.

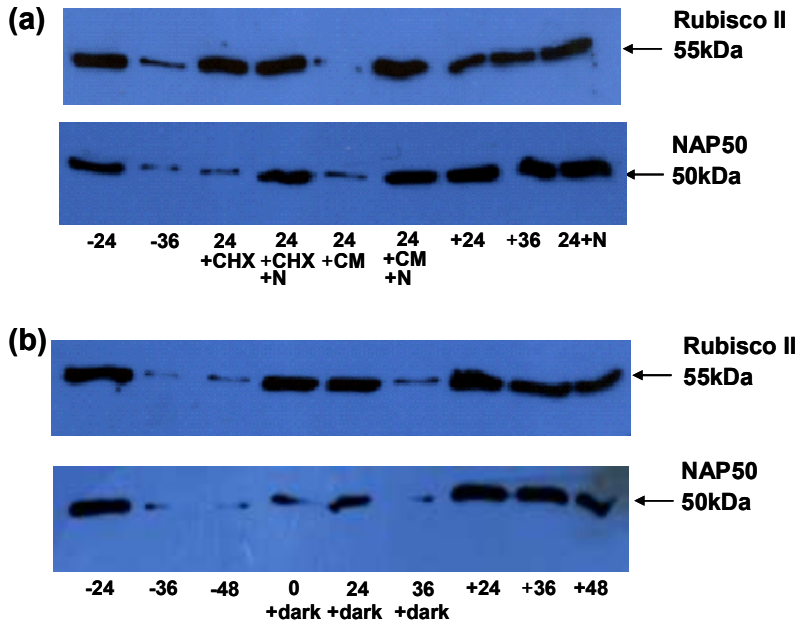


Figure 5.15 Degradation of rubisco II was prevented by protein synthesis inhibition and also under darkness. *A. affine* cells grown under N-depletion (-24, -36 and -48); N –repletion (+24, +36 and +48) and N-replenishment at 24 hour, during 48 hours were analyzed with anti-NAP50 or anti-rubisco II antibodies. (a) In addition to N-depletion and repletion, cells under N-depletion with the additions of 100 μM cycloheximide (24+CHX); cycloheximide together with nitrate (24+CHX+N); 100 μM chloramphenicol (24+CM) and chloramphenicol together with nitrate (24+CM+N), at 24 hour were also analyzed. (b) In addition to N-depletion and repletion, cells under N-depletion with the introduction of darkness at time 0 hour: (0+dark); 24 hour: (24+dark) and 36 hour: (36+dark) were also analyzed. Samples with the additions of CHX, CM, N at 24 hour were harvested and analyzed at 36 hour. Samples with the introduction of darkness at time 0 or 24 hour and at 36 hour were harvested and analyzed at 36 and 48 hour respectively.

Hence, taken overall, a schematic diagram summarizing the activation of rubisco II/ NAP50-degrading protease(s) and their subsequent proteolytic actions on the target proteins were shown in Figure 5.16. Although both rubisco II and NAP50 are located in chloroplast and the time when NAP50 was degraded coincided with that of rubisco II, several lines of evidences show that the degrading mechanisms of both proteins were completely different. Firstly, types of protease(s) involved in rubisco degradation (serine proteases) are different from that of NAP50 degradation (serine and

metalloproteases). Secondly, protein synthesis is required for activation of protease(s) in rubisco II degradation, but not in NAP50. Lastly, incubating N-deprived cells in the dark could completely inhibit the proteolysis of rubisco II, but it only partially inhibited the proteolysis of NAP50.

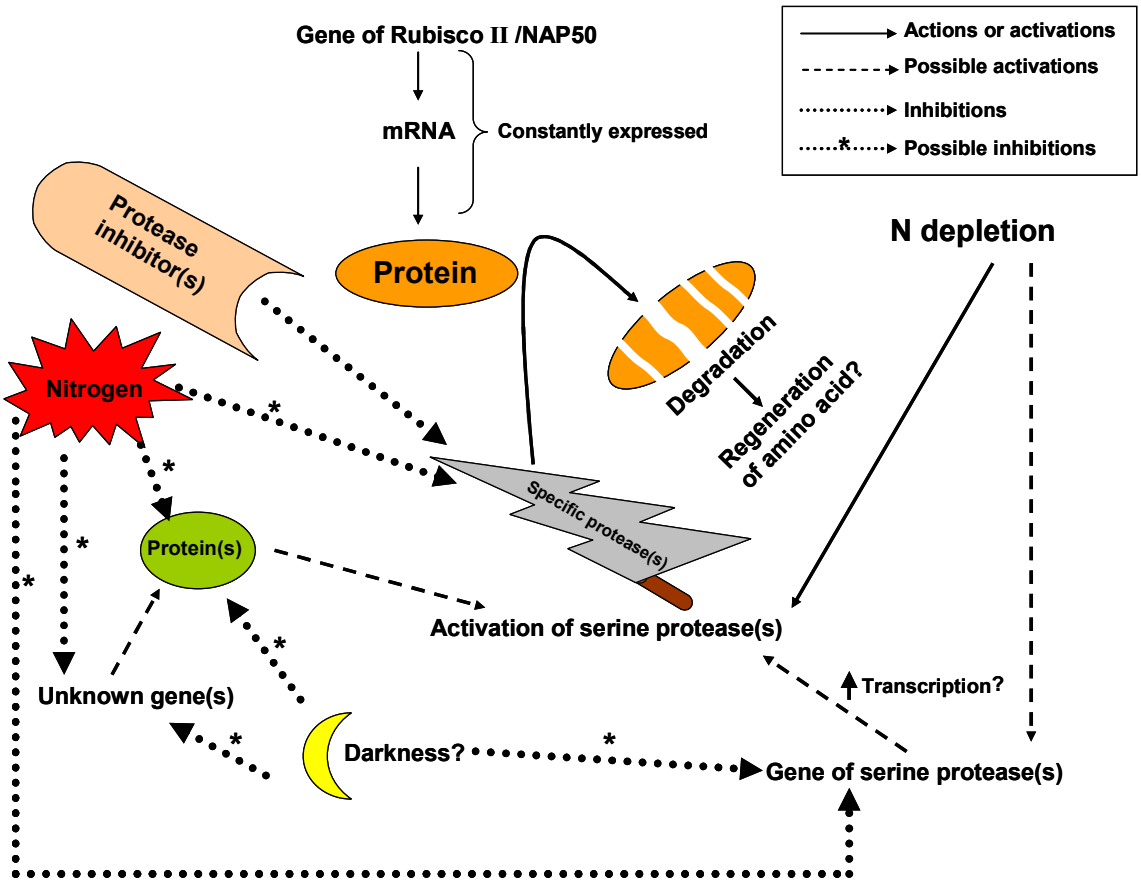


Figure 5.16 Schematic diagram showing the activation of rubisco II/NAP50-degrading protease(s) and their subsequent proteolytic actions on the target proteins.

5.3.7 Partial purification of rubisco-degrading serine protease(s) by benzamidine column

To further investigate the protein degradation mechanisms under nitrogen stress, an attempt to purify and identify the rubisco II-degrading protease(s) was made. To confirm the target protease(s) is a serine protease(s), another serine protease inhibitor (benzamidine) was used to test whether it can inhibit the degradation of rubisco II (Figure 5.17). These results suggested that the rubisco II-degrading protease(s) is a serine protease(s) and the protein degradation can be inhibited by adding the serine protease inhibitor either before (24+AE, 24+Ben) or after (36+AE, 36+Ben) the activation of protease(s).

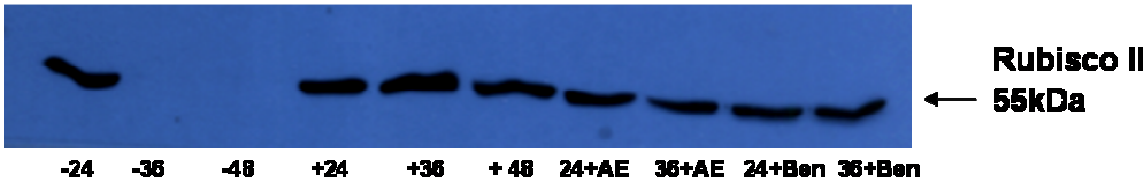


Figure 5.17 Inhibition of rubisco-degrading serine protease(s) by benzamidine under N-depletion. *A.affine* cells grown under N-depletion (-24, -36 and -48); N –repletion (+24, +36 and +48) and N-depletion with specific protease inhibitor including AE: AEBSF and Ben: Benzamidine added at 24 hour (24+AE and 24+Ben) and 36 hour (36+AE and 36+Ben) were analyzed with anti-Rubisco II antiserum. Samples with the additions of AE or Ben at 24 and 36 hour were harvested and analyzed at 36 and 48 hour respectively.

In addition, to ascertain that the protease is still function after cell lysis, an in vitro rubisco proteolytic assay of the cell extracts was constructed (Figure 5.8).

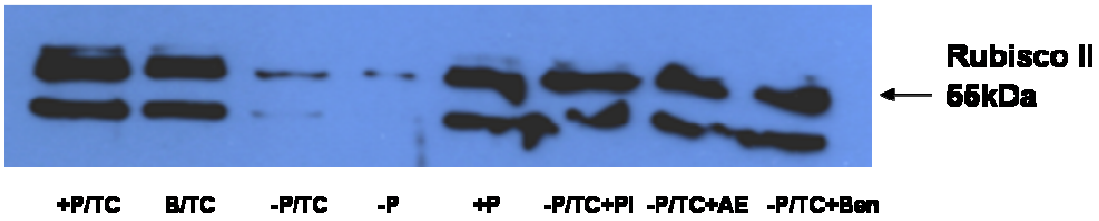


Figure 5.18 In vitro assay of the rubisco-degrading serine protease(s). +P/TC: cell extracts from N-repleted cells was added to testing cells; B/TC: Buffer (0.05M Tris pH8.2 with 0.02M $CaCl_2$) was added to testing cells; -P/TC: cell extracts from N-depleted cells was added to testing cells; -P: cell extracts from N-depleted cells only; +P: cell extracts from N-repleted cells only; -P/TC+PI: cell extracts from N-depleted cells was added to testing cells and protease inhibitor cocktails was also added; -P/TC+AE: cell extracts from N-depleted cells was added to testing cells and protease inhibitor AEBSF was also added; -P/TC+Ben: cell extracts from N-depleted cells was added to testing cells and protease inhibitor Benzamidine was also added. (Testing cells were cell pellet with $1.2x10^5$ exponential growing cells.)

From the results (Figure 5.18), it was obvious that rubisco was degraded in the N-deprived cell extracts (-P/TC), but not in the buffer (B/TC) and N-repleted cell extracts (+P/TC). It indicated that the protease is stable after cell lysis. Furthermore, the degradation could be completely inhibited by the additions of serine protease inhibitors (-P/TC+PI, -P/TC+AE, -P/TC+Ben) (i.e AEBSF or Benzamidine), suggesting that the rubisco-degrading protease is a serine protease. Thus, an attempt to purify the target protease was made using N-deprived *A. affine* cells and an benzamidine affinity column (Figure 5.19).

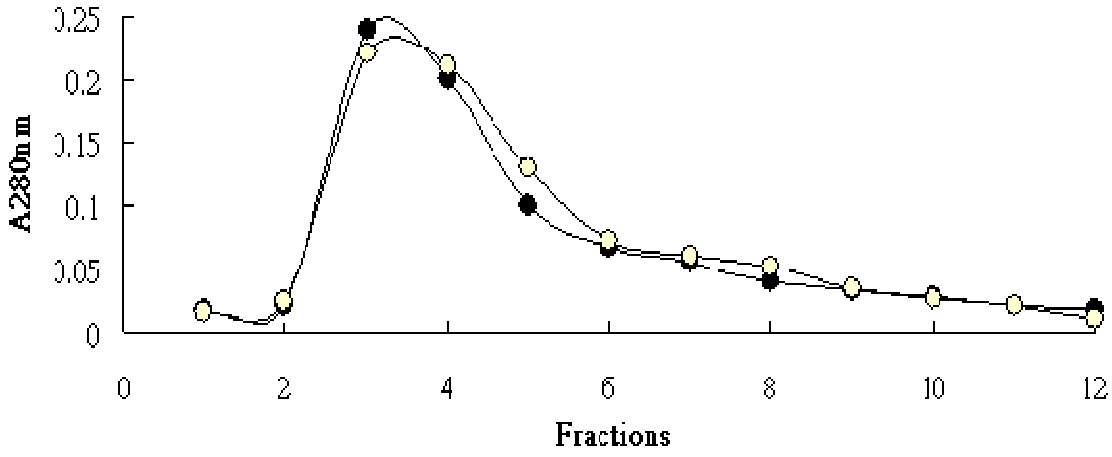


Figure 5.19 Elution profiles of the partial purification of rubisco-degrading serine protease(s) of *A.affine* under N-depletion. Proteins were eluted from Benzamidine-column with glycine-HCl pH 2.7. Fractions 3-5 were pooled for subsequent analysis. Open circle indicates elution profile of column eluted with glycine-HCl only (corresponding to Figure 5.19a). Closed circle indicates elution profile of column eluted with Benzamidine before the glycine-HCl elution (corresponding to Figure 5.19b).

The purification was performed by using benzamidine column with two slightly different eluting methods. Method 1: Proteins bound to benzamidine column were first eluted with acidic solution (glycine-HCl pH2.7) from the column, and then followed by competitive elution with benzamidine. Method 2: Proteins bound to benzamidine column were first eluted with stepwise concentrations of benzamidine, and then followed by acidic elution (glycine-HCl pH2.7). The proteolytic activities of the partially purified fractions were detected by the degradation of rubisco II in vitro assay. Interestingly, degradation of rubisco II was only observed in the acidic eluted fractions in both methods, suggesting that either the concentrations of benzamidine used were not enough to elute the target protease or the binding interaction between the target protease and the benzamidine ligands were too strong to be eluted. The SDS-PAGE and corresponding western blots of the crude homogenate compared with the partially purified sample showed that a partial purification was achieved (Figure 5.20). The purification factor was around 16-20 and the yield was around 3-3.6% (Table A1, Appendix XI). However, the partially purified fractions contain more than 10 discrete bands appeared in the SDS-PAGE, suggesting that identification of the target protease will be difficult to be identified.

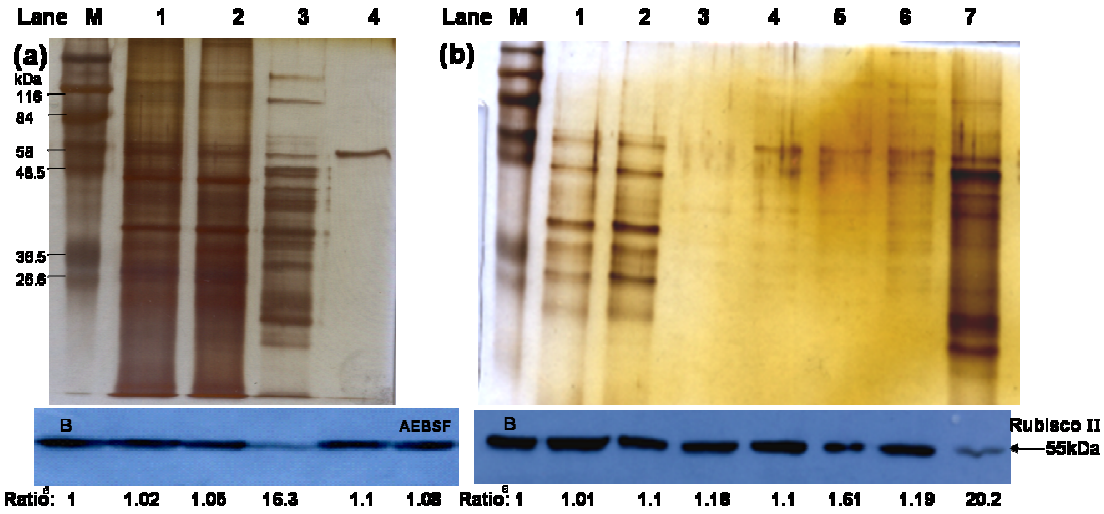


Figure 5.20 SDS-PAGE and western blotting analysis of the partial purified fraction eluted from the benzamidine column. (a) Proteins bind to benzamidine column were first eluted with glycine-HCl pH2.7 from the column, and then followed by elution with binding buffer containing 20mM benzamidine. Lane M: protein markers; lane 1: original protein extracts; lane 2: flow-through; lane 3: pooled fractions from the elution with glycine-HCl and lane 4: elute using binding buffer containing 20mM benzamidine. Each lane was loaded with 2 µg of proteins. For the western blot analysis, 1 µg of each samples were added to the testing cells and assay the present of the rubisco-degrading protease(s). Protein bands of the protein blot image below the SDS-PAGE were corresponding to the sample in each lane, except B: Buffer (0.05M Tris pH8.2 with 0.02M $CaCl_2$) was added to testing cells and AEBSF: elutant from the glycine-HCl elution together with AEBSF were added to the testing cells. (b) Proteins bind to benzamidine column were first eluted with stepwise binding buffer containing various concentrations of benzamidine, and then followed by elution with glycine-HCl pH2.7. Lane M: protein markers; lane1: original protein extracts; lane 2: flow-through; lane 3: elutant eluted from 4mM benzamidine; lane 4: elute using 12mM benzamidine; lane 5: elute using 20mM benzamidine; lane 6: elute using 40mM benzamidine; and lane 7: pooled fraction from the elution with glycine-HCl. Each lane was loaded with 0.5 µg of proteins. For the western blot analysis, 1 µg of each samples were loaded. Protein bands of the protein blot image below the SDS-PAGE were corresponding to the sample in each lane, except B: Buffer (0.05M Tris pH8.2 with 0.02M $CaCl_2$) was added to testing cells. Ratio[a] indicates the ratio of intensity (optical intensity x area) of the control protein band (testing cells added with buffer only) to each individual protein bands. The intensities of protein blots were detected by gel analysis software Melanie 3.0 (GeneBio, Switzerland).

The attempt to purify the rubisco II-degrading protease(s) was not successful and it can be attributed to several reasons. Firstly, amount of initial sample (N-depleted cells) was not enough for a series of purification steps. However, it should be emphasized that the target protease(s) is present only in N-deprived cells and cell growth in such nitrogen deprived condition are inhibited. Therefore, accumulating and the preparations of N-deprived samples are tedious, time-consuming and labor intensive. Secondly, it was found that the protease(s) would lose their degrading activity after freeze and thaw. The instability of the protease(s), leading to the storage of the samples during the purification becomes very difficult. Thirdly, to the best of my knowledge, there has never been any report on protease(s) purification in dinoflagellates. Since there is no references and background information of the target protease(s), more time is required for the optimization of the purification steps.

In fact, I had attempted to purify the target protease(s) of NAP50. The purification of NAP50 by the affinity column packed by the anti-NAP50 antibodies was performed (Figure A1, Appendix X). It was found that NAP50 is very unstable upon cell lysis even with the addition of an excess amount of protease inhibitors. The unavoidable fragmentation of NAP50 after cell lysis, suggesting it is impossible to have a functional assay for the purification of NAP50-degrading protease. Therefore, no further attempt to purify the NAP50-degrading protease was made.

In summary, a preliminary attempt to purify the rubisco II-degrading serine protease(s) from the N-deprived dinoflagellate *A. Affine* was made. Partial purification was achieved with more attempts needed. Sample fractionated with one-step benzamidine affinity column was not satisfactory. However, there are many limitations for the purification of this target protease.

5.4 Conclusions

In the present study, degradation mechanisms of two proteins (rubisco II and NAP50) under nitrogen depletion was investigated. NAP50 was found to be specific to *Alexandrium* species and immunohistochemical experiments suggested that both rubisco and NAP50 were located in the chloroplast. In the N-depleted conditions, it was revealed that both rubisco II and NAP50 have degraded to at least 16-times its original levels. There were no marked changes in both protein levels during the growth, daily cycles as well as phosphate-depletion, suggesting that the degradation of both proteins were strongly attributed to the N-deprived condition. Degradation of both proteins can be inhibited by the replenishment of nitrogen sources, suggesting that the protein degradation processes are reversible and highly regulated by nitrogen. There is no relation of rubisco II and NAP50 mRNA abundance with the availability of nitrogen, suggesting the degradation processes were not controlled at the transcriptional level. Protein degradation was triggered by specific proteases, which were activated in N-depletion. Different types of proteases were found to be responsible for the degradation of rubisco II and NAP50. Serine protease(s) was found to be responsible for the degradation of rubisco II. Metallo-protease(s) and serine protease(s) were found to be responsible for the degradation of NAP50. Although the detail degradation mechanisms of both proteins were unknown, preliminary results suggested that the protein degrading pathways of both proteins were different. Parial purification of the rubisco II-degrading protease was achieved. However, because of the time limit, further attempts were not made in the course of this thesis. Nevertheless, the preliminary results provide important information for the purification of the protease(s) in the near future. It should be stressed that protein

degradation by specific proteolytic protease during nitrogen stress in dinoflagellates has never been reported. The present study provides the first insight into protein degradation machineries of dinoflagellates in response to nitrogen stress.

CHAPTER VI

Rapid identification of dinoflagellates using protein/peptide profiles obtained with matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS)

6.1 Introduction

As said all along, HABs is a global problem (Hallegraeff, 1993). It occurs globally ranging from Europe, America, Asia to the Pacific regions (Premazzi and Volterra, 1993). The ever increasing risk of occurrence of HABs represents expanding threats to human health, fishery resources, and the tourism industries. Toxin-secreting species posted additional risk of intoxication when consumed either in seafood or directly. Rapid and accurate identification of the HAB species is critical for minimizing or controlling the damage. Microscope-based taxonomic identification methods have long been the standard protocols by which this task is accomplished. However, this type of taxonomic identification can be time-consuming and requires a high level of expertise to discriminate key morphological features indicative of HAB species. Furthermore, different criteria were used to classify different species by different taxonomists (Steidinger and Moestrup, 1990; Taylor, 1984). Therefore, taxonomic confusion and arguments on similar looking HAB species are common.

To resolve the taxonomic debate, different types of identification methods are being introduced for identification of dinoflagellates. One of the common identification methods is to analyze the sequences of the ribosomal RNA genes, including the small-subunit (16S rDNA, 18S rDNA) (Scholin and Anderson, 1994; Scholin et al., 1993; Scholin et al., 1994), large-subunit (28S rDNA) (Scholin et al., 1995; Scholin et al., 1994), and the internal transcribed spacers (ITS1 and ITS2) regions including the 5.8S rDNA (Adachi et al., 1994; Adachi et al., 1996). However,

the success of this method depends greatly on the primers design as they must bind effectively to the target. In addition, an incomplete description of the algal genome has limited the success of these approaches.

On the other hand, protein/peptide mass fingerprint profiles obtained by mass spectrometry (MS) is widely used for identification and characterization of microorganisms and has recently been extensively reviewed (Fenselau and Demirev, 2001). The use of matrix-assisted-laser-desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) to obtain these characteristic protein/peptide profiles (sometimes called biomarker profiles) for identification purposes has been applied to various microorganisms, such as viruses (Yao and Fenselau, 2001), bacteria (Donohue et al., 2006; Krishnamurthy and Ross, 1996; Krishnamurthy et al., 1996; Winkler et al., 1999), fungus(Amiri-Eliasi and Fenselau, 2001), parasites (Magnuson et al., 2000), bacterial spores (Dickinson et al., 2004; Hathout et al., 1999; Ryzhov et al., 2000) and fungal spores (Li et al., 2000; Welham et al., 2000). This type of identification method is praised as objective, fast, simple and reliable. Nevertheless, to the best of our knowledge, successful application of MALDI-TOF MS to obtain protein/peptide mass fingerprints profiles of HAB causative agents such as dinoflagellates for identification purposes has never been reported.

Therefore, attempts to use the protein/peptide profiles obtained by MALDI-TOF MS for identification purposes were made. In this chapter, it is aimed to demonstrate the application of MALDI-TOF MS for rapid, simple and accurate identification of dinoflagellate species. Results of this investigation clearly demonstrated the capability of this method in rapidly identification of different species of dinoflagellates (namely *A. affine, Prorocentrum minimum, Scrippsiella rotunda, Karenia brevis* and a yet to be

identified species*)*, as well as between individual species of *Alexandrium sp. and Scrippsiella sp*. This approach shows great potentials to be used for the continuous monitoring of water samples for the occurrence of HABs.

6.2 Materials and methods

6.2.1 Dinoflagellate species and strains

Dinoflagellate species used in this study are listed in Table 6.1. *A. affine, Prorocentrum minimum, Scrippsiella rotunda,* and a yet unidentified species were kindly provided by Prof. John I. Hodgkiss previously of The University of Hong Kong. The other species, including *Alexandrium catenella* (CCMP1598), *Alexandrium minutum* (CCMP113) and *Alexandrium tamarense* (CCMP116) and *Karenia brevis* (CCMP2281) were purchased from The Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP). Identities of all species used, except the unidentified species and *Karenia brevis* (CCMP2281), were confirmed either by the sequence of the ribosomal RNA genes or the internal transcribed spacers (ITS1 and ITS2).

6.2.2 Culture conditions

Seawater based K or f/2 media were used for culturing the dinoflagellates (Keller et al., 1987). Stock cultures of all dinoflagellates were kept at exponential growth phase by transferring to new medium every five or six days in a ratio of 1:10 v/v. Vegetative cells from cultures in mid- or late-exponential phase of growth were inoculated into freshly prepared culture medium. Possible contamination of algal culture was monitored by regular microscopic examination. The cultures were grown at 22℃ under 16: 8 hours light: dark cycle at a light intensity of 120μE Lux $m^{-1}s^{-1}$ provided by cool white fluorescent tubes in a Conviron growth chamber (Model EF7).

6.6.3 Cell counts

Cell density was counted in the same time everyday; 1ml of each culture was taken and fixed with 10µl Lugol's solution and counted under light microscope with a Sedgwick-Rafter cell counter.

6.6.4 Ribosomal gene and Internal Transcribed Spacers (ITS) sequencing

Cells were collected by centrifugation (1500 x g for 10 min at room temperature) from mid-exponential culture and frozen in liquid nitrogen prior to DNA extraction. DNA was extracted by an extraction kit (Roche, Switzerland) following mechanical cell disruption by a quick homogenization. ITS regions containing the 5.8S rDNA were amplified from the extracted genomic DNA with PCR by using the ITSA and ITSB primers (Adachi et al., 1994; Adachi et al., 1996), or using ITSF1 (forward primer): 5'TgAACCTTAYCACTTAgAggAAggA3' and ITSR1 (reverse primer): 5'gCTRAgCWDHTCCYTSTTCATTC3', which these two primers were designed by the alignment of the 3' end of 18S rDNA sequences and the 5' end of 28S rDNA sequences from the database of different dinoflagellate species. PCR were performed under conditions: 95℃ 5min; 35 cycles of 94℃ 45s, 50℃ 45s and 72℃ 2min; 72℃ 10min. PCR products were cloned into pGEM-T easy vectors (Promega, USA) prior to DNA sequencing. DNA sequencing of all cloned plasmids were performed by commercial facilities using traditional dideoxy-methodolgy.

6.6.5 Sample preparation to obtain protein/peptide mass fingerprints

About 250 ml cultures were grown to mid-exponential growth phase. Around 10^6 cells were collected by centrifugation (1500 x g for 10 min at room temperature). Cell pellets were resuspended in 0.1 % trifluoroacetic acid (TFA) (Aldrich, USA). These cells were broken by sonication for 20 seconds on ice. Cell debris was removed by centrifugation at 14000 x g at 4^oC for 5 minutes. Inorganic salts in the samples were cleaned up by absorbing the proteins/peptides onto C-18 zip tips (Millipore, USA) and subsequently washed with several volumes of 0.1 % TFA and 5% methanol in water. Proteins/peptides were eluted from the zip-tip with 0.1% TFA in 50% acetonitrile. 1 µl of eluted proteins/peptides solution were mixed with 1 µl of matrix solution. Martix solution contained saturated sinapinic acid (SA) in 50/ 50 (v:v) 0.1 % TFA/ acetonitrile. The resulting mixtures were then vortexed before spotting 0.5 µl volume onto a mass spectrometer target plate (MTP AnchorChip™ 600/384 T F) (Bruker, Germany). For testing the effects of different preparation steps, above procedures were repeated but with some changes including (1) without sonication, (2) without cleaning up by zip tips, (3) without cleaning up by zip tips but samples were dried by speed vacuum and resuspended with 0.1% TFA in 50% acetonitrile and (4) dried by speed vacuum and resuspended with 0.1% TFA in 50% acetonitrile, then clean-up with zip tips. After that, effects of using two different matrices (HCCA and SA) and different concentration of ACN were also evaluated.

6.6.6 MALDI-TOF MS analysis

Proteins/peptides mass fingerprints in all samples were obtained with a MALDI-TOF mass spectrometer (Autoflex, Bruker, Germany) in linear mode at an accelerating voltage of 20 kV by using a 300ns delay time and over a mass range of 2000–20000Da. For each sample, spectra from 500 laser shots at several different positions were combined to generate a mass spectrum. The mass spectra were calibrated using Protein Calibration Standard I (Bruker, Germany) and were used to provide a mass accuracy of 1 part in 3000. The calibrant mixture contains Insulin (5734.51 Da), Ubiquitin (8565.76 Da), Cytochrom c (12360.97 Da) and Myoglobin (16952.3 Da). Fresh calibration was performed for everyday for different samples and for different experiments.

6.3 Result and discussion

6.3.1 Comparison of MALDI mass spectrum obtained by different sample preparatory methods

Although there were many studies on microorganisms using MALDI, there is no golden sample preparation method for MALDI analysis. Different optimized methods have to be developed for different types of samples. Thus, it is important to determine how a good mass spectrum that with discrete peaks from the dinoflagellate samples could be produced prior to the protein profiling studies on different dinoflagellates. Five sample preparations of the dinoflagellate samples (*A. affine*) for MALDI analysis were evaluated (Figure 6.1). In Figure 6.1a where the cells were suspended in 0.1% TFA and without cell lysis before MALDI analysis, there were no obvious discrete peaks observed in the spectrum. Two very tiny peaks appeared in 5000-6000 m/z indicates that little proportion of the cells were lysed in 0.1 % TFA. Although the intensities of these peaks were increased after cells lysis with sonication, the intensities and numbers of the peaks were still small (Figure 6.1b). This may be attributed either to the presence of inhibitors/salts that prevent the ionization of the mass ions (peptides), or simply because there is not enough amount of dissolved peptides. Therefore, the protein/peptides extracts were either concentrated by speed vacuum (Figure 6.1c) or cleaned-up with zip tips (Figure 6.1d) prior to MALDI analysis. Although the intensities and numbers of peaks in both spectrums were increased, peaks in the spectra with cleaned-up procedures using zip tips were better in terms of both intensity and number. In addition, there was no further improvement if both concentrating (speed vacuum) and cleaned-up (zip tips) procedures were combined (Figure 6.1e). These indicate that the removal of salts/contaminants from the peptides were very important for the subsequent ionization which in turn affect

quality of the MALDI mass spectrum. It should also be noted that concentrating the samples with speed vacuum will not improve the spectrum even if the samples were cleaned-up after the concentrating step. This is mainly due to difficulties in re-solublization of the dried peptides.

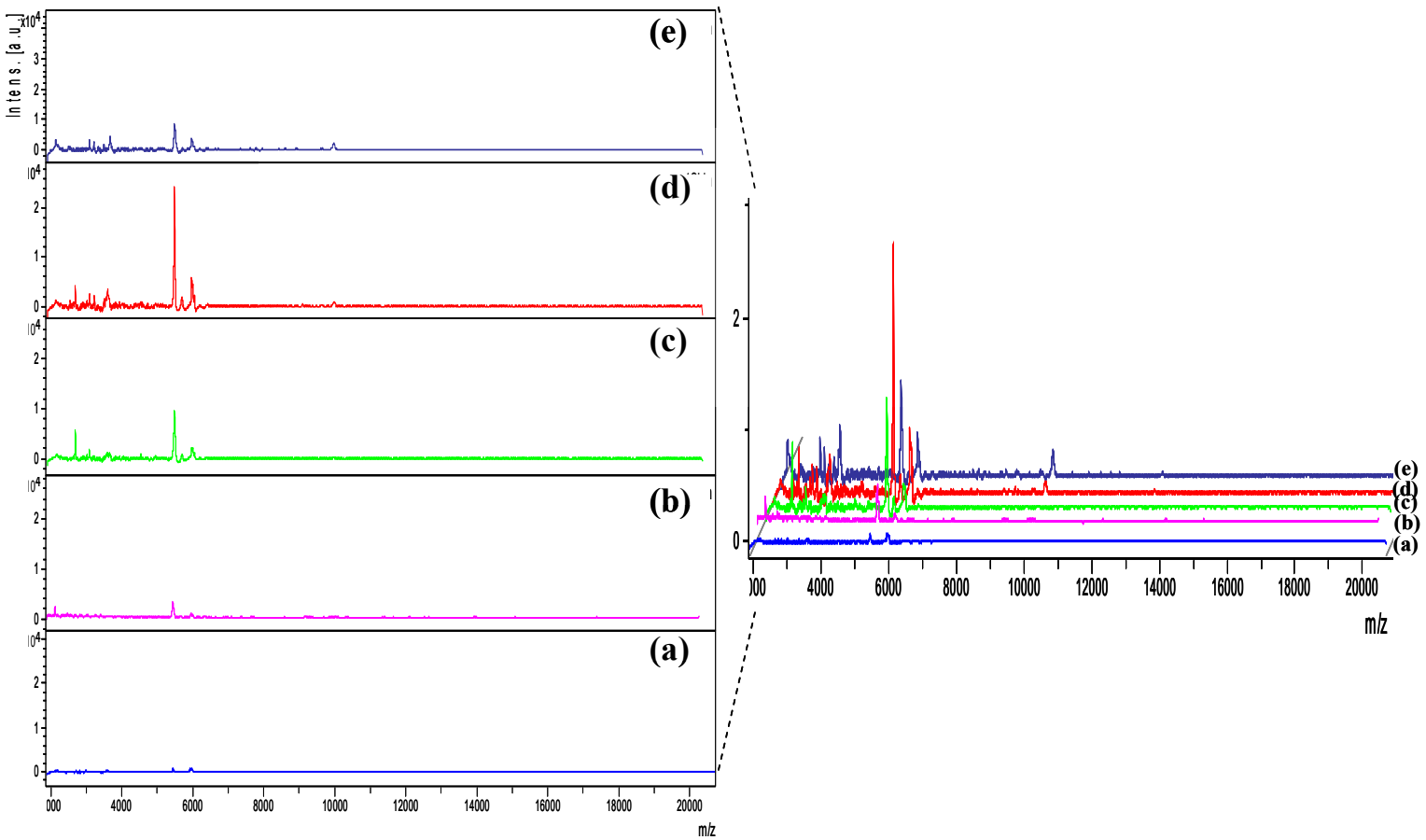


Figure 6.1 MALDI mass spectra of *A.affine* that with different sample preparation methods. The mass range depicted is from m/z 2000 to 20000. (a) cell suspensions (in 0.1% TFA) without cell lysed by sonication; (b) supernatant of cell lysate (in 0.1% TFA); (c) same as (b) but supernatant was dried by speed vacuum and resuspended in 50% ACN with 0.1% TFA; (d) same as (b) but sample was cleaned-up with zip tips and (e) same as (c) but sample was cleaned-up with zip tips.

Although some peptides are dissolved in aqueous solutions readily, a significant number of peptides (especially those containing hydrophobic amino acids) have very low solubility or even insoluble. Therefore, solubility of peptides with different concentrations of acetonitrile (ACN) was evaluated. The number of peaks increased with the percentage of ACN (Figure 6.2). More than 10 discrete peaks were observed when 70% of ACN used (Figure 6.2d). This indicated that the peptides/proteins of dinoflagellates were relatively hydrophobic and a higher concentration of ACN is required to solubilize the peptides/proteins.

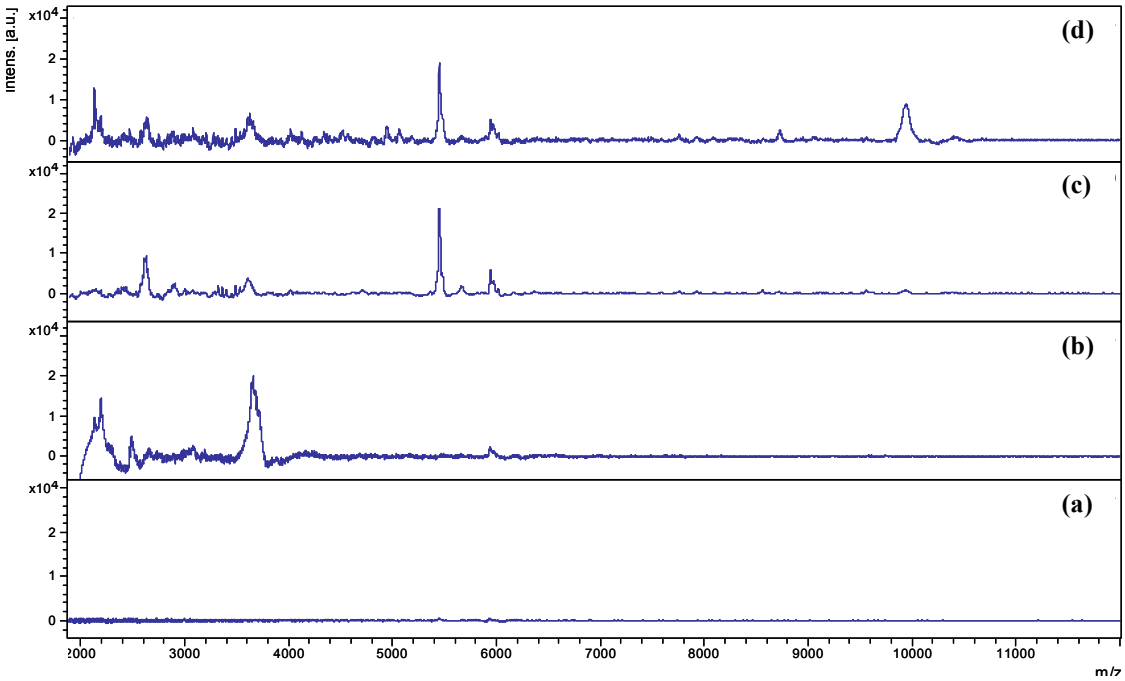


Figure 6.2 MALDI mass spectra of *A. affine* that samples were resuspended in different concentration of ACN with 0.1% TFA. The mass range depicted is from m/z 2000 to 12000. (a) 10% ACN; (b) 30% ACN; (c) 50% ACN and (d) 70% ACN.

In the MALDI analysis, the ionization process is triggered by a laser beam (nitrogen laser). A matrix is used to protect the biomolecule from being destroyed by direct laser beam and to facilitate vaporization and ionization. Therefore, choice of matrix is also one of the important determinant for high quality MS spectrum. Two commonly used matrices, α-cyano-4-hydroxycinnamic acid (HCCA) and sinapinic acid (SA), were compared (Figure 6.3). It was obviously that the intensities and numbers of discrete peaks from SA (Figure 6.3b) were much higher than that of HCCA (Figure 6.3a). SA is generally more suitable for ionization of peptides/proteins with higher molecular mass. Therefore, SA was used in the later part of the experiments.

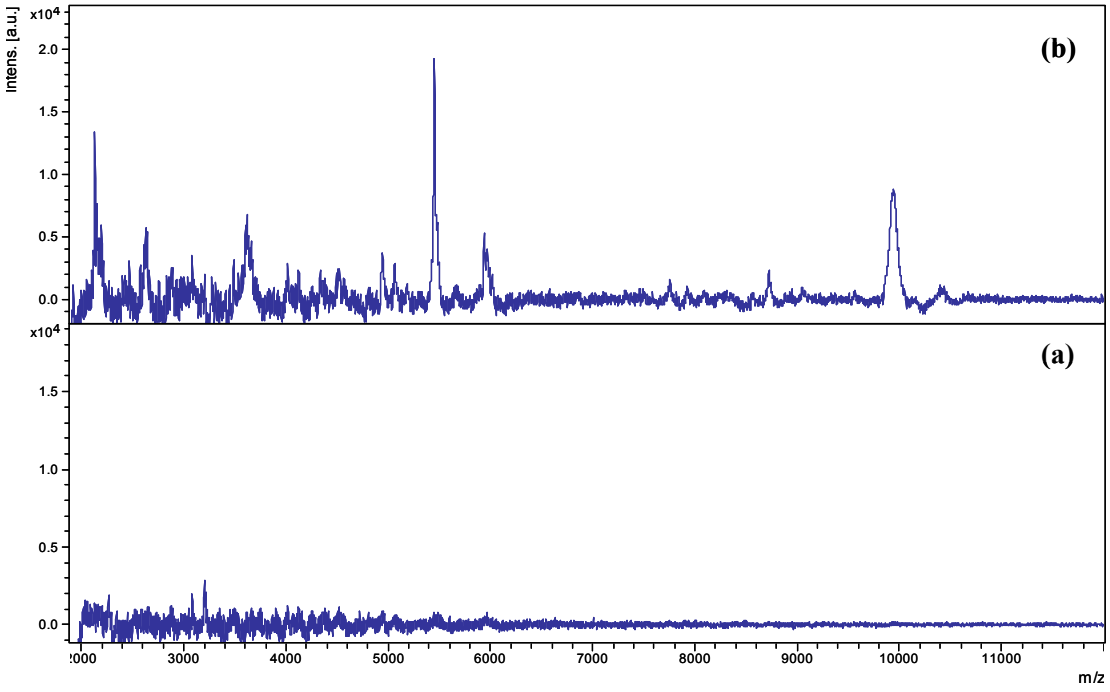


Figure 6.3 MALDI mass spectrums of *A. affine* that with two different matrixes. The mass range depicted is from m/z 2000 to 12000. (a) saturated α-cyano-4-hydroxycinnamic acid (HCCA) and (b) saturated sinapinic acid (SA)

6.3.2 Protein profiling and PCR-based identification

As elaborated earlier, the dinoflagellates cultures used in this study were from 2 sources. Some of the dinoflagellates were purchased from CCMP (The Provasoli-Guillard National Center for Culture of Marine Phytoplankton) and their identities had been validated. All the other cultures used in this study were a kind gift from Prof. John I. Hodgkiss, previously of The University of Hong Kong. Identities of these dinoflagellates were provided by taxonomists and were never validated by molecular biological tools. Hence, these dinoflagellates were subjected to PCR-based identification by analyzing the ITS sequences (Table 6.1) (Appendix V-IX). The readers are reminded of the fact that one of the supposedly *A. tamarense* turned out to be *A. affine* (Chapter 2). Therefore, identification with molecular biological tools is necessary. However, PCR of the ribosomal genes of one species (temporary called an unidentified species) was not successful with the three different sets of universal primers; ITSA and ITSB (Adachi et al., 1994; Adachi et al., 1996), ITSF1: 5'TgAACCTTAYCACTTAgAggAAggA3' (forward primer) and ITSR1: 5'gCTRAgCWDHTCCYTSTTCATTC3' (reverse primer), ITS 1 and ITS 4 (D'Onofrio et al., 1999). It should be stressed that choices of primers are very important for the success of the PCR-based identification. In addition, the annotation process of this perhaps new/different species could be time-consuming and certainly not within the theme and scope of this thesis, therefore, I would to leave that as it is at the moment. Furthermore, in contrast to PCR-based methodology, the protein profiling based identification method needs no initial assessment of unknown HAB samples which result in a simple and fast identification method.

Table 6.1 Dinoflagellate species and strains used in this study

Species/strains	Source	Collection site (Date)	Confirmed sequences	Accession
Karenia brevis(CCMP2281)	CCMP	Gulf of Mexico, Florida, USA	nil	nil
Alexandrium catenella (CCMP1598)	CCMP	Da-ya Bay Guan-dong (Canton) Province, Hong Kong	18S*	**AY027906**
Alexandrium minutum (CCMP113)	CCMP	Ria de Vigo, Spain (September,1987)	18S and ITS*	**AY831408**
Alexandrium tamarense (CCMP116)	CCMP	Ria de Vigo, Spain (June, 1984)	ITS*	**AJ005047**
Alexandrium affine	IJH	Junk Bay, Hong Kong (August,1998)	ITS	**EF579793**
Prorocentrum minimum	IJH	Junk Bay, Hong Kong (1998)	ITS	**EF579797**
Scrippsiella rotunda 1	IJH	Junk Bay, Hong Kong (March,1997)	ITS	**EF579794**
Scrippsiella rotunda 2	IJH	Kat O, Hong Kong (October,1998)	ITS	**EF579795**
Scrippsiella rotunda 3	IJH	Junk Bay, Hong Kong (1998)	ITS	**EF579796**
Unidentified species	IJH	Junk Bay, Hong Kong (1997)	#	#

CCMP: The Provasoli-Guillard National Center for Culture of Marine Phytoplankton
IJH: Donated by Prof. John I. Hodgkiss previously of The University of Hong Kong
ITS: Internal transcribed spacers
* Ribosomal DNA sequencing was done by other authors
# PCR of the ribosomal gene regions was not successful by using primers mentioned in the materials and methods section and the universal primers (ITS 1 and ITS 4) described by other authors (D'Onofrio et al., 1999)

6.3.3 MALDI-TOF mass spectra

The key success of this methodology is the ability to see either different sets of characteristic protein/peptide mass fingerprint spectra or individual signature mass biomarker (individual species specific mass fingerprint) for different species of dinoflagellates. Further, the differentiation between species with different sets of characteristic protein/peptide mass fingerprint spectra or individual signature mass biomarker should ideally be seen in a desirable mass/charge (*m/z*) range in the MALDI analysis. In this study, mass spectra (protein/peptide mass fingerprints) of the dinoflagellate samples generally exhibited around ten discrete mass peaks ranging from *m/z* 2000 to 20000. MALDI-TOF identification of other microbes showed similar numbers of discrete mass peaks over the same m/z range (Fenselau and Demirev, 2001). Moreover, MALDI-TOF MS analysis of f/2 and K medium returned no significant peak signals in the 2000 to 20000 *m/z* range (data not shown). MALDI-TOF MS peak mass fingerprint spectra obtained from the different genus of dinoflagellates studied are easily distinguishable by visual inspection (Figure 6.4) and each set of peak mass fingerprint spectra from different species are distinct. In addition to the unique peak mass spectral patterns, a number of unique and consistently occurring signature peak mass ions (biomarkers) can be identified that are characteristic at the genus and species level (Table 6.2). Therefore, either the peak mass spectral fingerprints or the individual characteristic peak mass biomarker can be used for identification purposes. Software and algorithms may be designed and applied to aid in the analysis of such spectral markers (Jarman et al., 2000; Jarman et al., 1999). Coupled with a universally accepted taxonomic database with DNA information, this methodology will allows unambiguous identification of HABs

causative agents.

Table 6.2 Biomarkers observed in MALDI-MS of dinoflagellate species

Species	Signature peak mass biomarkers (m/z)
Alexandrium canetella (CCMP1598)	2194, 2519, 2897, 3123, 3561, 8399, 8618, 12381, 17230
Alexandrium minutum (CCMP113)	2673, 4984, 5520, 10580, 12545, 15614, 17257
Alexandrium tamarense (CCMP116)	2418, 4094, 4473, 6169, 8024, 9050, 10085, 15494
Alexandrium affine	2615, 3603, 5445, 5656, 5936, 10388
Prorocentrum minimum	4852, 6662, 9704,
Scrippsiella rotunda	2175, 3803, 5017, 8724, 10045, 10256, 16089
Karenia brevis (CCMP2281)	3910, 4139, 6665, 7462, 9866, 9974, 12086, 19101
Unidentified species	2753, 4802, 7916, 9817, 10716

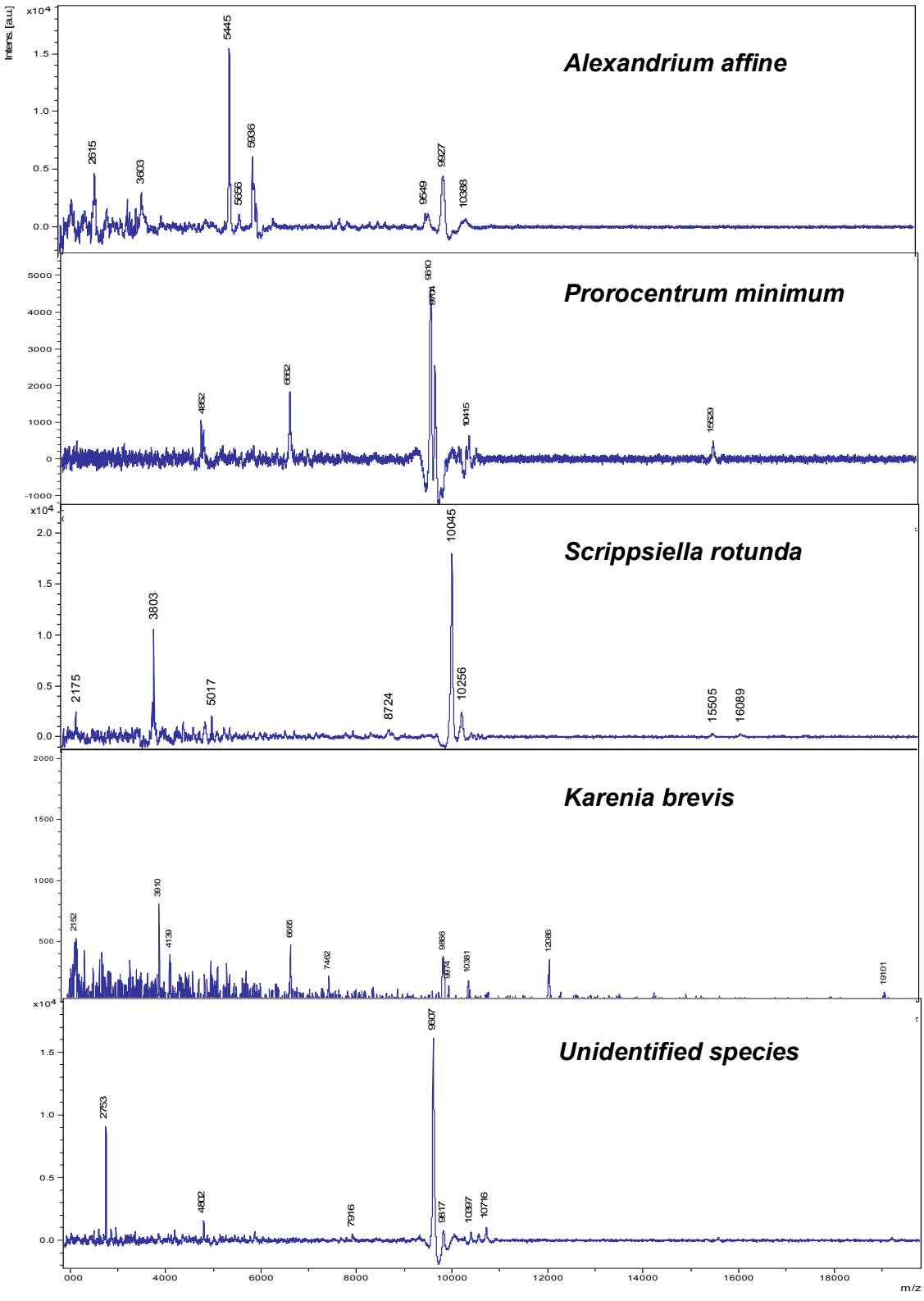


Figure 6.4 MALDI-TOF MS protein profiles of dinoflagellate species in different genus. The mass range depicted is from m/z 2000 to 20000. Individual species are listed.

6.3.4 Specificity and sensitivity

Another important issue associated with this developed methodology is the ability to differentiate between species that have similar morphologies. Dinoflagellate species within the genus of *Alexandrium* are highly similar in their morphological appearances when observed under microscope. They are difficult to be distinguished by their morphological features. Hence, this is not surprising that there are taxonomic confusion and argument on classification within this genus. In this study, however, the peak mass fingerprint spectral patterns obtained for different *Alexandrium* species are different (Figure 6.5). As mentioned previously, characteristic signature biomarker mass peaks that permit differentiation and identification of a particular *Alexandrium sp.* are also identified (Table 6.2). Therefore, this MALDI-TOF MS identification technology is an excellent alternative to classical microscopic identification techniques for the identification of closely-related HAB species.

Specificity of identification by using MALDI-TOF MS mass fingerprint spectral pattern methodologies on microorganism had been shown down to individual strains or even sub-species level (Krishnamurthy and Ross, 1996). However, in this study, dinoflagellates could only be distinguished from each other down to the species level. In my hands, there are three different local strains of *Scrippsiella rotunda* (Table 6.1) which were collected separately at different time. Three strains have different growth rate (data not shown). However, when these three strains were analyzed by the MALDI-TOF MS under identical conditions, the peak mass fingerprint spectral patterns of the three strains obtained were almost identical (Figure 6.6).

With respect to amount of samples required for the analysis, it was found that 10^5 of dinoflagellates cells are enough to generate a reasonable peak mass fingerprint

spectrum with the presence of all the representative peaks. We have tried to use 10^4 cells for the MALDI-TOF MS analysis, but only the most abundant peak masses were seen. Nevertheless, although this is not within the scope of this thesis, modifications of sample preparatory procedures before MALDI-TOF MS analysis could be studied further aiming to improve on the sensitivity of this method.

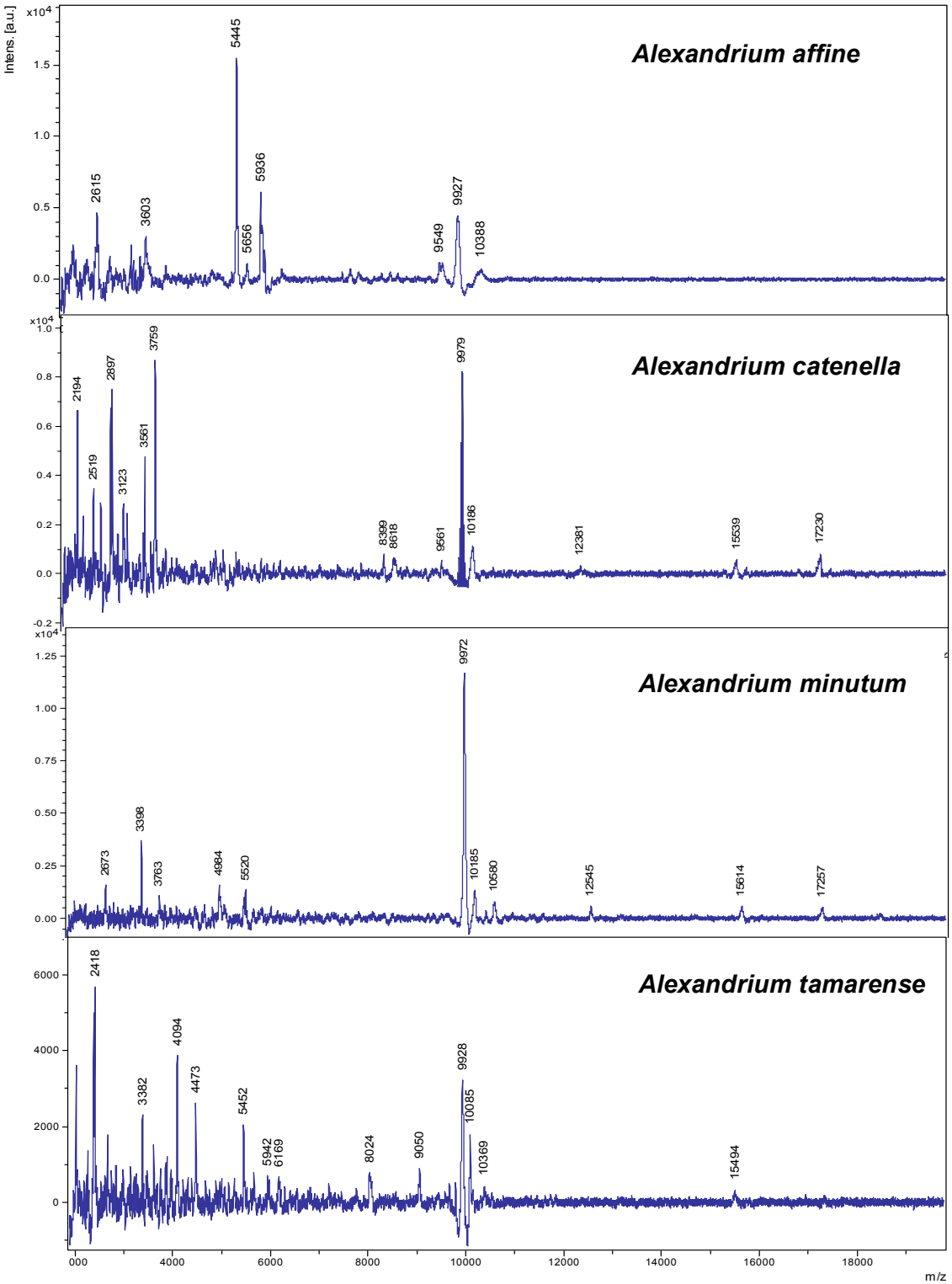


Figure 6.5 MALDI-TOF MS protein profiles of dinoflagellate species in same genus (*Alexandrium*). The mass range depicted is from m/z 2000 to 20000. Individual species are listed.

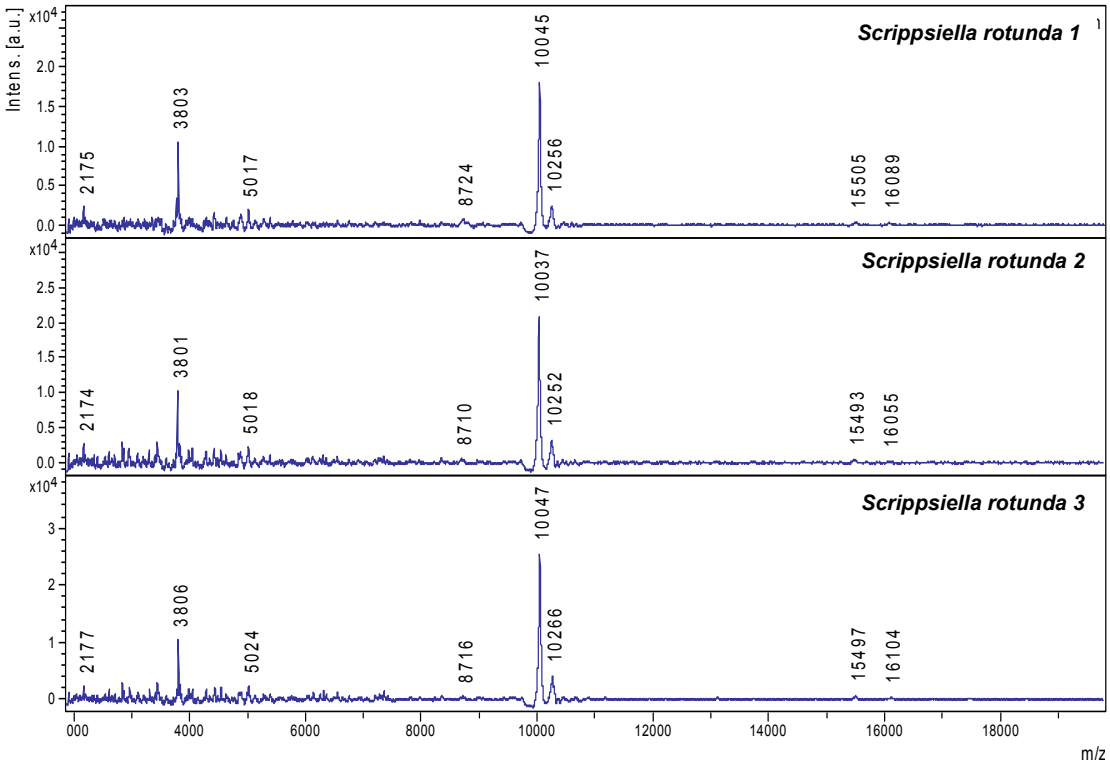


Figure 6.6 MALDI-TOF MS protein profiles of different strains of *Scrippsiella rotunda*. The mass range depicted is from m/z 2000 to 20000.

6.3.5 Reproducibility

The mass profiles presented in Figures 6.4 and 6.5 were representatives of three different batches of the same samples and each experiment was performed in different date. Given that sample preparation and measurement were performed under the same conditions, the acquired peak mass fingerprint spectra are the same between different batches of the same samples.

Further, in order to be qualified as a rapid and reliable identification method, it is important that a reproducible peak mass fingerprint spectral pattern can be obtained with the MALDI-TOF MS analysis irrespective of specific stages in the growth cycle and growth conditions. When applying this methodology to other microbes, it is reported that peak mass fingerprint spectral pattern distributions were dependent on different compositions of growth media (Evason et al., 2000; Valentine et al., 2005).

However, species and genus level distinctions are possible when the growth and experimental conditions had been properly controlled (Evason et al., 2000; Valentine et al., 2005). Therefore, in order to investigate if growing conditions have any effects on the MALDI-TOF mass spectra, peak mass fingerprint spectral patterns of a dinoflagellate species grown under two different culture media (f/2 and K medium) were compared. Highly similar peak mass spectral patterns were observed for both samples (data not shown). It seems that growth media has no observable effect in the peak mass spectral patterns observed. In addition, we had found that an identical amount of *A. affine* cells (about 10^5 cells) collected at different growth states exhibited the near-identical sets of specific peak mass spectral patterns (Figure 6.7). Cells in the lag phase of growth (Day 1) show a very similar peak mass spectral pattern as cells in mid-log (Day 3), late-log (Day 6), stationary or early death phase (Day13). The remarkable similarity of the peak mass spectral patterns were based on the measurement of constantly expressed highly abundant proteins in the dinoflagellate proteomes, for example the ribosomal proteins (Ryzhov and Fenselau, 2001). Therefore, it is not surprising that most of the peak mass ions, especially major ones such as 5445, 5656, 5936, 9527, 9949, 10388 *m/z* are not affected by growth states of the cells (Figure 6.7).

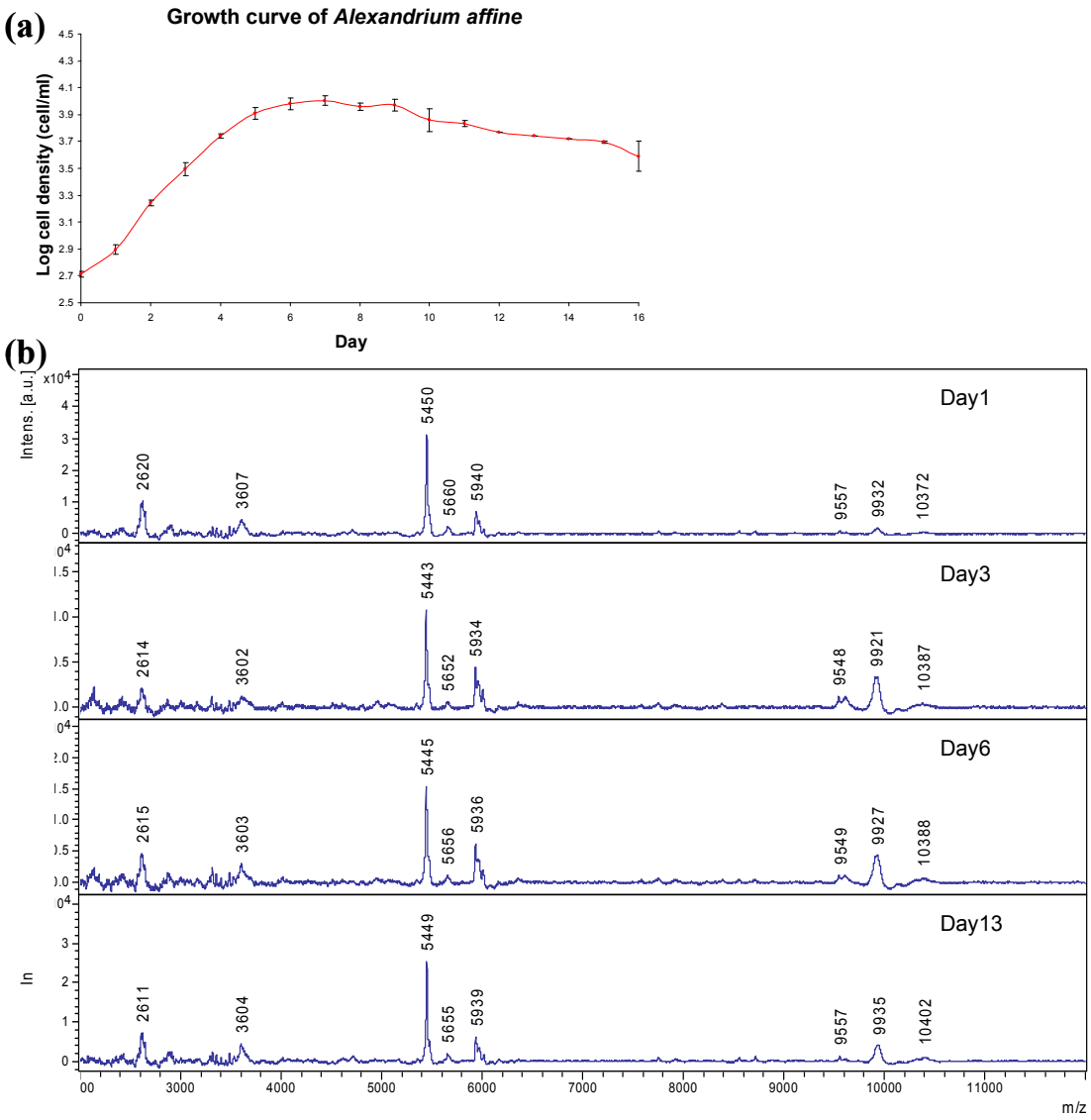


Figure 6.7 MALDI-TOF MS protein profiles of *Alexandrium affine* in different growth phases. (a) Growth curve of *Alexandrium affine*. Cells were counted every day, each data point was from the means of triplicate counts. (b) Protein profiles of *Alexandrium affine* in different growth phases. Day 1: lag phase; Day 3: mid-log phase; Day 6: late-log phase and Day 13: stationary phase. The mass range depicted is from m/z 2000 to 12000.The corresponding day of cells harvest are listed.

6.3.6 Recognition of different species of dinoflagellates in a mixed culture

In the real world with a real red tide, usually one or two HAB species from different taxonomic groups would predominate (Hallegraeff, 1993). Therefore, it is important to investigate whether it is possible to differentiate different dinoflagellate species from a mixed-culture. Two different species, *Prorocentrum minimum* and *Scrippsiella rotunda* (in different genus) (Figure 6.8a); *Alexandrium canetella* and *Alexandrium affine* (in same genus) (Figure 6.8b) were grown in a mixed culture. The mixed culture was harvested and analyzed by the MALDI-TOF MS under the same conditions as described in the Methods section. The results obtained showed that several species-specific notable signature peak masses can be easily identified from the peak mass fingerprint spectrum of the mixed cultures. In Figure 6.8a, signature biomarker peak masses of *Prorocentrum minimum* (P) *m/z* 4852, 6662, 9610, 9704, 10415 and biomarker peak masses of *Scrippsiella rotunda* (S) of *m/z* 2175, 3803, 5017, 10045, 10256; are easily identified from the peak mass spectrum of the mixed culture (P+S). In addition, individual biomarker peak mass can still be identified from the mixed culture containing two looking-alike species, *Alexandrium sp* (Figure 6.8b). Further, other notable biomarker peak masses of *m/z* 2194, 2897, 3759, 9979 from *Alexandrium canetella* (Ac) and *m/z* 2615, 5445, 5656, 5936, 9549, 9927 from *Alexandrium affine* were also easily identified from the mixed culture spectrum (Ac+Af).

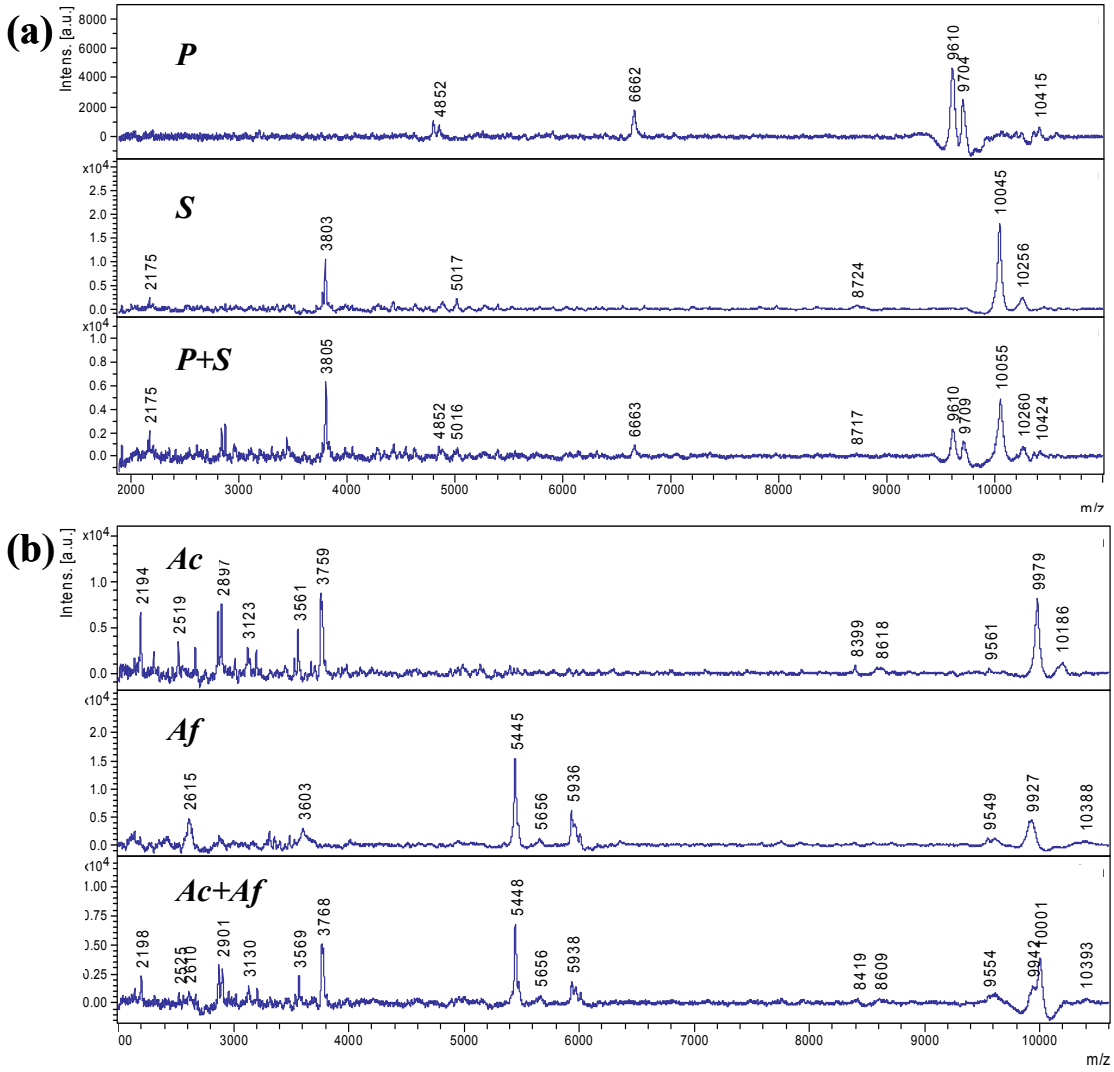


Figure 6.8 MALDI-TOF MS protein profiles of mixed populations of different dinoflagellate species. (a) Protein profiles of *P*: *Prorocentrum minimum; S*: *Scrippsiella rotunda* and *P+S*: *Prorocentrum minimum* and *Scrippsiella rotunda*. (b) Protein profiles of *Ac*: *Alexandrium canetella*; *Af*: *Alexandrium affine* and *Ac+Af*: *Alexandrium canetella* and *Alexandrium affine*. The mass range depicted is from m/z 2000 to 12000.

6.3.7 Potential of the technique used in the identification of field samples

Although ability of the peptide/protein profiling-based identification in different cultured dinoflagellate samples were successfully demonstrated, one may have concerns on the potential of successful application of this technique in the field or real red tide samples. I strongly believed that the proposed identification method having great potential applied to the actual situation after the fulfillment of some requirements. Firstly, the fingerprint spectrum and the signature peaks of different existing HAB species should be well identified and annotated. Secondary, all the identified signature peaks obtained should be well documented and developed into a database with a software program, for example MALDI BioTyper (Bruker, Germany). Thirdly, a "standard" sample preparation procedure including all the instrumental settings should be developed for all users to avoid any variations when different methods were applied by different users. By having more studies on this issue and having the above requirements fulfilled, the technique may be transferred to technical personnel in the Government or Monitoring Agencies for identification and monitoring purposes.

6.4 Conclusions

I had demonstrated that it is possible to identify dinoflagellate species either in a single cell culture or in a mixed population of different dinoflagellate species based on characteristic peak mass spectral patterns or species-specific signature biomarkers in the spectrum. Identification and differentiation of closely related species such as *Alexandrium affine, Alexandrium catenella, Alexandrium tamarense, Alexandrium minutum,* can be accomplished easily by the MALDI-TOF MS analysis. Although the identification would become complicated when more than two species are presented in the mixed culture, it may still be possible to identify the species compositions with the aid of computer software based on the analysis of the specific signature biomarker peak ions. The workflow of HAB species protein/peptide peak mass fingerprint spectral profiling is a straightforward approach. The method is simple, rapid, accurate and reproducible. It represents an excellent alternative to classical microscopic-based identification techniques. The peak mass fingerprint spectral pattern is unique for different dinoflagellate species and is easily distinguishable by visual inspection. In addition to the whole peak mass spectra, several specific signature biomarkers were identified from the mass spectra. Therefore, the signature biomarker ions and the peak mass fingerprinting spectral profiles form an unambiguous set of criterion for identification of dinoflagellate species.

CHAPTER VII

Concluding remarks

HAB is a global problem and the incidence of its occurrence is rising. Although the mechanism of of the sudden bloom is unknown, it has long been speculated that pollution caused by human activities and industrialization is root of the evil. Among the excessive nutrients presented in the polluted water system, nitrogen is believed to be an important factor in the initiation and maintenance of phytoplankton blooms. Although there are many studies on the effects of nitrogen on the growth of dinoflagellates, very little is known about the changes inside the cells at the protein level. Dissection into the proteome of dinoflagellate was not reported. It would be of great interest to pinpoint proteins that are either directly related to the blooming process or growth in response to various nitrogen concentration. Proteomic studies on these causative organisms of HABs have the potential to uncover cellular pathways and mechanisms involved in blooming at the molecular level.

A fast-growing dinoflagellate species *Alexandrium affine* was chosen as the model to study. *A. affine* has the ability to assimilate different kind of nitrogen sources including nitrate, ammonium, urea, glycine and aspartate for cell growth. The highest maximum growth rate (0.9 day^{-1}) and cell abundance (12000 cells ml^{-1}) obtained in nitrate-enriched cultures showed that nitrate is the preferred nitrogen source. The nitrogen-dependent growth experiments showed that when nitrogen was depleted, the growth of these cells stopped and was maintained at a basal cell density of around 500-700 cell ml^{-1} throughout the entire period of 14 to 18 days. After N was re-introduced as the N source, these N-repleted cells would undergo lag phase for the first day after N addition afterwhich the growth of these cells resumed. In the

nitrogen-depleted cultures, there was no detectable nitrate. From the results of nitrate measurement and assay on the nitrate reductase (NaR) activity, it is implicated that nitrate was not available in the N-depleted cultures and a rapid uptake of N occurred in the N-enriched cultures.

On another front, a high quality 2-DE gel with well resolved protein spots is a pre-requisite for finding differentially expressed proteins and subsequent success MALDI-TOF MS identification. In comparison with 2-DE electrophoretograms obtained using protein samples prepared with the usual urea-thiourea lysis buffer method or acetone/TCA/acetone precipitation methods, those obtained using a Trizol extraction method showed the best results. The 2DE gels obtained subsequently have a higher number of resolved protein spots than those prepared by using the other four methods. The results were consistently observed in two different dinoflagellates species (*Alexandrium affine* and *Scrippsiella spp*). The Trizol-sample preparation strategy is simple and fast. There are only a few steps to perform and the preparation time is a few hours. In addition, sonication was better than homogenization for cell disruption. Most of the endogenous proteases were inhibited in the Trizol reagent. It was also found that the 2-DE patterns and resolved spots obtained with the Trizol-extracted samples in rehydration loading mode was similar to that obtained with the cup loading mode. Thus, the Trizol method allows larger amount of proteins to be loaded onto the strips without limitations on the sample volumes and would greatly facilitate the production of high-quality and high protein–loaded 2-DE gels (mg of proteins loaded) for downstream analysis that require a larger amount of proteins. The excellent quality of 2-DE patterns obtained by this Trizol-extraction method would allow comprehensive protein expression studies on dinoflagellates to

be performed.

After finding an optimized method for sample preparation for high quality 2DE gels, the next step was to study differential protein expression with or without nitrogen. *A. affine* grown in N-depleted and repleted conditions were harvested and analyzed by 2-DE. By comparing the 2-DE of N-depleted and N-repleted samples, a total of 33 differentially expressed proteins were found. These proteins were consistently detected to exhibit at least 5-folds of differential expression. Moreover, 12 of them showed at least 15-fold of differential expression. However, of all 33 differentially expressed proteins, only 5 protein spots were successfully identified. Using the PMFs obtained and the Mascot search engine to search against the NCBInr database, these 5 proteins were identified as isoforms of ribulose 1,5-bisphosphate carboxylase/oxygenase II (rubisco II). These groups of proteins have an apparent molecular weight of around 55kDa and *pI* ranged from 5.3-5.7. On the other hand, a few of the peptides obtained from these 5 spots were analyzed with tandem mass spectrometry. Two amino acid sequences (YW[I/L]STEED[I/L][I/L]R and QFI/LHYHR) which also corresponded to rubisco were found. In addition, with the provision of sulfonation technology and post-source decay analysis of the MALDI-TOF MS, proteins that consistently exhibited a 15-fold or more differential expression were subjected to *de novo* amino acid sequencing. Some peptide sequences were obtained using these differentially expressed proteins. Bioinformatic search using these partial amino acid sequences against the NCBInr database revealed no homology in the literature. However, it should be stressed that one of the major difficulties in protein identification of dinoflagellate origin was the lack of genome sequence data for this organism.

By PCR amplification with suitable degenerate primers, an amino acid sequence of NAP50 was successfully determined. However, there was no protein in the NCBInr database that matches a significant portion of the protein sequence of NAP50.

Degradation mechanism of two proteins (rubisco II and NAP50) under nitrogen depletion was investigated. NAP50 was found to be specific to *Alexandrium* species and immunohistochemical experiments suggested that both rubisco and NAP50 were located in the chloroplasts. It was found that both rubisco II and NAP50 had been degraded more than 16-folds when compared to their original expression level in the N-depleted condition. Degradation of both proteins can be inhibited by the replenishment of nitrogen sources, suggesting that the protein degradation processes are reversible and highly regulated by nitrogen. There is no corelation of the mRNA abundance of rubisco II and NAP50 with the availability of nitrogen, suggesting the degradation processes were not controlled at the transcriptional level. Protein degradation was found to be triggered by specific protease(s). However, because of the lack of starting materials, attempts to purify a rubisco II-degrading protease to homogeneity was not successful. Only a partial purification was achieved.

Protein degradation by specific proteolytic proteases during nitrogen stress in dinoflagellates has never been reported. Characterization of the proteins involved in the response to nitrogen will provide an unambiguous picture of which pathways are involved in the rapidly growth of this organism. Nevertheless, there is still a long way to go in our understanding of the "blooming" mechanism.

Lastly, given that no effective method for controlling blooming is currently available, the best strategy for control is prevention. Therefore, quick identification of HABs causative agents is another important area in the study of HABs. A rapid and

accurate identification method will minimize the damage caused. My study had demonstrated that it is possible to identify dinoflagellate species based on characteristic peak mass spectral patterns or species-specific signature biomarkers in the MALDI-TOF mass spectrum. The peak mass fingerprint spectral pattern is unique for different species of dinoflagellates and is easily distinguishable by visual inspection. In addition, differentiation of closely related species such as *Alexandrium affine, Alexandrium catenella, Alexandrium tamarense, Alexandrium minutum,* can be accomplished easily by this MALDI-TOF MS analysis. It has also been found that it is possible to identify individual dinoflagellate species in a mixed population of different dinoflagellate species based on the presence of characteristic peak mass spectral patterns and species-specific signature biomarkers in the spectrum. Coupled with a universally accepted taxonomic database underpinned by genome information of dinoflagellates, this methodology will allows unambiguous identification of HABs causative agents. The newly developed method represents an excellent alternative to classical microscopic-based identification techniques.

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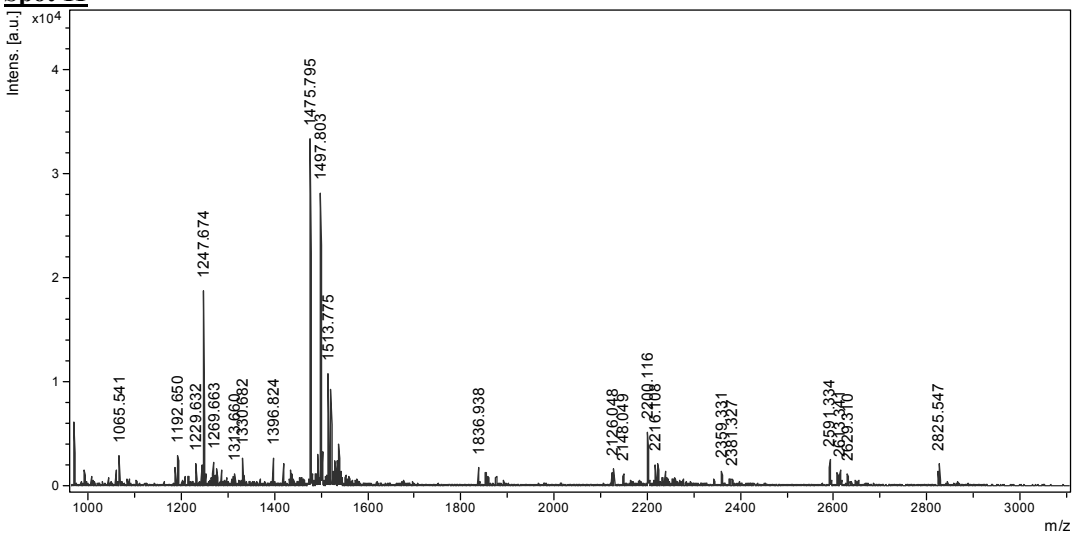
Appendix I: Peptide mass fingerprints (PMFs)

Spot 11



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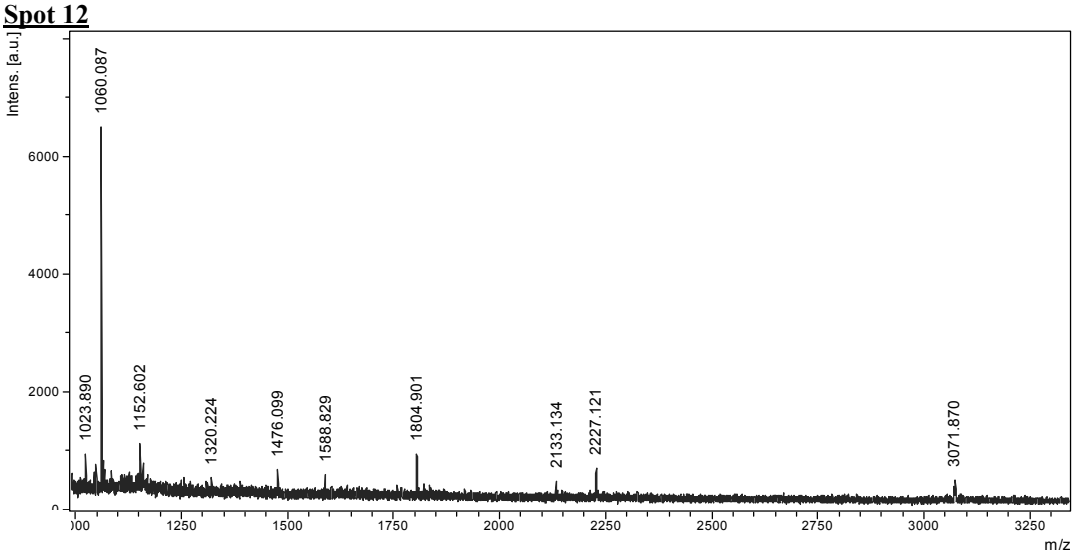

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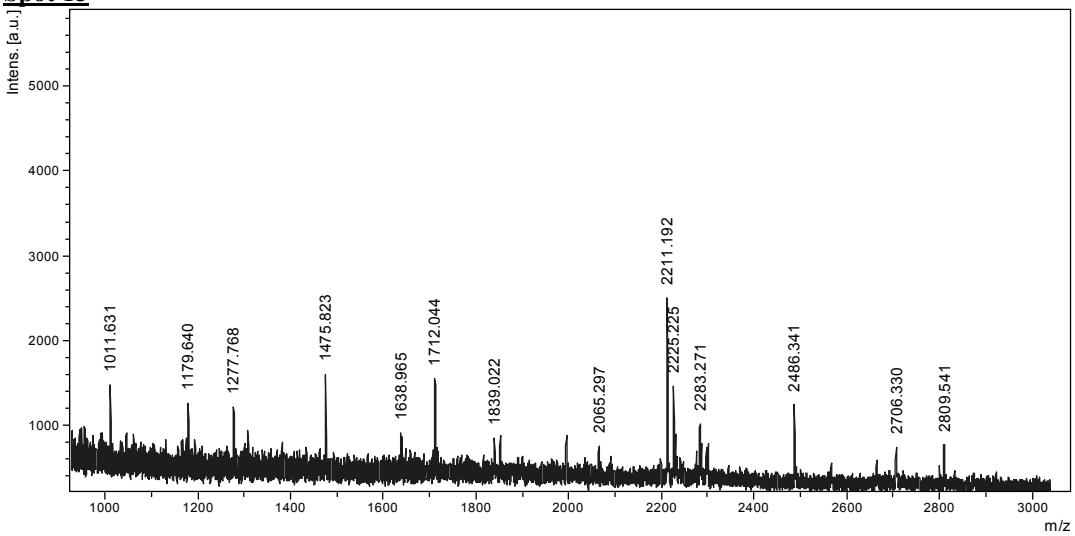

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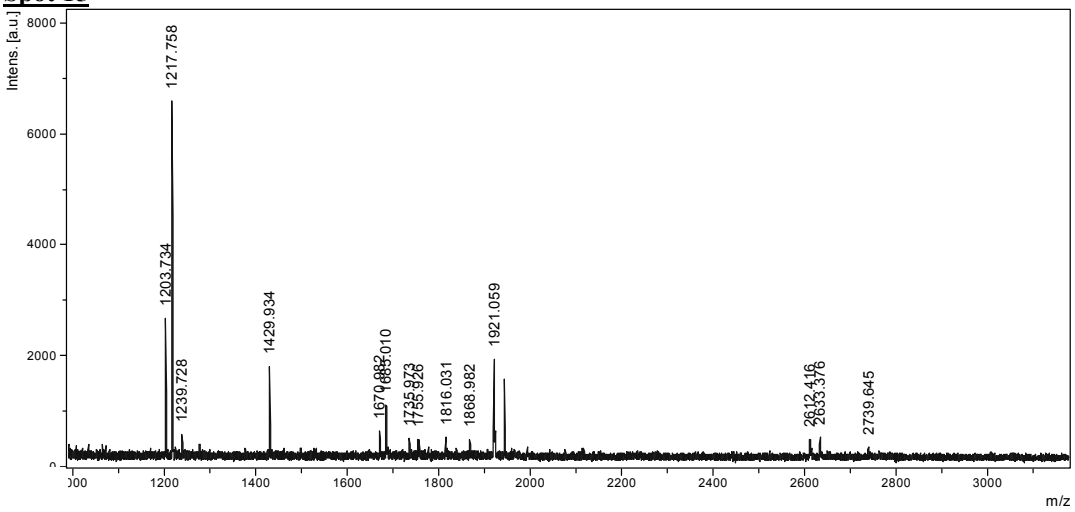

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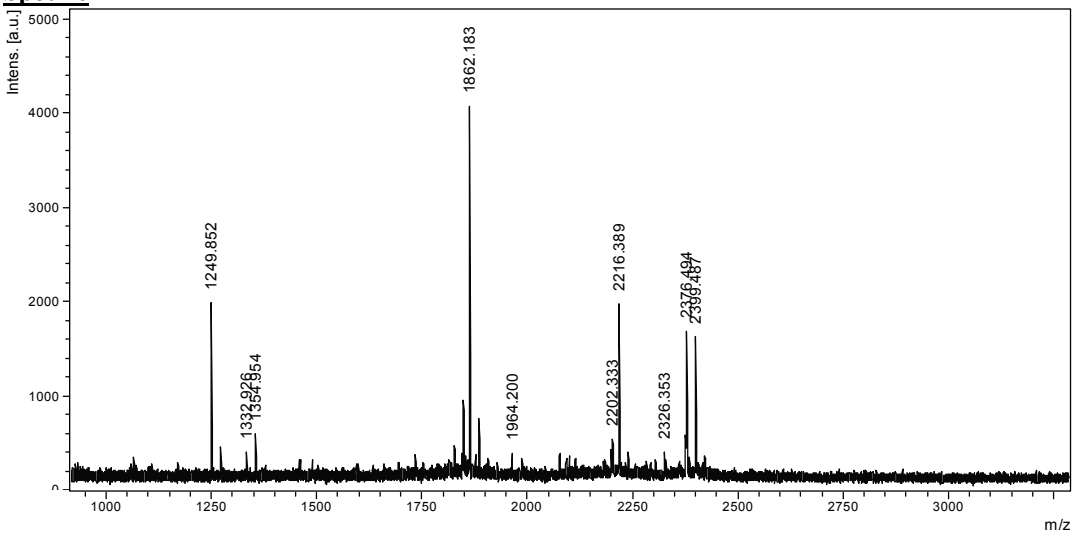

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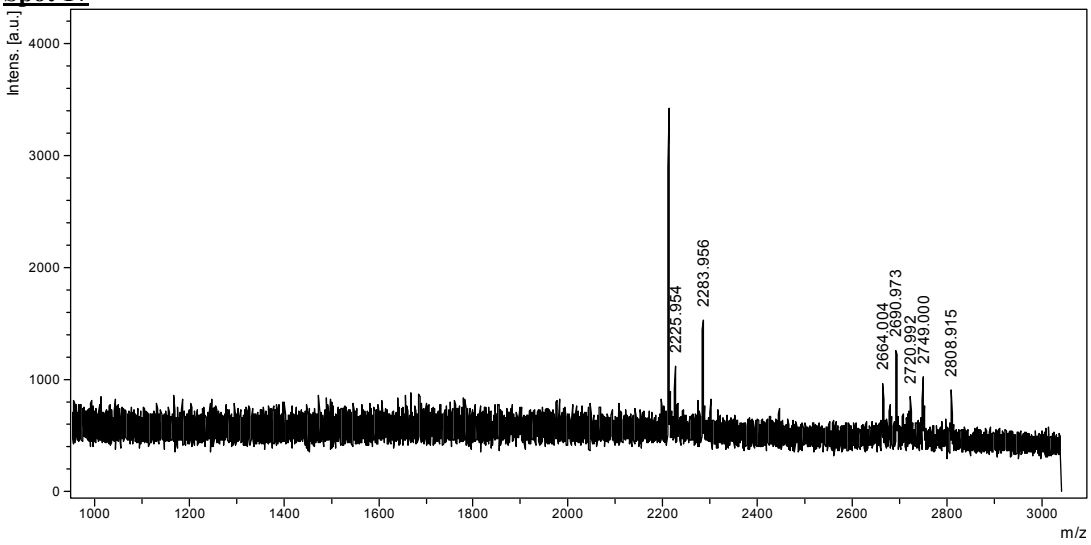

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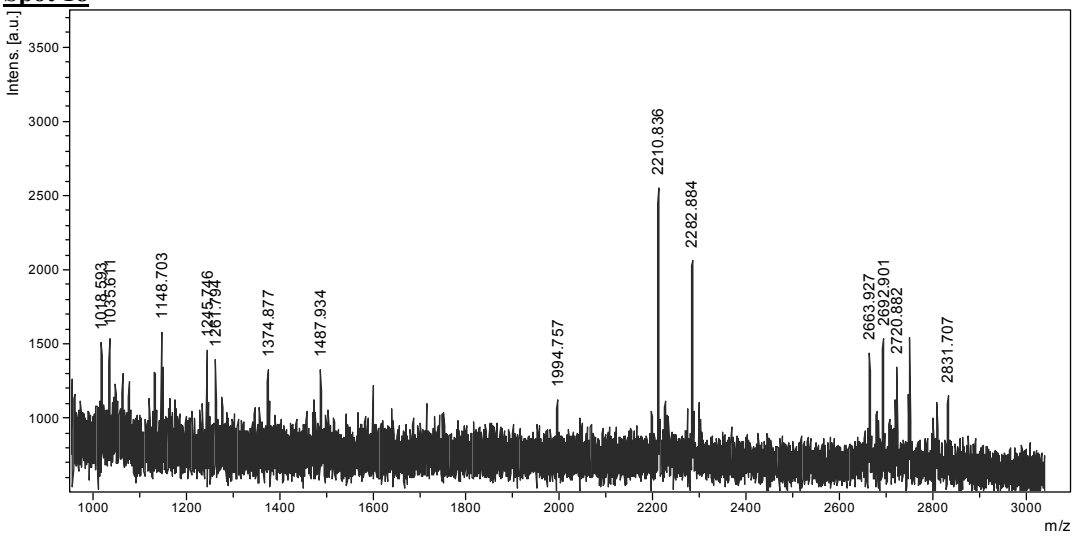

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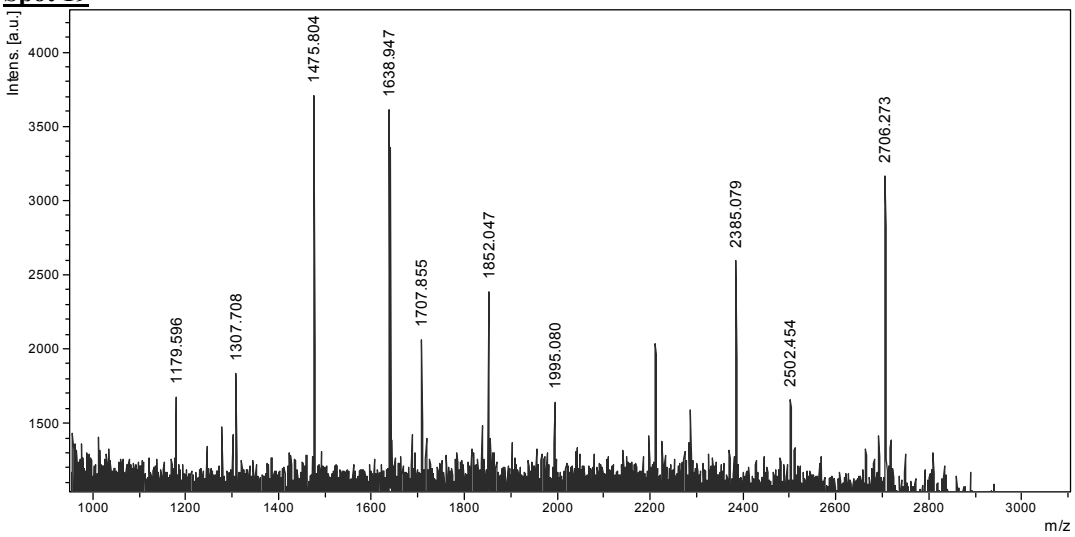

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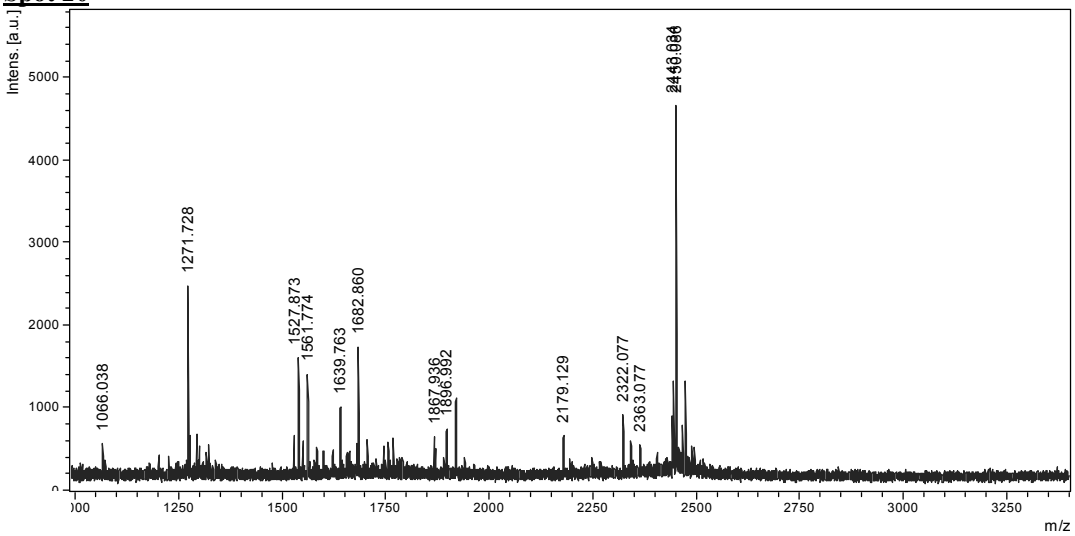

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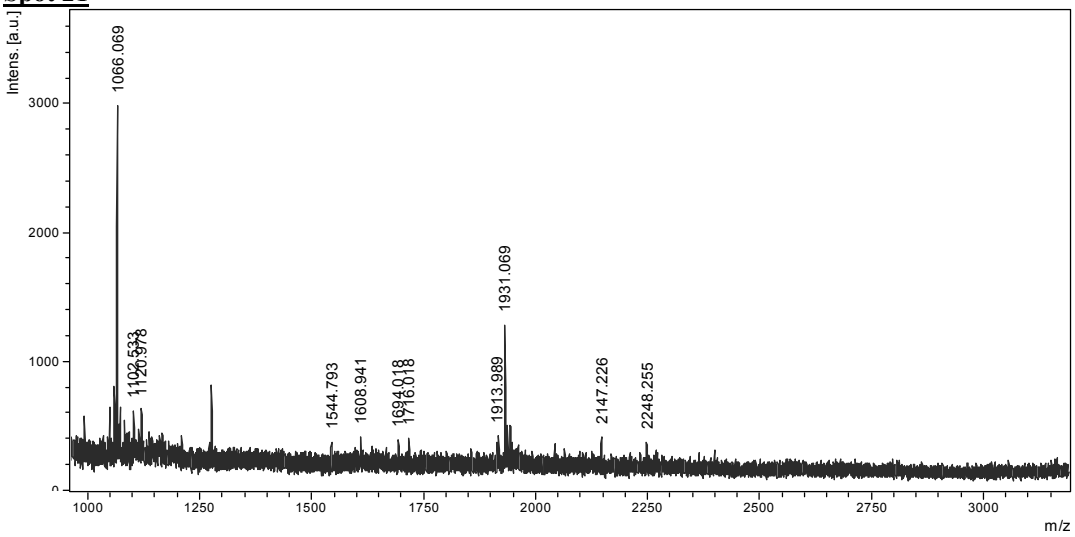

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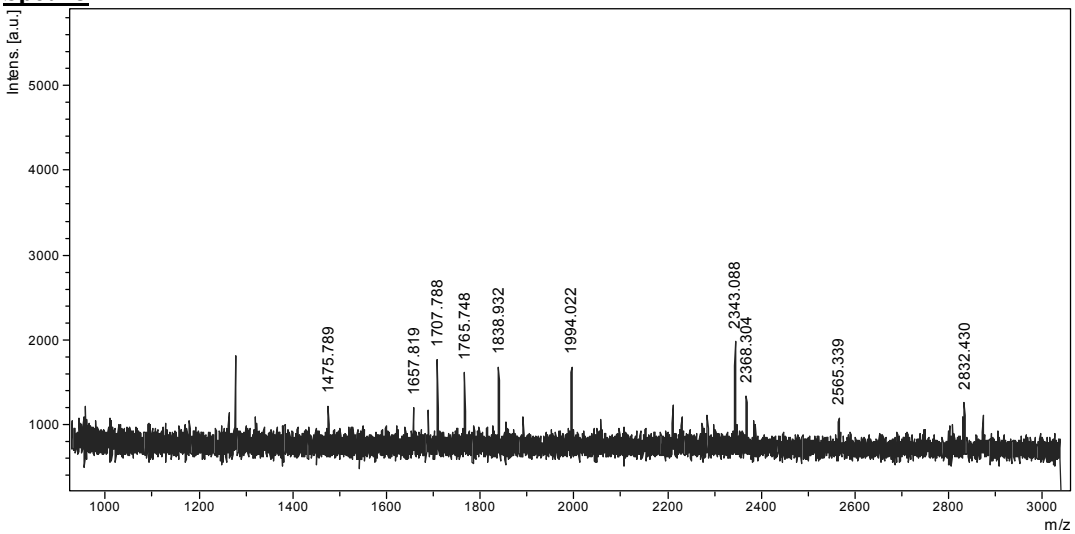

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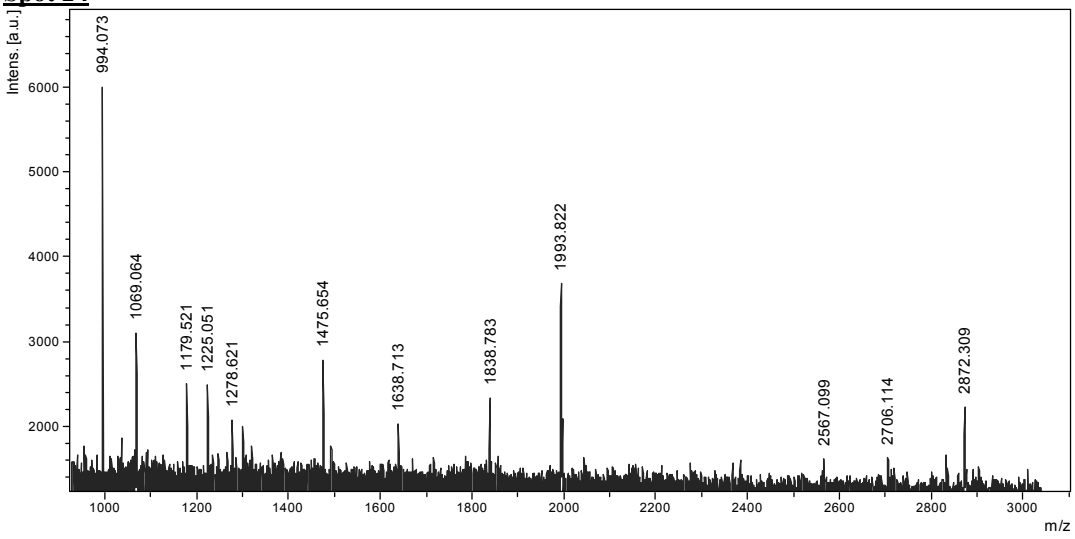

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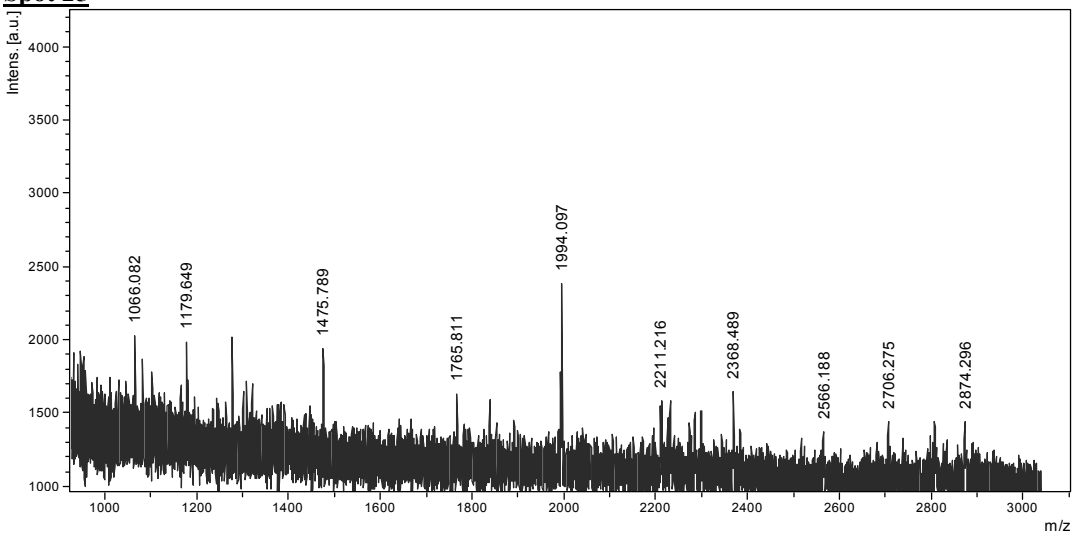

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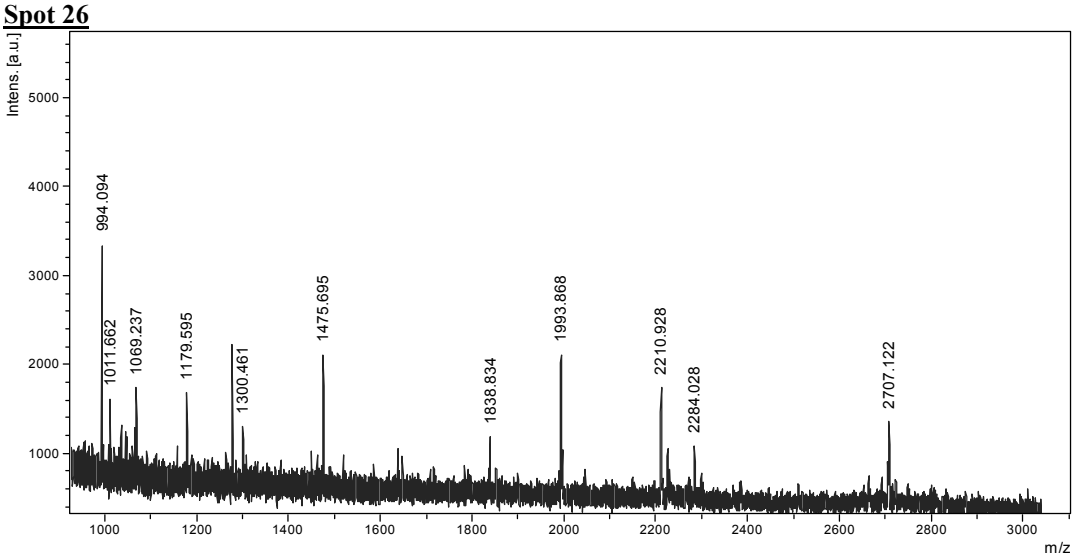

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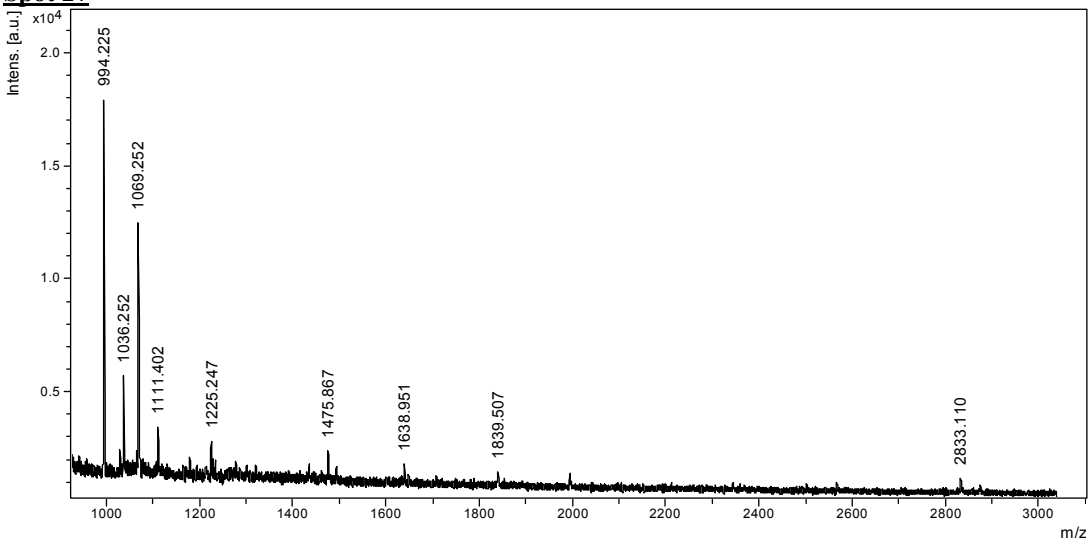

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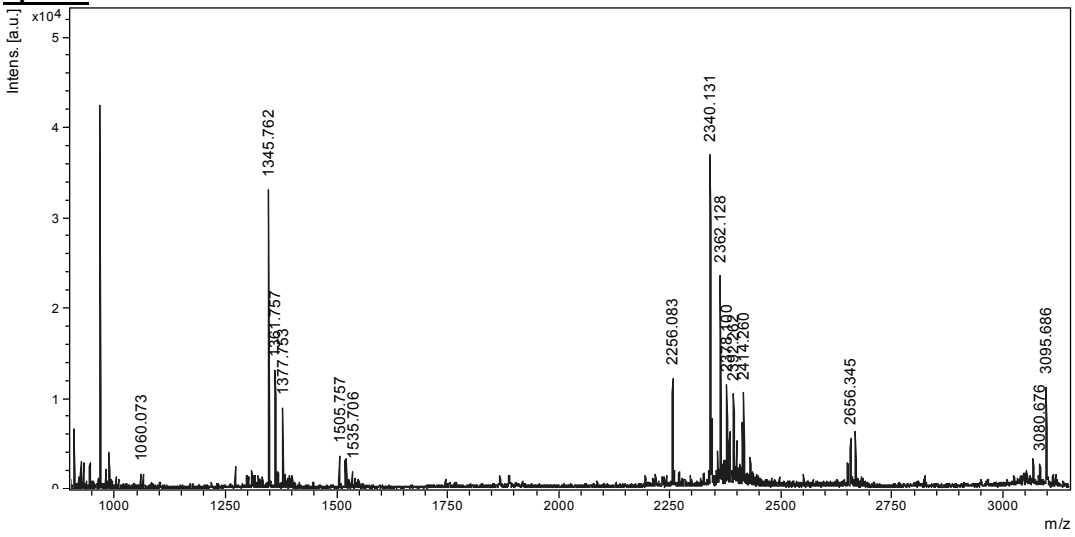

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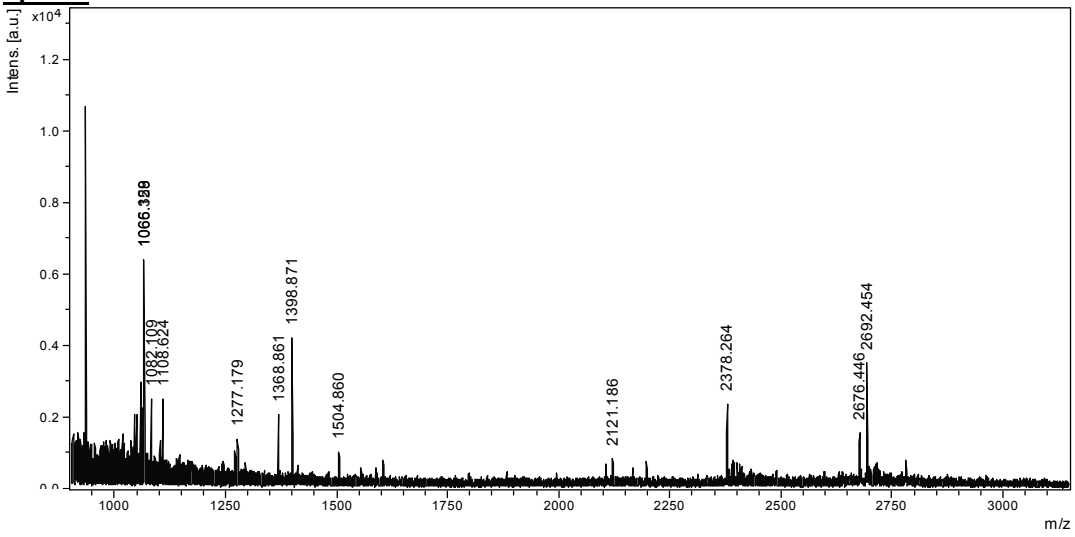

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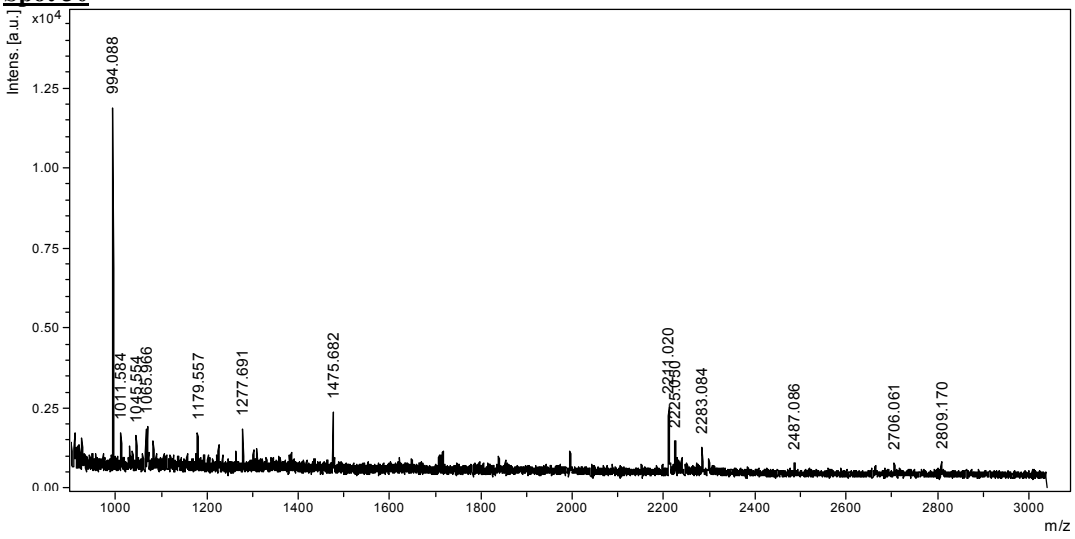

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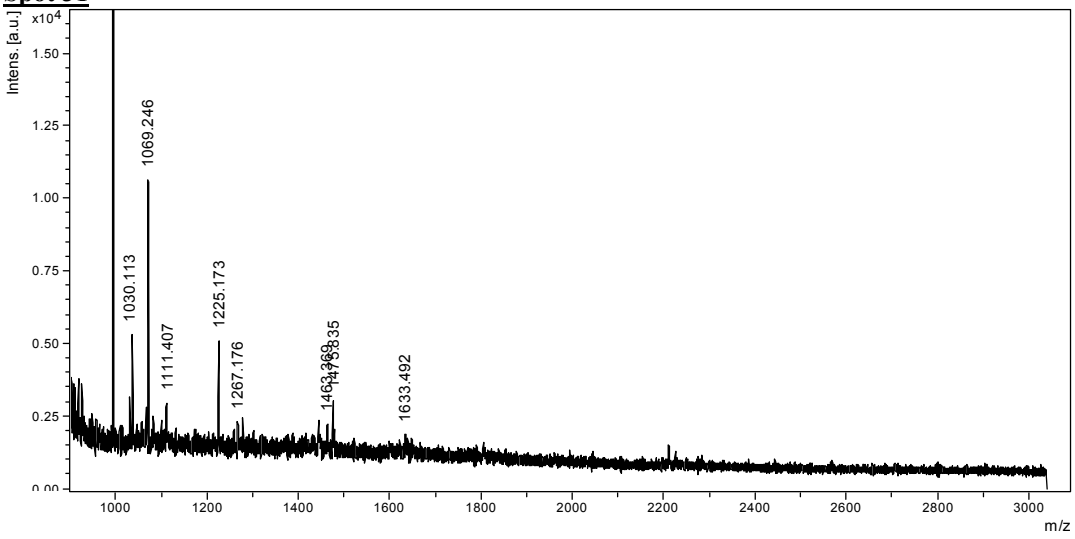

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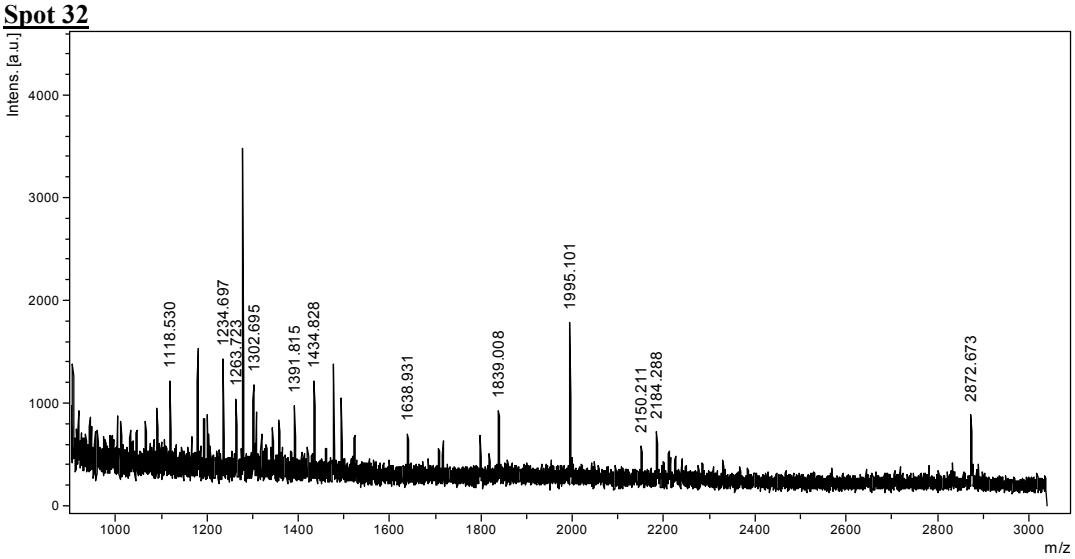

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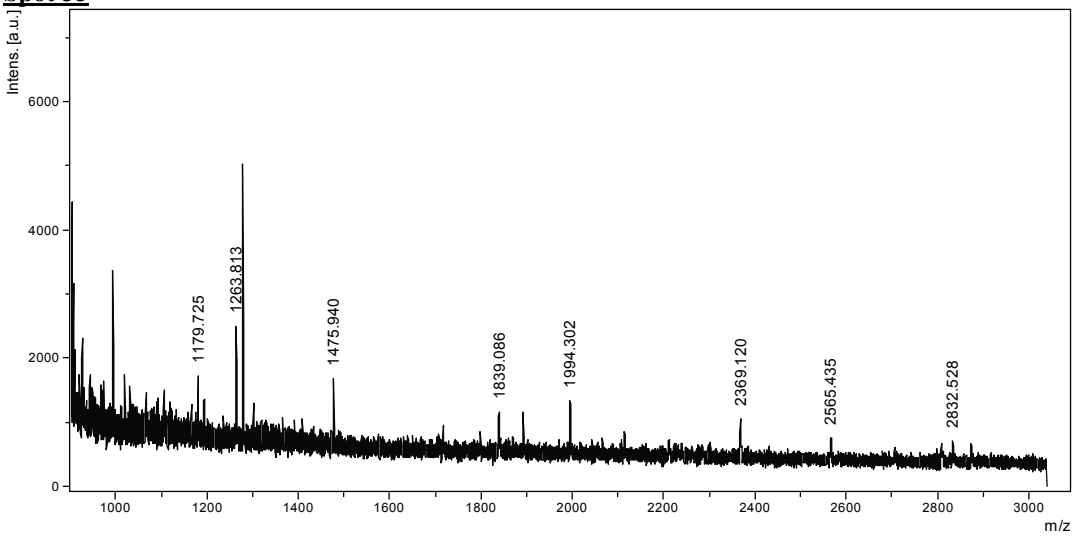
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1800
2000
2200
2400
2600
2800
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m/z
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1475.940
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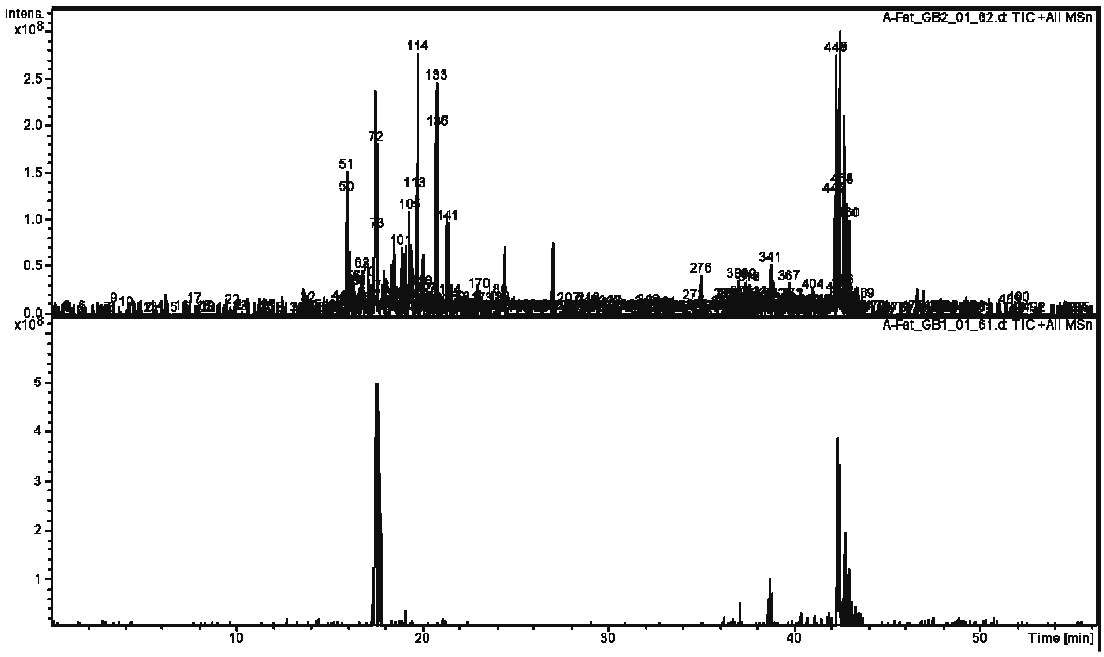
1994.302
2369.120
2565.435
2832.528

Appendix II:

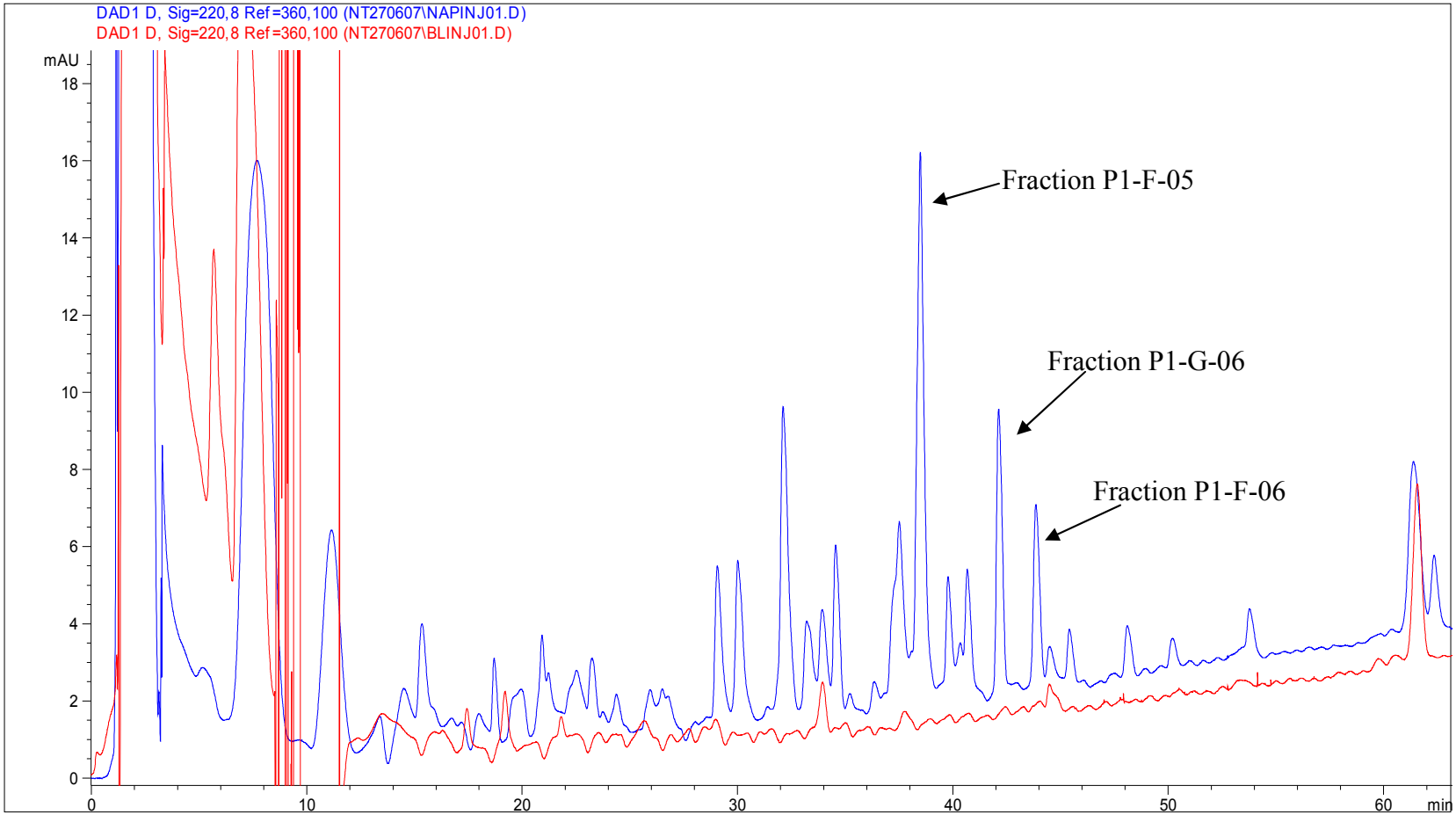
Chromatogram of the tryptic peptides of NAP50 for LC-Ms/MS



The upper panel is the elution profile of the tryptic peptides of NAP50. The lower panel is the elution profile of the tryptic peptides of blank gel (control)

Appendix III:

Chromatogram of the tryptic peptides of NAP50 from RP-HPLC for Edman sequencing



Appendix IV: Edman sequencing results from APAF

Analysis performed by: Judith Lysaght (Australian Proteome Analysis Facility)

Result: **P1-F-05**

CYCLE #	MAJOR SIGNAL	MINOR SIGNAL	COMMENTS
1	A		
2	F	S, E, L	
3	A	W, P	
4	G, D, I		
5	T, W, D		
6	A	N	
7	A	F, D	
8	V, E, G		
9	L,Y, M		
10	Y	E, K, I	
11	K		
12	-		
13	-		

Comments:

- There appears to be 3 sequences present in this peak fraction. It was possible on some cycles to see a single main sequence but on others it was not possible to clearly call and 3 amino acids have been included.

Result: **P1-G-06**

CYCLE #	MAJOR SIGNAL	MINOR SIGNAL	COMMENTS
1	G		
2	F		
3	K		
4	D	E	
5	D		
6	F		
7	D		
8	A		
9	(W)		( ) = tentative call
10	R		

Comments:

- There was a clear main sequence with an initial yield of about 6 pmol. Only the W in cycle 9 is not a definite call but it is the most likely amino acid.

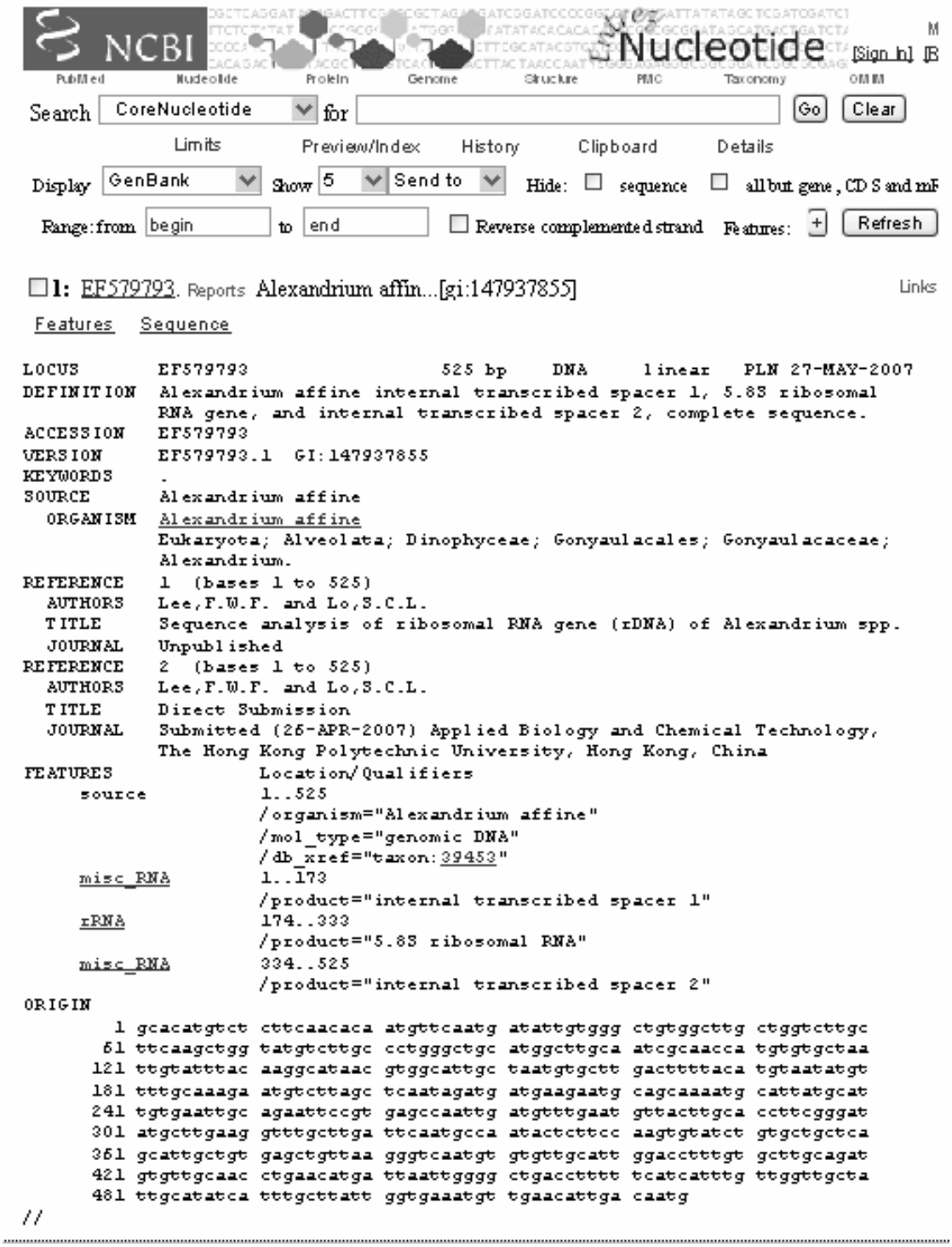
Result: **P1-F-06**

CYCLE #	MAJOR SIGNAL	MINOR SIGNAL	COMMENTS
1	G		
2	I		
3	(W)	E, A, K	
4	E		
5	E		
6	L	N	
7	P		
8	K	A	
9	-		
10	-		
11	-		
12	-		
13	-		

Comments:

- There was a clear main sequence with an initial yield of about 2 pmol. Only the W in cycle 3 is not a definite call but it is the most likely amino acid.

Appendix V: ITS sequences of *Alexandrium affine* (EF579793)



NCBI Nucleotide [Sign In]

PubMed Nucleotide Protein Genome Structure PMC Taxonomy OMIM

Search CoreNucleotide for Go Clear

Limits Preview/Index History Clipboard Details

Display GenBank Show 5 Send to Hide: sequence all but gene, CDS and mR

Range: from begin to end Reverse complemented strand Features: Refresh

1: EF579793. Reports Alexandrium affin...[gi:147937855] Links

Features Sequence

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VERSION     EF579793.1  GI:147937855
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  ORGANISM  Alexandrium affine
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            Alexandrium.
REFERENCE   1  (bases 1 to 525)
  AUTHORS   Lee,F.W.F. and Lo,S.C.L.
  TITLE     Sequence analysis of ribosomal RNA gene (rDNA) of Alexandrium spp.
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 525)
  AUTHORS   Lee,F.W.F. and Lo,S.C.L.
  TITLE     Direct Submission
  JOURNAL   Submitted (26-APR-2007) Applied Biology and Chemical Technology,
            The Hong Kong Polytechnic University, Hong Kong, China
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      121 ttgtatttac aaggcataac gtggcattgc taatgtgctt gacttttaca tgtaatatgt
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      241 tgtgaattgc agaattccgt gagccaattg atgtttgaat gttacttgca ccttcgggat
      301 atgcttgaag gtttgcttga ttcaatgcca atactcttcc aagtgtatct gtgctgctca
      361 gcattgctgt gagctgttaa gggtcaatgt gtgttgcatt ggacctttgt gcttgcagat
      421 gtgttgcaac ctgaacatga ttaattgggg ctgacctttt tcatcatttg ttggttgcta
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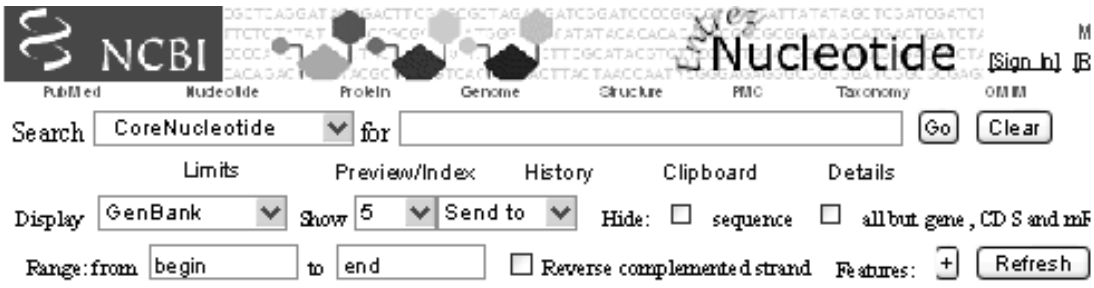
Appendix VI: ITS sequences of *Scrippsiella rotunda* 1 (EF579794)



1: EF579794. Reports Scrippsiella rotu...[gi:147937865] Links

Features Sequence

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VERSION     EF579794.1  GI:147937865
KEYWORDS    .
SOURCE      Scrippsiella rotunda
  ORGANISM  Scrippsiella rotunda
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            Scrippsiella.
REFERENCE   1  (bases 1 to 572)
  AUTHORS   Lee,F.W.F. and Lo,S.C.L.
  TITLE     Sequence analysis of ribosomal RNA gene (rDNA) of Scrippsiella spp.
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 572)
  AUTHORS   Lee,F.W.F. and Lo,S.C.L.
  TITLE     Direct Submission
  JOURNAL   Submitted (26-APR-2007) Applied Biology and Chemical Technology,
            The Hong Kong Polytechnic University, Hong Kong, China
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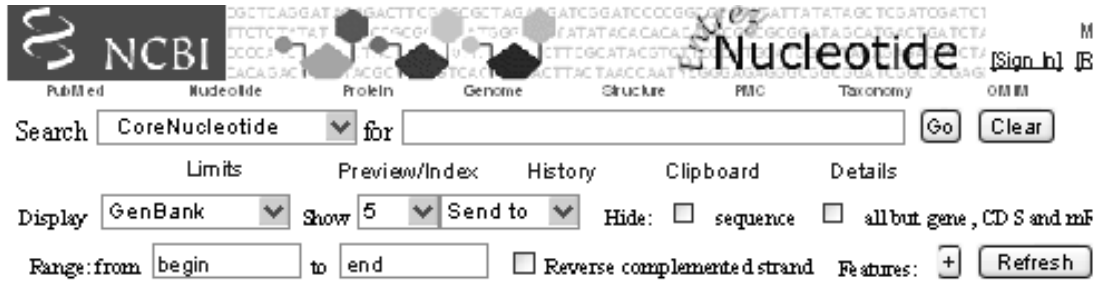
Appendix VII: ITS sequences of *Scrippsiella rotunda* 2 (EF579795)



1: EF579795. Reports Scrippsiella rotu...[gi:147937866] Links

Features Sequence

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DEFINITION  Scrippsiella rotunda strain 2 internal transcribed spacer 1, 5.8S
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ACCESSION   EF579795
VERSION     EF579795.1  GI:147937866
KEYWORDS    .
SOURCE      Scrippsiella rotunda
  ORGANISM  Scrippsiella rotunda
            Eukaryota; Alveolata; Dinophyceae; Peridiniales; Peridiniaceae;
            Scrippsiella.
REFERENCE   1  (bases 1 to 572)
  AUTHORS   Lee,F.W.F. and Lo,S.C.L.
  TITLE     Sequence analysis of ribosomal RNA gene (rDNA) of Scrippsiella spp.
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 572)
  AUTHORS   Lee,F.W.F. and Lo,S.C.L.
  TITLE     Direct Submission
  JOURNAL   Submitted (26-APR-2007) Applied Biology and Chemical Technology,
            The Hong Kong Polytechnic University, Hong Kong, China
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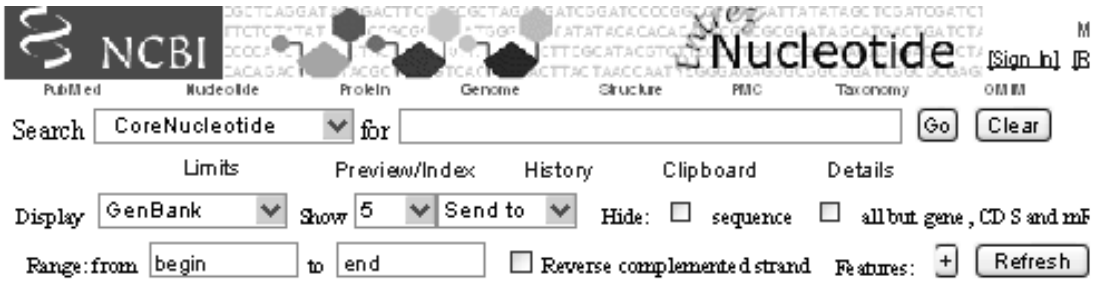
Appendix VIII: ITS sequences of *Scrippsiella rotunda* 3 (EF579796)



1: EF579796. Reports Scrippsiella rotu...[gi:147937867] Links

Features Sequence

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DEFINITION  Scrippsiella rotunda strain 3 internal transcribed spacer 1, 5.8S
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ACCESSION   EF579796
VERSION     EF579796.1  GI:147937867
KEYWORDS    .
SOURCE      Scrippsiella rotunda
  ORGANISM  Scrippsiella rotunda
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            Scrippsiella.
REFERENCE   1  (bases 1 to 572)
  AUTHORS   Lee,F.W.F. and Lo,S.C.L.
  TITLE     Sequence analysis of ribosomal RNA gene (rDNA) of Scrippsiella spp.
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 572)
  AUTHORS   Lee,F.W.F. and Lo,S.C.L.
  TITLE     Direct Submission
  JOURNAL   Submitted (26-APR-2007) Applied Biology and Chemical Technology,
            The Hong Kong Polytechnic University, Hong Kong, China
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      421 cagtgtgttt gtgcgttaga gtgctttgcg gcctctgacg cgctaaattc atagggattt
      481 cctcttgcgg cacgcaactt gctaaacatc ttggatgtaa cctgttgctg tgctttcatg
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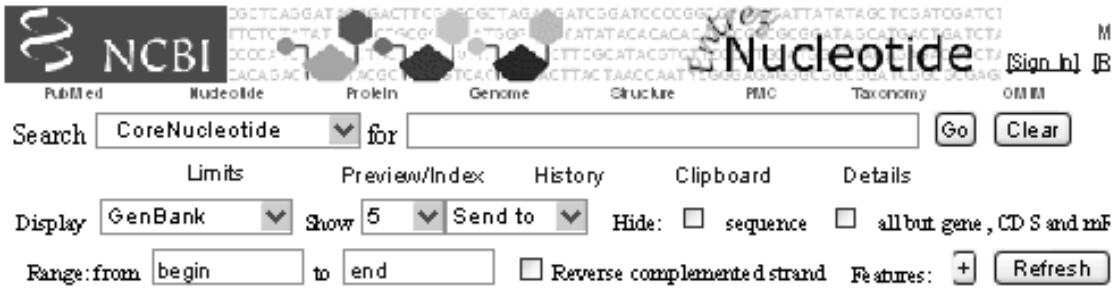
Appendix IX: ITS sequences of *Prorocentrum minimum* (EF579797)



1: EF579797. Reports Prorocentrum mini...[gi:147937868] Links

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  AUTHORS   Lee,F.W.F. and Lo,S.C.L.
  TITLE     Sequence analysis of ribosomal RNA gene (rDNA) of Prorocentrum spp.
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 555)
  AUTHORS   Lee,F.W.F. and Lo,S.C.L.
  TITLE     Direct Submission
  JOURNAL   Submitted (26-APR-2007) Applied Biology and Chemical Technology,
            The Hong Kong Polytechnic University, Hong Kong, China
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Appendix X: Purification of NAP50

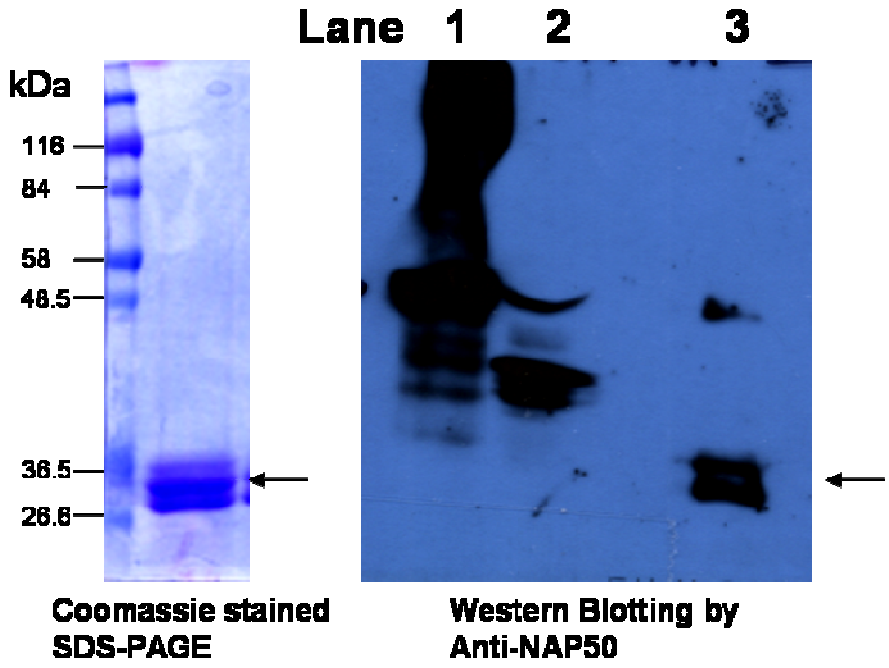

Figure A1. Fragmentation of NAP50 during cell lysis and purification. Lane 1: boiled *A.affine* cell extracts. Lane 2: cell extract after cell lysis by sonication. Lane 3: eluted fractions from the affinity column (packed with anti-NAP50 antibody). The size of the product shown in lane 3 from the western blotting is same as that from the coomassie stained SDS-PAGE (arrow), suggesting that the fragmented product detected is NAP50.

Appendix XI:

Table A1. Partial purification of Rubisco II-degrading protease(s) from *A.affine*

(a)

Fraction	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification fold	Yield (%)
Protein extract	75	0.075	0.001	1	100
Benzamidine -column	0.167	0.0027	0.0163	16.3	3.6

(b)

Fraction	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification fold	Yield (%)
Protein extract	104.4	0.104	0.001	1	100
Benzamidine -column	0.157	0.0032	0.0202	20.2	3.076923077

Appendix XII: Gordon research conference –poster presentation

Protein identification using *de novo* sequencing of N-terminal sulfonated peptides from the toxic dinoflagellate (*Alexandrium spp.*) species with post-source decay on a matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI-TOF)

Fred Wang-Fat Lee, John Hon-Kei Lum, Samuel Chun-Lap Lo *

The Proteomic Task Force, Department of Applied Biology and Chemical Technology, The Hong Kong Polytechnic University, Hong Kong SAR, China. (*Email of SCL Lo: bcsamlo@)

Introduction

Protein sequence information is fundamentally important for understanding many physiological processes at the molecular level. With minute amount of sample available for protein identification, methods such as using peptide mass fingerprints (PMF) obtained by matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI-TOF-MS) has become more and more popular. This work well when protein sequence of the target protein is available in protein sequence databases. However, protein sequence data of dinoflagellates (the causative agents of harmful algal bloom) is very limited. Therefore, the success rate in identifying proteins obtained from dinoflagellates is very low with PMF and *de novo* protein or peptide sequencing of the target protein would be extremely helpful or mandatory. Post source decay (PSD) analysis of precursor ions generated from MALDI-TOF-MS is a powerful tool for amino acid sequencing, but the result is very difficult to interpret due to the formation of different types (a,b,c,x,y,z-series) of ions in the PSD [1]. N-terminal sulfonation has become an effective derivatization strategy in facilitating *de novo* peptide sequencing by the formation of predominate single-type ion series (y-ions) in MALDI-TOF PSD spectra [1,2]. Here, we demonstrate the *de novo* peptide sequencing of different proteins isolated from a toxic dinoflagellate (*Alexandrium spp.*) by using PSD-data obtained.

Methods and Materials

10^6 dinoflagellate cell was harvested and lysed by sonication. Proteins were extracted and dissolved in lysis buffer. 0.5mg proteins were loaded for two dimensional gel electrophoresis (2-DE). The 2-D gel was then coomassie blue stained. Proteins of interested (spots) were picked and cut from the 2-D gel. Proteins in the gel plug were in-gel digested with trypsin into peptides. 10mg/ml 4-sulfophenyl isothiocyanate (SPITC) was added into the tryptic peptides and incubated at 55°C for 30min for the sulfonation reaction [2]. Sulfonated peptides were cleaned by zip-tips and analyzed by MALDI-TOF-MS. Amino acid sequences were deduced from the PSD spectrum obtained.

Results and Disscussion

Proteins of a toxic dinoflagellate species, *Alexandrium spp.*, were separated by two-dimensional electrophoresis (2-DE) (Figure1c). Different sulfonated peptides from the corresponding protein (Table 1) were selected for post-source decay (PSD). Our result show that PSD spectra dominated with y-ions series were successfully generated (Figure 2) and the amino acid sequences of the peptides selected from different proteins were easily obtained from the spectra (Table 1).

To conclude, N-terminal sulfonation of tryptic peptides with MALDI-PSD provides an effective, fast and easy way for *de novo* protein sequencing in dinoflagellate. This strategy provides a useful method in the proteome study of the toxic dinoflagellate species.

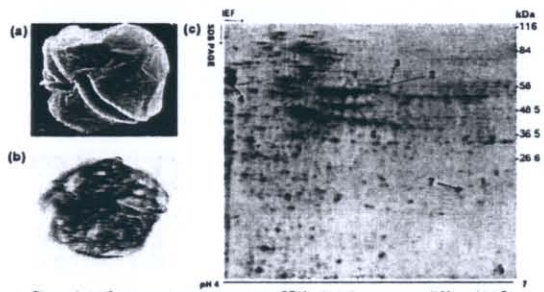

Figure 1. (a) Scanning electron microscopy (SEM), (b) light microscopy (LM) and (c) 2-DE of *Alexandrium spp.* Seven proteins were selected from the 2-DE for *de novo* peptide sequencing.

Spot	Peptide (m/z)	Sulfonated peptide (m/z)	Amino acid sequence
1	1000.43	1215.55	QFI/LHYHR
	1166.74	1381.12	XAI/LFDR
	1537.84	1752.02	YWI/LSI/LTEEDI/LI/R
2	886.39	1101.13	YFAGCVAR
	1681.91	1896.89	XNADI/LPI/LAADHFR
3	1010.59	1225.21	XWRSPHATR
4	1028.61	1243.56	SHVGI/LTFI/LR
	1053.21	1268.11	XVEFYR
5	1211.11	1426.33	XI/LI/LPGFEA
6	1152.51	1367.47	XYHFATMX
7	1813.45	2028.56	XSQI/LTYNQVR

Table 1. *De novo* peptide sequence of different peptides from the corresponding protein spots in Figure1c. (X represents gap in the peptide sequence which contain one or more amino acids)

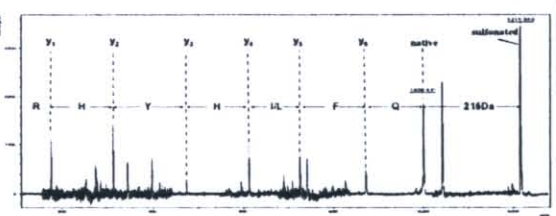

Figure 2. PSD spectrum, dominated with y-ions, of sulfonated peptide with 1215.55 m/z (digested from protein 1 in Figure 1c). The sulfonated peptide (1215.55m/z) was derived from sulfonation of the native peptide (1000.431m/z) with SPITC (215 Da). Amino acid sequences are deduced from the mass difference between adjacent y-ions of the spectrum (shown in red colour).

Acknowledgement

This research is supported by a grant from The Hong Kong Polytechnic University (A/C: RGD4)

References

[1] Keough T, Youngquist RS, Lacey MP. Proc Natl Acad Sci U S A. 1999; 96(13):7131-6.

[2] Wang D, Kalb SR, Cotter RJ. Rapid Commun. Mass Spectrom. 2004;18(1):96-102.

Appendix XIII:

Current Proteomics, 2007, 4, 67-78

Proteomic Study of Micro-Algae: Sample Preparation for Two-Dimensional Gel Electrophoresis and *De Novo* Peptide Sequencing Using MALDI-TOF MS

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Abstract: Proteins are the major players in most processes of living cells and study of the proteome has great relevance to investigations on cells and organisms at the molecular level. 2-DE is a core and powerful proteomic technique to study protein expression and function in living organisms and it allows a fast overview of changes that occurred in cells at the proteome level. Although data on DNA sequences from large scale genomic sequencing projects has greatly facilitated the identification and characterization of proteins, genomic sequences of many organisms, including that of many microalgae are still unknown. Thus, *de novo* protein/peptide sequencing techniques are important for acquiring amino acid sequences of proteins from organisms with incomplete genome data. Take dinoflagellates as an example of micro-algae for discussion purposes, they are the major causative agents of harmful algal blooms (HABs). Lack of well described genome information of dinoflagellates has limited progress of proteomic studies on these organisms. Nevertheless, 2-DE combined with *de novo* protein/peptide sequencing could provide an alternative route for identification and annotating proteins of interest in these organisms. However, preparation of high-quality samples of dinoflagellate cell extracts for 2-DE analysis is difficult due to the presence of high endogenous levels of nucleic acids, polysaccharides, salts, pigments, and other interfering compounds. Thus, an optimized protocol of sample preparation is a pre-requisite for obtaining satisfactory and reproducible 2-DE profiles. In light of these perceived problems, we had reviewed current methods available for preparing dinoflagellate samples for 2-DE analysis with some working alternatives from the authors' laboratory. Moreover, *de-novo* protein/peptide sequencing methods by MALDI-TOF MS assisted by effective derivatization protocols were also described.

Key Words: Dinoflagellates, *de novo* protein/peptide sequencing, MALDI-TOF MS, sulfonation, 2-DE.

1. INTRODUCTION

Proteomics is the large-scale study of proteins, particularly their structures and functions, in cells. Proteomics has firmly been established as a powerful tool for understanding various biological problems (Reinders *et al.*, 2004). Currently, for most proteomic investigations, proteins have first to be resolved and separated first before subjected to identification and annotation procedures. While two-dimensional gel electrophoresis (2-DE) is still an important protein resolving procedure in proteomics, it was invented more than 30 years ago (O'Farrell, 1975). The use and set up of 2-DE had recently been extensively reviewed (Gorg *et al.*, 2000; Gorg *et al.*, 2004). 2-DE is still one of the most commonly used techniques in proteomics research because it is relatively cheap to set up and it provides a fast overview of the proteome of interest. In addition, 2-DE allows the isolation of proteins with sufficient quantities (in nanograms or more) for subsequent protein identification by MALDI-TOF MS, ESI-MS, LC-MS/MS or Edman microsequencing (Celis and Gromov, 1999; Ong and Pandey, 2001; Graves and Haystead, 2002). Edman sequencing, sometimes also called N-terminal sequencing, is especially helpful in functional analysis of proteins isolated from organisms such as dinoflagellates whose genome information are not well described.

Dinoflagellates are single celled micro-algae. They are one of the most common causative agents of Harmful Algal Blooms (HABs). Some of these dinoflagellates can produce toxins. The ever increasing risk of occurrence of HABs represents expanding threats to human health, fishery resources and the tourism industries. However, many questions about these dinoflagellates are unknown. For examples, it is currently unknown why these autotrophs would need to produce toxins. Mechanisms of blooming and pathways by which these toxins are synthesized are unknown. Consequently, the study of global changes in protein expression in these dinoflagellates in different environmental and growth conditions using 2-DE gels can be very rewarding.

Proteomic studies on these dinoflagellates have the potential to uncover cellular pathways and mechanisms involved in toxin production and/or blooming at the molecular level. Despite of the emerging alternative proteomic technologies like multidimensional protein identification technology (Mud-PIT) and shotgun peptide sequencing (Hu *et al.*, 2007), 2-DE is still the only technique that can be rou-

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tinely conducted in parallel for the quantitative expression profiling of large sets of complex protein mixtures (Gorg *et al.*, 2004). The primary strength of 2-DE is the detection of proteins with post-translational modifications (PTMs) and they are easy observed in 2-DE gels. In many instances, post-translationally modified proteins appear as distinct spot trains in the horizontal/vertical axis of the 2-DE gels. In comparison with 2-DE protein expression map approach, LC-MS/MS based analytical methods cannot provide information on isoelectric points as well as molecular masses of intact proteins. Further, stable isotope labeling is required for quantitative analysis. More importantly, 2-DE analysis not only provides information on protein expression levels and their PTM, it also allows isolation of proteins in significant amounts (even up to mg levels if required) for downstream structural analysis or *de novo* sequencing by MS or Edman degradation. However, despite its popular applications in proteomic studies on animal, plant, yeast and bacterial cells, there is limited information on the use of 2-DE in research in dinoflagellates!

In order to proceed on proteomic investigations on dinoflagellates under different growth and environmental conditions, it is important to obtain high quality 2-DE images with good resolution first for finding differentially expressed proteins. However, the preparation of high-quality 2-DE gels from dinoflagellate samples are difficult due to the presence of high endogenous levels of salts, nucleic acids (estimated to be at least 10-100 times that of human), polysaccharides, phenolic compounds, pigments, and other interfering compounds. Presence of all these interfering substances would lead to a failure in the IEF-focusing process, the first electrophoretic phase in 2-DE. To establish routine procedures for running 2-DE on dinoflagellates, an efficient sample preparation protocol for producing high-quality 2-DE gels for dinoflagellates has to be developed. Further, protein identification utilizing peptide mass fingerprints (PMFs) obtained from MALDI-TOF MS analysis and bioinformatic searches has greatly improved efficiency of the identification process over classical Edman sequencing procedures. However, bioinformatic searches using PMFs data required a concomitant provision of large amount of genome information of the organisms of interest. Nonetheless, genomic sequences of many subclasses of *Dinophyceae* (dinoflagellates) are completely unknown. Thus, *de novo* protein/peptide sequencing is important for acquiring protein sequences on samples of interest from these organisms. It should be reiterated that incomplete genome information on dinoflagellates has limited applications of proteomic techniques in the studies on those organisms. However, 2-DE combined with *de novo* protein/peptide sequencing could greatly enhance the success of proteomic studies on these dinoflagellates.

In this review, we shall elaborate on current methods used for preparing protein extracts from dinoflagellates for 2-DE. Various strategies will be discussed and some working protocols from the authors' laboratory will be described. In addition, various commonly used protein/peptide sequencing methods will be discussed. We shall also focus on some effective derivatization strategies for *de novo* peptide sequencing which are compatible with MALDI-TOF MS. These illustrate how researchers can obtain novel protein/peptide sequences from micro-algae which have incompletely described genomes.

2. PROTEOMICS OF DINOFLAGELLATES

Although rare, there were several proteomic studies on dinoflagellates with the uses of 2-DE and MS approach. These studies can be divided into three broad categories: (1) the study of blooming and toxin- producing biomarker of dinoflagellates (Chan *et al.*, 2004a; Chan *et al.*, 2004b; Chan *et al.*, 2005; Chan *et al.*, 2006); (2) the study of cell surface proteins of dinoflagellates (Bertomeu *et al.*, 2003) and (3) the study of circadian expressed proteins in dinoflagellates (Milos *et al.*, 1990; Markovic *et al.*, 1996; Akimoto *et al.*, 2004).

Chan and co-authors have found different differentially expressed proteins of *Prorocentrum triestinum* grown under different growth phases and growth conditions. They found several preblooming (PB1, PB2, and PB3) and blooming proteins (BP1 and BP2) which were differentially expressed before and after blooming (Chan *et al.*, 2004). In addition, by comparing protein expression on 2DE gels from samples of toxic and non-toxic dinoflagellates, a differentially expressed toxin biomarker (T1) was found. N-terminal sequence tags of the blooming proteins and internal amino acid sequence of T1 were successfully obtained by Edman micro-sequencing (Chan *et al.*, 2006). DNA sequence of T1 was obtained subsequently. That series of proteomic studies was the first set of investigations on blooming as well as toxin-releasing mechanisms on dinoflagellates and some preliminary understanding on these aspects in molecular terms were achieved.

On the other hand, the first report on surface proteins of dinoflagellates was from Bertomeu and co-authors (2003) using I^{125} labeling. They had successfully obtained several peptide sequences of p43, a cell surface protein that was isolated from *Lingulodinium polyedrum* (a dinoflagellate) through 2-DE. Amino acid sequences of p43 was obtained by microsequencing performed with an unspecified mass spectrometer (Bertomeu *et al.*, 2003). Degenerate primers derived from these amino acid sequences could be designed and subsequent PCR amplifications aiming to obtain the full cDNA sequence could be performed. The complete cDNA sequence encoding p43 was obtained eventually.

Proteomic technologies had also been applied to study the circadian processes of dinoflagellates. Several cellular processes in the marine dinoflagellate *Lingulodinium polyedra* (formerly called *Gonyaulax polyedra*) were found to exhibit circadian rhythms. Some proteins involved in these processes were found to be regulated at the translational level only (Mittag, 2001). Because they are controlled only at the translational level, characteristics of these proteins have to be studied at the protein level. In their studies, Milos *et al.* (1990) labeled proteins *in vivo* using cultures from the day- and night- phases before being resolved by 2-DE. Several proteins were found to be differentially expressed at different time. This approach was taken further by amino acid microsequencing of the labeled proteins and four proteins were successfully identified from the peptide sequences obtained (Markovic *et al.*, 1996). These proteins are ribulose 1,5 bisphosphate carboxylase/oxygenase (RUBISCO); the nuclear encoded glyceraldehydes-3-phosphate dehydro-

genase (GAPDH), a protein component of the oxygen evolving complex; and the peridinin-chlorophyll a-binding protein (PCP). All these proteins are related to photosynthesis and under circadian regulation. In 2004, with the same *L. polyedra* as Bertomeu *et al.* (2003), Akimoto and his collaborators (2004) had study biological rhythmicity in terms of expressed proteins in these algae using proteomic technologies. In their study, cells were harvested at different time points of the light-dark regime and protein extracts from these cells were resolved by 2-DE. Out of about 900 protein spots detected, 28 were found to display differential expression in a diurnal pattern. 20 out of these 28 proteins were identified by LC-ESI-MS/MS. The remaining 8 proteins, which were also found to exhibit diurnal changes, were not identified at that time due to insufficient genome information of the dinoflagellates. With provision of more advanced technologies these days, *de novo* protein sequencing and bioinformatic search for protein annotation that relied on homologous or highly conserved protein sequences can be performed for identification of these proteins. Nevertheless, Akimoto and colleagues had demonstrated the feasibility of applying proteomic approach in studying differential protein expression in dinoflagellates. The combination of 2-DE, MALDI-TOF MS and amino acid sequencing by Edman degradation or other types of MS, has provided a useful approach for the identification and annotation of differentially expressed proteins in dinoflagellates.

3. SAMPLE PREPARATION FOR 2-DE

As elaborated earlier, efficient and reproducible sample preparation methods for samples of different origins are key to successful 2-DE (Rabilloud, 1999; Macri *et al.*, 2000; Molloy, 2000). There are four fundamental steps in sample preparation: (1) cell disruption, (2) inactivation of proteases, (3) removal of interfering substances and (4) solubilization of the proteins (Herbert, 1999; Rabilloud, 1999; Gorg *et al.*, 2000; Shaw and Riederer, 2003; Gorg *et al.*, 2004). To have a high resolution 2-DE gel with protein spots well resolved from each other, the sample have to be adequately denatured, disaggregated, reduced and solublized to achieve complete disruption of molecular interactions among the proteins (Gorg *et al.*, 2004). Obviously, the major effort for obtaining a high-quality 2-DE gel is to remove all the interfering substances during the protein extraction procedures. Although there are many 2-DE sample preparation protocols that were published, there is no single method of sample preparation that can be universally applied to all types of samples. Therefore, one has to optimize their samples based on the published protocols.

In general, sample preparations for 2-DE starts with cell disruption and it can be achieved by methods like osmotic lysis (Dignam, 1990), freeze-thaw cycling (Toda *et al.*, 1994), detergent or enzymatic lysis (Cull and McHenry, 1990; Jazwinski, 1990), sonication (Gorg *et al.*, 1988), homogenization (Dignam, 1990; Gegenheimer, 1990), French press high pressure disruption (Cull and McHenry, 1990; Jazwinski, 1990) and grinding with liquid nitrogen. These methods can be used individually or in combination. After cell lysis, the next thing to do is to block activation of endogenous proteases which would lead to protein degradation. Two of the most common methods used are (1) to inactivate proteases by adding protease inhibitors (Rabilloud, 1996) and (2) to precipitate proteins by adding either TCA/acetone (Gorg *et al.*, 2004) or acetone only (Holloway and Arundel, 1988; Fiengsrud and Kobro, 1989). If protease inhibitors were added to control protease activation, the intrinsically presented interfering substances (including salts, nucleic acids, lipids, polysaccharides etc.) had to be removed before performing 2-DE. Presence of these substances would lead to unsuccessful IEF-focusing which eventually yielded aberrant patterns with streaking and unfocused spots in the 2-DE gels obtained (also see below). The protein precipitation methods control protease activation and also separate proteins from these interfering substances in the sample (Gorg *et al.*, 2004). Among these methods, acetone precipitation and TCA/acetone precipitation (Granier, 1988; Tsugita *et al.*, 1996), were the most commonly used precipitation protocol. Lastly, the proteins precipitated must be brought into an appropriate buffer and in a condition suitable for IEF electrophoresis (Josic *et al.*, 2005).

3.1. Sample Preparation of Dinoflagellates

As elaborated earlier, a high quality 2-DE gel with well resolved protein spots is the pre-requisite for finding differentially expressed proteins and success MALDI-TOF MS identification subsequently. For example, a protein spot could not be identified if it co-migrates together with another protein in a 2-DE gel. Presence of interfering substances in significant quantities also renders successful 2-DE of dinoflagellates difficult. Nevertheless, proteomic studies on dinoflagellates by using 2-DE are becoming more common. In the literature, there is only one comprehensive study on optimizating protocols for sample preparations of dinoflagellates for 2-DE studies (Chan *et al.*, 2002). The authors had compared different procedures and methods on cell disruption; extractions buffers; and pre-electrophoretic treatments (such as addition of protease inhibitor and RNase/DNase/endonuclease, ultrafiltration, desalting and protein precipitation etc.). They found that sonication was the most effective cell disruption method for breaking the dinoflagellate cells, especially the thecae forms. From their results with different independent extraction procedures, Tris-extraction buffer and TCA/acetone precipitation enabled good protein resolution on subsequent 2-DE. Between the two extraction procedures compared, Tris buffer extraction procedure was found to yield a greater number of spots (407 spots) than that of TCA/acetone precipitation (387 spots). Although the authors claimed that there were minor differences when the extraction were done with or without the presence of protease inhibitors, nearly 40 spots were found to be degraded when protease inhibitors were not added. Therefore, addition of protease inhibitors to extraction buffer was recommended in order to minimize endogenous proteolytic degradation of proteins in samples before 2-DE. Further, in order to remove nucleic acids, DNase/RNase or endonucleases were added to the extraction buffer. A clearer 2-DE protein expression profile was observed after the removal of nucleic acids. Moreover, three desalting protocols (desalting by Biospin column, ultrafiltration and acetone precipitation) were evaluated for their abilities to remove interfering substances prior to IEF. The authors claimed that Biospin column is easier to carry with and gave good 2-DE protein expression pattern.

Fig. (**1a**) summarized the optimized method that Chan *et al.* had developed and used in subsequent proteomic studies on dinoflagellates (Chan *et al.*, 2002; Chan *et al.*, 2004a; Chan *et al.*, 2004b; Chan *et al.*, 2005; Chan *et al.*, 2006). The optimized protein extraction method by Chan and co-authors enabled the production of good quality 2-DE gels with reasonable resolution of spots. Fig. (**1b**) is a representative 2-DE gel prepared by the optimized methodology with several differentially expressed blooming-related proteins (PB1, PB2, PB3, BP1 and BP2) found (Chan *et al.*, 2004). The additional desalting and concentrating steps could improve quality of the final results, but each additional step would result in selective losses of some proteins. Samples prepared by methodologies described by Chan and coworkers yield only a few hundred of spots. Therefore, it seems that there was a significant loss of proteins with that sample preparatory protocol. Proteins lost may be a consequence of the lengthy desalting and concentrating steps. Although there is no one-step procedure for sample preparation for 2-DE, one should always aim for a simple sample preparatory protocol in order to minimize protein losses and ensure reproducibility.

After these earlier studies (Chan *et al.*, 2002; Chan *et al.*, 2004a; Chan *et al.*, 2004b; Chan *et al.*, 2005), we had since embarked on studies aiming to find a more simplified method for sample preparation of dinoflagellates. Trizol, a commercial reagent of monophasic mixture of phenol and guanidine isothiocyanate which is typically used to isolate RNA from cell and tissue samples, had been studied. We found that Trizol allowed a much simpler and fast sample preparation protocol for dinoflagellates for producing high quality 2-DE gels (Fig. **2a**). This extraction method is effective in produce high-quality 2-DE gels with more than 1800 distinctly resolved protein spots found after silver staining (Fig. **2b**). Successful application of Trizol-extraction in studying proteins from a halophilic archaea (*Haloferax volcanii*) had been reported (Kirkland *et al.*, 2006). Therefore, we had adopted the method with modifications. Subsequently, we compared the 2-DE gels of a model dinoflagellate –*Alexandrium spp* obtained using the three different protein extraction methods: including a buffer containing 7M urea, 2M Thiourea, 4% CHAPS, 40mM Tris pH 8.7 (which we called lysis buffer), acetone precipitation and Trizol extractions. In our hands, 2-DE gels of dinoflagellate protein samples prepared using Trizol extraction method revealed a higher resolution of protein spots when compared to the other methods (Fig. 3). On a 2-DE gel, protein extracted by directly homogenizing the dinoflagellate cells in Lysis buffer produced excessive horizontal and vertical streaking (Fig. **3a**). Many proteins were not being focused and appeared either as smearing or aberrant patterns. In addition, high background with relatively few proteins spots was seen. Obviously, the "unremoved" endogenous interfering substances in these dinoflagellate cells have greatly affected focusing of proteins during the IEF step in the 2-DE. As for the acetone precipitation method, the precipitation step served both to concentrate proteins in the samples and also to separate these

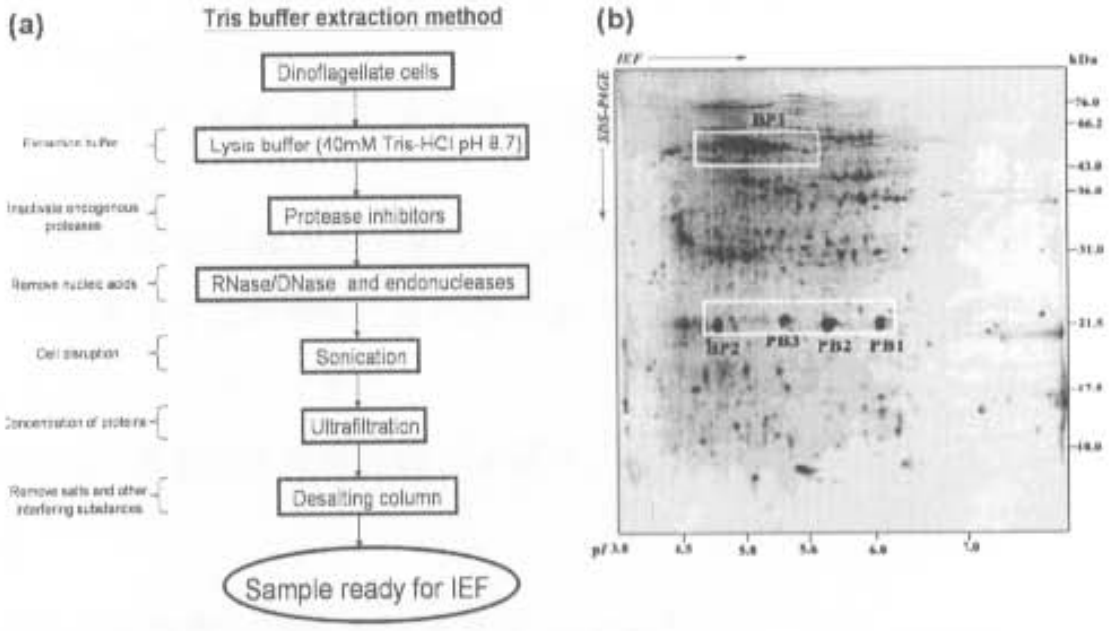

Fig. (1). (a) Workflow of Tris-buffer protein extraction procedures as described (Chan *et al.*, 2002). (b) 2-DE of total protein extracts of dinoflagellates (*Prorocentrum spp.*) using Tris –buffer extraction method as described (Chan *et al.*, 2002; Chan *et al.*, 2004). Differentially expressed proteins (BP1, BP2, PB1, PB2 and PB3) were found highly up-regulated in a peak blooming conditions. The 2-DE gel was loaded with 20µg of proteins into the pH3-10 range IEF strip before being resolved with a 15% resolving polyacrylamide gel.

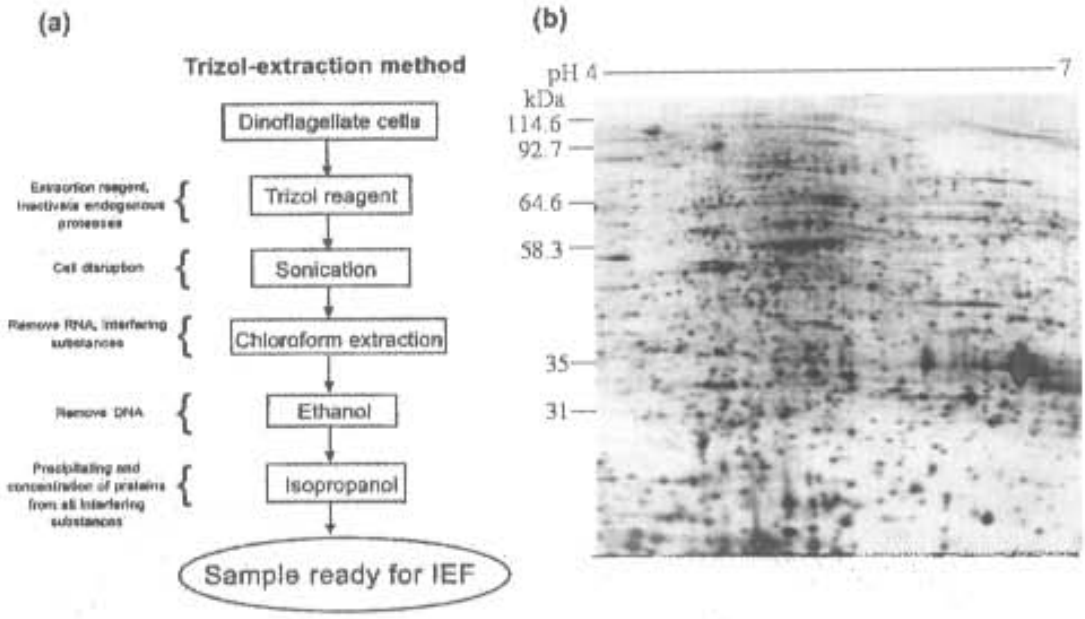

Fig. (2). (a) Workflow of Trizol protein extraction procedures for dinoflagellates. (b) 2-DE of total protein extracts of dinoflagellates (*Alexandrium spp.*) using Trizol-extraction methods. 2-DE was loaded with 60μg proteins in 10% polyacrylamide gel and with IEF in the pH range of 4-7.

proteins from potentially interfering substances. The number of protein spots resolved and intensity of these spots were better than that obtained using the lysis buffer extraction method (Fig. 3b). However, phenolic and some contaminants were co-extracted together with the proteins, resulting in several horizontal and vertical streaking with a high background. This is easily observed in the enlarged area of the gel (Fig. 3b). Further cleaning-up procedures are required to remove the substantial interfering substances that remained in the acetone precipitated sample. Taken overall, the Trizol-extraction method gave protein extracts that yielded the highest resolution of stained spots with a clear background in a 2-DE gel (Fig. 2b and 3c). The Trizol-sample preparation strategy is simple and fast as the procedure will finish in a few hours. The Trizol-extraction method is by far much superior to existing published protocols for sample preparation of dinoflagellate cells. For illustration purposes, spots in the rectangle and circle regions in Fig. (3) have shown a greater intensity in the Trizol prepared 2-DE sample when compared to those prepared with the other two methods. In addition, some spots were missing in the 2-DE prepared from protein extracts prepared by Lysis buffer (for example, see spots 1-7, Fig. 3). Although these spots (#1-7) were seen in both the acetone precipitation and Trizol prepared 2-DE (Fig. 3b and c), these spots were much focused and with a higher resolution in the Trizol prepared 2-DE sample than that of the acetone precipitation prepared.

Using this Trizol-protein extraction method, we are currently investigating several differentially expressed proteins from 2-DE gels obtained using proteins extracted from dinoflagellates exposed to different nutritional stress (Fig. 4).

4. DE NOVO PROTEIN/PEPTIDE SEQUENCING

An important prerequisite for efficient proteomic study in terms of protein identification is the presence of complete DNA sequences databases for the given class or species of organism of interest. These databases should ideally cover sequences of the entire genome as well as the EST sequences. However, when DNA sequences of the organism(s) of interest are either not available or are only available in a very limited numbers, one must rely on the homology of similar proteins in other organisms. Given that the genome sequences of dinoflagellates are incomplete, *de novo* protein sequencing is important for the protein identification process.

4.1. Edman Sequencing

In the 1950s, the Edman degradation chemistry which could be used for *de novo* protein sequencing, was described (Edman and Begg, 1967). It is a stepwise procedure to degrade proteins from their N-terminal end. Although details of Edman sequencing is outside the scope of this review, various technical developments and advances of microsequencing had enable fabrication of highly sensitive and fully automated protein sequencers (Tsugita, 1987; Walker, 1994; Shively, 2000). Although Edman sequencing continues to contribute to the field of protein biochemistry and proteomic research, it has a number of serious limitations. The most

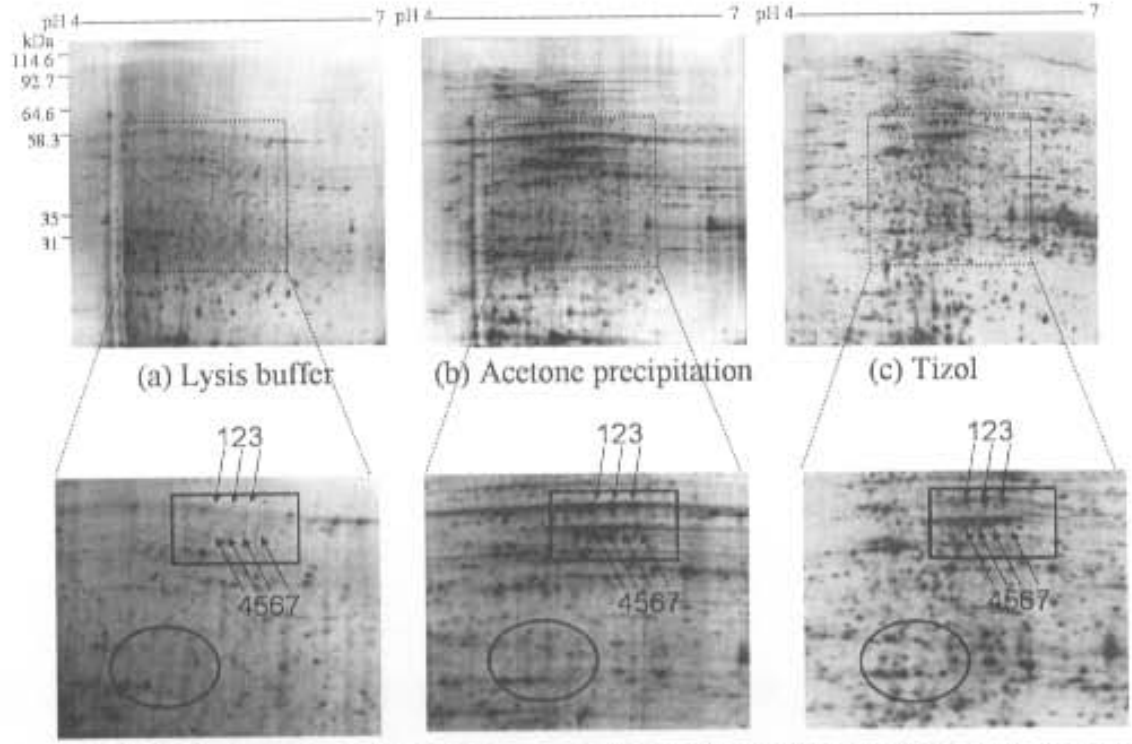

Fig. (3). 2-DE of total protein extracts of dinoflagellates (*Alexandrium spp.*) prepared using the (a) lysis buffer method, (b) acetone precipitation method and (c) the Trizol protein extraction method. Gels in lower panel are the enlargement of each 2-DE images from representative regions of the three protein extraction methods. Arrows indicate spots 1-7 and they were not visible in gel (a).

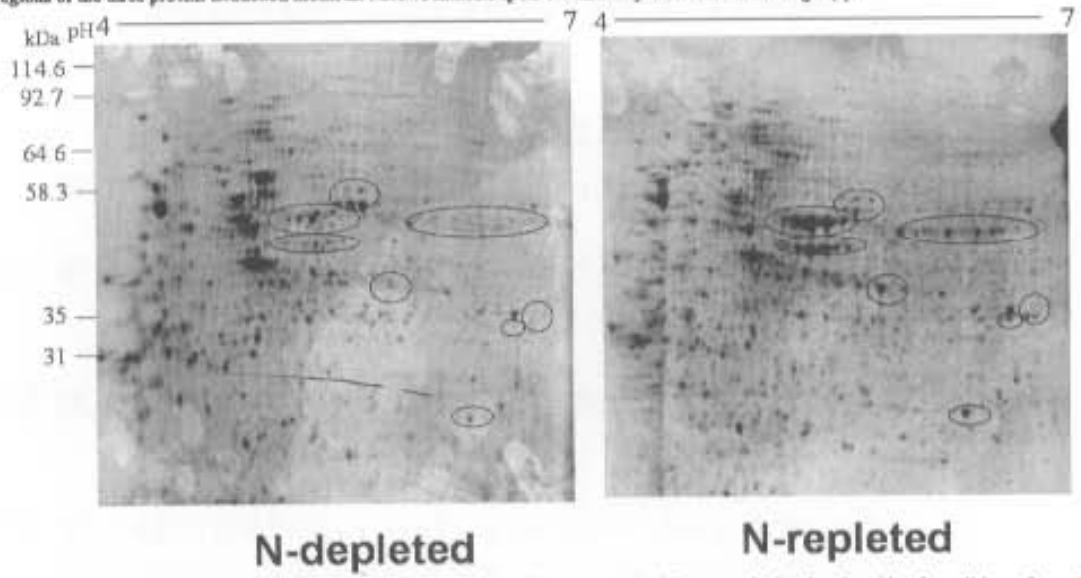

Fig. (4). Coomassie blue stained 2-DE of total proteins of *Alexandrium spp.* under Nitrogen –depleted and –repleted conditions. Several differentially expressed proteins were found to be down-regulated in Nitrogen–limited conditions. 2-DE was loaded with 500μg proteins in 10% polyacrylamide gel and IEF run with strips in the pH range of 4-7.

significant limitation is that the sequencing reaction is slow. One typical degradation cycle is around 45 minutes with a narrow-bore HPLC. Therefore, only a limited number of peptides can be sequenced every day. Furthermore, eukaryotic proteins are often blocked at their N-termini, i.e. without an α-carbon (Gevaert and Vandekerckhove, 2000). Deblocking procedures or enzymatic digestion to open up peptide chains with subsequent HPLC separation have to be performed before amino acid sequencing could be performed again. However, it should be noted that after a protein was blotted onto a PVDF membrane, each sample can only be used once in N-terminal sequencing. There is no chance for repeating the experiment using the same blotted sample. Inadequacy of Edman sequencing in these respects makes it less popular than other sequencing techniques (see below). Nonetheless, Edman sequencing still remains as a fallback peptide sequencing method when mass spectrometric methods failed to yield useful sequencing information.

4.2. Peptide Sequencing by MALDI-TOF Mass Spectrometry (MS)

Provision of MS has significantly reduced the need for Edman sequencing because it is more sensitive and provides higher sample throughput (Bienvenut *et al.*, 2002). MS itself can only give considerable information on the peptide mass as well as the amino acid compositions of peptides. MS does not determine the order of the amino acids (Standing, 2003). Therefore, *de novo* peptide sequencing usually requires tandem mass spectrometry or MS/MS. Generally, for MS/MS peptide sequencing, a particular precursor ion is selected in the first masses scan before being fragmented either by CID or metastable decay. Subsequently, the fragmented ions can be separated by their mass/charge during the second MS scan. This procedure may yield a series of ions with ladder like appearance which may contain sufficient information to determine the amino acids sequences. Further, it was found that the presence of matrix greatly facilitated the ionization process.

Matrix-assisted laser desorption ionization (MALDI) in MS was developed by Karas and Hillenkamp in the late 1980s (Karas and Hillenkamp, 1988). MALDI *de novo* sequencing has been carried out using CID and metastable decay (Spengler *et al.*, 1992; Schilling *et al.*, 1999; Medzihradszky *et al.*, 2000; Yergey *et al.*, 2002). There are several attractive properties for the analysis of peptides by using MALDI MS. Firstly, it has a relatively high tolerance to salts and impurities when compared to that of ESI. Therefore, requirement for sample purity for MALDI analysis is relatively low and extensive sample cleaning steps can be avoided. Secondly, the sample intended for MALDI analysis is co-crystallized with the matrix and dried on a target plate. This allows the sample to be used until it is depleted; so data can be obtained for different ion species produced from a single peptide mixture. Because of these advantages, *de novo* peptide sequencing using MALDI MS is becoming very popular (Spengler *et al.*, 1992; Yergey *et al.*, 2002); particularly after pretreatment of the sample by derivatization such as sulfonation (Keough *et al.*, 1999; Keough *et al.*, 2000; Keough *et al.*, 2001; Keough *et al.*, 2002), as described in section 4.2.2.

4.2.1. MALDI-TOF MS-PSD

There are two different fragmentation reactions in MALDI-MS. Firstly, fragmentation of ions occurred in-source following laser impact and it can occur within a few hundred nano-seconds (Lennon and Walsh, 1997). Another one is the Post-Source Decay (PSD) (Kaufmann *et al.*, 1993). It takes a longer time scale (μs) following extraction of MALDI-ions out of the source region into the field-free region (in the flight tube) and the peptide ions decayed because of the energy departed in the desorption process. Fragment ions maintain the same velocity as their intact precursor ions in the field-free region. However, the fragmented ions could not travel far as intact ions into the reflectron field due to their lesser kinetic energy. Therefore, the lower the mass of the ions, the sooner the ions will be ejected out of the reflectron field. It will then appear as a lower apparent mass in the spectra. Because the dynamic separation range is limited for most reflectron fields at a defined voltage, therefore, a regime of gradually stepped down voltages is required to collect the complete set of fragment masses in the range of interest (usually 10-15 segments are obtained). The final sequences could be deduced after stitching together the spectra from different voltage ranges. Alternatively, a "curved-field reflectron" in the TOF mass detector will enable simultaneous focusing of a wide mass range of fragment ions generated by metastable decay. The entire PSD fragment ions spectrum can be obtained in a single experiment (scan) without changing voltages. Provision of the 'curved-field reflectron' has since circumvented the time-consuming reflector voltage stepping procedures (Cornish and Cotter, 1994).

In addition, when delayed extraction is applied in the ion source of a MALDI MS, precursor ions are energetically cooled down which brought about a concomitant reduction of the rate of PSD fragmentation by at least an order of magnitude (Kaufmann *et al.*, 1996). Part of this loss is balanced by a better signal/noise ratio which results from a significantly improved mass resolution of the PSD fragment ions (M/ΔM up to 1800 compared with M/ΔM = 200-500 under prompt extraction). While this compensatory effect is true for the middle to high mass range of PSD fragment ions, it gradually vanishes towards the low mass end of the PSD mass scale where, in the case of linear peptides some important immonium ions are lost. Taken overall for most practical work with PSD, delayed extraction improves quality of the PSD spectra and that high energy collisional post-source activation can compensate for the occasional loss of analytical information (Kaufmann *et al.*, 1996).

It should be stressed that there are many types of fragment ions were produced during the fragmentation processes. Both the N-terminal fragment ions (a-, b-, c- and d-type ions) and C-terminal fragment ions (x-, y-, and z-type ions) as well as the internal fragment ions can be formed from double chains cleavages and they can be observed in the PSD spectra (Chaurand *et al.*, 1999) . [For the nomenclature of fragment ions, please refer to (Biemann, 1988)]. These ions are complementary to each other and can be used to validate accuracy of *de-novo* sequences obtained. However, interpretation of these spectra and deduce a "*de-novo*" peptide sequence is a highly skillful task. In fact, the major drawback of *de-novo* peptide sequencing using PSD is that

the output of fragment ions spectra is too complex. As mentioned, interpretation of the results is complicated, labor-intensive and certainly required a high level of proficiency. Therefore, MALDI-TOF-PSD cannot be used as a standard peptide sequencing method (Samyn *et al.*, 2004).

4.2.2. Derivatization and N-Terminal Sulfonation

As mentioned above, in order to produce a simple but informative mass spectrum, chemical modification or derivatization of the peptides is highly desirable. Several derivatization techniques had been developed (Keough *et al.*, 1999; Shen *et al.*, 1999; Keough *et al.*, 2000; Lindh *et al.*, 2000; Keough *et al.*, 2001; Hellman and Bhikhabhai, 2002; Keough *et al.*, 2002; Sonsmann *et al.*, 2002; Keough *et al.*, 2003). A notable derivatization that we would like to highlight is "sulfonation of the peptide N terminus". This is originated by Keough and his collaborators (Keough *et al.*, 1999; Keough *et al.*, 2000; Keough *et al.*, 2001; Keough *et al.*, 2002; Keough *et al.*, 2003). Their technique has facilitated *de novo* peptide sequencing by using MALDI-TOF-PSD. In their method, a sulfonic acid group was introduced at the N-terminus of a peptide. This chemical modification facilitates protonation of amide bonds of the protein backbone. Protonation destabilizes amide bonds which lead to only b- and y-type ions being produced during PSD fragmentation. However, the negative charge at the N-terminus neutralizes the positive charge normally located at the C-terminus; thereby suppressing the formation of b-ions in positive mode. Hence, a series of y-type ions dominated the PSD spectrum observed. The three most commonly used sulfonation reagents and their general advantages and disadvantages were listed in Table 1. Sulfonation was first accomplished by acylation with 2-sulfobenzoic acid cyclic anhydride and chlorosulfonylacetyl chloride (Keough *et al.*, 1999). This procedure was then refined by using a water soluble reagent, 3-sulfopropinic acid NHS-ester (Keough *et al.*, 2002) which had been commercialized as the CAF (chemically assisted fragmentation) sequencing kit by Amersham Biosciences (Hellman and Bhikhabhai, 2002). The procedures were further refined by guanidination of the ε-amino group of lysine-terminated tryptic peptides with O-methylisourea hydrogen sulfate (Keough *et al.*, 2000; Keough *et al.*, 2002). This step converts lysine residues into more basic homoarginine residues. This modification protects the ε-amino groups against unwanted reaction with sulfonation and therefore allows it to become suitable for *de novo* sequencing reaction by MALDI-TOF-PSD.

In the last few years, another effective sulfonation procedure was reported using 4-sulfophenyl isothiocyanate (SPITC) as the derivatization reagent (Gevaert *et al.*, 2001; Marekov and Steinert, 2003; Wang *et al.*, 2004). Wang and his collaborators (2004) have improved the operation procedures and increased sensitivity on the derivatized peptide. The advantage of this method is that SPITC is much less expensive (about $16 per gram with less than 1 mg required for one reaction) and it is really stable. Our laboratory found this method simple and fast in obtaining *de novo* peptide sequences of dinoflagellate proteins isolated by 2-DE gels. Fig. (5) summarized the routine procedures used to obtain *de novo* peptide sequences for novel proteins isolated from dinoflagellates. Briefly, after gel plugs containing the spots of interest were excised (visualized by stains such as Coomassie Brilliant blue), it was washed and dried. Trypsin digestion was performed on the protein inside the gel plug before dividing the tryptic digested mixture into two portions. One fraction was analyzed by MALDI-TOF MS to yield a peptide mass fingerprint (PMF) spectrum. This PMF spectrum can be used for identification purposes through

Table 1. Most Commonly Used N-Terminal Sulfonation Reagents for *de novo* Peptide Sequencing

Sulfonation Reagents	Advantages	Disadvantages	Useful References
chlorosulfonylacetyl chloride (CSAC)	• commercially available • quite reliable for the derivatization of low-level Arginine-terminated tryptic peptides • allows sulfonation of subpicomole quantities of tryptic peptides	• too reactive to used in water, it must be used in non-aqueous conditions • poor stability • more purification and cleaning steps are required	Keough *et al.*, 1999
3-sulfopropinic acid NHS-ester	• developed into a commercial available sequencing kit named chemically assisted fragmentation (CAF) (Amersham Biosciences, USA), which is more friendly to be used. • water compatible • unwanted sulfonation side products can be selectively reversed by using hydroxlamine	• relatively expensive • have to be freshly prepared	Keough *et al.*, 2002; Hellman and Bhikhabhai, 2002
4-sulfophenyl isothiocyanate (SPITC)	• commercially available • derivatization reaction is efficient in aqueous solution • stable, can be prepared into stock solution • much less expensive	• may need larger amounts of protein materials (nanomoles)	Gevaert *et al.*, 2001; Marekov and Steinert, 2003; Wang *et al.*, 2004

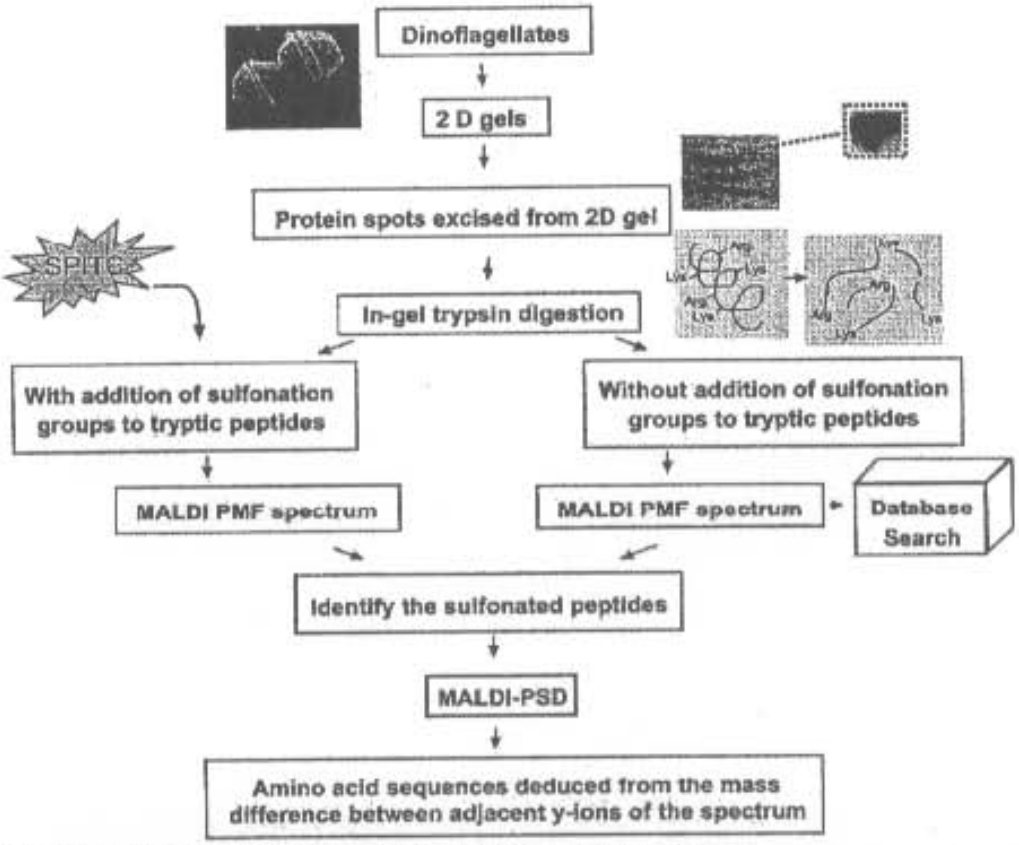

Fig. (5). Routine proteomic workflow used for the annotation of novel proteins in dinoflagellates.

bioinformatic searches in the relevant genome databases. If the PMF spectrum is not enough for identification of the novel protein, the other portion of the tryptic digest will be subjected to SPITC sulfonation as described (Wang *et al.*, 2004). After cleaning-up with zip-tips, the sulfonated tryptic peptide mixtures were subjected to another MALDI-TOF MS analysis. A PMF spectrum of these sulfonated peptides was obtained (from the analysis of this second fraction). When compared to the PMF spectrum obtained with the non-sulfonated portion (from the first fraction), the sulfonated peptide was identified as the same peptide will have a mass difference of 215 Da apart in between the two spectra. This 215 Da represents the mass of SPITC. Subsequently, a MALDI-TOF PSD will be performed on this particular SPITC tagged peptide. The *de novo* amino acids sequences would be easily deduced by the mass distance between adjacent y ions in the spectrum. For an example, a known protein (Rubisco II) of dinoflagellates was subjected to the SPITC-sulfonated MADLI-TOF PSD analysis as mentioned (Fig. 6). A sulfonated peptide from the Rubisco II protein spot was used to demonstrate peptide *de novo* sequencing with the MALDI-TOF PSD analysis. An amino acid sequence QFI/LHYHR was easily deduced from the mass difference between adjacent y-ions in the spectrum. The tag sequence was used to search in the NCBInr database and it matched with ribulose 1,5-bisphosphate carboxylase oxygenase (Rubisco II) of a *Symbiodinium sp.* (accession no. AAY51977). Therefore, this sulfonation technique performed with MALDI-TOF PSD analysis provides a fast and simple way to obtain *de novo* peptide/protein sequences of dinoflagellate proteins.

After some peptide amino acid sequences were obtained, the next question to ask is how to get the entire amino acid sequences of the protein. Peptide sequences obtained earlier could provide the raw information required for determining the overall protein sequence. Further, a protein can be digested with different specific proteases to yield different set of peptide fragments. Sequence coverage of this protein will be increased when different types of proteases are used to generate different sets of peptides. By getting sequences of these peptide fragments, these sequences can be stitched together and extended. However, a complete protein sequence can only be obtained by this method if the peptides coverage is very close to 100%. Completeness of the coverage can be checked by a MALDI measurement of the overall protein mass. On the other hand, we can determine the com-

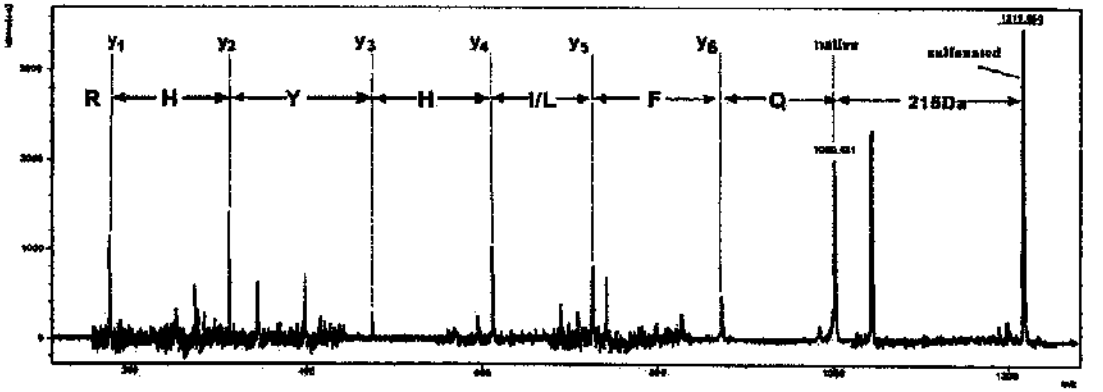

Fig. (6). *De novo* sequencing of a major dinoflagellate proteins (Rubisco II) from 2-DE gels that were used to resolve Trizol-based extracted proteins. PSD spectrum, of sulfonated peptide ((digested from a Rubisco II containing gel spot resolved earlier with another 2-DE gel) which was dominated with y-ions) with a m/z ratio of 1215.55. The sulfonated peptide 1215.55 m/z was derived from sulfonation of the native peptide 1000.431m/z with SPITC (215 Da). The amino acid sequence was deduced from the mass difference between adjacent y-ions of the spectrum. The sequence tag: QFI/LHYHR obtained was searched against the NCBIar database. Then, the sequences matched with ribulose 1,5-bisphosphate carboxylase oxygenase (Rubisco) of the *Symbiodinium spp.* (accession no. AAY51977) with 100% match, Score = 28.6 bits (60), Expect value = 0.006.

plete protein sequence even if only limited or partial peptide sequence information is available. It is because a single peptide sequence (if it is long enough with 7 amino acid is the minimum) allows the construction of a short oligonucleotide probe. This probe can be used for isolation of the gene that encodes this target protein. However, success of this approach is limited when the peptide sequence at hand is not long enough. A long peptide sequence will allow us to have a higher chance of success as we can design primers that have low degeneracy (i.e amino acids encoded by one or two base triplet). As mentioned previously, Bertomeu *et al.* (2003) had successfully obtained the full cDNA sequence of a surface protein p43 by using degenerate primers derived from the peptide sequences obtained from MS.

CONCLUSION

Although it is generally believed that HABs is induced by eutrophication which in turn is caused by various kinds of human activities, many questions relating to blooming is unknown. With the advance of proteomic technologies including 2-D gels and *de novo* sequencing by MS, researchers in the discipline can collectively aim to decipher cellular pathways and mechanisms of blooming and toxin-producing at the whole proteome level. The suggested Trizol-protein-extraction method has greatly simplified protein extraction and cleaning-up procedures for dinoflagellate samples. High quality 2-DE gels can be generated using these "cleaner" protein samples. The excellent 2-DE patterns obtained by this method would enable comprehensive protein expression studies on dinoflagellates to be performed. Different derivatization procedures have greatly enhanced the case of obtaining *de novo* peptide sequences by mass spectrometry. Of these derivatization methods, sulfonation provides a fast and simple way to obtain *de novo* peptide/protein sequences. Although the limited DNA databases information of dinoflagellates makes proteomic study of this class of organism challenging, the 2-DE/derivatization/MS procedures provide a practical and workable proteomic workflow for dinoflagellates research.

ACKNOWLEDGEMENTS

Mr. Fred Lee is supported by a Ph.D. studentship awarded by the Research Committee of The Hong Kong Polytechnic University.

ABBREVIATIONS

2-DE	=	Two dimensional gel electrophoresis
CAF	=	Chemically assisted fragmentation
CID	=	Collision induced dissociation
ESI	=	Electrospray ionization
EST	=	Expressed sequence tag
GAPDH	=	Glyceraldehydes-3-phosphate dehydrogenase
HABs	=	Harmful algal blooms
HPLC	=	High performance liquid chromatography
IEF	=	Isoelectric focusing
LC	=	Liquid chromatography
MALDI	=	Matrix assisted laser desorption/ ionization
MS	=	Mass spectrometry
PCP	=	Peridinin-chlorophyll a-binding protein
PCR	=	Polymerase chain reaction
PITC	=	Phenylisothiocyanate
PMF	=	Peptide mass fingerprint
PSD	=	Post source decay

RP	=	Reverse phase
RUBISCO	=	Ribulose -1,5 bisphosphate carboxylase/ oxygenase
SPITC	=	4-Sulfophenyl isothiocyanate
TCA	=	Trichloroacetic acid
TOF	=	Time-of-flight

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Appendix XIV: Decision letter of manuscript (HARALG-D-07-00008R1)

Date: Dec 03, 2007
To: "Samuel Chun Lap Lo" bcsamlo@
From: sandra.shumway@
Subject: Your Submission

Ms. Ref. No.: HARALG-D-07-00008R1
Title: Rapid Identification of Dinoflagellates Using Protein Profiling With Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry
Harmful Algae

Dear DR. Lo,

FORM LETER FOLLOWS:

I am pleased to confirm that your paper "Rapid Identification of Dinoflagellates Using Protein Profiling With Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry" has been accepted for publication in Harmful Algae.

Comments from the Editor and Reviewers can be found below.

Thank you for submitting your work to this journal.

With kind regards,

Sandra E. Shumway, Ph.D.,D.Sc
Editor-in-Chief
Harmful Algae

Appendix XV:

Reviewer's comments on (HARALG-D-07-00008R1) (original fax)

Ms. Ref. No.: HARALG-D-07-00008R1

Title: Rapid Identification of Dinoflagellates Using Protein Profiling With Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry

Review of Harmful Algae manuscript #S-07-00011[1]

I find no major problems with the scientific content of this manuscript. The idea is interesting and potentially promising. However, before publication, the writing needs to be edited for grammar and, in some areas, content.

In addition, I have two suggestions for the authors, both of which are directed at making the information more useful for other scientists. First, it would be very useful to include more discussion about what is needed to take the technology presented here and make it work in anything other than a cultured sample. For readers who are not familiar with proteomic methods, it is difficult for them to assess the potential utility of the method without a clear explanation of the other information necessary to interpret complex samples (e.g. a library of known algal MALDI patterns). Second, it would be very interesting to see the results of a MALDI –TOF analysis using a "background" phytoplankton assemblage. Even the analysis of a single, dominant HAB species in a sample with naturally occurring, nontoxic phytoplankton, would be informative. This also would help readers to understand the necessary steps for the transition from lab to field analysis.

Review of Manuscript # HARALG - D - 07 - 00008 Rapid Identification of Dinoflagellates Using Protein Profiling with Matrix-Assited Laser Desorption/Ionization Mass Spectrometry.
Author: Sam Lo et. al.

Absolutely great paper. This work has been proposed by a number of people and these guys did a bang up job demonstrating the efficacy of this work. In my view, folks like Karen S., Steve M will be put out of business when this technology matures.
Corrections: Abstract : Third line down from top: posted should be "post"
- fifth line from the bottom: Fingering should be "fingerprinting"

Page 2: line 20- posted should be "post"
Line 26 - fingering should be "fingerprint"
Page 3: line 37 every should be "ever"
Page 10: line 160 - 162: the sentence beginning "Although....." doesnt make any sense.

Highlights of this paper:
* technique requires no initial assessment of unkown HAB samples (simple!)
* Spectral fingerprints of individual species make excellent biomarkers and can be used for ID purposes
* Has the unique ability to distinguish between species with similar morphologies (ie Alexandrim) - circumventing the current confusion that arises when two phycologists do not agree on morphological differences/similarities.
*It is simple, rapid, accurate and due to its analytical nature, very reproducible

This paper is one of the best I have seen in a long time. Great work.

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Rapid identification of dinoflagellates using protein profiling with matrix-assisted laser desorption/ionization mass spectrometry

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Abstract

The occurrence of harmful algal blooms (HABs) or red tides is an important and expanding threat to human health, fishery resources, and the tourism industries. Toxic species post an additional treat of intoxication when consumed either in seafood or directly swallowed. Rapid and accurate identification of the HAB species is critical for minimizing or controlling the damage. We report the use of protein/peptide mass fingerprint profiles obtained with matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) for the identification of dinoflagellates, common causative agents of HABs. The method is simple, fast and reproducible. The peptide mass fingerprint spectral patterns are unique for different dinoflagellate species and are easily distinguishable by visual inspection. In addition to the whole mass spectra, several specific biomarkers were identified from the mass spectra of different species. These biomarker ions and the mass spectral patterns form an unambiguous basis for species discrimination.

Keywords: Dinoflagellates; Harmful algal blooms (HABs); Identification; MALDI; Protein profiling

1. Introduction

Harmful algal blooms (HABs) or red tide is a global problem (Hallegraeff, 1993). It occurs globally ranging from Europe, America, Asia to the Pacific regions (Premazzi and Volterra, 1993). The ever increasing risk of occurrence of HABs represents expanding threats to human health, fishery resources, and the tourism industries. Toxin-secreting species posted additional risk of intoxication when consumed either in seafood or directly. Dinoflagellates are one of the most common causative agents of HABs. Rapid and accurate identification of the HAB species is critical for minimizing or controlling the damage. Microscope-based taxonomic identification methods have long been the standard methods by which this task is accomplished. However, this type of taxonomic identification method can be time-consuming and requires a high level of expertise to discriminate key morphological features indicative of HAB species. Furthermore, different criteria were used to classify different species by different taxonomists (Taylor, 1984; Steidinger and Moestrup, 1990). Therefore, taxonomic confusion and arguments on similar looking HAB species are common.

To resolve the taxonomic debate, different types of identification methods are being introduced for identification of dinoflagellates. One of the common identification methods is to analyze the sequences of the ribosomal RNA genes, including the small-subunit (16S rDNA, 18S rDNA) (Scholin et al., 1993, 1994; Scholin and Anderson, 1994), large-subunit (28S rDNA) (Scholin et al., 1994, 1995), and the internal transcribed spacers (ITS1 and ITS2) regions including the 5.8S rDNA (Adachi et al., 1994, 1996). However, the success of this method depends greatly on the primers design as they must bind effectively to the target. In addition, an incomplete description of the algal genome has limited the success of these approaches.

On the other hand, protein/peptide mass fingerprint profile obtained by mass spectrometry (MS) is widely used for identification and characterization of microorganisms and has recently been extensively reviewed (Fenselau and Demirev,

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2001). The use of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) to obtain these characteristic protein/peptide profiles (sometimes called biomarker profiles) for identification purposes has been applied to various microorganisms, such as viruses (Yao and Fenselau, 2001), bacteria (Krishnamurthy and Ross, 1996; Krishnamurthy et al., 1996; Winkler et al., 1999; Donohue et al., 2006), fungus (Amiri-Eliasi and Fenselau, 2001), parasites (Magnuson et al., 2000), bacterial spores (Hathout et al., 1999; Ryzhov et al., 2000; Dickinson et al., 2004) and fungal spores (Li et al., 2000; Welham et al., 2000). This type of identification method is praised as objective, fast, simple and reliable. Nevertheless, to the best of our knowledge, successful application of MALDI-TOF MS to obtain protein/peptide mass fingerprints profiles of HAB causative agents such as dinoflagellates for identification purposes has never been reported.

This is the first report for the identification of dinoflagellates species by using protein/peptide mass fingerprint profiles obtained with MALDI-TOF MS. In this paper, we demonstrate the application of MALDI-TOF MS for rapid, simple and accurate identification of dinoflagellate species. Results of our investigations clearly demonstrated the capability of this method in rapidly identification of different species of dinoflagellates (namely *Alexandrium affine*, *Prorocentrum minimum*, *Scrippsiella rotunda*, *Karenia brevis* and a yet to be identified species), as well as between individual species of *Alexandrium* sp. *and Scrippsiella* sp. This approach shows great potentials to be used for the continuous monitoring of water samples for the occurrence of HABs.

2. Materials and methods

2.1. Dinoflagellate species and strains

Dinoflagellate species used in this study are listed in Table 1. *A. affine*, *P. minimum*, *S. rotunda*, and a yet unidentified species were kindly provided by Prof. John I. Hodgkiss previously of The University of Hong Kong. The other species, including *Alexandrium catenella* (CCMP1598), *Alexandrium minutum* (CCMP113) and *Alexandrium tamarense* (CCMP116) and *K. brevis* (CCMP2281) were purchased from The Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP). Identities of all species used, except the unidentified species and *K. brevis* (CCMP2281), were confirmed either by the sequence of the ribosomal RNA genes or the internal transcribed spacers (ITS1 and ITS2).

2.2. Culture conditions

Seawater-based K or f/2 media were used for culturing the dinoflagellates (Keller et al., 1987). Stock cultures of all dinoflagellates were kept at exponential growth phase by transferring to new medium every 5 or 6 days in a ratio of 1:10 (v/v). Vegetative cells from cultures in mid- or late-exponential phase of growth were inoculated into freshly prepared culture medium. Possible contamination of algal culture was monitored by regular microscopic examination. The cultures were grown at 22 °C under a 16-h light:8-h dark cycle at a light intensity of 120 μE lx m^{-1} s^{-1} provided by cool white fluorescent tubes in a Conviron growth chamber (Model EF7).

2.3. Cell counts

Cell density was counted in the same time everyday; 1 mL of each culture was taken and fixed with 10 μL Lugol's solution and counted under light microscope with a Sedgwick-Rafter cell counter.

2.4. Ribosomal gene and internal transcribed spacers (ITS) sequencing

Cells were collected by centrifugation (1500 × *g* for 10 min at room temperature) from mid-exponential culture and frozen in liquid nitrogen prior to DNA extraction. DNA was extracted by an extraction kit (Roche, Switzerland) following mechanical cell disruption by a quick homogenization. ITS regions containing the 5.8S rDNA were amplified from the extracted genomic DNA using ITSA and ITSB primers (Adachi et al., 1994, 1996), or

Table 1
Dinoflagellate species and strains used in this study

Species/strains	Source	Collection site (Date)	Confirmed sequences	Accession
Karenia brevis (CCMP2281)	CCMP	Gulf of Mexico, Florida, USA	Nil	Nil
Alexandrium catenella (CCMP1598)	CCMP	Da-ya Bay Guan-dong (Canton) Province, Hong Kong	18S[a]	AY027906
Alexandrium minutum (CCMP113)	CCMP	Ria de Vigo, Spain (September, 1987)	18S and ITS[a]	AY831408
Alexandrium tamarense (CCMP116)	CCMP	Ria de Vigo, Spain (June, 1984)	ITS[a]	AJ005047
Alexandrium affine	IJH	Junk Bay, Hong Kong (August, 1998)	ITS	EF579793
Prorocentrum minimum	IJH	Junk Bay, Hong Kong (1998)	ITS	EF579797
Scrippsiella rotunda 1	IJH	Junk Bay, Hong Kong (March, 1997)	ITS	EF579794
Scrippsiella rotunda 2	IJH	Kat O, Hong Kong (October, 1998)	ITS	EF579795
Scrippsiella rotunda 3	IJH	Junk Bay, Hong Kong (1998)	ITS	EF579796
Unidentified species	IJH	Junk Bay, Hong Kong (1997)	[b]	[b]

CCMP: The Provasoli-Guillard National Center for Culture of Marine Phytoplankton. IJH: Donated by Prof. John I. Hodgkiss previously of The University of Hong Kong. ITS: Internal transcribed spacers.

[a] Ribosomal DNA sequencing was done by other authors.

[b] PCR of the ribosomal gene regions was not successful by using primers mentioned in Section 2 and the universal primers (ITS 1 and ITS 4) described by other authors (D'Onofrio et al., 1999).

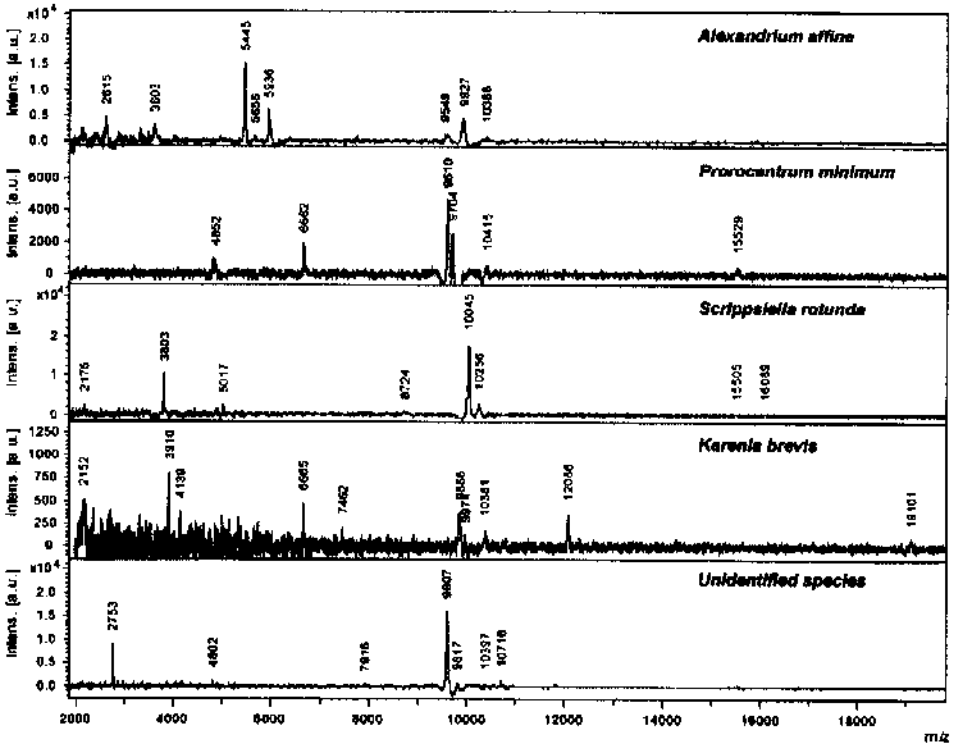

Fig. 1. MALDI-TOF MS protein profiles of dinoflagellate species in different genus. The mass range depicted is from *m/z* 2000 to 20,000. Individual species are listed.

ITSF1: 5′TgAACCTTAYCACTTAgAggAAggA3′ (forward primer) and ITSR1: 5′gCTRAgCWDHTCCYTSTTCATTC3′ (reverse primer), which these two primers were designed by the alignment of the 3′ end of 18S rDNA sequences and the 5′ end of 28S rDNA sequences from the database of different dinoflagellate species. PCR were performed under conditions: 95 °C 5 min; 35 cycles of 94 °C 45 s, 50 °C 45 s and 72 °C 2 min; 72 °C 10 min. PCR products were cloned into pGEM-T easy vectors (Promega, USA) prior to DNA sequencing. DNA sequencing of all cloned plasmids were performed by commercial facilities using traditional dideoxy-methodolgy.

2.5. Sample preparation to obtain protein/peptide mass fingerprints using MALDI-TOF MS

About 250 mL cultures were grown to mid-exponential growth phase. Around 10^5 cells were collected by centrifugation (1500 × *g* for 10 min at room temperature). Cell pellets were resuspended in 0.1% trifluoroacetic acid (TFA) (Aldrich, USA). These cells were broken by sonication for 20 s. Cell debris was removed by centrifugation at 14,000 × *g* at 4 °C for 5 min. Inorganic salts in the samples were cleaned up by absorbing the proteins/peptides onto C-18 zip tips (Millipore, USA) and subsequently washed with several volumes of 0.1%

Table 2
Biomarkers observed in MALDI-MS of dinoflagellate species

Species	Signature peak mass biomarkers (*m/z*)
Alexandrium catenella (CCMP1598)	2194, 2519, 2897, 3123, 3561, 8399, 8618, 12,381, 17,230
Alexandrium minutum (CCMP113)	2673, 4984, 5520, 10,580, 12,545, 15,614, 17,257
Alexandrium tamarense (CCMP116)	2418, 4094, 4473, 6169, 8024, 9050, 10,085, 15,494
Alexandrium affine	2615, 3603, 5445, 5656, 5936, 10,388
Prorocentrum minimum	4852, 6662, 9704,
Scrippsiella rotunda	2175, 3803, 5017, 8724, 10,045, 10,256, 16,089
Karenia brevis (CCMP2281)	3910, 4139, 6665, 7462, 9866, 9974, 12,086, 19,101
Unidentified species	2753, 4802, 7916, 9817, 10,716

TFA and 5% methanol in water. Proteins/peptides were eluted from the zip tip with 0.1% TFA in 50% acetonitrile. 1 μL of eluted proteins/peptides solution were mixed with 1 μL of matrix solution. Matrix solution contained saturated sinapinic acid (SA) in 70:30 (v/v) 0.1% TFA/acetonitrile. The resulting mixtures were then vortexed before spotting 0.5 μL volume onto a mass spectrometer target plate (MTP AnchorChip™ 600/384 T F) (Bruker, Germany).

2.6. MALDI-TOF MS analysis

Proteins/peptides mass fingerprints in all samples were obtained with a MALDI-TOF mass spectrometer (Autoflex, Bruker, Germany) in linear mode at an accelerating voltage of 20 kV by using a 300 ns delay time and over a mass range of 2000–20,000 Da. For each sample, spectra from 500 laser shots at several different positions were combined to generate a mass spectrum. The mass spectra were calibrated using Protein Calibration Standard I (Bruker, Germany) and were used to provide a mass accuracy of 1 part in 3000. The calibrant mixture contains insulin (5734.51 Da), ubiquitin (8565.76 Da), cytochrome *c* (12360.97 Da) and myoglobin (16952.3 Da). Fresh calibration was performed for everyday for different samples and for different experiments.

3. Results and discussions

3.1. Protein profiling and PCR-based identification

All the species kindly donated by Prof. John I. Hodgkiss previously of The University of Hong Kong used in this study have been subjected to the PCR-based identification by analyzing the ITS sequences (Table 1). However, the PCR of the ribosomal genes of a species (temporary called an unidentified species) was not successful by using three different set of universal primers; ITSA and ITSB (Adachi et al., 1994, 1996), ITSF1: 5′TgAACCTTAYCACTTAgAggAAggA3′ (forward primer) and ITSR1: 5′gCTRAgCWDHTCCYTSTTCATTC3′ (reverse primer), ITS 1 and ITS 4 (D'Onofrio et al., 1999). We are not sure if this tentatively unidentified species is a novel species and hence cannot be identified by existing universal primers. Nevertheless, we would like to point out that choices of primers are very important for the success of the PCR-based identification. In addition, the annotation process of this perhaps new/different

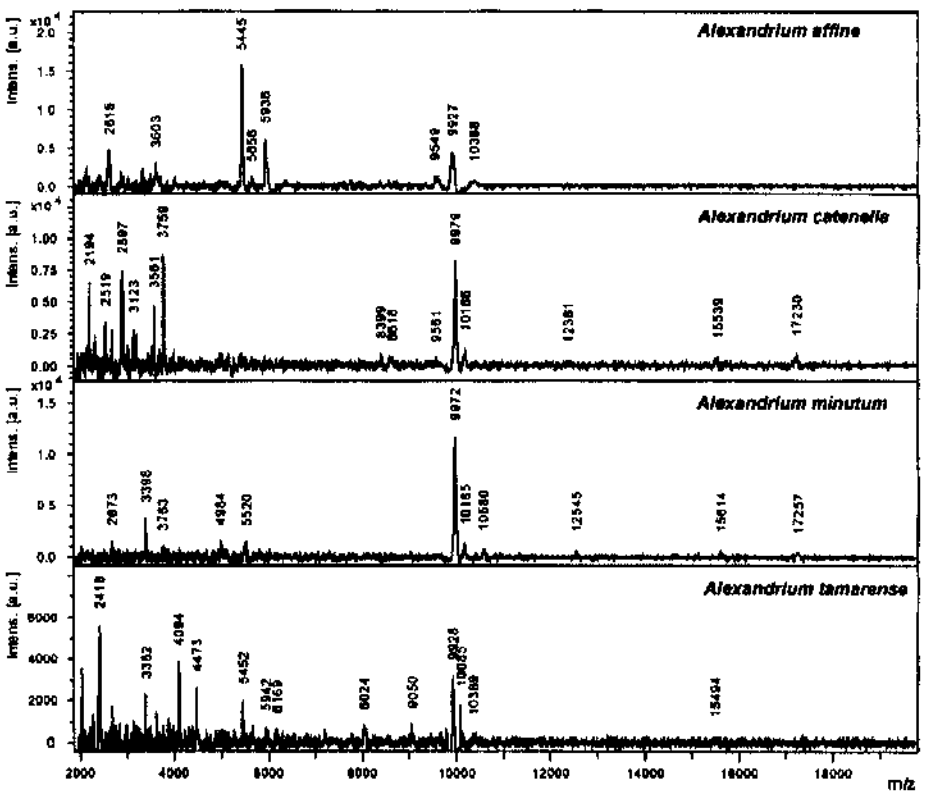

Fig. 2. MALDI-TOF MS protein profiles of dinoflagellate species in same genus (*Alexandrium*). The mass range depicted is from *m/z* 2000 to 20,000. Individual species are listed.

species could be time-consuming and certainly not the theme of this paper, we would like to leave that as it is at the moment. Further, in contrast to PCR-based methodology, the protein profiling based identification method needs no initial assessment of unknown HAB samples which result in a simple and fast identification method.

3.2. MALDI mass spectra

The key success of this methodology is the ability to see either different sets of characteristic protein/peptide mass fingerprint spectra or individual signature mass biomarker (individual species-specific mass fingerprint) for different species of dinoflagellates. Further, the differentiation between species with different sets of characteristic protein/peptide mass fingerprint spectra or individual signature mass biomarker should ideally be seen in a desirable mass/charge (m/z) range in the MALDI analysis. In this study, mass spectra (protein/peptide mass fingerprints) of the dinoflagellate samples generally exhibited around ten discrete mass peaks ranging from m/z 2000 to 20,000. MALDI-TOF identification of other microbes showed similar numbers of discrete mass peaks over the same m/z range (Fenselau and Demirev, 2001). Moreover, MALDI-TOF MS analysis of f/2 and K medium returned no significant peak signals in the 2000–20,000 m/z range (data not shown). MALDI-TOF MS peak mass fingerprint spectra obtained from the different genus of dinoflagellates studied are easily distinguishable by visual inspection (Fig. 1) and each set of peak mass fingerprint spectra from different species is distinct. In addition to the unique peak mass spectral patterns, a number of unique and consistently occurring signature peak mass ions (biomarkers) can be identified that are characteristic at the genus and species level (Table 2). Therefore, either the peak mass spectral fingerprints or the individual characteristic peak mass biomarker can be used for identification purposes. Software and algorithms may be designed and applied to aid in the analysis of such spectral markers (Jarman et al., 1999, 2000). Coupled with a universally accepted taxonomic database with DNA information, this methodology will allows unambiguous identification of HABs causative agents.

3.3. Specificity and sensitivity

Another important issue associated with this developed methodology is the ability to differentiate between species that have similar morphologies. Dinoflagellate species within the genus of *Alexandrium* are highly similar in their morphological appearances when observed under microscope. They are difficult to be distinguished by their morphological features. Hence, this is not surprising that there are taxonomic confusion and argument on classification within this genus. In this study, however, the peak mass fingerprint spectral patterns obtained for different *Alexandrium* species are distinct (Fig. 2). As mentioned previously, characteristic signature biomarker mass peaks that permit differentiation and identification of a particular *Alexandrium* sp. are also identified (Table 2). Therefore, this MALDI-TOF MS

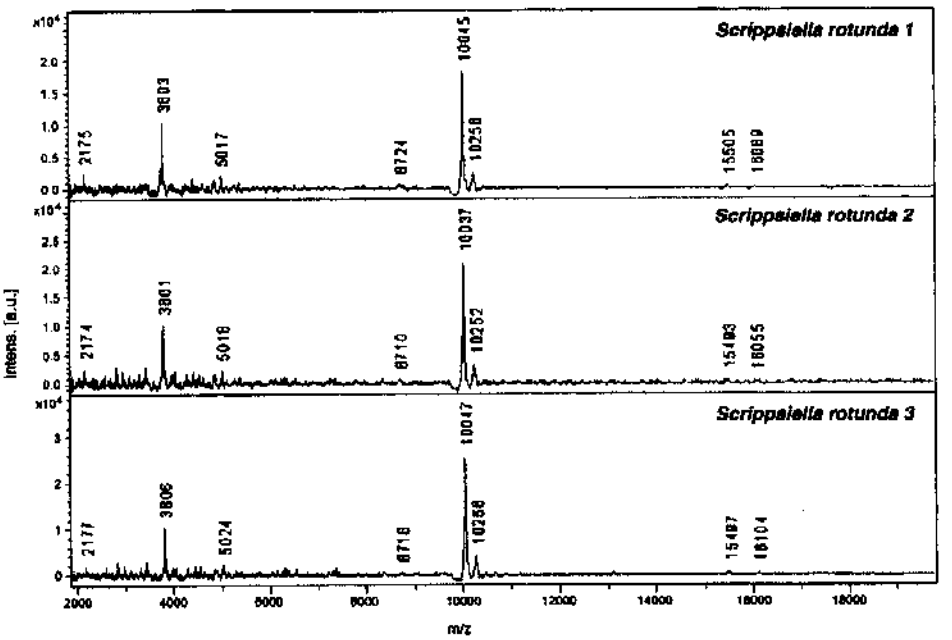

Fig. 1. MALDI-TOF MS protein profiles of different strains of *Scrippsiella rotunda*. The mass range depicted is from m/z 2000 to 20.

identification technology is an excellent alternative to classical microscopic identification techniques for the identification of closely related HAB species.

Specificity of identification by using MALDI-TOF MS mass fingerprint spectral pattern methodologies on microorganism had been shown down to individual strains or even sub-species level (Krishnamurthy and Ross, 1996). However, in our study, we can only distinguish dinoflagellates species down to the species level. In the three different local strains of *S. rotunda* (Table 1) which were collected separately at different time. Three strains have different growth rate (data not shown). However, when these three strains were analyzed by the MALDI-TOF MS under identical conditions, the peak mass fingerprint spectral patterns of the three strains obtained were almost identical (Fig. 3).

With respect to amount of samples required for the analysis, we found that 10^5 of dinoflagellates cells are enough to generate a peak mass fingerprint spectrum with the presence of all the representative peaks. We have tried to use 10^4 cells for the MALDI-TOF MS analysis, but only the most abundant peak masses were seen. Nevertheless, although this is not within the scope of this paper, modifications of sample preparatory procedures before MALDI-TOF MS analysis should be able to improve on the sensitivity of the assay.

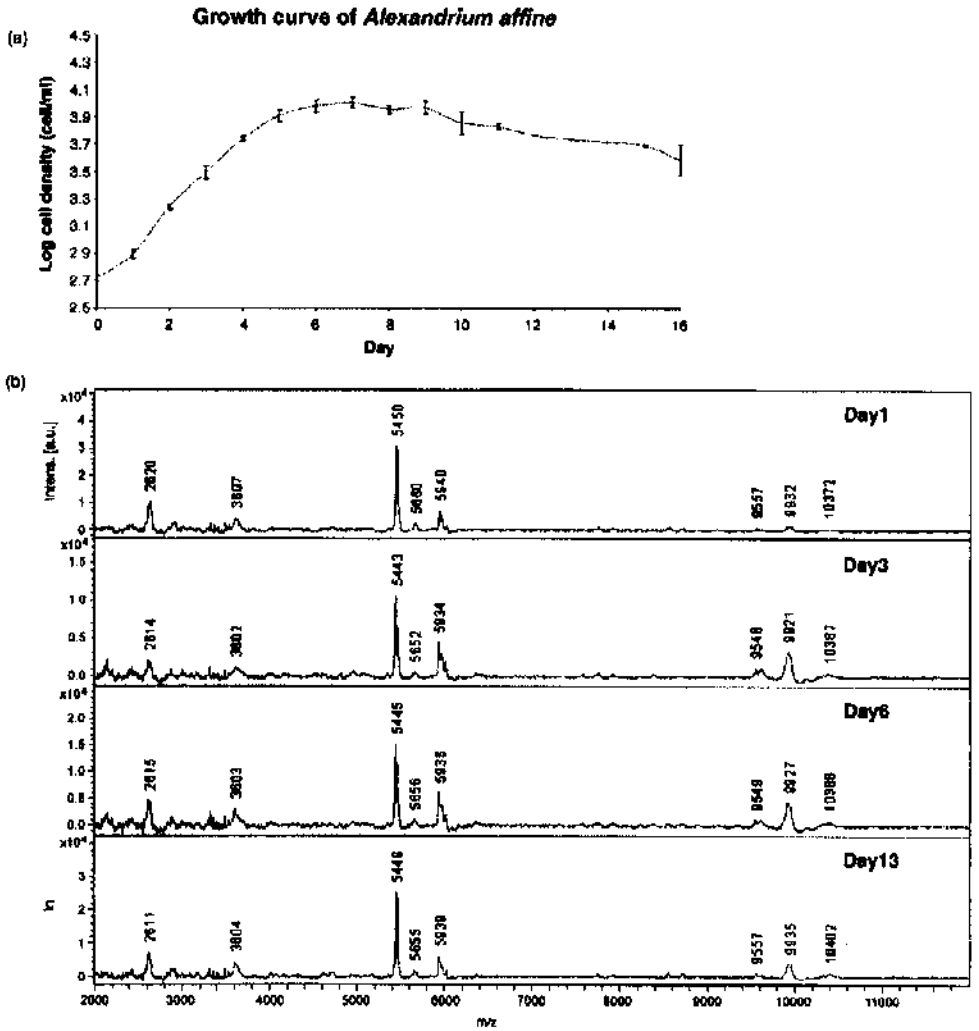

Fig. 4 MALDI-TOF MS protein profiles of *Alexandrium affine* in different growth phases. (a) Growth curve of *Alexandrium affine*. Cells were counted every day, each data point was from the means of triplicate counts. (b) Protein profiles of *Alexandrium affine* in different growth phases. Day 1: lag phase; Day 3: mid-log phase; Day 6: late-log phase and Day 13: stationary phase. The mass range depicted is from *m/z* 2000 to 11,000. The corresponding day of cells harvest are listed.

3.4. Reproducibility

The mass profiles presented in Figs. 1 and 2 were representatives of three different batches of the same samples and each experiment was performed in different date. Given that sample preparation and measurement were performed under the same conditions, the acquired peak mass fingerprint spectra are the same between different batches of the same samples.

Further, in order to be qualified as a rapid and reliable identification method, it is important that a reproducible peak mass fingerprint spectral pattern can be obtained with the MALDI-TOF MS analysis irrespective of specific stages in the growth cycle and growth conditions. When applying this methodology to other microbes, it is reported that peak mass fingerprint spectral pattern distributions were dependent on different compositions of growth media (Evason et al., 2000; Valentine et al., 2005). However, species and genus level distinctions are possible when the growth and experimental conditions had been properly controlled (Evason et al., 2000; Valentine et al., 2005). Therefore, we tried to compare peak mass fingerprint spectral patterns of a dinoflagellate species grown under two different culture media (f/2 and K medium). Highly similar peak mass spectral patterns were observed for both samples (data not shown). It seems that growth media has no observable effect in the peak mass spectral patterns observed. In addition, we had found that same amount of *A. affine* cells (about 10^5 cells) collected at different growth states and analyzed by the MALDI-MS (Fig. 4) exhibited the near-identical sets of specific peak mass spectral patterns. Cells in

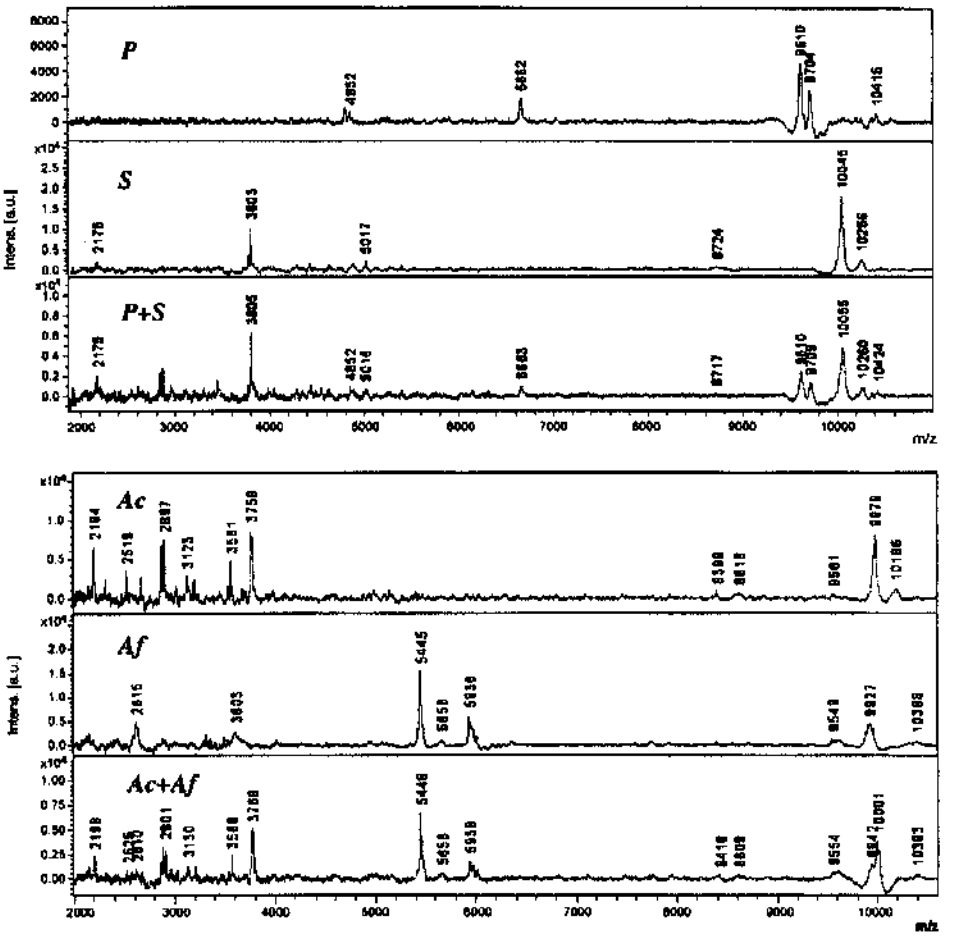

Fig. 5. MALDI-TOF MS protein profiles of mixed populations of different dinoflagellate species. (a) Protein profiles of P: *Prorocentrum minimum*; S: *Scrippsiella rotunda* and P + S: *Prorocentrum minimum* and *Scrippsiella rotunda*. (b) Protein profiles of Ac: *Alexandrium catenella*; Af: *Alexandrium affine* and Ac + Af: *Alexandrium catenella* and *Alexandrium affine*. The mass range depicted is from *m/z* 2000 to 11,000.

the lag phase of growth (Day 1) show a very similar peak mass spectral pattern as cells in mid-log (Day 3), late-log (Day 6), stationary or early death phase (Day13). The remarkable similarity of the peak mass spectral patterns were based on the measurement of constantly expressed highly abundant proteins in the dinoflagellate proteomes, for example the ribosomal proteins (Ryzhov and Fenselau, 2001). Therefore, it is not surprising that most of the peak mass ions, especially major ones such as 5445, 5656, 5936, 9527, 9949, 10,388 *m/z* are not affected by growth states of the cells (Fig. 4).

3.5. Recognition of different species of dinoflagellates in a mixed culture

In the real world with a real red tide, usually one or two HAB species from different taxonomic groups would predominate (Hallegraeff, 1993). Therefore, it prompts us to investigate whether it is possible to differentiate different dinoflagellate species from a mixed-culture. Two different species, *P. minimum* and *S. rotunda* (in different genus) (Fig. 5a); *A catenella* and *A. affine* (in same genus) (Fig. 5b) were grown in a mixed culture. The mixed culture was harvested and analyzed by the MALDI-TOF MS under the same conditions as described in Section 2. Our results shown that several species-specific notable signature peak mass can be easily identified from the peak mass fingerprint spectrum of the mixed cultures. In Fig. 5a, signature biomarker peak masses of *P. minimum* (P) *m/z* 4852, 6662, 9610, 9704, 10,415 and biomarker peak masses of *S. rotunda* (S) of *m/z* 2175, 3803, 5017, 10,045, 10,256; are easily identified from the peak mass spectrum of the mixed culture (P + S). In addition biomarker peak mass can still be identified from the mixed culture containing two looking-alike species, *Alexandrium* sp. (Fig. 5b). Further, other notable biomarker peak masses of *m/z* 2194, 2897, 3759, 9979 from *A. catenella* (Ac) and *m/z* 2615, 5445, 5656, 5936, 9549, 9927 from *A. affine* were also easily identified from the mixed culture spectrum (Ac + Af).

3.6. Potential of applying the identification technique in field samples

Although we have successfully demonstrated the ability of protein/peptide mass fingerprints profiling-based identification in different cultured dinoflagellate samples, one may concern the efficacy of this technique when applied to field or real harmful algal bloom samples. We strongly believed that the identification method described have great potentials when applied in real world scenarios. Nevertheless, there are several pre-requisites to ensure success and general application of this methodology. Firstly, protein/peptide mass fingerprints spectra and signature peaks of different HAB causative agents should be adequately identified and annotated. This will involve *de novo* amino acid sequencing of characteristic biomarker peaks for proper annotation. Secondly, all the identified and annotated signature peaks obtained should be well documented, stored in a database or library aided by bioinformatic softwares and freely available to interested parties. Thirdly, a unified and generally agreed-upon "standard" sample preparation procedure including all the instrumental settings should be developed for all users to follow aiming to minimize unnecessary variations when different versions of the methodology are applied by different users. Fourthly, already it is easily said than done, studies on real life HAB causative agent samples should be done to study the effects of various environmental conditions, salinity and presence of co-existing microbes, etc. on the test results. By having more studies on this methodology and having the above requirements fulfilled, the technique can be easily implemented by technical personnel in local governments, monitoring agencies and/or large scale fish-farming and aquaculture companies.

4. Conclusion

Here we have demonstrated that it is possible to identify dinoflagellate species either in a single cell culture or in a mixed population of different dinoflagellate species based on characteristic peak mass spectral patterns or species-specific signature biomarkers in the spectrum. Identification and differentiation of closely related species such as *A. affine*, *A. catenella*, *A. tamarense*, *A. minutum*, can be accomplished easily by the MALDI-MS analysis. Although the identification would become complicated when more than two species are presented in the mixed culture, it may still be possible to identify the species compositions with the aid of computer software based on the analysis of the specific signature biomarker peak ions. The workflow of HAB species protein/peptide peak mass fingerprint spectral profiling is a straightforward approach. The method is simple, rapid, accurate and reproducible. It represents an excellent alternative to classical microscopic-based identification techniques. The peak mass fingerprint spectral pattern is unique for different dinoflagellate species and is easily distinguishable by visual inspection. In addition to the whole peak mass spectra, several specific signature biomarkers were identified from the mass spectra. Therefore, the signature biomarker ions and the peak mass fingerprinting spectral profiles form an unambiguous set of criterion for identification of dinoflagellate species.

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Appendix XVII: Decision letter of manuscript (MIMET-D-07-00444)

From: afox@
To: bcsamlo@
Subject: Your Submission
Date: Wed, 9 Jan 2008 16:00:25 +0000

Ms. Ref. No.: MIMET-D-07-00444R1
Title: The use of Trizol reagent (phenol/guanidine isothiocyanate) for producing high-quality two-dimensional gel electrophoretograms (2-DE) of dinoflagellates
Journal of Microbiological Methods

Dear Prof. Lo,

I am pleased to inform that your paper "The use of Trizol reagent (phenol/guanidine isothiocyanate) for producing high-quality two-dimensional gel electrophoretograms (2-DE) of dinoflagellates" has been accepted for publication in Journal of Microbiological Methods.

Thank you for submitting your work to this journal.

With kind regards,

Alvin Fox Ph.D.
Professor &
Editor-in-Chief
Journal of Microbiological Methods

Appendix XVIII:

Reviewer's comments on (MIMET-D-07-00444)

Ms. Ref. No.: MIMET-D-07-00444
Title: The use of Trizol reagent (phenol/guanidine isothiocyanate) for producing high-quality two-dimensional gel electrophoretograms (2-DE) of dinoflagellates
Journal of Microbiological Methods

Dear Prof. Lo,

Your manuscript received favorable reviews regarding publication in the Journal of Microbiological Methods. A revision has been recommended.
Final acceptance is subject to successfully answering the criticisms raised by the reviewers whose comments are below for your review. In addition to the reviewers coments.

1. Tone down the emphasis on dinitroflagellates. Presumably the improvements in 2D gel resolution will have general applicability and be of interest to the wide readership of JMM.

2. Overall the article is extremely long-winded and needs to be tightened up, including removing any extraneous figures.

3. The section "MALDI analysis of selected spots" requires greater breadth and depth. As I am sure the authors are aware, MALDI TOF MS (tryptic mass profiling)is routine but nowadays the proteomics community prefers at least one peptide(preferably two or more) to be sequenced by MALDI TOF-TOF MS-MS. MALDI PSD MS has to this point been the poor man's substitute (for MS-MS) and rarely used; e.g. if access to a MALDI TOF-TOF lacking. Furthermore, the vast bulk of proteomics labs to do not use derivatitization, i.e. it is also non-standard. Most readers of JMM will not be aware of the above and so your work should clearly explain/illustrate what you have achieved and and describe how it relates to other standard (or non-standard) mass spectrometry methodology with appropriate references.

For your guidance, reviewers' comments are appended below.

If you decide to revise the work, please submit a list of changes describing point-by-point your comments to the reviewers or a rebuttal against each point which is being raised when you submit the revised manuscript. Please paste each reviewers comment above each of your responses.

Your revised article should be received by Jan 29, 2008.

To submit a revision, please go to http://ees.elsevier.com/mimet/ and login as an Author.

Your username is: ******
Your password is: ******
On your Main Menu page is a folder entitled "Submissions Needing Revision". You will find your submission record there.

Yours sincerely,

Alvin Fox Ph.D.
Professor &
Editor-in-Chief
Journal of Microbiological Methods

Reviewers' comments:

Reviewer #1:
This article will provide an alternative method for protein preparation from dinoflagellates. A few problems were identified.

1. In figures 2 and 3 panel C Trizol is misspelled as Tizol.

2. The discription of the difference between cup loading and rehydration loading is adequate, the figure is not necessary.

3. page 18, lines 325-327 "Recently, derivatization technique was found to facilitate de novo peptide sequencing with the MALDI-TOF (Keough et al., 1999; Keough et al., 2000)." The english in this sentence needs to be revised, "Recently, a derivatization technique was found to facilitate de novo peptide sequencing with the MALDI-TOF"

4. The Mass spectra in Figure 7b has numbers, I think, but they were not readable. This needs to be fixed.

Reviewer #2: Major comments:

This paper addresses two aspects of 2-D gel-based proteomics. The first is comparing the quality and number of spots on the gel with different protein precipitation methods, the second comparing sample loading on the IEF gel. The combination of methods combined with an N terminal derivatization of tryptic peptides to aid identification by tandem mass spectrometry. This combination of methods seems to be beneficial for protein identification from these samples.

However, several places in this manuscript need to be rewritten to improve the grammar. The authors are encouraged to consult a native English speaker to improve this aspect of the manuscript.

Figure 2 and 3 are redundant for demonstrating the effect of Trizol precipitation over the lysis buffer or acetone precipitation methods. One of the two figures should be removed.

Minor Comments

Page 15 line 301-302: The sentences: "The enablement of using rehydration…" and "It allows larger amount of proteins.." need to be rewritten for clarity and number agreement.

Page 16 line 310: the sentence containing "….one may concern the usefulness of the 2-DE…" likewise needs to be rewritten for clarity.

Figure 2C, Trizol is misspelled as "Tizol".

Figure 7 legend is a paragraph and entirely too long. The majority of this information needs to be placed within the results section.

Appendix XIX: Accepted Manuscript (MIMET-D-07-00444)

Title: The use of Trizol reagent (phenol/guanidine isothiocyanate) for producing high-quality two-dimensional gel electrophoretograms (2-DE) of dinoflagellates

Authors: Fred Wang-Fat Lee[1] and Samuel Chun-Lap Lo[1,2*]

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Abstract

Two-dimensional gel electrophoresis (2-DE) is one of the most efficient ways of resolving complex protein mixtures based on the isoelectric point (*pI*) and molecular mass (M_r). Although it has been extensively used in proteomic studies of animal, plant, yeast and bacterial cells, there is limited information on the use of 2-DE in research with dinoflagellates (harmful algal blooms causative agents). The preparation of high-quality samples from dinoflagellate cells for 2-DE is difficult due to high endogenous levels of salts, nucleic acids, polysaccharides, phenolic compounds , pigments, and other interfering compounds. Desalting and concentrating steps are usually required for the preparation of dinoflagellate protein sample prior to the IEF and these steps can be lengthy and complicated. In this study, we report the use of Trizol (a monophasic solution of phenol and guanidine isothiocyanate) for the extraction of proteins from dinoflagellate cells for 2-DE. The method is simple and fast. 2-DE profiles obtained from this Trizol treatment are of very high-quality in terms of resolution, spot number and spot intensity. The method greatly simplify the protein extraction procedures on dinoflagellate samples for obtaining a high quality and reproductive 2-DE profile and therefore greatly contributes to proteomic works in these algal samples.

Keywords: Dinoflagellate; HABs; protein extraction; proteomics; Trizol; Two-dimensional gel electrophoresis; 2-DE

Introduction

Dinoflagellate is one of the most common causative agents of Harmful Algal Blooms (HABs). Although it is generally believed that HABs are induced by eutrophication which in turn is caused by various kinds of human activities, many questions relating to blooming/toxin-producing is unknown. With the advance of proteomic technologies including 2-D gels, researchers in the field can collectively aim to decipher cellular pathways and mechanisms of blooming and environmental effects at the whole proteome level. Study of the proteome or protein expression in HAB species is therefore important for understanding more about their blooming and toxin-producing mechanisms.

Of the limited number of publications on studies on proteomes of dinoflagellates, mostly rely on the high power of resolution of 2-DE (Akimoto et al., 2004; Chan et al., 2005; Chun et al., 2004; Chun et al., 2004; Chun et al., 2006). Since its description more than 30 years ago (O'Farrell, 1975), it has remained one of the most commonly used techniques in proteomics research today. It allows a fast and relatively inexpensive overview of changes in cell processes by analyzing of the entire proteome of cells. However, to obtain a high quality protein samples subsequent to 2-DE required tedious sample preparatory steps (Chan et al., 2004; Chan et al., 2004; Chan et al., 2002). The lengthy preparatory steps are necessary to remove high endogenous levels of salts, nucleic acids (estimated at least 10-100 times more than human), polysaccharides, phenolic compounds, pigments, and other interfering compounds of dinoflagellates. All these compounds will interfere with the IEF- focusing process, the first step of 2-DE. The classical method includes sample cleaning up steps which take a few days and the amount of proteins loaded cannot be in excess of 100 μg. In this study, we report the use of a commercially available reagent Trizol (phenol/guanidine isothiocyanate) for the extraction of proteins from dinoflagellate cells for 2-DE. There are several advantages in using this method. Firstly, the method is simple, fast and will only take several hours. Secondly, most of the interfering materials (nucleic acids, salts, pigment, non-proteinaceous components etc.) will be effectively removed. Thirdly, proteins extracted with the procedures can be used directly to the first dimension IEF step without any further treatments like protein concentrating and desalting steps. Fourthly, proteins in the Trizol reagent are well protected from

proteases degradation and adding of proteases inhibitors is therefore not required. Lastly, extracted protein samples can be used in in-gel rehydration loading mode in the IEF step which yielded high-quality 2-DE profiles that are comparable with those obtained with the cup-loading method.

To demonstrate our point, two dinoflagellate species, *Alexandrium spp* and *Scrippsiella spp*, were used in this study. The 2-DE profiles obtained with this Trizol treatment gave high-quality 2-DE electrophoretograms in terms of resolution, spot numbers and spot intensity. Further, we had also attempted and successfully completed the identification of a major protein in the dinoflagellate 2-DE electrophoretogram obtained using peptide mass fingerprint (PMF) and *de novo* peptide sequencing with MALDI-TOF mass spectrometry. Taken overall, our method described here has greatly simplified the protein extraction and cleaning-up procedures for dinoflagellate samples for 2-DE and it would greatly contributes to proteomic works in this field.

Materials and Methods

Dinoflagellate species used and Culture conditions

Unialgal cultures of *Alexandrium spp* and *Scrippsiella spp* were kindly donated by Prof John I. Hodgkiss previously of the University of Hong Kong. Seawater based K or f/2 media were used for culturing the dinoflagellates (Keller et al., 1987). Stock cultures of all dinoflagellates were kept at exponential growth phase by transferring to new medium every five or six days in a ratio of 1:10 v/v. Vegetative cells from cultures in mid- or late-exponential phase of growth were inoculated into freshly prepared culture medium. Possible contamination of algal culture was monitored by regular microscopic examination. The cultures were grown at 22℃ under 16: 8 hours light: dark cycle at a light intensity of 120μE Lux $m^{-1}s^{-1}$ provided by cool white fluorescent tubes in a Conviron growth chamber (Model EF7, USA).

Cell counts

Cell density was counted in the same time everyday. Briefly, 1ml of each culture was taken and fixed with 10μl Lugol's solution and counted under light microscope with a Sedgwick-Rafter cell counter.

Preparation of protein extracts

4L of mid-log phase cells of both dinoflagellate species were harvested by centrifugation at 1000 x g for 15 minutes at 4℃. The cell pellets were then equally divided into three 2ml micro-centrifuge tubes (for the 3 different types of extraction procedures, see below) and equal amount of cells in each tube were checked by cell counts under light microscope. Pellets were rinsed with sterile seawater and centrifuged at 1000 x g again for 15 minutes at 4℃. Cell pellets were stored at -80℃ until used. Proteins were extracted according to the three different extraction methods including lysis buffer (7M urea, 2M thiourea, 4% CHAPS, 40mM Tris pH 8.7), acetone precipitation and Trizol extractions.

For protein extraction using lysis buffer (7M urea, 2M thiourea, 4% CHAPS, 40mM Tris pH 8.7), 500µl of lysis buffer was added to the cell pellets as previously described (Chan et al. 2002). Cells were disrupted by sonication (a total of 3 minutes with short pulses of 5-10 seconds each) on ice. Lysis of cells was confirmed using light microscope. Cell lysate was then centrifuged at 14000 x g for 15 minutes at 4℃ to collect the supernatant. Subsequently, the supernatant collected was placed into a new eppendorf tube and store at -80℃ until use.

For sample preparation relying on acetone precipitation, 2.5ml of ice-cold acetone was added to a 500µl protein extracts in lysis buffer and kept at -20℃ overnight to allow precipitation of proteins. Proteins precipitated were washed twice with ice-cold acetone before air dry. Subsequently, 500µl of fresh lysis buffer was added to re-solubilize the protein pellet. The protein solution is ready for 2-DE.

In protein extraction using Trizol (Roche, Switzerland), preparation was performed according to manufacturer's instructions with some modifications. Briefly, 1ml Trizol reagent was added to the cell pellet and subjected to sonication (a total of 3 minutes with short pulses of 5-10 seconds) on ice. Lyses of cells were confirmed using light microscope. Subsequently, 200µl of chloroform was added to the cell lysate before shaking vigorously for 15 seconds. The mixture was allowed to stand for 5 minutes in room temperature before being centrifuged at 12000 x g for 15 minutes at 4℃. The top pale-yellow or colorless layer was removed, 300µl of ethanol was added to resuspend the reddish bottom layer and the mixture centrifuged at 2000 x g for 5 minutes at 4℃. Supernatant was transferred to a new tube and 1.5 ml of

isopropanol was added. The mixture was allowed to stand for at least 20 minutes for precipitation of proteins at room temperature. It was then centrifuged at 14000 x g for 10 minutes at 4℃. Pellet obtained was briefly washed with 95% ethanol before allowed to air dry. 500µl of lysis buffer was added to solubilize the protein pellet before loading onto the first dimension IEF.

Protein determination

Protein quantification in the urea-containing protein samples was performed using a modified Bradford protein assay (Bio-Rad, USA) (Ramagli and Rodriguez, 1985).

Two dimensional gel electrophoresis (2DE) and imaging analysis

Typically, 340µl sample containing 60µg of sample proteins (for silver staining gel while 500µg proteins are needed for coomassie staining gel) in rehydration buffer (containing 7M urea, 2M thiourea, 4% CHAPS, 0.2% DTT and 3.4µl of IPG buffer pH 4-7) was used to rehydrate the IPG strip (18 cm) pH 4-7 (Bio-Rad, USA) for 16 hours. For samples to be cup-loaded (for comparative studies), protein samples were added in the sample cup after rehydration of the IEF strips. IEF was performed using a Protean-IEF cell (Bio-Rad, USA). Voltage was applied according to the following: 1 hour at 100V, 2 hour at 300V, 2 hours 1000V, 2 hours 4000V and 5 hours 8000V. Following IEF, the gel strip was equilibrated with equilibration buffer (50mM Tris pH 8.8, 6M urea, 30% glycerol, 2% SDS, 1% DTT and trace amount of bromophenol blue) for 15 minutes. The gel strip was then placed in fresh equilibration buffer containing 1% iodoacetamine (instead of DTT) for another 15 minutes. The second dimension SDS-PAGE was performed using 10% polyarylamide gel running at a constant current of 15 mA/gel until the bromophenol blue dye reached the end of the gel. After electrophoresis, the gel was either silver stained or coomassie blue (R250) stained. Stained gels were scanned and the images were analyzed by Melanie III (GeneBio, Switzerland) as described in the user manual.

Immunoblotting analysis

Subsequent to 2-DE, separated proteins were electroblotted onto a nitrocellulose membrane at 100V for 1 hour at 4℃. Subsequently, the blotted membrane was rinsed with TBS buffer (20mM Tris-HCl, pH 7.6 and 137 mM NaCl) and blocked with 5% BSA in TBS buffer for 2 hours. The membrane was then probed with rabbit antibodies

to Ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco II) (kindly provided by Professor David Morse, Montreal University, Canada) diluted 1:4000 with 1% BSA in TBS for 1 hour at room temperature. After washing six times with TBS buffer with 1% Tween 20 (TBST buffer), the membrane was incubated with peroxidase conjugated anti-rabbit IgG antibodies (diluted 1:100000 with 1% BSA in TBS; Santa Cruz Biotechnology, USA) for 1 hour. After washing six times with TBST buffer, the chemiluminescent signal was detected using Supersignal Chemiluminescent Substrate kit (Pierce Chemical, USA) following the manufacturer's instructions.

MALDI-TOF mass spectrometry analysis

Guided by the results of Western-blots with samples extracted with the Trizol-extraction method described above and resolved by 2-DE with subsequent probing with anti-Rubisco II antibodies, location of the protein Rubisco II could be recognized and matched to a corresponding coomassie blue stained 2-DE gel. This protein spot was excised from the coomassie blue stained gel and transferred to a microcentrifuge tube. The gel plug was washed twice in 25mM NH_4HCO_3 in 50% acetonitrile (ACN). Subsequently, the gel plug was washed with 100% ACN and dried under vacuum for 10-15 minutes. In-gel trypsin digestion was performed by adding 20ng/ml trypsin in 25mM NH_4HCO_3 overnight at 37°C. For MALDI-TOF mass spectrometry analysis, 1µl peptide mixture was mixed with 1µl matrix solution (CHCA, saturated solution in ACN: 0.1% TFA (1:1)) on the target plate before being dried and analyzed with a MALDI-TOF mass spectrometer (Autoflex; Bruker, Germany in reflector mode over a mass range of 1000-3000 Da and using external mass calibration with the manufacturers' calibration standards). Spectra from 150 shots at several different positions on the target plate were combined to generate a peptide mass fingerprint (PMF) for database searches. The PMF obtained was searched against the NCBI non-redundant database using the search engine MASCOT.

De-novo sequencing was performed with the aid of N-terminal sulfonation (Wang et al., 2004). Briefly, 10mg/ml 4-sulfophenyl isothiocyanate (SPITC) was added into the tryptic peptides and incubated at 55°C for 30min for the sulfonation reaction to occur. Sulfonated peptides were cleaned by absorption onto and subsequently eluted from zip-tips before being analyzed with MALDI-TOF mass

spectrometer. Amino acid sequences were deduced from the ladder sequences (spectrum) obtained and were searched against the NCBI non-redundant database.

Results and Discussion

Comparison of 2DE electrophoretograms obtained using the acetone precipitation method and Trizol–protein extraction method

The success of obtaining high-quality 2-DE electrophoretograms is greatly dependent on sample preparations before the IEF run. Typically, IEF is tolerant of salt concentrations up to 50mM (Kirkland et al., 2006). Therefore, in order to have good quality IEF for 2-DE, a thorough desalting of the sample is required. In dinoflagellates, there are quite a large amount of interfering substances including salts, nucleic acids, polysaccharides, phenolic compounds and pigments. If untreated, these interfering substances will cause aberrant patterns in the 2-DE electrophoretograms. Thus, sample preparation should include steps to get rid of these substances. Using Trizol-extracted proteins for 2-DE has been reported in halophilic proteins with satisfactory results (Kirkland et al., 2006). In comparison with 2-DE electrophoretograms obtained using protein samples prepared either with the lysis buffer method or acetone precipitation method, those obtained using Trizol extraction method showed better results. They have higher resolution of protein spots than those prepared by other two methods. The results were consistently observed in the two dinoflagellates species (*Alexandrium spp* and *Scrippsiella spp*) used in this study (Figure 1).

Proteins extracted by direct homogenization of the *Alexandrium* cells in lysis buffer produced 2-DE electrophoretograms that have excessive horizontal and vertical streakings (Figure 2a). Most of the proteins were not being focused properly and appeared either as smearing or in aberrant patterns. In addition, high backgrounds with relatively fewer spots were seen. Obviously, the "unremoved" endogenous interfering substances in these dinoflagellate cells have greatly affected focusing of the IEF step in the 2-DE. Therefore, this method is not the best choice for 2-DE of dinoflagellates.

As for the acetone precipitation method, the precipitation step was used both to concentrate proteins in the samples and also to separate the proteins from potentially

interfering substances. Therefore, resolution and intensity of the protein spots were better than those obtained with the lysis buffer extraction method (Figure 2b). However, phenolic and some other contaminants were coextracted together with the proteins, resulting in several horizontal and vertical streakings with a high background. This is easily observable in enlarged areas of the gel (Figure 3). Therefore, further cleaning-up procedures are required to remove the substantial interfering substances that remained in the acetone precipitated samples.

Of the three protein extraction procedures, Trizol-extraction resulted in the best resolution of stained spots with a clear background (Figure 2c). The Trizol-sample preparation strategy is simple and fast. There are only a few steps to perform and the preparation time just requires a few hours. When compared to the lengthy and complicated extraction procedures described previously (Chan et al., 2004; Chan et al., 2004; Chan et al., 2002), the shorter time of sample preparation decreased the risk of protein degradation and losses. The beneficial effects that Trizol extraction method has on the improvement of protein yield, spot resolution, numbers and intensity in 2-DE, as compared with the other two methods in this study, are attributed to the great efficiency in the removal of all the interfering substances. Similar results were observed in *Scrippsiella spp* (data not shown). The excellent 2-DE patterns obtained by this method would allow comprehensive protein expression studies on dinoflagellates and thus greatly contributes to proteomics working.

Total numbers and intensity of spots

With the help of the Melanie III software, it was found that 2D gels generated using proteins extracted from the *Alexandrium spp.* with the lysis buffer-extraction method, the acetone precipitation and Trizol-extraction methods yielded an average of 1233, 1741 and 1835 protein spots respectively (Figure 4). On the other hand, those 2D gels using *Scrippsiella spp.* sample yield an average of 1147, 1527 and 1627 spots for the three extraction methods respectively (Figure 4). Excessive streaking, smearing and weakening appearance of the 2D gels would make it difficult to discern any appreciable number of spots in some regions in the 2-DE developed either with the lysis buffer or the acetone precipitation methods (Figure 3). However, the corresponding region in the gel developed using Trizol method had shown a numbers of spots that could be discerned individually (Figure 3c and 3f). There were not only

an obvious increase in spot numbers in these regions, but also many faint protein spots can now be seen clearly with the use of Trizol extraction method, presumably due to a clearer background. For example, spots in the rectangle and circle regions in Figure 3 had shown a greater intensity of the Trizol prepared 2-DE than that of the other two methods. In addition, some spots were missing in the 2-DE prepared by Lysis buffer (for example, see spot 1-7, Figure 3). Although these spots were appeared in both the acetone precipitation and Trizol prepared 2-DE (Figure 3b and 3c), spots were much focused and with a higher resolution in the Trizol prepared 2-DE than that of the acetone precipitation prepared 2-DE.

Rehydration versus Cup-loading method

Cup loading has been reported to improve the quality of 2-DE electrophoretograms obtained and frequently yields better resolution, especially when sample was applied in the anode side (Gorg et al., 2000). However, it has limited sample volumes to be loaded of up to 100µl. Larger sample loads inevitably lead to protein precipitation in the sample cup. Rehydration loading instead of cup-loading could accommodate larger sample volumes (greater than 100µl) and protein loading without the protein precipitation problems. In this study we found that the 2-DE pattern and spot resolution obtained with the Trizol-extracted samples in rehydration loading mode was similar to that obtained with the cup loading mode in the pH 4-7 range. Further, we have experienced that some other samples (e.g bacteria) extracted with lysis buffer will not yield high-quality 2-DE gels without using cup loading. In addition, there are significant differences in the 2-DE gel patterns obtained from samples prepared with the two sample loading methods. However, a high-quality 2-DE gel can be obtained relatively straight-forward with sample proteins extracted by the Trizol method. Samples could be added directly onto the IEF tray, i.e. without cup-load. The 2-DE pattern as well as resolution from rehydration loading obtained is similar to that of cup loading (data not shown). Using rehydration loading can avoid the problems of the sample cup leak and protein precipitation that would be likely happened when cup loading method is used. In addition, without the limitation on the sample loading volumes (more than 100µl), larger amount of sample can be loaded onto the IEF strips. Thus, it will greatly facilitates the production of high-quality and high protein-loaded 2-DE gels (mg of proteins loaded) for some downstream analysis

which usually require a larger amount of proteins, for example the MALDI-TOF mass spectrometry in Post-Source-Decay (PSD) mode.

MALDI analysis of selected spot (Rubisco II)

A known protein (Rubisco II) of dinoflagellates was subjected to the MALDI-TOF mass spectrometry analysis (Figure 5). Location of this protein spot on the 2-DE chromatogram was confirmed by western blotting (Figure 5a). Further, after in-gel tryptic digestion and MALDI-TOF mass spectrometric analysis, the corresponding peptide mass fingerprints (PMFs) generated were subjected to searches in the NCBInr database (Figure 5b). With the MASCOT probability obtained, it was suggested that the protein spot was indeed Ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco II) (accession no. AAO13075). Identification through PMF search is only workable when the genome of the organism is available in the database. Since the protein sequence database of dinoflagellates is very limited, determination of the peptide/protein sequences (*de novo* sequencing) is therefore very important for identification and description of novel proteins. The PSD fragmentation of the tryptic peptide ions generated from the MALDI-MS always results in complex spectra and interpreting these data can frequently be a challenge. Therefore, MALDI-MS-PSD is less popular than other sequencing techniques such as the LC-MS/MS and edman sequencing. Nonetheless, N-terminal sulfonation coupled with MALDI-MS-PSD can act as fallback sequencing method when the other sequencing methods are not available. Recently, an N-terminal sulfonation technique was found to facilitate *de novo* peptide sequencing with the MALDI-TOF-PSD (Keough et al., 2000; Keough et al., 1999). Here, 4-sulfophenyl isothiocyanate (SPITC) was used to sulfonate the tryptic peptides from the selected spot and provide the amino acid sequences of the sulfonated peptide with the MALDI-TOF-PSD analysis (Wang et al., 2004). In addition, this method was successfully applied to enhance the protein identification efficiency of an organism that with unknown genome (Leon et al., 2007). Two sulfonated peptides from the Rubisco II protein spot were used to demonstrate peptide *de novo* sequencing with the MALDI-TOF-PSD analysis (Figure 5c). An amino acid sequence QFI/LHYHR and YWI/LSI/LTTEEDI/LI/LR were easily deduced from the mass difference between adjacent y-ions in the two spectra. Sequence tag: QFI/LHYHR and YWI/LSI/LTTEEDI/LI/LR were searched against with the NCBInr

database. Sequence QFI/LHYHR matched with sequence QFLHYIR of Ribulose 1,5-bisphosphate carboxylase oxygenase II (Rubisco II) of the *Symbiodinium spp.* (accession no. AAY51977). While sequence YWI/LSI/LTEEDI/LI/LR matched with sequence Y(AD)LSLTEEDLIK of Ribulose 1,5-bisphosphate carboxylase oxygenase II (Rubisco II) of the *Prorocentrum minimum* (accession no. AAO13049.1). Therefore, beside the other standard amino acid sequencing methods such as LC-MS/MS and edman sequencing, this N-terminal sulfonation technique provides an alternative way to decipher the *de novo* peptide/protein sequences of dinoflagellate proteins.

Conclusion

Trizol, a monophasic mixture of phenol and guanidine isothiocyanate that is commercially available, is typically used to isolate RNA from cell and tissue samples. In this study, it is shown that Trizol extraction is a simple, fast and effective method for preparing proteins samples for 2-DE analysis of dinoflagellates. With the model organisms used: *Alexandrium* and *Scrippsiella* species, it was shown that proteins were effectively separated from various types of IEF interfering substances. The cleaner protein samples prepared by Trizol-extraction protocol was able to produce high-quality 2-DE gels in which more than 1600-1800 distinctly resolved protein spots were detected after sliver staining. We also demonstrated the feasibility of an identification method of a selected protein spot (Rubisco II) by PMF search and *de novo* sulfonated peptide sequencing with MALDI-TOF-PSD. This work provides an efficient and feasible 2-DE/MS workflow for proteomic analysis of dinoflagellates.

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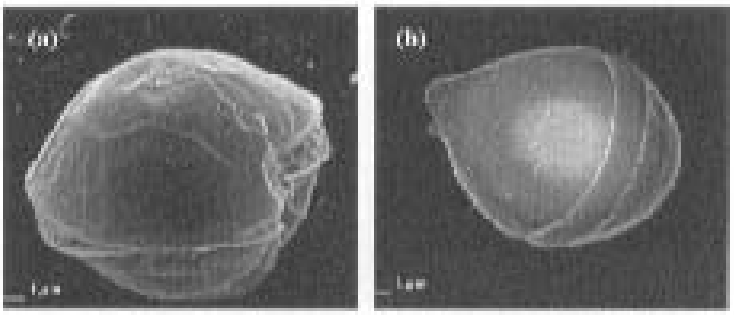

Figure 1. Scanning electron micrographs (SEM) of *Alexandrium spp* and *Scrippsiella spp* isolated from Hong Kong waters. SEM was taken at x 2120 (scale bar = 1μm) of (a) *Alexandrium spp* and (b) *Scrippsiella spp*.

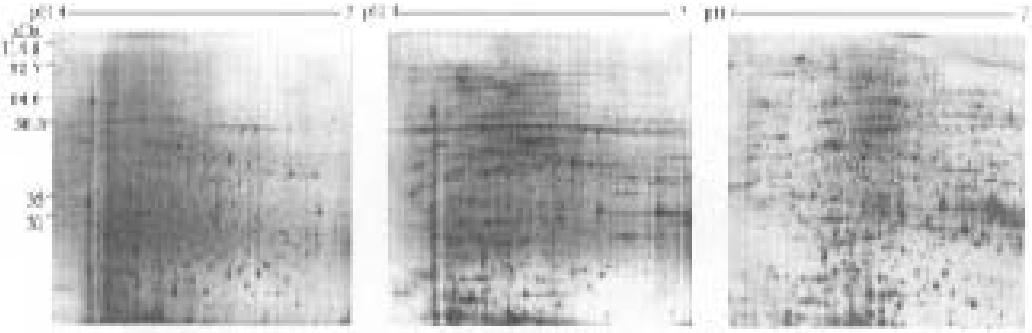

Figure 2. 2-DE of total protein extracts of *Alexandrium spp* prepared using the (a) lysis buffer method, (b) acetone precipitation method and (c) the Trizol protein extraction method.

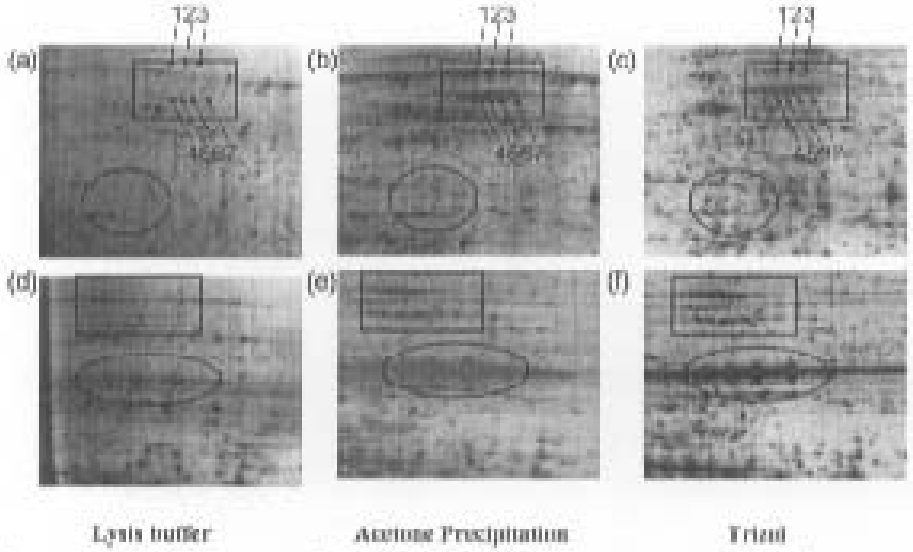

Figure 3. Enlargement of 2-DE images from representative regions among the three protein extraction methods: lysis buffer; acetone precipitation and Trizol-extraction method. (a)-(c) and (d)-(f) are gels from *Alexandrium spp* and *Scrippsiella spp* samples respectively. Arrows indicate spots 1-7 and they were missing in the 3(a) gel.

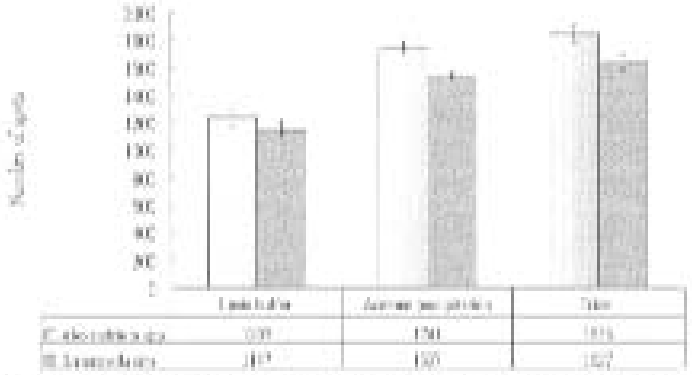

Figure 4. Comparison of total number of spots of the 2-DE from three different extraction methods of the 2-DE of the two dinoflagellates species, *Alexandrium spp.* and *Scrippsiella spp.* Results were the mean values from three independent experiments.

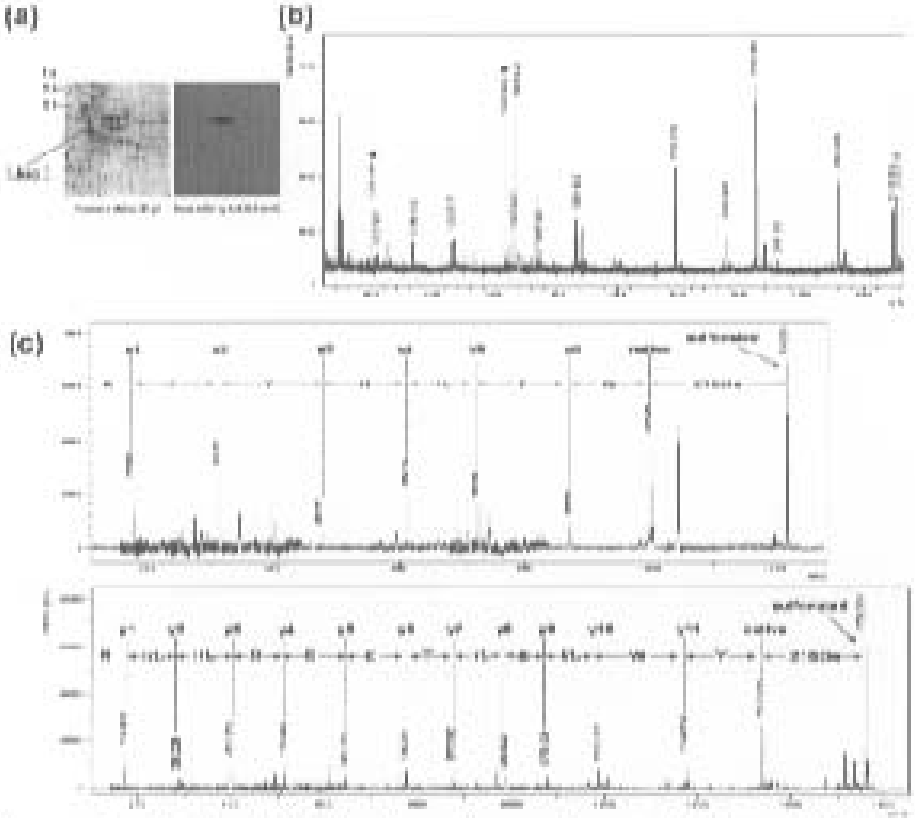

Figure 5. Protein identification of a gel spot (Rubisco II) from the 2-DE electrophoretogram obtained with Trizol-based extraction method. (a) Gels either stained with coomassie blue or immunoblotted with Anti-Rubisco II antibodies on the 2DE gels prepared from proteins extracted from the *Alexandrium spp.* (b) Peptide mass fingerprint (PMF) of Rubisco II. (c) PSD spectrum of sulfonated peptides 1215.55 m/z and 1753.05 m/z . The sulfonated peptide1215.55 m/z and 1753.05 m/z were derived from sulfonation of the native peptides 1000.4 m/z and 1537.9 m/z respectively (asterisk in 5b) with SPITC (215 Da).