# The Development of Human Recombinant Arginase as a Novel Agent in the Treatment of Human Cancer

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## Certificate of Originality

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Tsui Sam Mui

September, 2004

## Abstract

Some tumour cells have been shown to be auxotrophic for arginine. Enzyme degradation of extracellular arginine becomes a possible mean for inhibiting tumour growth but harmless to normal cells. Arginase is one of the arginine depriving enzymes that catalyses the hydrolysis of arginine to urea and ornithine.

Arginase was shown by others to have anti-tumour activity and is potentially useful for treatment of cancer. In 1990, recombinant human arginase was produced successfully from an *Escherichia coli* expression system. However, *E. coli* is pathogenic and the yield of arginase was low. To overcome these problems, we produced recombinant human arginase using a non-pathogenic prophage-based *Bacillus subtilis* expression system. In addition, the arginase was tagged with 6 histidines at the N-terminus to allow single-step purification, by which about 100 mg of arginase was obtained from 1 L of fermentation culture in unprecedented high purity as well as activity. In phosphate-buffered saline buffer (pH 7.4), the purified enzyme is a dimer with $K_m$ and $k_{cat}$ values of 1.89 mM and 1.80 $s^{-1}$, respectively, and the specific activity is 500-600 I.U. $mg^{-1}$.

The blood circulation half-life of the recombinant arginase was found to be too short and was not effective to deplete serum arginine. To overcome this problem, the enzyme was modified with polyethylene glycol (PEG) to produce pegylated arginase, arginase-SPA-$PEG_{5000}$. Arginase-SPA-$PEG_{5000}$ is fully active, with substantially increased circulation half-life, and retains all the enzyme kinetic parameters as the native arginase. When given a single intraperitoneal (i.p.) injection of 1,500 I.U. of arginase-SPA-$PEG_{5000}$ to rats, the plasma arginine level was decreased to a non-detectable level for about six days.

These data indicate that the pegylated human arginase, arginase-SPA-$PEG_{5000}$, is safe and could be further developed to function as an effective arginine depriving anti-cancer drug.

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## Abbreviations

| | |
|---|---|
| 6xHis | 6 histidines |
| ADC | Arginine decarboxylase |
| ADI | Arginine deiminase |
| ARG | Arginase |
| $A_x$ | Absorbance at a wavelength of x nm |
| *B. subtilis* | *Bacillus subtilis* |
| BHY medium | Bovine heart infusion with yeast extract |
| BSA | Bovine serum albumin |
| CD | Circular dichroism |
| DNA | Deoxyribonucleic acid |
| *E. coli* | *Escherichia coli* |
| h | Hour/ hours |
| I.U. | Unit/units |
| IEF | Isoelectric focusing |
| min | Minute/ minutes |
| mPEG-ALD | Methoxy-polyethylene glycol - propionaldehyde |
| mPEG-CN | Cyanuric chloride activated methoxy-polyethylene glycol |
| mPEG-MAL | Methoxy-polyethylene glycol -maleimide |
| mPEG-NHS | Methoxy-polyethylene glycol -N-hydroxysuccinimide |
| mPEG-SPA | Methoxy-polyethylene glycol succinimidyl propionate |
| MW | Molecular weight |
| NADPH | Reduced form of nicotinamide adenine dinucleotide phosphate |

| | |
|---|---|
| Ni-NTA | Nickel-nitrilotriacetic acid |
| OD | Optical density |
| PBS | Phosphate buffered saline |
| PCR | Polymerase chain reaction |
| PEG | Polyethylene glycol |
| psi | Pounds per square inch |
| rpm | Revolution per minute |
| s | Second/ seconds |
| SDS-PAGE | Polyacrylamide gel electrophoresis in the presence of SDS |
| TEMED | N, N, N', N'-Tetramethyl-ethylenediamine |
| TFF | Tangential flow filtration |
| Tris-HCl | Tris(hydroxymethyl)aminomethane, pH adjusted with HCl |
| UV | Ultra violet light |

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# Chapter One:
# Introduction

## 1.1 Arginine

L-arginine (2-amino-5-guanidinovaleric acid) is a vitally important amino acid. It is characterized as a nonessential amino acid in a healthy adult human but as an essential amino acid for young growing animals (Barbul A., 1986). It serves as a substrate for protein synthesis. Besides, arginine plays a number of critical physiological roles, including creatine synthesis and protein degradation. Arginine is also a precursor of nitric oxide, a free radical with diverse physiological and pathological actions, such as regulation of vasodilation, immune response, neurotransmission and inflammation.

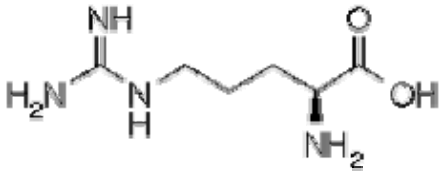



Fig. 1.1: The structure of arginine.

### 1.1.1 Urea cycle

One of the major functions of arginine within the body is to act as an intermediate in the urea cycle. The urea cycle is an essential metabolic pathway responsible for the removal of toxic compounds from the body. It is the route for disposal of surplus nitrogen, providing the means of detoxification of neurotoxic ammonia. Hydrolytic cleavage of arginine to urea and ornithine is catalyzed by

arginase. Arginase removes the guanidine group from arginine to produce urea and ornithine. The urea produced by the urea cycle is transported in the blood to the kidneys for excretion in the urine. In human, 80-90% of ingested nitrogen was excreted as urea, whereas the ornithine synthesized is used to regenerate arginine.

Urea cycle occurs in the liver (Fig. 1.1). Arginase is one of the five key enzymes of the urea cycle; the others are ornithine carbamoyltransferase, argininosuccinate synthetase, argininosuccinate lyase and carbamoyl phosphate synthase. Of various urea cycle intermediates, only arginine can be incorporated into protein.

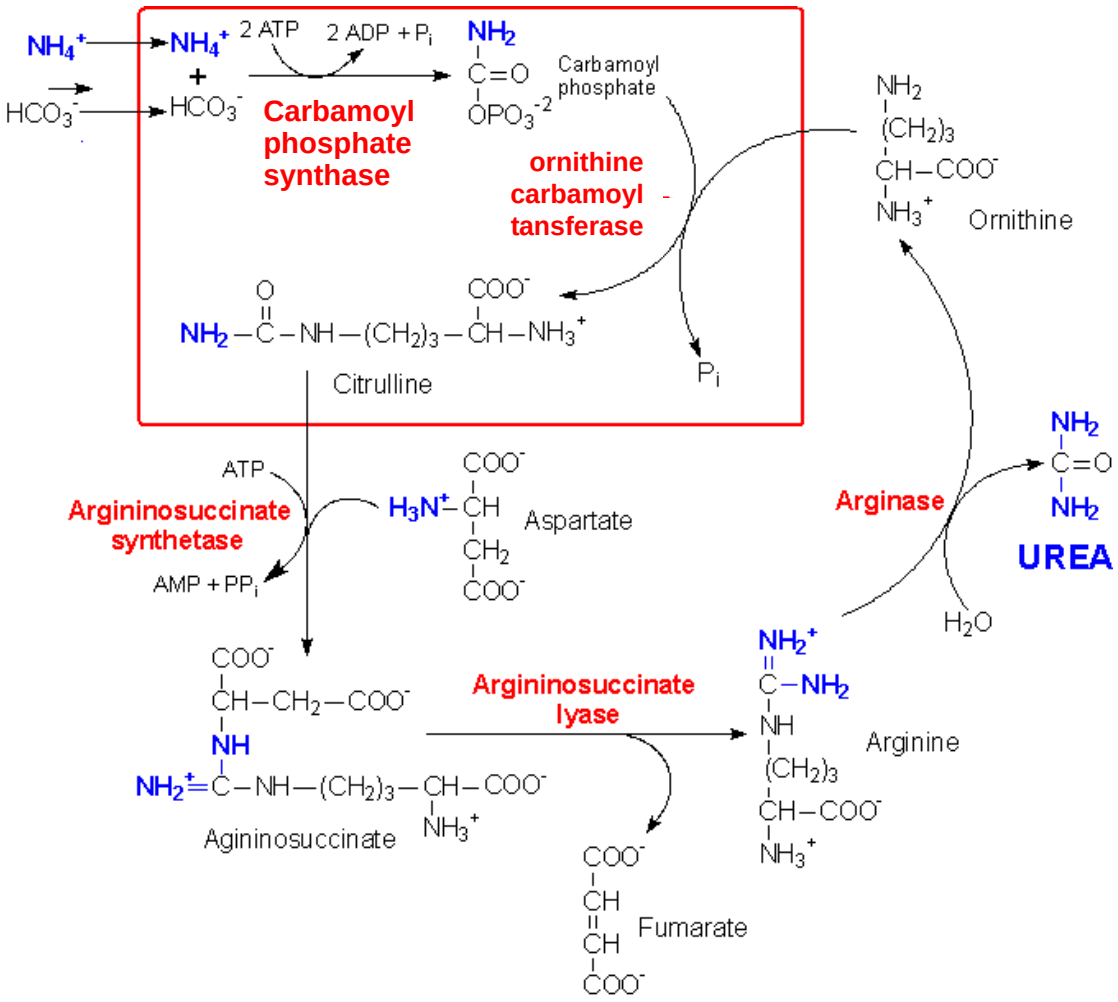



Fig. 1.2: Reactions and enzymes of the urea cycle. The enzymes are argininosuccinate synthetase, argininosuccinate lyase, arginase, ornithine carbamoyltransferase and carbamoyl phosphate synthase. The five enzymatic reactions of the urea cycle result in the net conversion of two ammonium ions and one bicarbonate ion into a molecule of urea.

### 1.1.2 Protein synthesis

Another function of arginine is protein synthesis. Arginine can be converted into proline, glutamate and glutamine, all of which are common amino acids found within most proteins. Synthesis of these amino acids also begins with arginine, which is then converted into the ornithine, an intermediate of these amino acids.

### 1.1.3 Essential non-protein compounds

Arginine metabolism also generates some essential non-protein, nitrogen-containing compounds. Some examples of these compounds are creatine, polyamines and nitric oxide.

Creatine is first synthesized in the liver and then transported through the blood stream to muscle tissue. Functions of creatine are the carrier for phosphate and are required for the rapid regeneration of adenosine triphosphate in the muscles.

Another metabolic pathway of arginine is the synthesis of polyamines. Polyamines function in membrane transport, cell growth, cell proliferation and cell differentiation. Arginine and products of arginine metabolism are necessary in both the regulation and the synthesis of polyamines (Schuber F., 1989).

In addition to regulating polyamine levels, agmatine and arginine also regulate production of nitric oxide (Dillon *et al.*, 2002). Nitric oxide is an antimicrobial agent that is effective against intracellular pathogens, extracellular parasites and bacteria. Nitric oxide is also a neurotransmitter and vasodilator. The enzyme that produces nitric oxide is nitric oxide synthase. The substrate for this reaction is arginine and the products are nitric oxide and citrulline (Fig. 1.2). There are three isoforms of nitric oxide synthase; these are NOS-1, NOS-2 and NOS-3.

NOS-1 (also known as nNOS, NOS-I and Type I NOS) is constitutive and is predominately located in neuronal tissue. NOS-2 (also known as iNOS, NOS-II and Type II NOS) is inducible and is located in a variety of tissues. NOS-3 (also known as eNOS, NOS-3 and Type III) is constitutive and is primarily localized in endothelial tissue (Bermudez *et al*., 1993; Gobert *et al*., 2000).

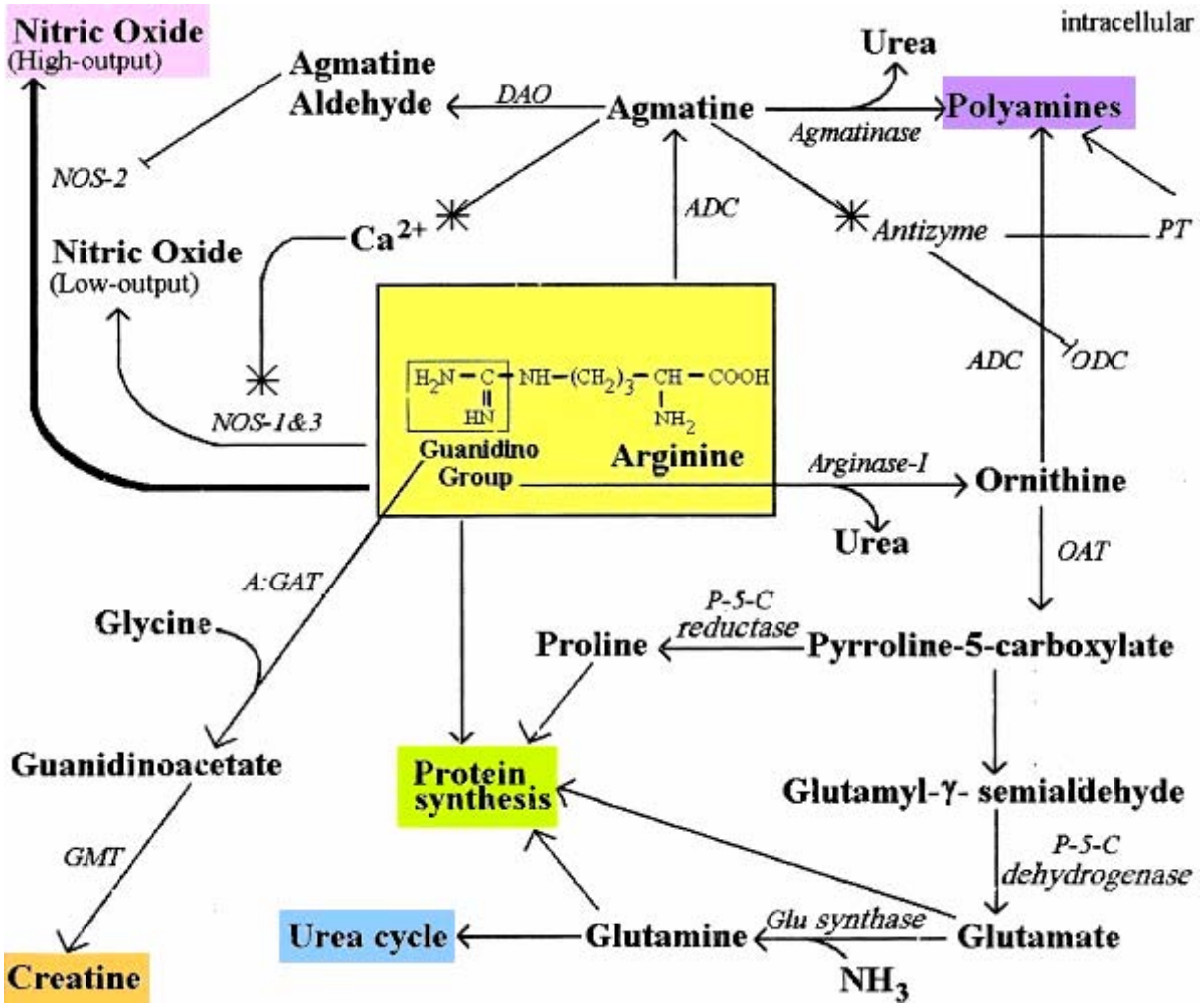



Fig. 1.3: Arginine metabolisms (Nieves and Langkamp-Henken, 2002). Abbreviations: ADC, arginine decarboxylase; A:GAT, arginine: glycine amidinotransferase; DAO, diamine oxidase; Glu synthase, glutamine synthase; GMT, guanidinoacetate-*N*-methyltransferase; NOS-1, nitric oxide synthase-1; NOS-2, nitric oxide synthase-2; NOS-3, nitric oxide synthase-3; OAT, ornithine aminotransferase; ODC, ornithine decarboxylase; P-5-C dehydrogenase, pyrroline-5-carboxylate dehydrogenase; P-5-C reductase, pyrroline-5-carboxylate reductase; and PT, polyamine transporter.

## 1.2 Amino acid availability and cancer

Potential of amino acid deprivation as a means of control for growth of tumour cells has caught research interest. Selective deprivation of amino acid has been used to treat some forms of cancers. The best example is the use of L-asparaginase as a treatment for acute leukemia. In which, L-asparaginase depletes L-asparagine and converts it into L-aspartic acid. Normally, L-aspartic acid can be converted back to L-asparagine by the enzyme asparagine synthase. However, this enzyme was transcriptionally absent in most malignant lymphoid cells. As a consequence, malignant lymphoid cells die from the lack of asparagines (Ronghe *et al*., 2001). This example demonstrates that the use of amino acid deprivation in cancer treatment is feasible.

### 1.2.1 Arginine deprivation

Early in 1940, Bach and Lasnitzki (1947) have reported that arginine stimulated mitotic activity in tumour cells; it was observed that rapidly growing tumours had a greater demand for arginine than that normally required by the body. And then in 1970s, the apoptotic effect of arginine depletion on tumour cells by arginase rich liver extract was demonstrated (Storr and Burton, 1974). The potential of arginase as a new therapy for cancer treatment was studied and

further investigations have been carried out (Scott *et al*., 2000).

Wheatley *et al*. (2000) analyzed the effect of deprivation of several essential amino acids in tumour cells and found that apoptotic cell death occurred more quickly during arginine deprivation than other amino acids. Twenty-four common cancer cell lines died after arginine deprivation for 5 days. When arginase was removed from co-cultures of normal and tumour cells, the normal cells survived and the tumour cells died. The results showed that normal cells remained surviveed after 5 weeks of arginine deprivation. The normal cells generally switched into Go arrest from G1 and stayed there for weeks and re-entered the cycle when arginine was replenished. The anti-proliferation effect of arginine deprivation in cancer cells is probably due to the defective control of the G1 checkpoint which inhibits the initiation of DNA synthesis under unfavorable conditions. Without arginine, which is an irreplaceable amino acid in cancer cells, protein synthesis was deranged and finally, cells died (Wheatley *et al*., 2000).

As modulation of the cell cycle may precede cell death, Gong *et al*. (1999) investigated the effect of arginine deprivation by arginine deiminase (ADI) on apoptosis. When ADI was applied to the tumour cells (melanomas and hepatomers), signs of apoptosis like cell shrinkage, nuclear fragmentation and

chromatin condensation, were observed. Induction of apoptosis by ADI was further verified by the ability to generate DNA breaks in the tumour cells. These results suggested that the effect of arginine deprivation in tumour cells may be due to the inhibition of cell cycle progression and the induction of apoptosis.

Although arginine deprivation can cause cell death *in vitro*, in our body, citrulline can be converted and used as the source to produce arginine by the urea cycle (Fig. 1.1). Therefore, it is difficult to achieve the required arginine deprived condition *in vivo* because of our body homeostasis. In 1992, Sugimura *et al.* (1992) reported that four out of five human melanoma cell lines could not be rescued with citrulline, but grew when argininosuccinate was supplied under arginine depriving condition. The data suggested that these melanoma cell lines were deficient in argininosuccinate synthetase (ASS) and therefore could not recycle citrulline to arginine. Another tumour type that is characteristically deficient in ASS is the human hepatocellular carcinoma (Ensor *et al*., 2002). The inability of these tumour cell lines to utilise citrulline makes them to become the preferred targets for eradication by arginine deprivation (Wheatley and Campbell, 2003). According to these observations, arginine deprivation not only induced selective death of cultured malignant cells, but it is potentially useful in the treatment of a wide range of common cancers.

## 1.3 Arginine depriving enzymes

Three enzymes were demonstrated to have the ability of arginine deprivation. They are arginase, arginine deiminase and arginine decarboxylase. For these three enzymes, arginase is the one that originally presents in human body. In this project, arginase was studied, characterized and developed as a potential cancer treatment drug.

### 1.3.1 Arginase

Arginase is a hydrolytic enzyme responsible for converting arginine to ornithine and urea in the final step of urea biosynthesis. It accomplishes this by cleaving the guanidino group from arginine (Jenkinson *et al.*, 1996). In doing so, it converts one positively charged amino acid into another to yield urea. Liver arginase is one of the most important enzymes of mammalian nitrogen metabolism, since it comprises the principal route for disposal of excess nitrogen resulting from amino acid and nucleotide metabolism (Jenkinson *et al.*, 1996).

Two isozymes of the mammalian arginase are defined: type I and type II arginase. The identity between type I and type II is less than 60% (Hus-Citharel and Levillian, 1999; Que *et al*., 2002). Type I arginase is the most characterized form and is abundant in liver. It is cytosolic and an essential component of the

urea cycle (Jenkinson *et al.,* 1996). The mitochondrial type II arginase has little or no expression in liver, but is present in numerous extra hepatic tissues. The biological roles of type II arginase are postulated to be regulation of polyamines (Kim *et al*., 2002).

The crystal structure of the type I arginase from rat liver has been determined (Ash *et al*., 1998; Cama *et al*., 2003). The key features of the type I arginase included a trimeric quaternary structure, a binuclear manganese cluster, and an S-shaped tail composed 19 amino acids at the C terminus (Fig. 1.3). The S-shaped tails are located at the subunit–subunit interface. Since the amino acid sequence of the rat liver enzyme is 87% identical to that of the human liver enzyme, all principal structural features are expected to be conserved between the rat and human enzymes.

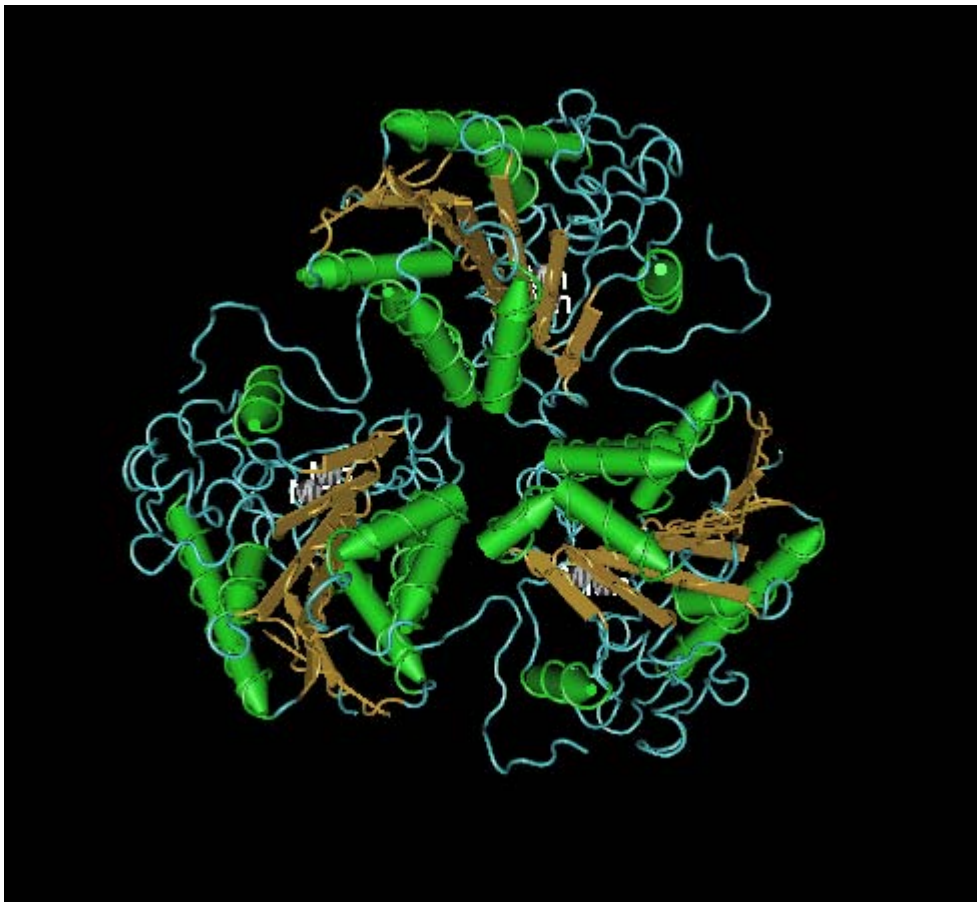


Fig. 1.4 : Trimeric structure of the rat liver arginase (Ash *et al*., 1998). The amino acid sequence of the rat liver enzyme is 87% identical to that of the human liver enzyme. Nearly all principal structural features are expected to be conserved between the rat and human enzymes.

### 1.3.2 Arginine deiminase

Arginine deiminase (ADI) is an enzyme secreted by several species of mycoplasma, e.g., *Mycoplasma arginine*. ADI catalyzes the hydrolysis of arginine into citrulline and ammonia (Miyazaki *et al*., 1990; Noh *et al*., 2002). ADI has been reported to have anti-cancer effect by degrading arginine into citrulline (Gong *et al*., 1999; Komada *et al.*, 1997; Beloussow *et al*., 2002). However, citrulline can be converted back to arginine by most of mammalian cells, and therefore it is difficult to achieve a true arginine-deficient state. Also, products produced by ADI are citrulline and ammonia, which is toxic to human.

ADI was recently shown to suppress lipopolysaccharide-induced nitric oxide (NO) synthesis (Park *et al*., 2003). Arginine is the precursor of NO and ADI have the ability to deplete plasma arginine and thus suppress NO production (Thomas *et al*., 2002). A potential inhibitory role of ADI in NO-mediated angiogenesis was reported, which suggests that ADI will suppress angiogenesis by inhibiting tube formation of endothelial cells (Beloussow *et al*., 2002; Dillon *et al*., 2002).

### 1.3.3 Arginine decarboxylase

Arginine decarboxylase (ADC) is the key enzyme in the production pathway of polyamines (Philip *et al*., 2003). The presence of ADC activity has been widely reported in plants, as well as some bacteria, fungi and animals. The activity of arginine decarboxylase in plants has been associated with a range of physiological and developmental conditions, such as salt-stress tolerance, flowering and fruit ripening.

Products of ADC are agmatine and carbon dioxide. Agmatine is not particularly toxic, but when its concentration reaches millimolar level, cell growth is inhibited. However, when ADC undergoes modification with PEG, it almost loses all of its activities (Wheatley and Campbell, 2002).

## 1.4 Production of recombinant human liver arginase

Since arginase can be developed for the treatment of cancers, before people can study the properties and cancer killing ability of arginase, there is a need to isolate and purify the enzyme from the liver extracts. However, researchers reported that it was not easy to isolate pure arginase and the amount of arginase obtained was very low (Bach and Lasnitzki, 1947; Beruter *et al*., 1978). Therefore, recombinant technology was employed for the production of arginase (Ikemoto *et*

*al*., 1990).

Recombinant technology begins with the isolation of a gene of interest. The gene of interest (foreign DNA) is inserted into a plasmid or phage vector, and this is referred to as recombinant DNA.

Cloning is necessary to produce numerous copies of the DNA since the initial supply is inadequate to be inserted into the host cells. After cloning, vectors can be introduced into the desired host cells such as mammalian, yeast, or special bacterial cells. The host cells will synthesize the protein. When the cells are grown in vast quantities, the foreign or recombinant protein can be isolated and purified in large amounts. Insulin, growth hormone, follicle-stimulating hormone, as well as other proteins are now available as recombinant products.

### 1.4.1 Recombinant arginase from *Escherichia coli*

Recombinant human arginase has been successfully produced and purified from *Escherichia coli* (Ikemoto *et al*., 1990). The yield of the *E. coli*-expressed arginase was 10 mg (from 1 g of wet cells), and it was well characterized and its properties were found to be similar to the native arginase.

Using *E. coli* as the host for cloning genes and expressing recombinant

proteins is quite popular, because *E. coli* has been well characterized. However, the major drawbacks of using *E. coli* as the expression host include the presence of endotoxins and the inability of *E. coli* to secrete protein extracellularly.

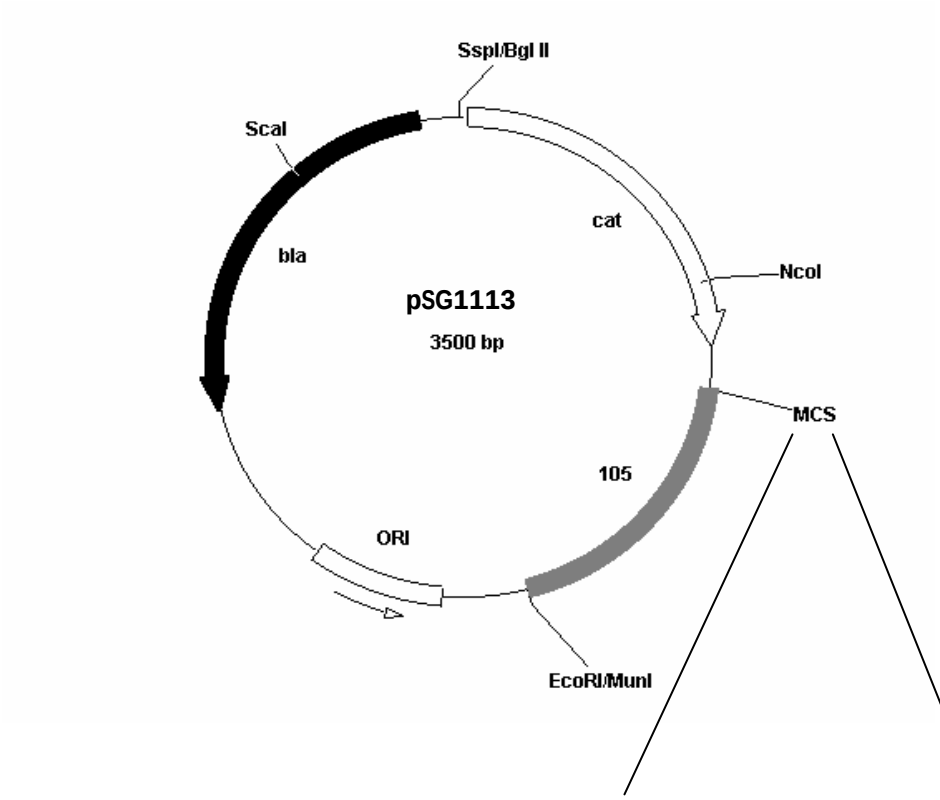
### 1.4.2 Recombinant arginase from *Bacillus subtilis*

In this project, recombinant human liver arginase was produced using the prophage-based *Bacillus subtilis* expression system (Thornewell *et al*., 1993). *B. subtilis* is a gram-positive microorganism, which has been used as a host for the production of recombinant proteins, such as amylases and proteases. *B. subtilis* is non-pathogenic, produces no endotoxins and has been granted the "Generally Regarded as Safe (GRAS)" status in the U.S.

To produce recombinant arginase from *B. subtilis*, expression vector with the human liver arginase gene was first constructed. The human liver arginase gene was isolated from a human cDNA library and its sequence was confirmed by DNA sequencing (Iyer *et al*., 1998). The purified arginase gene was inserted into an integration vector, pSG1113 (Fig. 1.4). Six histidine amino acid residues (6xHis tag) are encoded by the vector sequence. Thus, proteins produced using pSG1113 are tagged with 6xHis at their N-termini. The 6xHis tag usually does not interfere with the structure and the function of the expressed protein as this

has been demonstrated in other recombinant proteins.

The pSG1113 vector carrying the arginase gene has been transformed into the host cells of *B. subtilis*. Finally, the gene was targeted into the expression site in the chromosome of the *B. subtilis* cells. This recombinant *B. subtilis* strain was named LLC101.




**pSG1113**

8 bp HisHis HisHis HisHis

AATGGAGGGATTCATAGATGCATCACCATCACCAT**CATATG**AAAAGCGGGGATCCTCTAG**AGTCGA**CCCT

Fig. 1.5: Plasmid map of pSG1113. The size of the vector is about 3.5 kb, and six histidines are encoded by this vector.

## 1.5 Protein pegylation technology

Recombinant proteins have medical and pharmaceutical utility; after purification, they are administered for various therapeutic indications. However, parentally administered proteins may be immunogenic, and have short circulation half-life. Consequently, it is difficult to achieve therapeutically useful blood levels of the protein in patients.

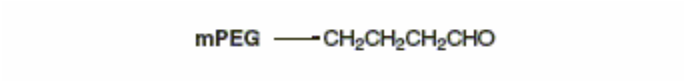
To overcome these problems, attachment of proteins to polymers such as polyethylene glycol (PEG) has been used (Harris *et al*., 2001). Pegylation is a technique that enhances the therapeutic and biotechnological potential of proteins (Roberts *et al*., 2002; Harris and Chess, 2003). Attachment of PEG molecules will modify the enzymatic activity or receptor recognition of the proteins. PEG conjugation masks the protein's surface and increases molecular size, thus reducing its renal ultrafiltration, preventing the approach of antibodies or antigen processing cells and reducing the degradation by proteolytic enzymes (Roberts and Harris, 1998). Examples of protein pegylation include mPEG-IFN-A (Wang *et al*., 2002) and mPEG-IL-2 (Goodson and Katre, 1990).

Pegylation of bovine liver arginase was carried out by Savoca *et al*., (1979; 1984). Methoxypolyethylene glycol (mPEG; MW=5,000) was attached covalently to arginase using 2,4,6-trichloro-s-triazine as the coupling agent. The

pegylated bovine arginase had PEG attached to 53% of the amino groups and retained 65% of its original enzymatic activity. The blood-circulating life of the pegylated bovine arginase was 12 h (half-life of the native arginase was less than 1 h) and the bovine arginase did not react with antisera raised with the native arginase (Savoca *et al*., 1984).

Since the recombinant human arginase in this project is developed for therapeutic use, the short circulating half-life of the native arginase may result in the use of high drug dosage as well as immunogenicity. Therefore, the recombinant human arginase was modified with PEG molecules in this project . There was an undesirable loss in enzyme activity of the bovine arginase after pegylation. To screen for the best PEG molecules, different PEG molecules were used for the pegylation of the recombinant human arginase, such as, mPEG-SPA (methoxypolyethylene glycol-succinimidyl propionate, MW= 5,000), mPEG-NHS (mPEG-N- hydroxysuccinimide, MW= 10,000), mPEG-MAL (methoxypolyethylene-maleimide, MW= 5,000), and mPEG-ALD (mPEG-propionaldehyde, MW= 5,000).

(A)

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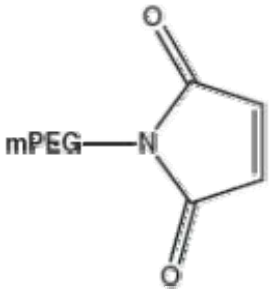
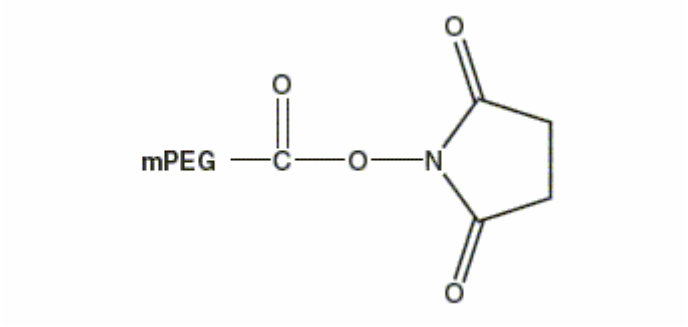



(C)

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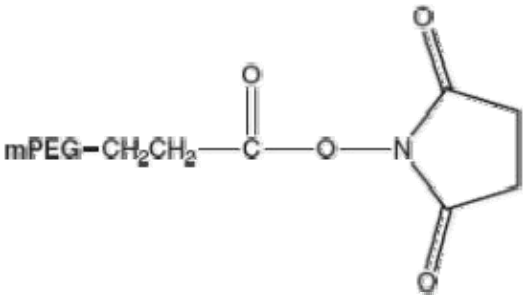



Fig. 1.6: Structures of PEG molecules with different derivatives.

(A) mPEG-ALD (methoxypolyethylene-propionaldehyde);

(B) mPEG-MAL (methoxypolyethylene-maleimide) ;

(C) mPEG-NHS (methoxypolyethylene-N-hydroxysuccinimide);

(D) mPEG-SPA (methoxypolyethylene glycol-succinimidyl propionate).

# Chapter Two:
# Methodology

## 2.1 Small scale production of arginase

### 2.1.1 Overexpression of arginase

*B. subtilis* (LLC101) harboring the expression vector was grown overnight (~12 h) at 37$^{o}$C with shaking (300 rpm) in 100 ml of BHY medium (3.7 % brain heart infusion and 0.625 % yeast extract) containing 5 μg/ml chloramphenicol in a 1-L flask. The overnight culture (6 ml) was added to 100 ml fresh BHY medium in a 1-L baffled flask and the bacteria were cultured at 37$^{o}$C with 300 rpm shaking. To introduce expression of arginase, heat shock (50 $^{o}$C, 3 min) was applied when the $OD_{600}$ was 3.5-4.5. After heat shock, the bacteria were further cultured at 37 $^{o}$C with 300 rpm shaking for 6 h before harvesting.

Cell pellet (collected from 1,600 ml culture) was resuspended in 100 ml solubilization buffer [50 mM Tris-HCl (pH 8.0), 0.1 M NaCl and 0.5 m $MnCl_2$] containing 75 μg/ml lysozyme. The resuspended cell culture was sonicated using the Soniprep 150 (MSE) apparatus and was then digested with about 1,000 units of deoxyribonuclease I (Sigma D4527) at 37°C for 10 min. The resuspended cell culture was centrifuged at 10,000 rpm for 20 min at 4°C. Supernatant was collected after centrifugation, the crude proteins of the cell culture present in the suspernatant was ready for purification.

### 2.1.1.1 Optimization of pH of BHY fermentation medium

*B. subtilis* (LLC101) harboring the expression vector was grown overnight (~12 h) at 37$^{o}$C with shaking (300 rpm) in 100 ml of BHY medium (3.7 % brain heart infusion and 0.625 % yeast extract) containing 5 μg/ml chloramphenicol in a 1-L flask. The overnight culture (6 ml) was added to 100 ml BHY medium with different pH conditions (pH 5, 6, 7, 8 and 9). The bacteria were cultured at 37$^{o}$C with 300 rpm shaking. Heat shock (50$^{o}$C, 3 min) was applied when the $OD_{600}$ was 3.5-4.5 and the bacteria were further cultured at 37$^{o}$C with 300 rpm shaking for 6 h. The growth of the bacteria in different pH media was compared by measuring the absorbance at $OD_{600}$.

### 2.1.1.2 Effect of the compositions of fermentation media

*B. subtilis* (LLC101) harboring the expression vector was grown overnight (~12 h) at 37$^{o}$C with shaking (300 rpm) either in 100 ml of BHY medium with 5 μg/ml chloramphenicol or in glucose medium (glucose 5 g/L, tryptone 10 g/L, yeast extract 3 g/L, citric acid 1 g/L, $KH_2PO_4$ 1.5 g/L, $K_2HPO_4$ 1.5 g/L, $(NH_4)_2SO_4$ 3 g/L). The overnight culture (6 ml) was added to 100 ml BHY medium and glucose medium, respectively. The bacteria were cultured at 37$^{o}$C with 300 rpm shaking. Heat shock (50 $^{o}$C, 3 min) was applied when the $OD_{600}$ was 3.5-4.5 and

the bacteria were further cultured at 37$^{o}$C with 300 rpm shaking for 6 h**.** The growth of the bacteria in different media was compared by measuring the absorbance at $OD_{600}$.

### 2.1.2 Purification of arginase from shake-flask fermentation

The purification of arginase from *B. subtilis* involved four steps: (1) Affinity chromatography (Hitrap chelating column); (2) Desalting chromatography ;(3) Ion-exchange chromatography; (4) Desalting chromatography. All chromatographic steps were done using $AKTA_{FPLC}$ with UNICORN 3.0 control (Green *et al*., 1990; Jenkinson *et al*., 1994).

#### 2.1.2.1 Affinity chromatography

Crude proteins were first purified using the HiTrap chelating chromatography (5 ml column). The crude sample was filtered before loading onto the column. The chelating column was charged with nickel ions and pre-equilibrated with 0.1 M $NiCl_2$ and then with the Start buffer A [0.2 M sodium phosphate buffer, 0.5 M NaCl (pH 7.4)] with flow rate of 5 ml/min.

After sample loading, the column was washed with Start buffer A to remove the nonspecific binding proteins. Arginase was eluted with 75 ml of 0.5 M

imidazole in Start buffer A (pH 7.4) in linear gradient. Peak fractions were collected and analyzed by the Bradford assay and SDS-PAGE. The activity of arginase was assayed based on the $A_{340}$ activity assay. Fractions containing arginase were pooled for subsequent purification.

#### 2.1.2.2 Desalting chromatography

The salt in the sample was removed by a HiPrep 26/10 desalting column (53 ml). Sample should be filtered before loading onto the column, which was previously equilibrated with 10 mM Tris-HCl (pH 7.5) with a flow rate of 10 ml/min. The protein was eluted by 80 ml (1.5 bed volume) of desalting buffer. Fractions containing proteins were pooled.

#### 2.1.2.3 Ion exchange chromatography

Since the fractions eluted from the chelating column still contained impurities, ion-exchange column (SP Sepharose Fast Flow ion exchange chromatography column) was performed for further purification. The column was pre-equilibrated with 10 mM Tris-HCl, pH 7.5. Arginase was eluted with 1 M NaCl in linear gradient. Peak fractions were collected and analyzed by the Bradford assay and SDS-PAGE. The activity of arginase was assayed based on

the $A_{340}$ activity assay. Fractions containing arginase were pooled for subsequent procedures.

#### 2.1.2.4 Desalting chromatography

The final step of arginase purification was to remove salt by a HiPrep 26/10 desalting column (53 ml). The column was pre-equilibrated with PBS buffer with a rate of 10 ml/min. Protein was eluted with 80 ml (1.5 bed volume) of PBS buffer and fractions containing arginase were pooled.

### 2.1.3 SDS-PAGE analysis

Proteins were separated according to their molecular weights by SDS-PAGE in a Mini-protein II/ III dual slab cell (bio-Rad Laboratories). The subunit molecular weight of a protein was determined under reducing condition. The sample was mixed with 10 μl loading buffer which contained the reducing agent β-mercaptoethanol and SDS. A standard low-range protein marker (Bio-Rad) was used. Each SDS-PAGE gel consisted of 12% separating gel (pH 8.8) and 12% stacking gel (pH 6.8) and was subjected to electrophoresis in 1x running buffer at 200 volts for 45-60 min. The gel was stained in Coomassie blue stain solution for 1-2 h with agitation to stain the protein bands. Then the gel was incubated with

destain solution with shaking; the solution were changed every 4-5 h until the background of gel become clear. The gel was air-dried by using the gel drying film (Promega).

### 2.1.4 Protein assay

Protein concentration was determined by the Bradford assay (Zor *et al.*, 1996). Using a micro-protein assay, 800 μl of the diluted samples were incubated with 200 μl Bradford Reagent Dye (Bio-Rad) for 10 min at room temperature. The protein concentration was measured spectrophotometrically at 595 nm against a standard protein curve. The bovine serum albumin (sigma) was used as a standard protein and the curve was a straight line in the range 0-20 μg/ml.

### 2.1.5 Activity assay

Two methods were used to determine the activity of arginase. They are the $A_{340}$ assay and the assay using amino acid analyzer. One unit (I.U.) of arginase is defined as the amount of enzyme that can produce 1 μmol of urea per min at 30$^{o}$C, pH 8.5.

### 2.1.5.1 Activity assay using a spectrophotometer

A coupled spectrophotometric assay was used to determine arginase activity (Ikemoto *et al*., 1989; Ozer *et al*., 1985). The system coupled arginase to glutamate dehydrogenase as shown in the following reactions:

$$\text{arginine} + H_2O \xrightarrow{\text{arginase}} \text{ornithine} + \text{urea}$$

$$\text{urea} + 2\,H_2O + H^+ \xrightarrow{\text{urease}} HCO_3^- + 2\,NH_4^+$$

$$NH_4^+ + \alpha\text{-ketoglutarate} + \text{NADPH} \xrightarrow{\text{glutamate dehydrogenase}} \text{glutamate} + NADP^+ + H_2O$$

Activity of arginase was determined by following the decrease in absorbance at 340 nm ($A_{340}$) because the amount of urea produced from arginase's reaction finally catalyzed the NADPH depletion.

0.1 M Tris-HCl (pH 8.5), 50 mM L-arginine, 5 mM α-ketoglutarate, 0.25 mM NADPH, 35 units of urease and 15 units of glutamate dehydrogenase were used in a 1 ml $A_{340}$ assay mixture. The enzyme reaction was initiated by addition of arginase and carried out at 30°C. A linear decrease in $A_{340}$ was observed. Activity of arginase was calculated by measuring the slope of decrease in $A_{340}$

Activity of arginase was calculated using the following equation:

$\Delta C/\Delta t = (\Delta A/\Delta t) \times (1/\varepsilon) \times 10^6 \times (1/2)$ I.U./ml (for 1 ml reaction volume)

$= (\Delta A/\Delta t) \times (1/6220) \times 10^6 \times (1/2)$ I.U./ml

**Specific activity (I.U./ mg)**

= [($\Delta A/ \Delta t$) x (1/6220) x $10^6$ x (1/2) I.U./ ml] / arginase concentration for reaction (mg/ml)

= [(slope) x (1/6220) x $10^6$ x (1/2) I.U./ ml] / arginase concentration for reaction (mg/ml)

$\Delta C$ = change of NADPH concentration

$\Delta A$ = change of absorbance at 340 nm

$\varepsilon$ = extinction coefficient for NADPH = 6,220 $M^{-1}cm^{-1}$

### 2.1.5.2 Activity assay using an amino acid analyzer

Arginase (1 mg/ml) was dissolved in PBS buffer (pH 7.4) with 5 mM $MnCl_2$. $4H_2O$. Sample was then incubated for 30 min at 37$^oC$. Arginine (1 mM) was added and the enzyme solution was incubated for 10 min. After 10 min, same volume of 10% trichloroacetic acid was added to stop the enzymatic reaction. Sample was then spun down to remove protein (10,000 rpm, 10 min), supernatant was filtered with a 0.45 μm spring filter and was then analyzed using the amino acid analyzer (Model L-8800, Hitachi).

The activity of arginase was calculated using the following equation:

$$\text{Activity} = \frac{\text{In}\{\frac{C_{arg(i)} + [C_{arg(f)}+C_{orn(f)}]-[C_{arg(i)}+C_{orn(i)}]}{C_{arg(f)}}\}}{\text{Reaction time (min)}}$$

Where $C_{arg(i)}$ = initial concentration of arginine (mM)

$C_{arg(f)}$ = final concentration of arginine (mM)

$C_{orn(i)}$ = initial concentration of ornithine (mM)

$C_{orn(f)}$ = final concentration of ornithine (mM)

## 2.2 Production of arginase from fermentation of 8 L of culture

### 2.2.1 Fed-batch fermentation

The fed-batch fermentation was carried out by Mr. Law Kin Ho. *B. subtilis* was incubated in nine 1-L baffled flasks with 100 ml basic fermentation medium (5 g glucose, 10 g typtone, 3 g yeast extract, 1 g citric acid, 1.5 g $KH_2PO_4$, 1.5 g $K_2HPO_4$ and 3 g $(NH_4)_2SO_4$ in 1 L medium) with chloramphenicol (5 μg/ml) and pH 7.0 on an orbital shaker rotating at 280 rpm and 37°C. The cultivation was terminated when $A_{600}$ reached 5 – 6 at about 9 – 11 h growth time. Then the 900 ml culture was introduced into 15-L fermenter containing 8 L basic fermentation medium, pH 7.0.

Fed-batch fermentation was carried out at 37$^{o}$C, pH 7. A 20% dissolved oxygen content was maintained during fermentation. The medium feeding rate was controlled with the pH-stat control strategy. In this strategy, if the pH = 7.1, 4.5 g (0.5 g/L) glucose in Fed Medium was introduced into the fermenter. After the addition of glucose, the pH value would decrease below 7.1 rapidly. And when the value of pH increase back to 7.1, another 4.5 g glucose was added again.

Heat shock was applied to *B. subtilis* culture when $A_{600}$ reached 12-13. The culture was heated from 37°C to 50°C and immediately cooled to 37°C. The

culture was harvested at 4.5 h after heat shock. The cells were centrifuged at about 12, 000 x g and stored at -80°C.

The cells were then resuspended in ~ 1,250 ml solubilization buffer and 10 ml $MnCl_2$ stock (0.12 g/ml) was added (final $MnCl_2$ conc. = 5 mM). Lysozyme digestion was done by 75 μg/ml lysozyme (10 mg/ml) at 30$^{o}$C for 20 min. Sonication was then carried out with the use of Soniprep 150 (MSE) Apparatus. The crude sample was divided into four portions, each portion of sample was sonicated for 2 min (10 sec each time). The ample was digested with 5,000 units of DNase I for 15 min at 37$^{o}$C. After DNase I digestion, the protein was centrifuged at 9, 000 rpm and 4$^{o}$C for 30 min. The supernatant was centrifuged again at 9, 000 rpm and 4$^{o}$C for 30 min and then filtered for purification.

### 2.2.2 Purification of arginase from 8 L of culture

For purification of arginase from 8 L of culture, two steps were involved: Ni-NTA affinity chromatography and desalting chromatography. All chromatographic steps were done using the AKTA Purifier with UNICORN 4.0 control.

#### 2.2.2.1 Ni-NTA affinity chromatography

For purification of arginase from 8-L fermentation culture, the Ni-NTA affinity chromatography (130 ml) was used. The column was pre-equilibrated with 0.1 M $NiCl_2$ and then with the Start buffer A [0.2 M sodium phosphate buffer, 0.5 M NaCl (pH 7.4)] at a flow rate of 5 ml/min.

The crude protein sample was filtered before loading onto the column. After sample loading, the column was washed with Start buffer A to remove the nonspecifically bound proteins. Arginase was eluted with 75 ml of 0.5 M imidazole in Start buffer A (pH 7.4) using a linear gradient. Peak fractions were collected and analyzed by the bradford assay and SDS-PAGE. The activity of arginase was assayed based on the $A_{340}$ activity assay. Fractions containing arginase were pooled for subsequent purification.

#### 2.2.2.2 Desalting

Tangential flow filtration system (TFF) was used for the desalting of arginase samples (Millipore). Membrane PXB00850 (Millipore) with nominal cutoff of 8 kDa was used for the desalting of our protein. TFF system was first flushed with 500 ml MilliQ water and the normalized water permeability of the membrane was checked. The system was then pre-equilibrated with PBS buffer

(pH 7.4). The feed pressure and the retentate pressure for the TFF system were adjusted to about 30 psi and 10 psi, respectively, and the rate of permeate collected was about 3 ml/min. The dilution factor of the arginase sample was adjusted to about 500 for removal of the salts.

## 2.3 Pegylation of purified arginase

Purified arginase was pegylated with mPEG-SPA (Methoxypolyethylene Glycol Succinimidyl Propionate, MW 5000) to generate arginase-SPA-$PEG_{5000}$. One hundred arginase was dissolved in 10 ml PBS buffer (pH 7.4). A 70 mg portion of activated mPEG-SPA (in 1: 50 mole ratio) was added to the solution, and was stirred gently. Pegylation was allowed to take place in room temperature. The stirring condition was maintained at pH 7.4 for 2.5 h. Free PEG molecules were removed by extensive dialysis against 20 L PBS buffer with the use of FX 50s High–Flus Dialyzer (Fresenius Polysulfone).

The effect of arginase pegylation was monitored with 12% SDS-PAGE, and protein concentration was determined by the Bradford assay using a bovine serum albumin standard. The enzymatic activity of arginase-SPA-$PEG_{5000}$ was also measured. The dialyzed arginase-SPA-$PEG_{5000}$ was stored at 4°C.

### 2.3.1 Pegylation of arginase with various PEG molecules

Argianse was pegylated with five different PEG molecules; they are: mPEG-N-hydroxysuccinimide, MW=10,000 (mPEG-NHS); mPEG-propionaldehyde, MW= 5,000 (mPEG-ALD); mPEG-maleimide MW= 5,000 (mPEG-MAL); mPEG-succinimidyl propionate MW= 5,000 **(**mPEG-SPA) and cyanuric chloride

activated PEG (mPEG-CN).

Each type of PEG molecules was added to arginase in mole ratio of 1:20 and 1:50. Samples were then stirred for 3 h to allow reaction between the PEG molecules and the arginase. The effect of arginase pegylation was monitored with 12% SDS-PAGE, and protein concentration was determined by the Bradford assay using a bovine serum albumin standard. The enzymatic activity of arginase-SPA-$PEG_{5000}$ was also measured.

### 2.3.2 Pegylation of arginase with mPEG-SPA in different mole ratios

Arginase was pegylated with mPEG-SPA, which was added to arginase in different mole ratios (1:10, 1:20, 1:30, 1:40, 1:50, 1:100, 1:200 and 1:500). Samples were then stirred for 3 h to allow the attachment of PEG molecules to arginase. The effect of arginase pegylation was monitored with 12% SDS-PAGE, and protein concentration was determined by the Bradford assay using a bovine serum albumin standard. The enzymatic activity of arginase-SPA-$PEG_{5000}$ was also measured.

## 2.4 Characterization of arginase

### 2.4.1 Enzyme kinetic analysis

To obtain kinetic data, the formation of ornithine from arginine catalyzed by arginase was measured by following the change in absorbance at 340 nm, based on the $A_{340}$ assay method (Ikemoto *et al*., 1990). At 340 nm, the absorption at pH 8.5 was measured by varying the concentration of arginine in the range 0 – 50 mM (0.25, 0.5, 0.75, 1, 2, 4, 8, 14, 20 and 50 mM). The change in absorbance at 340 nm ($A_{340}$) was measured for 5 – 10 min in a double-beam spectrophotometer (Perkin Elmer Lambda Bio20), set at 0.1 absorbance full scale. All assays were performed in duplicate with the $A_{340}$ assay mixture (0.1 M Tris-HCl (pH 8.5), 50 mM L-arginine, 5 mM α-ketoglutarate, 0.25 mM NADPH, 35 units of urease and 15 units of glutamate dehydrogenase) at 30$^{o}$C. The initial velocity data were fitted by the nonlinear regression plots using the program of Stanislawski.

### 2.4.2 Determination of the optimum $Mn^{2+}$ concentration

The optimum $Mn^{2+}$ concentrations for the native arginase and arginase-SPA-$PEG_{5000}$ were investigated based on the $A_{340}$ assay method. Activity assays of arginase were performed with the $A_{340}$ assay mixture in addition of different $Mn^{2+}$ concentrations (from 0- 2 mM). The effect of the $Mn^{2+}$

concentration on arginase activities was observed by following the change in absorbance at 340 nm. All assays were performed with the double-beam spectrophotometer (Perkin Elmer Lambda Bio20).

### 2.4.3 Determination of the optimum temperature and pH conditions

The optimum temperature and pH conditions for arginase and arginase-SPA-$PEG_{5000}$ were investigated based on the assay method using amino acid analyzer (Kuhn *et al*., 1991; Kuhn *et al*., 1995).

#### 2.4.3.1 Determination of the optimum pH condition

Arginase-SPA-$PEG_{5000}$ (1 mg/ml) was dissolved in PBS buffer with different pH values. For each sample, 1 mM arginine was added. The samples were then incubated at 37$^{o}$C for 10 min. After 10 min, equal volume of 10% trichloroacetic acid was added. Samples were then spun down at 10,000 rpm for 10 min, supernatant was collected and filtered with a 0.45 μm spring filter. The samples were analyzed with the amino acid analyzer for concentration of arginine and ornithine.

#### 2.4.3.2 Determination of the optimum temperature

Arginase and arginase-SPA-$PEG_{5000}$ (7 mg) were dissolved in PBS buffer (7 ml). The dissolved arginase sample was then aliquoted into 7 tubes, 1 ml sample per tube.

For each sample, 1 mM arginine was added. The samples were then incubated at different temperatures ($0^oC$, $20^oC$, $37^oC$, $50^oC$, $60^oC$, $70^oC$ and $80^oC$) for 10 min. After 10 min, equal volume of 10% trichloroacetic acid was added. Samples were then spun down at 10,000 rpm for 10 min; supernatant was collected and filtered with a 0.45 μm spring filter. The samples were analyzed with the amino acid analyzer for measuring the concentrations of arginine and ornithine.

### 2.4.4 Determination of pI value

Acrylamide solution (T=25%, C=3%) and ampholyte (Pharmacia Biotech, 3/10 pharmalyte) were used to prepare the monomer-ampholyte solution for isoelectric focusing (IEF) gel. The monomer-ampholyte solution was then cast on the gel support film. The IEF gel was irradiated with fluorescent lamp to allow photopolymerization. Sample template was then placed on top of the polymerized gel. The isoelectric marker (pI) marker and arginase were applied

onto the wells of the template. When all the samples were ready, the template was removed from the gel.

Model 111 Mini IEF Cell (Bio-Rad) was used to run the IEF gel. The gel was first focused at 100 V for 15 min. Voltage was then increased to 200 V for 15 min, and finally increased to 450 V for an additional 75 min. Focusing was completed when the current was decreased.

After electrofocusing, proteins were detected by fixing and staining. Coomassie Blue G-250 was used for protein staining. The gel was immersed in the straining solution for 1 h, and was intensified in 7% (v/v) acetic acid until the background of the gel was clear. The gel was finally dried overnight at room temperature.

### 2.4.5 Gel filtration chromatography

Gel filtration chromatography was done using the HiLoad 16/60 Superdex gel filtration column (Amersham Bioscience). Both low and high molecular weight gel filtration kits (Amersham Biosciences) were used as molecular weight standards (Standard proteins were purchased from Amersham Biosciences: Thyroglobulin 669 kDa, Ferritin 440 kDa, Catalase 232 kDa, Aldolase 158 kDa, Ribonuclease A 13.7 kDa, Chymotrypsinogen A 25 kDa, Ovalbumin 43 kDa and

Albumin 67 kDa). The standard proteins were dissolved in the PBS buffer (2.5 mg/ ml). Elution time of each standard protein was recorded and used in column calibration. Calibration curve was then generated from these standard proteins.

The native arginase (5.7 mg/ml) and arginase-SPA-$PEG_{5000}$ (5.4 mg/ml) were dissolved in PBS buffer in concentration at about 2.5 mg/ml to 5 mg/ml. For each protein, 2 ml sample was subjected to gel filtration chromatography. Elution time and volume of the native and the pegylated arginase were recorded (Kanda *et al*., 1992).

### 2.4.6 Circular dichroism (CD) spectroscopic analysis

For CD experiment, the arginase concentration was adjusted to 0.3 mg/ml and dissolved in 10 mM potassium phosphate at pH 7.0. The CD measurements were carried out on a Jasco J-810 spectropolarimeter at 25 $^{o}$C using cuvettes of 1 mm path length. Each CD spectrum represented the average of two scans. Spectra were acquired over the far-UV region 250-190 nm under constant nitrogen flush. The instrument was regularly calibrated with a 1 mg/ml solution of 10-camphorsulfonic acid. The CD signals were expressed in terms of mdeg.

## 2. 5 Pharmacological studies of arginase-SPA-PEG$_{5000}$

### 2.5.1 *In vitro* half-life studies of arginase-SPA-PEG$_{5000}$

Arginase-SPA-PEG$_{5000}$ (1 mg/ml) was incubated in human plasma at 37$^{o}$C for 17 days. The activities of the arginase-SPA-PEG$_{5000}$ were assayed each day during incubation using the $A_{340}$ activity assay. Time required for the decrease of activity to half of the original activity was used for the determination of the *in vitro* half-life of arginase-SPA-PEG$_{5000}$.

### 2.5.2 *In vivo* arginine depletion by the pegylated arginase in rats

The in vivo study was performed by Miss W. M. Lam. Four groups of rats (one male and one female in each group) were given dosages of various amounts of arginase-SPA-PEG$_{5000}$ (500 I.U., 1,000 I.U., 1,500 I.U. and 3,000 I.U.). At day 0, arginase-SPA-PEG$_{5000}$ was administrated to each group of rats. Blood samples were drawn from their tail veins before and after administration, from day 0 to day 6. Blood samples collected were then analyzed using the amino acid analyzer for measuring the plasma arginine and ornithine levels.

# Chapter Three:
# Results

## 3.1 Overexpression of arginase in *Bacillus subtilis*

### 3.1.1 Shake-flask fermentation

The *B. subtilis* strain LLC101 was constructed as described in Section 1.4. Shake-flask fermentation was carried out to investigate the expression of arginase. The *B. subtilis* strain without the human liver arginase gene was used as a negative control in this experiment. The growth of these two strains is shown in Fig. 3.1. The crude proteins isolated from *B. subtilis* strain LLC101 were analyzed by SDS-PAGE. The results are shown in Fig. 3.2. The molecular weight of the subunit of arginase expressed was about 35 kDa.

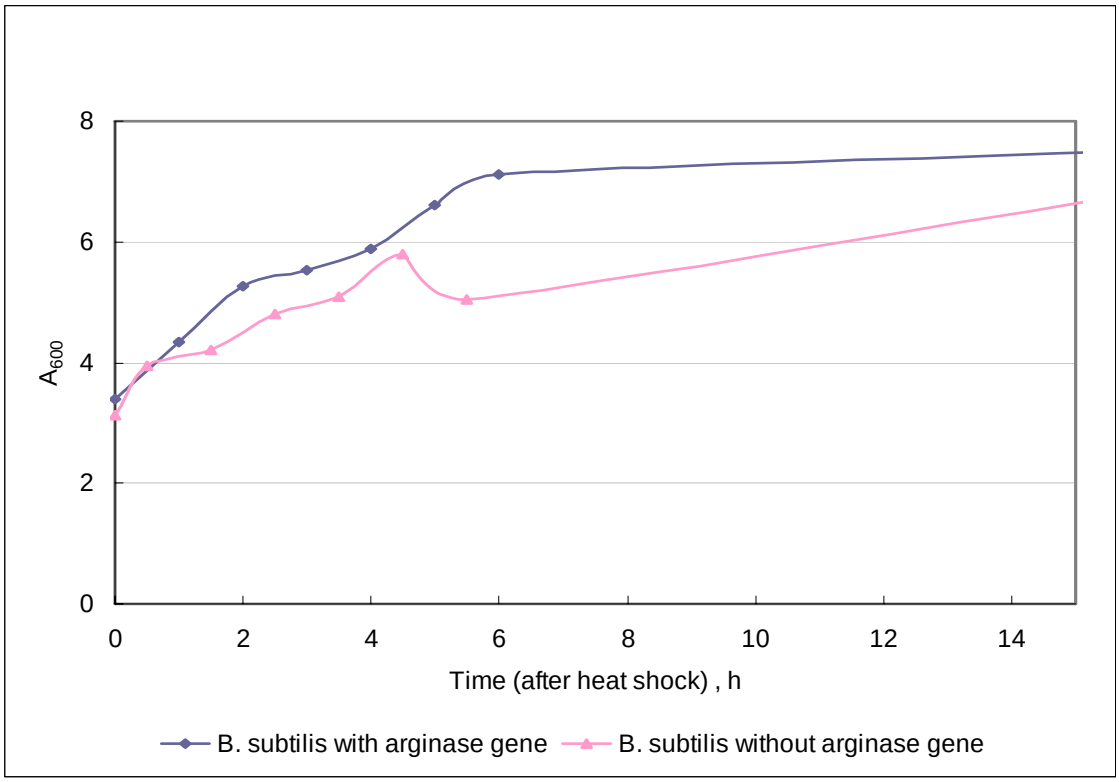



Fig. 3.1: Growth of the *B. subtilis* strain LLC101 in shake-flask. *B. subtilis* without the arginase gene was used as a negative control. The bacterial growth was monitored by measuring the absorbance at 600 nm of wavelength ($A_{600}$).

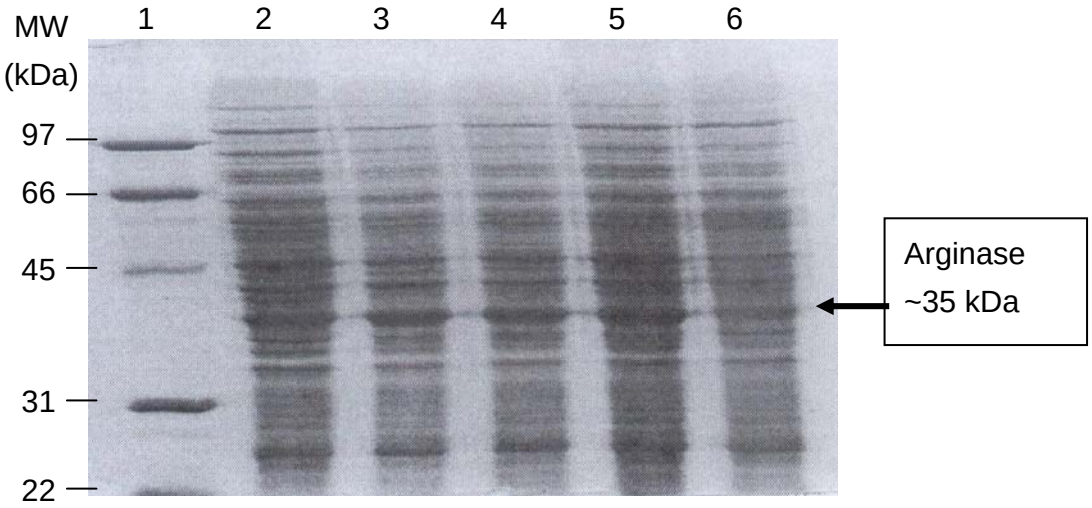



Fig. 3.2: 12% SDS-PAGE analysis showing total proteins extracted from the *B. subtilis* strain LLC101. Protein extracts were obtained at different time points during fermentation. The molecular size of arginase was about 35 kDa. Lane 1: low range molecular weight marker (1 µg per band); Lanes 2-6: contain 2 µl of crude extract obtained at 0 h, 2 h, 4 h, 6 h and 14 h after heat shock, respectively.

#### 3.1.1.1 Optimization of pH of the BHY culture medium

The optimum pH value of the culture medium used for the growth of *B. subtilis* and arginase expression was investigated. BHY medium was prepared at different pH values (pH 5-9). The bacteria were cultivated at different pH and the results are shown in Fig. 3.3.

The results showed that the growth of the bacteria was greatly inhibited when the pH value was low (pH 5). However, the growth of the bacteria increased significantly when the pH value of the medium was increased to 6. Further increase of the pH values of the medium from 6 up to 9 did not further increase the growth of the bacterial cells.

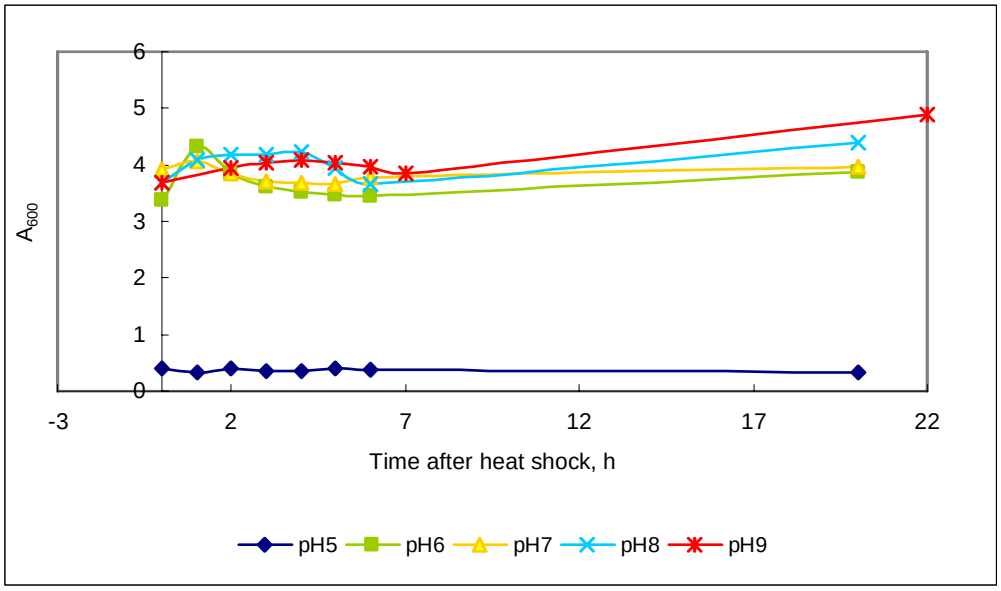



Fig. 3.3: Growth of the *B. subtilis* strain LLC101 in BHY medium of different pH values. The growth of bacteria was monitored by measuring the absorbance at 600 nm wavelength ($A_{600}$).

### 3.1.1.2 Effect of the compositions of fermentation media

Fermentation media with different compositions were used for the growth of *B. subtilis.* The BHY medium (3.7 % brain heart infusion and 0.5 % yeast extract) and the glucose medium (0.5% glucose) were used in this experiment. The growth of bacteria in these two media is compared in Fig. 3.4.

The results showed that the richer BHY medium, which contained 3.7% brain heart infusion, could achieve a higher final bacterial cell concentration than the 0.5 % glucose medium.

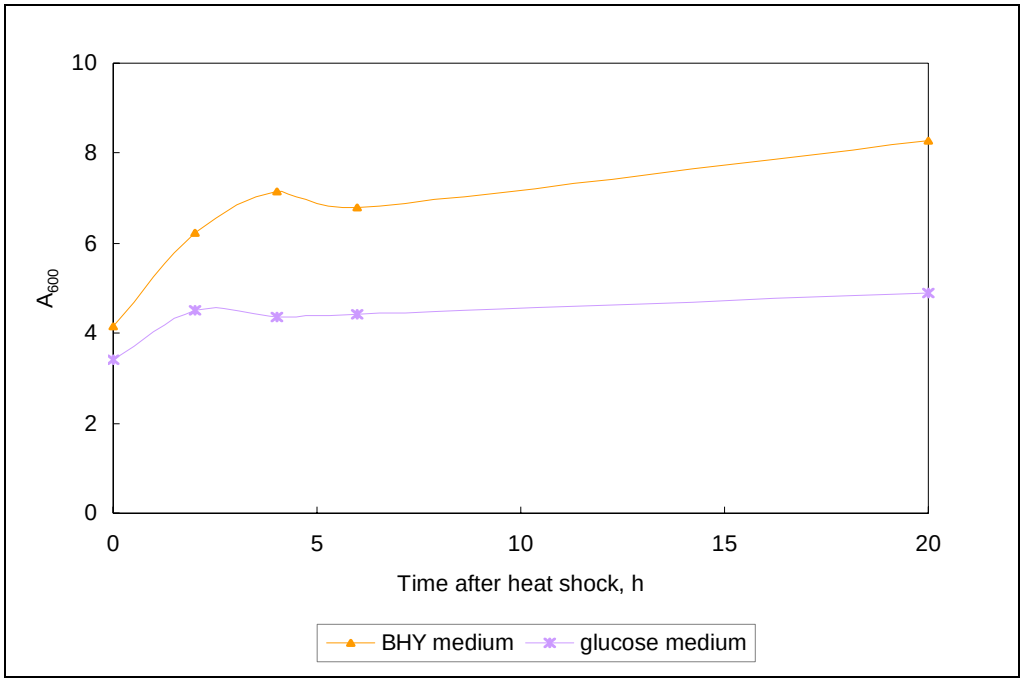



Fig. 3.4: Growth of *B. subtilis* in the BHY medium and the glucose medium. The growth of bacteria was monitored by measuring the absorbance at 600 nm wavelength ($A_{600}$).

### 3.1.2 Purification of arginase from shake-flask fermentation

The protocol for purifying arginase was developed in this experiment. Four steps were required to purify the arginase enzyme: (1) affinity chromatography; (2) desalting step; (3) ion-exchange chromatography; (4) desalting step.

#### 3.1.2.1 Affinity chromatography

The recombinant arginase produced in *B. subtilis* strain LLC101 was tagged with 6 histidines at the N-terminus. HiTrap chelating affinity chromatography was used in the first step of protein purification. 6xHis-arginase was bound to the nickel ions in the column to allow purification of the arginase. After that, arginase was eluted from the column by using imidazole, which would dissociate the binding between nickel ions and 6xHis-arginase.

Fig. 3.5 shows the chromatogram of HiTrap chelating chromatography. Fractions containing arginase were pooled together for further purification. Purities and concentrations of the recombinant arginase eluted from the HiTrap chelating column were analyzed by SDS-PAGE and the Bradford assay, respectively. Fig. 3.6 shows the results of the 12% SDS-PAGE analysis of different fractions eluted from the HiTrap chelating column.

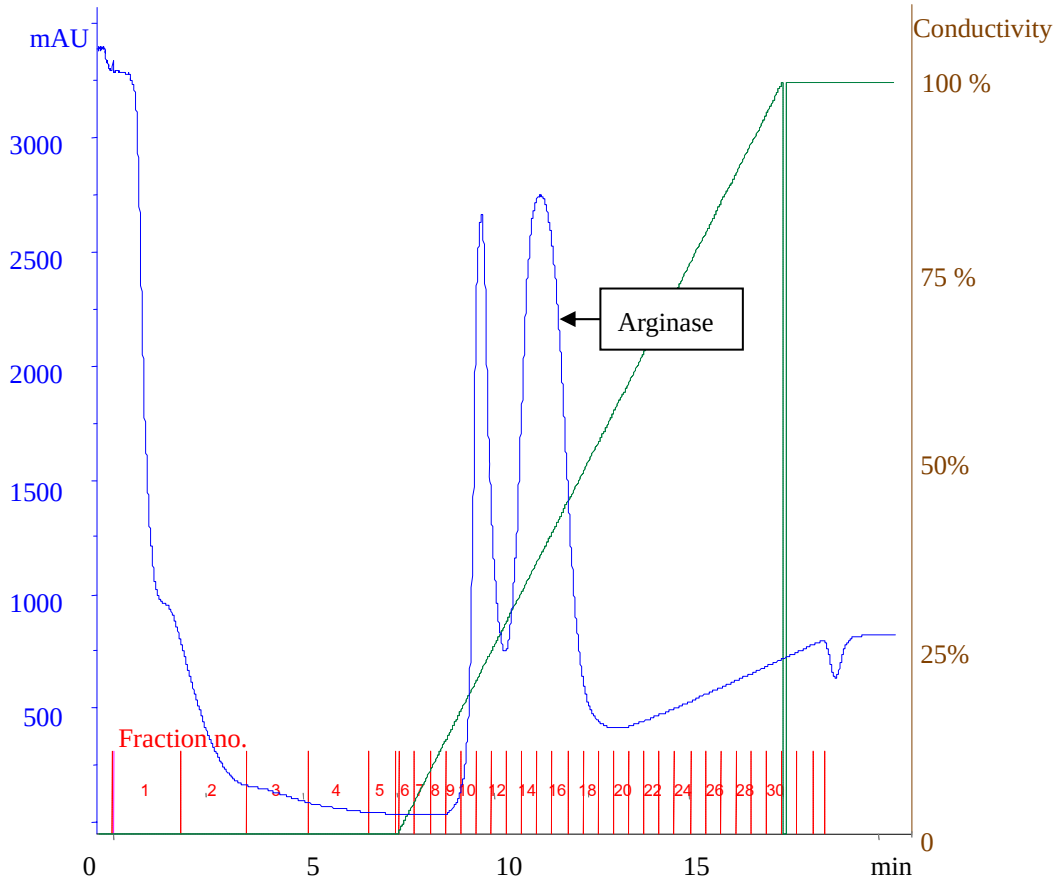



Fig. 3.5: Elution profile of arginase from the HiTrap chelating chromatography column. The protein concentration of the sample was measured by absorbance at 280 nm, while the salt concentration was determined by measuring the conductivity. All the measurements were performed using the Pharmacia AKTA $_{FPLC}$. mAU, milli absorption units. The elution concentration was detected by the conductivity flow cell, and it was represented by conductivity, i.e. 100%=0.5 M imidazole.

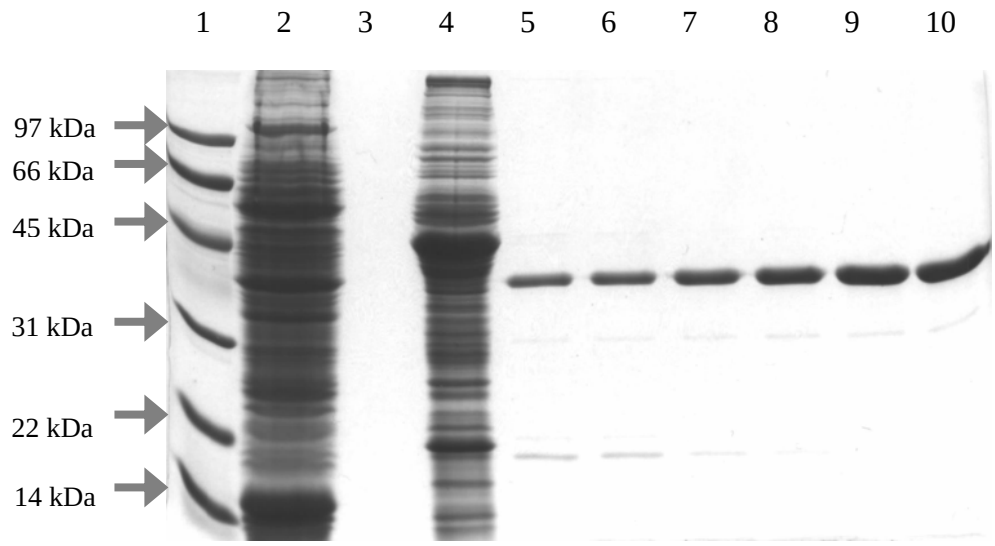



Fig. 3.6: 12% SDS-PAGE analysis showing fractions eluted from the HiTrap chelating chromatography column. The molecular size of arginase was about 35 kDa. Lane 1: low range molecular weight marker (1 µg per band); Lane 2: crude protein extract (2 µl); Lane 3: empty; Lane 4: flowthrough (2 µl); Lane 5: fraction 14 (5 µl); Lane 6: fraction 15 (5 µl); Lane 7: fraction 16 (5 µl); Lane 8: fraction 17 (5 µl); Lane 9: fraction 18 (5 µl); Lane 10: fraction 19 (5 µl).

#### 3.1.2.2 Desalting

After HiTrap chelating chromatography, it was necessary to remove the salts and exchange buffer solution prior to the subsequent purification step. The salts in the protein samples were removed by means of a desalting column (HiPrep 26/10, 53 ml). The pooled fractions were exchanged from 0.2 M sodium phosphate buffer (pH 7.4) to 10 mM Tris-HCl buffer (pH 7.5) during the desalting step. Arginase was eluted first from the desalting column followed by the salts. Fractions containing proteins were collected, pooled together and applied to the ion-exchange chromatography.

### 3.1.2.3 Ion exchange chromatography

Some weak minor bands were observed in the SDS gel (Fig. 3.6), which indicated that impurities were still present in the protein sample. Since the purity of arginase collected after the affinity column was not high enough, the third stage of purification was preformed with the ion exchange SP sepharose fast flow chromatography column using the pharmacia AKTA system. The SP sepharose column was pre-equilibrated with 10 mM Tris-HCl (pH 7.5) and arginase was eluted with 0.3 M NaCl. The elution profile of the SP sepharose chromatography column is shown in Fig. 3.7.

The purity of the purified arginase was analyzed by 12% SDS-PAGE and the results are shown in Fig. 3.8. The estimated purity of purified arginase was about 95% high. Also, it was observed that the molecular weight of the subunit of the purified arginase was approximately 35 kDa.

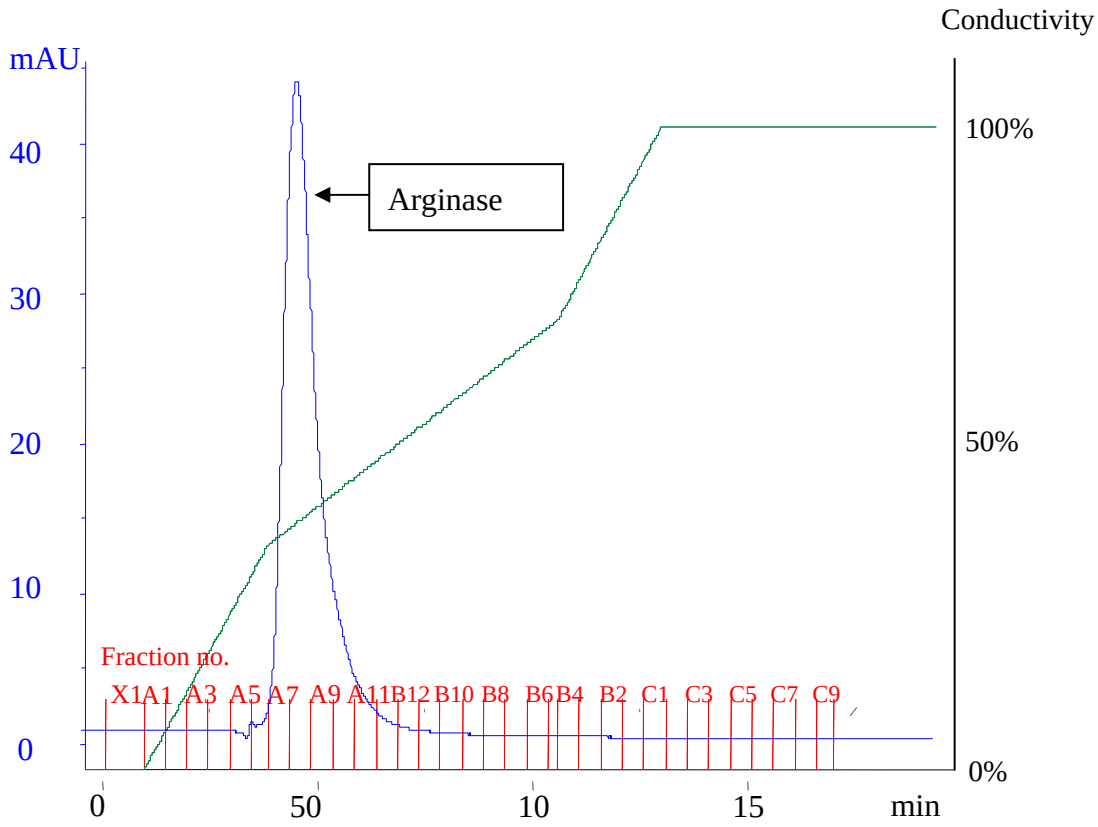



Fig. 3.7: Elution profile of arginase from the SP Sepharose Fast Flow ion exchange chromatography column. The elution process was carried out at room temperature for about 3 h. The protein concentration of each fraction was determined by absorbance at 280 nm and the unit used was mAU, milli absorption unit. The elution salt concentration was determined by the conductivity flow cell, and it was represented by conductivity, i.e., 100% =1 M NaCl.

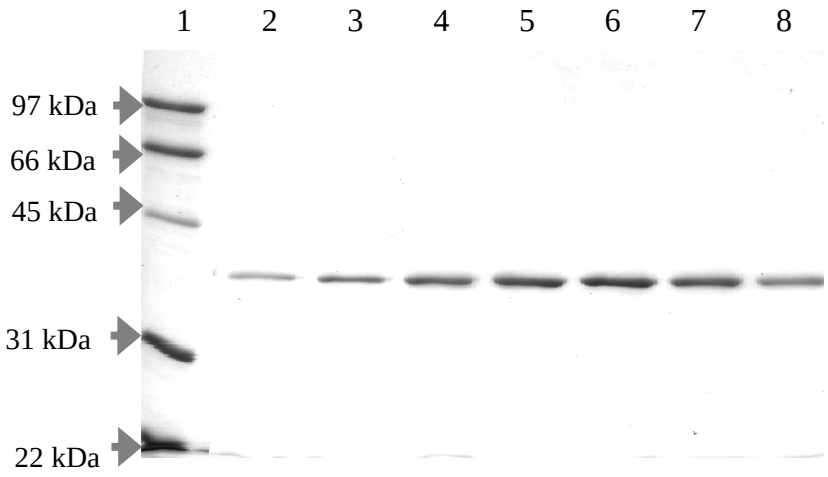



Fig. 3.8: 12% SDS-PAGE showing the fractions collected from the SP Sepharose Fast Flow ion exchange chromatography. Lane 1: low range molecular weight marker (1 µg per band); Lane 2: Fraction A6 (5 µl); Lane 3: Fraction A7 (5 µl); Lane 4: Fraction A8 (5 µl); Lane 5: Fraction A9 (5 µl); Lane 6: Fraction A10 (5 µl); Lane 7: Fraction A11 (5 µl); Lane 8: Fraction A12 (5 µl).

#### 3.1.2.4 Desalting

After ion exchange chromatography, desalting and buffer exchange were carried out with the use of the HiPrep 26/10 desalting column (53 ml). Desalting column was pre-equilibrated with PBS buffer (pH 7.4) with a flow rate of 10 ml/min. Proteins were eluted and fractions containing arginase were pooled. Arginase was eluted first from the desalting column followed by the salt. Fractions containing arginase were collected.

### 3.1.3 Overview of purification of shake-flask fermentation

The purification process for arginase is summarized in Table 3.1. The purification of arginase was started from a 1.6-L of cell culture. In general, about 30 mg of purified arginase was obtained

**Table 3.1:** Overview of the small scale purification of arginase from shake-flask culture. One unit of activity (I.U.) is defined as the amount of protein which catalyzes the formation of 1 μmole urea per min at 30$^{o}$C, pH 8.5.

| | Volume (ml) | arginase concentration (mg/ml) | Total arginase (mg) | Specific activity (I.U./mg) | Total activity (I.U.) |
|---|---|---|---|---|---|
| Nickel column | | | | | |
| Nickel chelating column | 62 | 0.85 | 52.85 | -------- | -------- |
| Desalting column | | | | | |
| Desalting column | 81 | 0.532 | 43.1 | 224.86 | 9691.73 |
| SP Sepharose FF column | | | | | |
| SP Sepharose FF column | 24 | 1.277 | 30.65 | 205.55 | 6300.22 |
| Total protein yielded = 30 mg | | | | | |

### 3.1.4 SDS-PAGE analysis

The purity of arginase was determined by 12% SDS-PAGE analysis and the result is shown in Fig. 3.9. The purity of all the purified proteins estimated was about 99%, indicating that our purification method was efficient. As shown in Fig. 3.9, the molecular weight of the purified arginase was about 35 kDa.

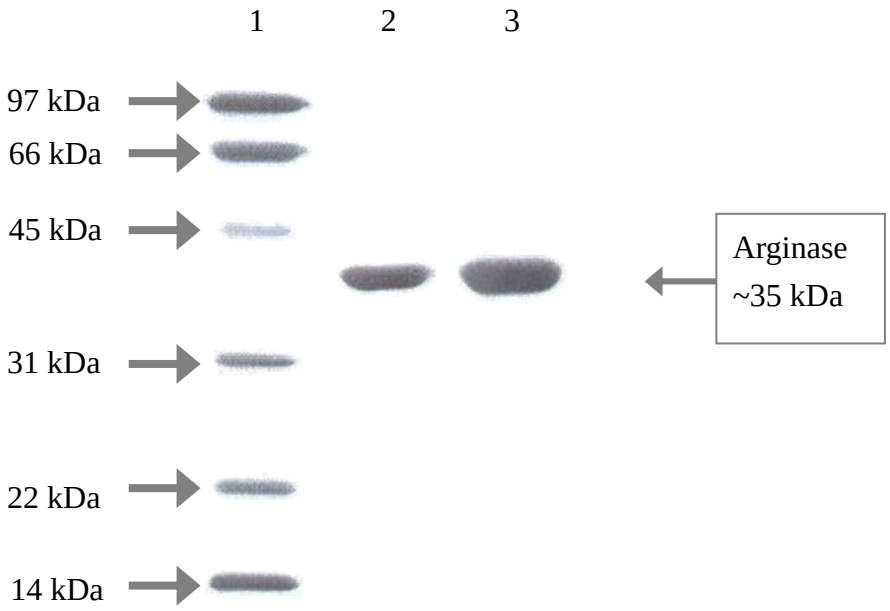



Fig. 3.9: 12% SDS-PAGE showing the purified arginase from shake-flask culture. Lane 1: low range molecular weight marker (1 μg per band); Lane 2: purified arginase (1.2 μg); Lane 3: purified arginase (2.4 μg).

### 3.1.5 Protein standard curve

During arginase purification, the protein concentrations were determined by the Bradford Assay in which the bovine serum albumin (BSA) was used as the standard. A protein standard curve was constructed for measuring the sample protein concentration. The standard curve shown in Fig. 3.10 is a straight line in the standard range 0-15 μg/ml.

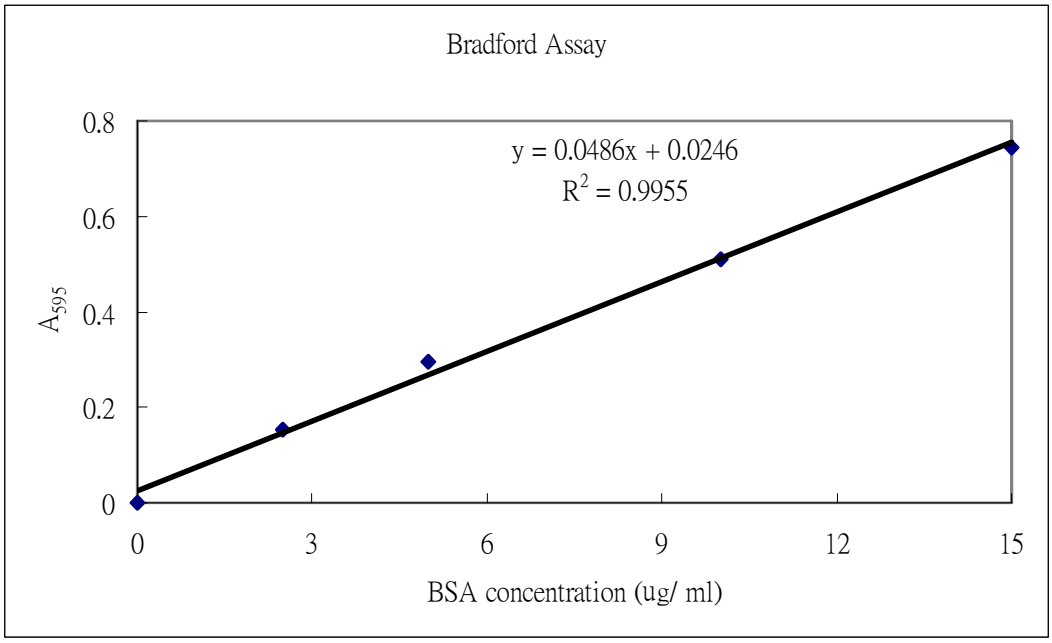



Fig. 3.10: Bradford assay standard curve (BSA was used as a standard).

### 3.1.6 Activity assay

Two methods were used to determine the activity of arginase. They are the $A_{340}$ assay and the assay using an amino acid analyzer. Activity of the purified arginase was measured in every purification process. One unit (I.U.) of arginase is defined as the amount of enzyme that can produce 1 μmol of urea per min at 30$^{o}$C, pH 8.5.

#### 3.1.6.1 Activity assay using a spectrophotometer

A coupled spectrophotometric assay for arginase was used to determine arginase activity (Ikemoto *et al*., 1989; Ozer, 1985). By following the decrease in absorbance at 340 nm ($A_{340}$), the activity of arginase was determined. A typical reaction is shown in Fig. 3.11; the reaction was monitored with the Lambda Bio 20 UV/Vis Spectrometer (Perkin Elmer Lambda Bio20).

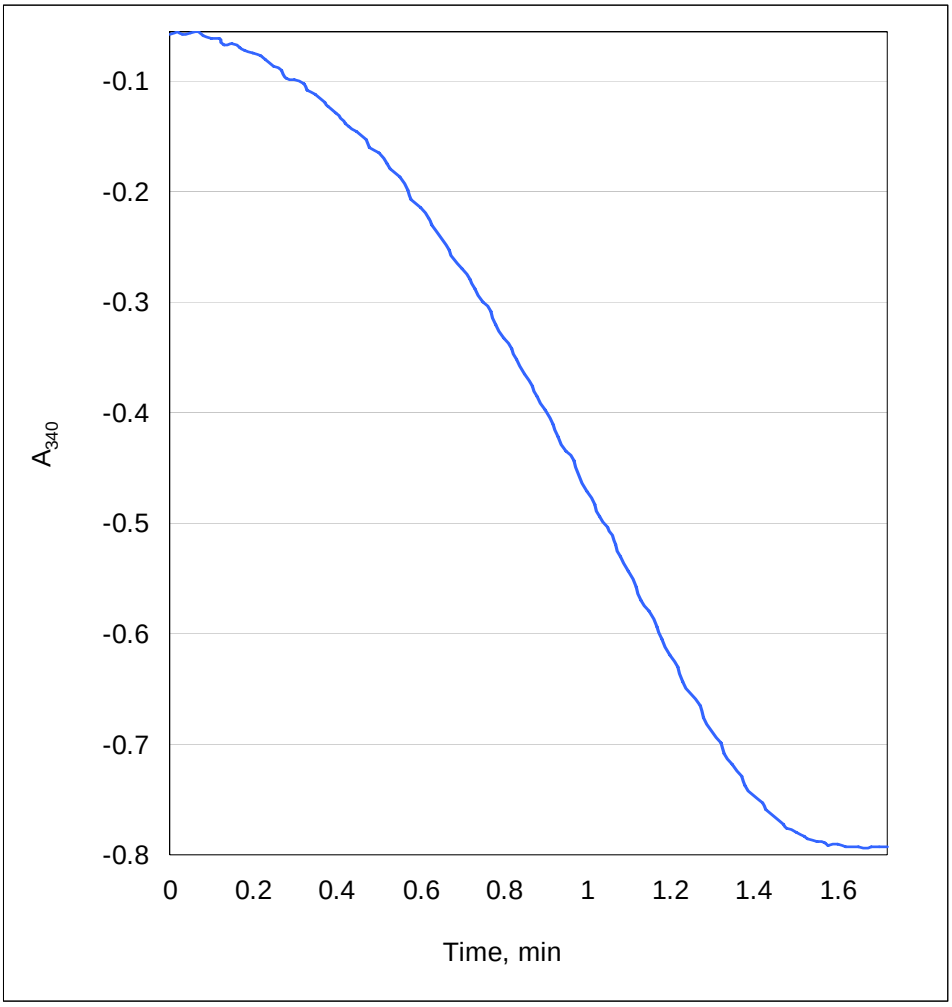



Fig. 3.11: Spectrophotometric activity assay for arginase. Arginase was coupled to glutamate dehydrogenase and the decrease in $A_{340}$ was recorded. Measurement was performed in 0.1 M Tris-HCl (pH 8.5) at 30 $^{o}$C. The formation of the product $NADP^{+}$ was monitored spectrophotometrically at 340 nm for 1-2 min.

### 3.1.6.2 Activity assay using an amino acid analyzer

Amino acid analyzer was also used to monitor the activity of arginase (Bastone *et al*., 1990). The amount of arginine depleted and the amount of ornithine produced can be determined directly using the amino acid analyzer (Model L-8800, Hitachi). Typical results obtained from the amino acid analyzer are shown in Fig. 3.12. Concentrations of arginine and ornithine were measured and the activity of arginase was calculated from these concentrations.

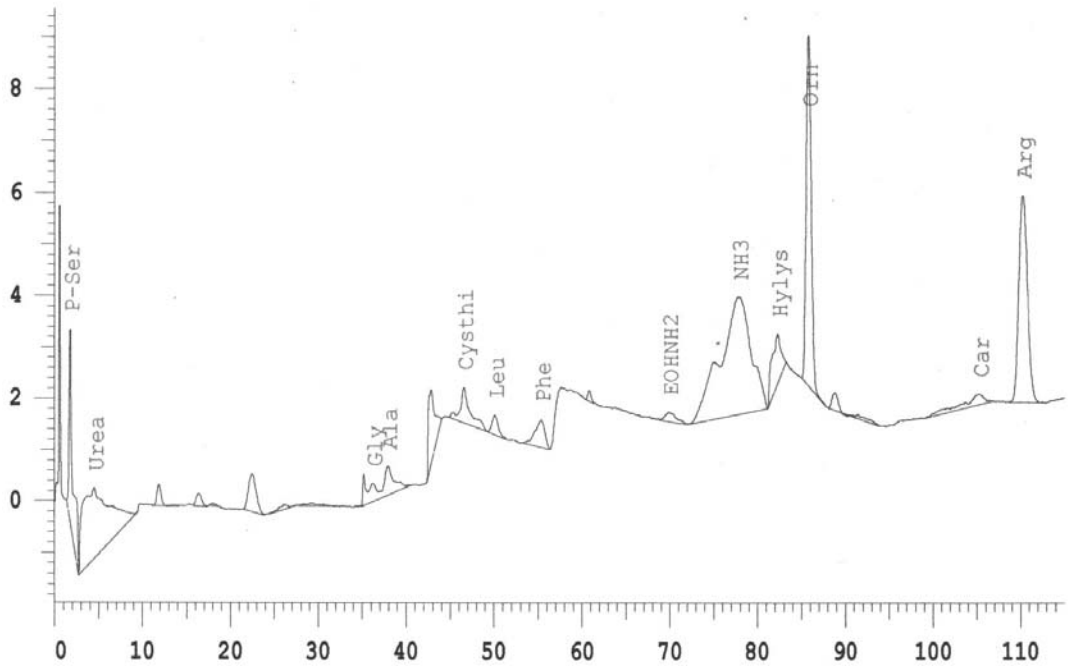



Fig. 3.12: Typical results obtained from the amino acid analyzer. The x-axis represents the time (min) and the y-axis represents the intensities of the amino acid. Abbreviation: P-Ser, serine; Gly, glycine; Ala, alanine; Cysthi, cysteine & thioglycolic; Leu, leucine; Phe, phenylalanine; $NH_3$, ammonia; Hylys, Hydroxylysine; Orn, ornithine; Car, Carboxymethylcysteine; Arg, arginine.

## 3.2 Production of arginase from fed-batch fermentation

When arginase was purified successfully from 1-L shake-flask fermentation, the scale of fermentation was increased from 1-L to 8-L. Fermentation was now carried out in a fermentor instead of using shake flasks.

### 3.2.1 Purification of arginase enzyme

The strategy of arginase purification from 8-L fermentation medium was similar to that of l-L arginase purification. Two steps were involved when purifying arginase from 8-L of cell culture: (1) affinity chromatography, (2) desalting step.

#### 3.2.1.1 Affinity chromatography

A self packed nickel-nitrilotriacetic acid (Ni-NTA) metal-affinity chromatography column (130 ml) was used to purify arginase from 8-L of culture The advantage of using Ni-NTA affinity column is to allow a one-step purification. After loading the arginase enzyme onto the Ni-NTA column, 0.5 M imidazole was used to elute the arginase. When the concentration of imidazole was higher than 0.2 M, binding between the Ni-NTA resins and the 6xHis-arginase became dissociated and arginase was eluted from the column.

Fig. 3.13 shows the elution profile of Ni-NTA affinity chromatography column. Fractions containing arginase were pooled together for the next purification step. Purities and concentrations of recombinant arginase after Ni-NTA affinity chromatography were analyzed by 12 % SDS-PAGE and Bradford assay, respectively. The result of SDS-PAGE analysis is shown in Fig. 3.14. The purity of arginase eluted from the Ni-NTA column was very high since only one single band (35 kDa) could be observed on the gel.

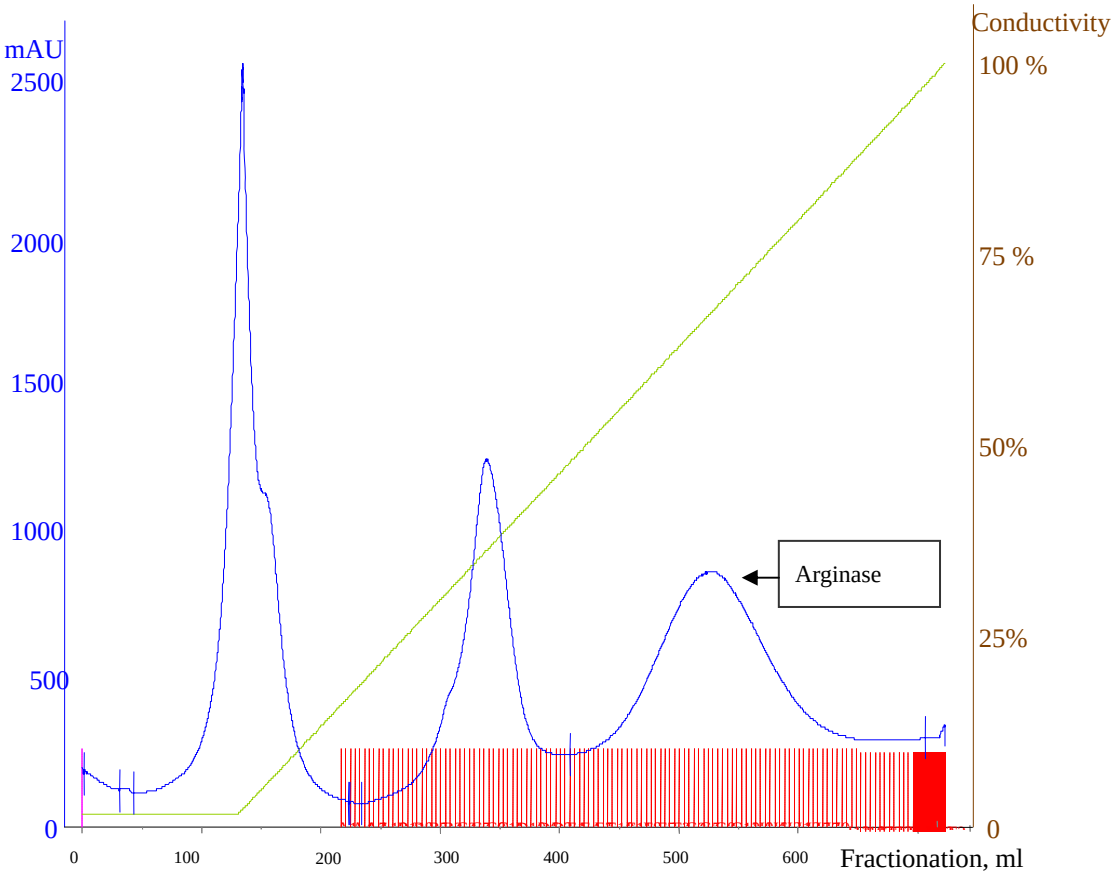



Fig. 3.13: Elution profile of arginase from the Ni-NTA affinity column. The protein concentration of the sample was measured by absorbance at 280 nm, while the salt concentration was determined as the conductivity. All the measurements were performed using the Pharmacia AKTA $_{FPLC}$ or AKTA $_{purifier}$. The elution salt concentration was determined by the conductivity flow cell, and it was represented by conductivity, i.e., 100% =1 M NaCl.

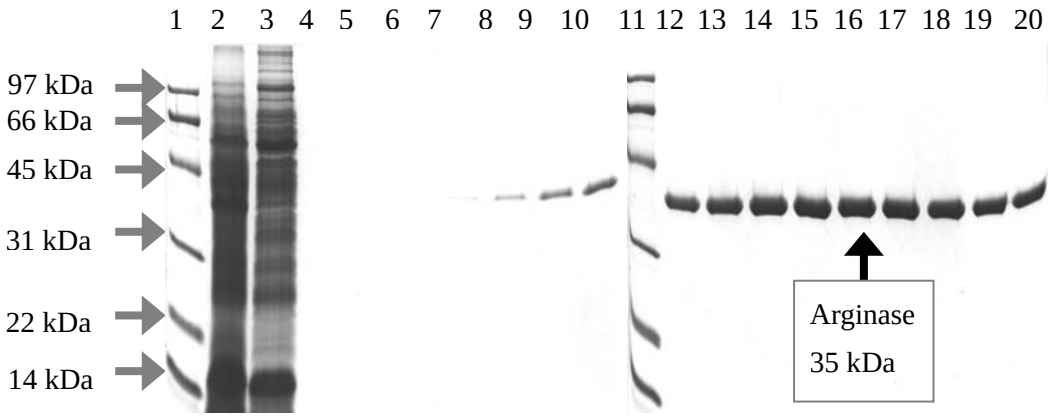



Fig. 3.14: 12% SDS-PAGE showing the fractions eluted from Ni-NTA affinity column. Lane 1: 1 µg low-range protein marker (Bio-rad); Lane 2: 5 µl crude protein; Lane 3: 5 µl flowthrough after 2$^{nd}$ Ni-NTA column; Lane 4: 1 µl fraction B1; Lane 5: 1 µl fraction C3; Lane 6: 1 µl fraction C6; Lane 7: 1 µl fraction C9; Lane 8: 1 µl fraction C12; Lane 9: 1 µl fraction D10; Lane 10: 1 µl fraction D7; Lane 11: 1 µg low-range protein marker (Bio-rad); Lane 12: 1 µl fraction D4; Lane 13: 1 µl fraction D1; Lane 14: 1 µl fraction E3; Lane 15: 1 µl fraction E6; Lane 16: 1 µl fraction E9; Lane 17: 1 µl fraction E12; Lane 18: 1 µl fraction F10; Lane 19: 1 µl fraction F7; Lane 20: 1 µl fraction F4.

#### 3.2.1.2 Desalting

Fractions containing arginase were collected after Ni-NTA affinity chromatography and desalting was done by using the tangential flow filtration (TFF) system. Imidazole was removed by TFF system and the buffer was changed from 0.2 M sodium phosphate buffer (pH 7.4) to PBS buffer (pH 7.4). The dilution table of TFF system is shown in Table 3.2. The feed pressure and the retentate pressure for the TFF system were 30 psi and 10 psi, respectively. Membrane PXB00850 was used and the nominal cutoff of the membrane was 8 kDa. The rate of permeate collected was about 3 ml/min.

Table 3.2. Dilution table for the TFF system.

| | | |
|---|---|---|
| 220 ml arginase<br>(in sodium phosphate buffer)<br>↓ | → | 170 ml permeate collected |
| ~50 ml retentate | + | Dilute with 450 ml PBS buffer |
| ↓<br>500 ml<br>↓ | → | 450 ml permeate collected |
| ~50 ml retentate | + | Dilute with 450 ml PBS buffer |
| ↓<br>500 ml<br>↓ | → | 400 ml permeate collected |
| ~100 ml retentate | + | Dilute with 120 ml PBS buffer |
| ↓<br>220ml<br>↓ | → | 110 ml permeate collected |
| ~100 ml retentate | + | Dilute with 100 ml PBS buffer |
| ↓<br>**196 ml**<br>**(in PBS buffer)** | | |
| **Dilution factor for salts = ~430 times** | | |

### 3.2.2 SDS-PAGE analysis

The purity of arginase produced from fermentation of 8-L of culture was determined by 12% SDS-PAGE and the result is shown in Fig 3.15. It is observed that the purity of all the purified proteins was very high, indicating that the purification method was practical. As shown in Fig. 3.15, the molecular mass of the subunit of the purified arginase was about 35 kDa when compared with the low range molecular weight marker.

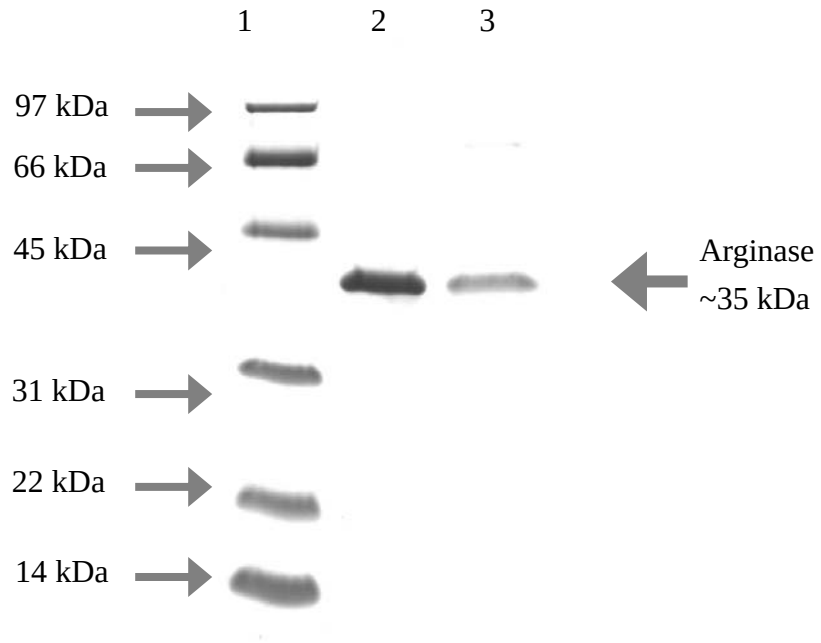



Fig. 3.15: 12% SDS-PAGE showing the purified arginase from fed-batch fermentation. Lane 1: 1 μg of low range molecular weight marker; Lane 2: 1 μg of purified arginase; Lane 3: 0.5 μg of purified arginase.

### 3.2.3 Protein standard curve

Protein concentrations were determined by the Bradford Assay in which bovine serum albumin (BSA) was used as the standard. A protein standard was constructed for measuring the sample protein concentration. The standard curve shown in Fig. 3.16 is a straight line in the standard range 0-20 μg/ml.

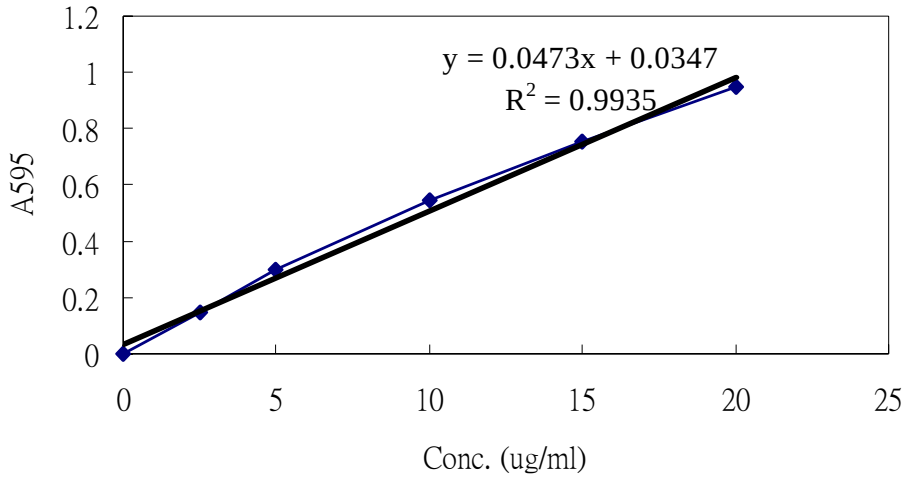



Fig. 3.16: Bradford assay standard curve (BSA was used as a standard).

### 3.2.4 Overview of purification of fed-batch fermentation

The purification table for arginase from fed batch fermentation of 8-L of culture is shown in Table 3.3. The purification of arginase was started from 8-L of cell culture and the purified enzymes showed 7.3 purification folds and 76.6 % yield. In general, about 700 mg of purified arginase was obtained from 8-L of *B. subtilis* culture. Thus, about 88 mg of arginase was purified from one liter of bacterial culture.

Table 3.3: Overview of the fed-batch fermentation and purification of arginase.

| Sample | Total protein conc. (mg/ml) | Total volume (ml) | Total proteins (mg) | Specific activity (I.U./mg) | Total activity (I.U.) | Fold of purification | Yield of purification (%) |
|---|---|---|---|---|---|---|---|
| | | | | | | | |
| Crude | 5.3 | 1325 | 7067 | 59 | 416953 | 1 | 100 |
| | | | | | | | |
| Ni-NTA columns | 1.3 | 568 | 735 | 405 | 297675 | 6.9 | 70 |
| | | | | | | | |
| TFF | 1.1 | 626 | 672 | 479 | 321888 | 7.3 | 76.6 |

Fold of purification:

= Specific activity of Sample (I.U./mg) / Specific activity of Crude proteins (I.U./mg)

Yield of purification:

= {Total activity of the Sample (I.U.) / Total activity of Crude proteins (I.U.)} x 100%

## 3.3 Pegylation of arginase

Arginase has a short blood-circulating half-life and will raise immune response. Modification of arginase with polyethylene glycol (PEG) would overcome these problems. Thus, arginase was modified with mPEG-SPA (Methoxypolyethylene Glycol Succinimidyl Propionate, MW 5000) to produce arginase-SPA-$PEG_{5000}$. Modification of arginase by PEG molecules increased the blood-circulating half-life of arginase and reduced immunogenicity as well as antigenic properties.

The pegylated arginase was analyzed with 12% SDS-PAGE and the result is shown in Fig. 3.17, which indicated that the size of arginase was increased after conjugated with mPEG-SPA.

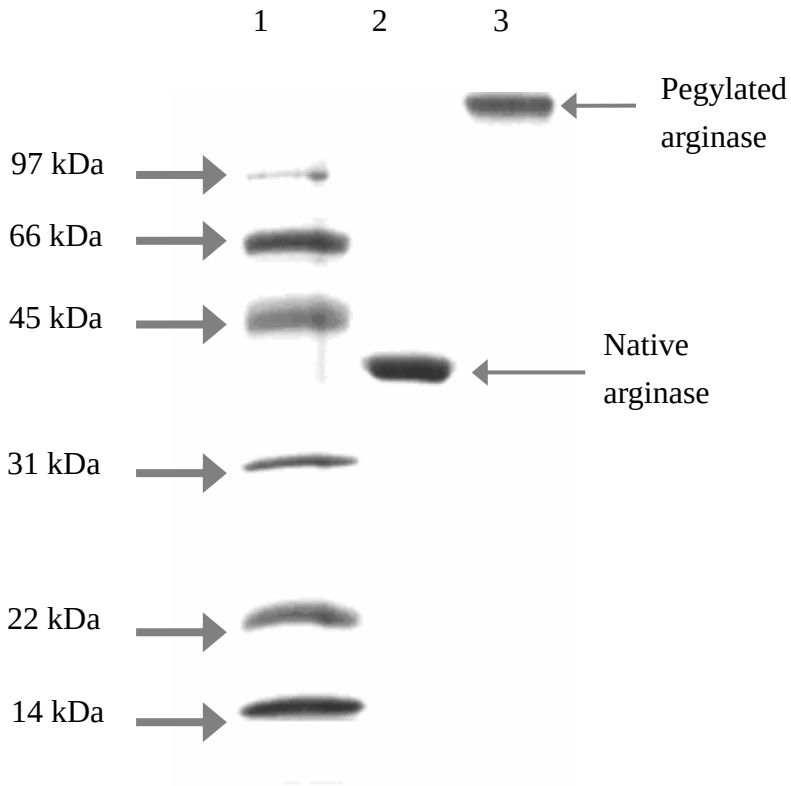



Fig. 3.17: 12% SDS-PAGE analysis of the purified arginase and the pegylated arginase (arginase-SPA-$PEG_{5000}$). Arginase was pegylated with mPEG-SPA in mole ratio of 1: 50 for 2.5 h. Lane 1: low range protein marker; Lane 2: native arginase; MW= 35 kDa; Lane 3: arginase-SPA-$PEG_{5000}$, much larger than 35 kDa.

### 3.3.1 Pegylation of arginase with different PEG molecules

Pegylated arginase was generated by derivatization of arginase with PEG molecules of various sizes, structures and linkers. Five PEG molecules were used for arginase pegylation; they are: mPEG-N-hydroxysuccinimide, MW= 10,000 (mPEG-NHS); mPEG-propionaldehyde, MW= 5,000 (mPEG-ALD); mPEG-maleimide, MW= 5,000 (mPEG-MAL); mPEG-succinimidyl propionate, MW= 5,000 **(**mPEG-SPA) and cyanuric chloride activated PEG, MW= 5,000 (mPEG-CN).

A 15% SDS-PAGE was used to analyze the modification of arginase with various PEGs. The results are presented in Figs. 3.18- 3.23, which indicate that mPEG-SPA and mPEG-CN reacted with the native arginase more efficiently. Therefore, they were more suitable for pegylating arginase. In Fig. 3.24, it is observed that the size of the PEG or the linker used to attach it to the primary amines of the arginase did not significantly affect the specific activity of the pegylated arginase, except pegylation using mPEG-CN, which decreased the activity by 70% after pegylation. Consequently, mPEG-SPA (MW= 5000) was selected for the pegylation of arginase.

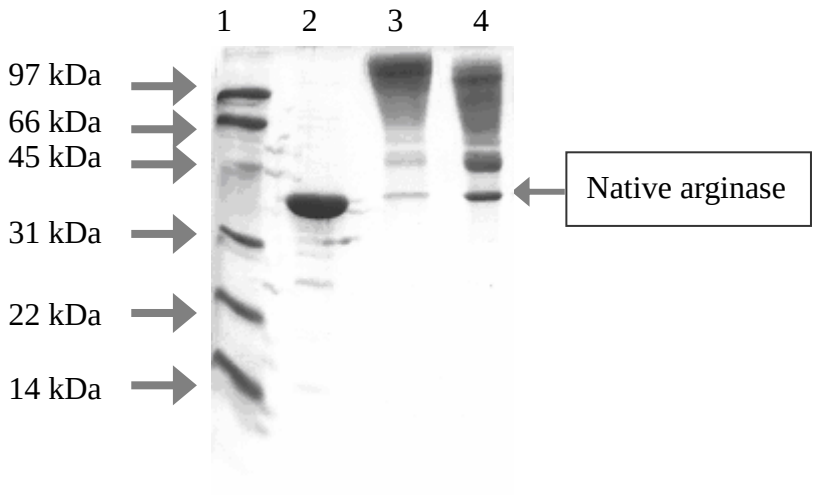



Fig. 3.18: Purified arginase was modified with mPEG-SPA. 15% SDS-PAGE was used to analyze the pegylated arginases. Lane 1: low-range protein marker; Lane 2: native arginase; Lane 3: Arginase-SPA-$PEG_{5000}$ (1:50); Lane 4: Arginase-SPA-$PEG_{5000}$ (1:20).

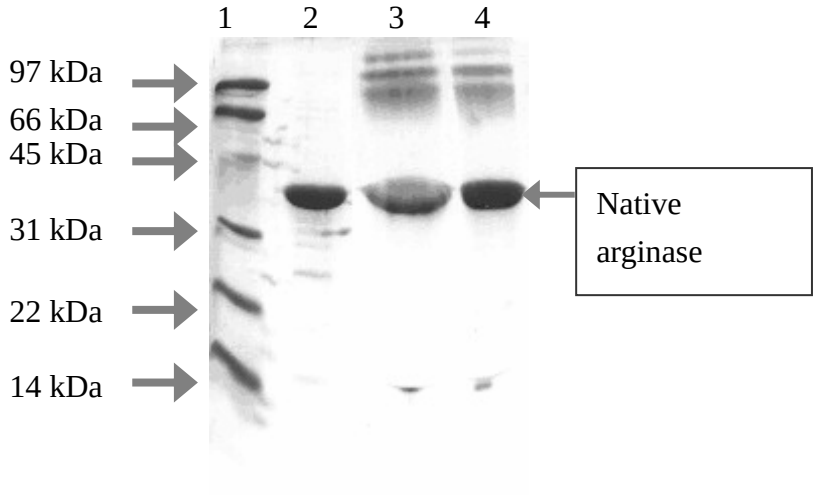



Fig. 3.19: Purified arginase was modified with mPEG-NHS. 15% SDS-PAGE was used to analyze the pegylated arginases. Lane 1: low-range protein marker; Lane 2: native arginase; Lane 3: Arginase-NHS-$PEG_{10,\ 000}$ (1:50); Lane 4: Arginase-NHS-$PEG_{10,\ 000}$ (1:20).

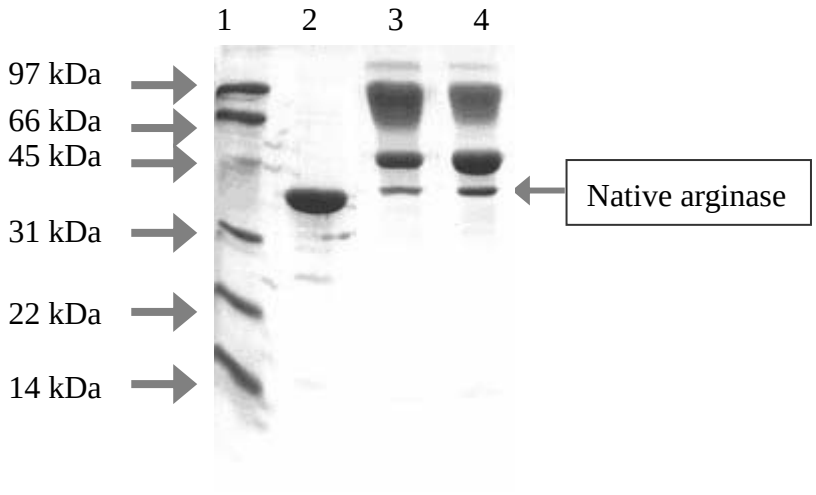



Fig. 3.20: Purified arginase was modified with mPEG-MAL. 15% SDS-PAGE was used to analyze the pegylated arginases. Lane 1: low-range protein marker; Lane 2: native arginase; Lane 3: Arginase-MAL-$PEG_{5000}$ (1:50); Lane 4: Arginase-MAL-$PEG_{5000}$ (1:20).

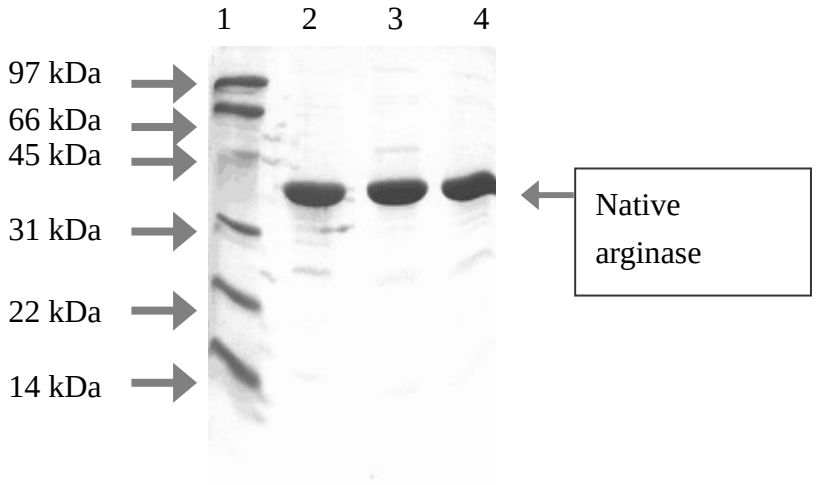



Fig. 3.21: Purified arginase was modified with mPEG-ALD. 15% SDS-PAGE was used to analyze the pegylated arginases. Lane 1: low-range protein marker; Lane 2: native arginase; Lane 3: Arginase-ALD-$PEG_{5000}$ (1:50); Lane 4: Arginase-ALD-$PEG_{5000}$ (1:20).

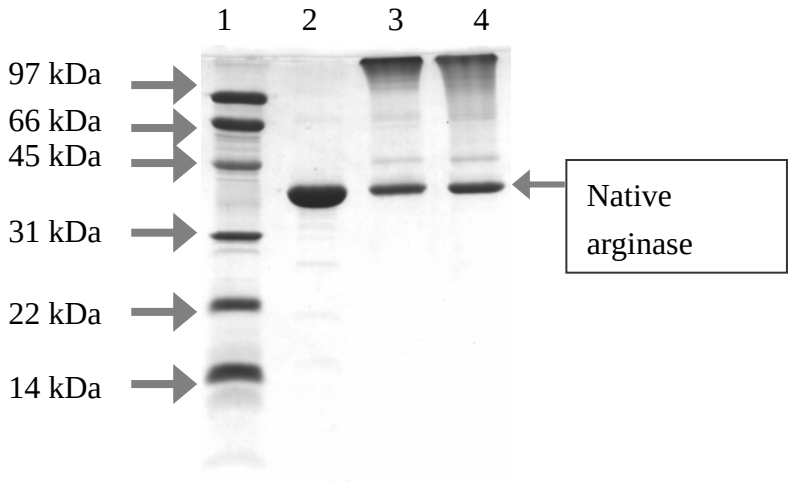



Fig. 3.22: Purified arginase was modified with CN-PEG. 15% SDS-PAGE was used to analyze the pegylated arginases. Lane 1: low-range protein marker; Lane 2: native arginase; Lane 3: Arginase-CN-$PEG_{5000}$ (1:50); Lane 4: Arginase-CN-$PEG_{5000}$ (1:20).

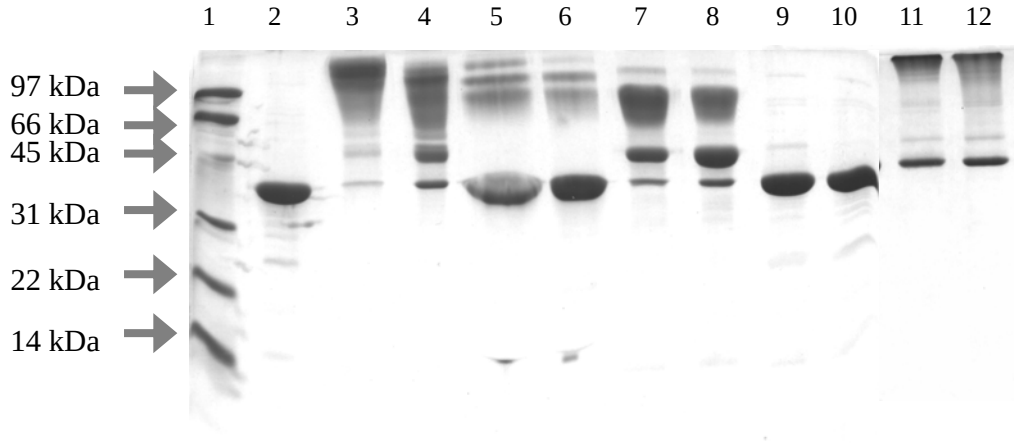



Fig. 3.23: Pegylation of arginase with various PEG molecules. 15% SDS-PAGE was used to analyze the pegylated arginases. Lane 1: low-range protein marker; Lane 2: native arginase; Lane 3: Arginase-SPA-$PEG_{5000}$ (1:50); Lane 4: Arginase-SPA-$PEG_{5000}$ (1:20); Lane 5: Arginase-NHS-$PEG_{10,000}$ (1:50); Lane 6: Arginase-NHS-$PEG_{10,000}$ (1:20); Lane 7: Arginase-MAL-$PEG_{5000}$ (1:50); Lane 8: Arginase-MAL-$PEG_{5000}$ (1:20); Lane 9: Arginase-ALD-$PEG_{5000}$ (1:50); Lane 10: Arginase-ALD-$PEG_{5000}$ (1:20); Lane 11: Arginase-CN-$PEG_{5000}$ (1:50); Lane 12: Arginase-CN-$PEG_{5000}$ (1:20).

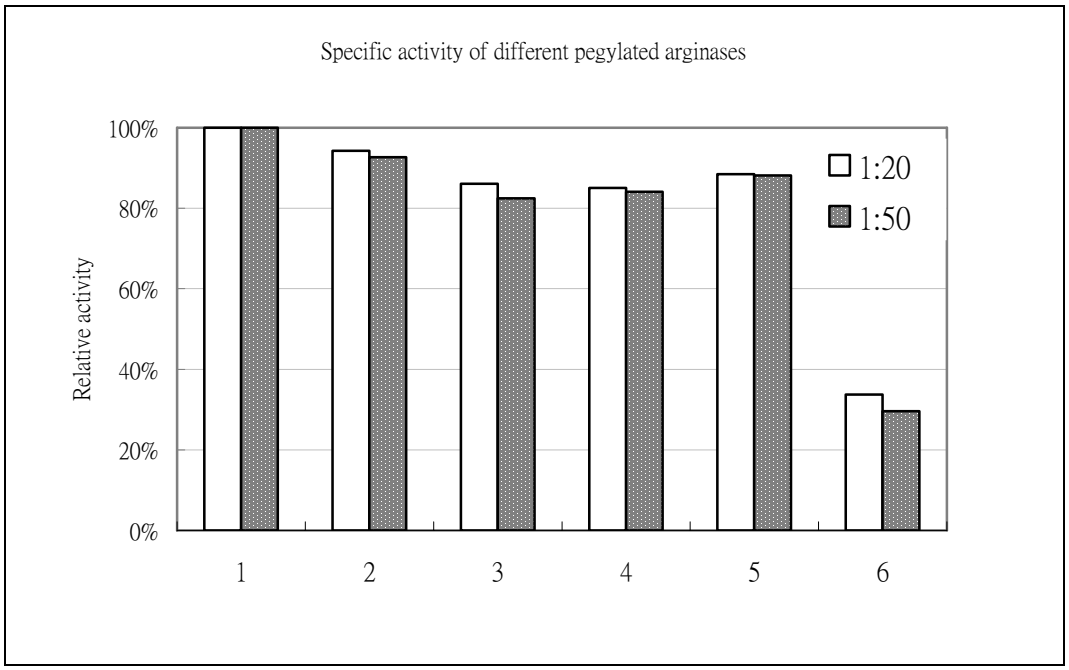



Fig. 3.24: The effect of various PEGs attached to arginase on the enzyme activity. Bar 1: Native arginase; Bar 2: arginase-SPA-$PEG_{5000}$; Bar 3: arginase-NHS-$PEG_{10,000}$; Bar 4: arginase-MAL-$PEG_{5000}$; Bar 5: arginase-ALD-$PEG_{5000}$; Bar 6: arginase-CN-$PEG_{5000}$. The white and dotted bars represent arginase pegylated with PEG molecules in different mole ratios (white bar, 1: 20; dotted bar, 1:50).

### 3.3.2 Pegylation of arginase with PEG molecules in different mole ratios

mPEG-SPA (MW=5000) was selected to be used in arginase pegylation. Experiments were carried out to find out the effect of variation of the number of moles of PEG attached to each mole of arginase. As the number of moles of PEG per mole of arginase increased, the amount of native arginase remained was decreased. The results of the time course of pegylation using mPEG-SPA in different mole ratios were analyzed by 15% SDS-PAGE, and are shown in Fig. 3.25 to Fig. 3.29. These results suggested that the optimum PEG-to-arginase mole ratio was 50:1 and the optimum time used for pegylation was 3 h.

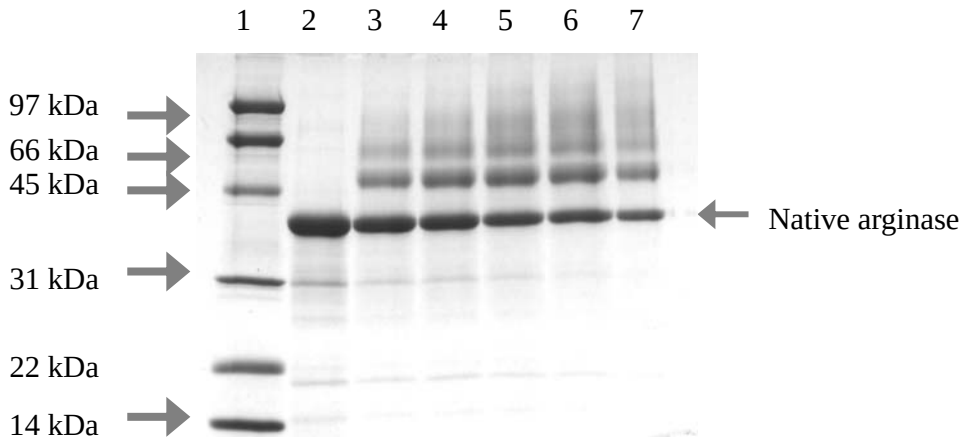



Fig. 3.25: Time course of pegylation of arginase-SPA-$PEG_{5,000}$ (1:10 mole ratio). Lane 1: low-range protein marker; Lane 2: 5.12 µg native arginase; Lane 3: 5.12 µg arginase-SPA-$PEG_{5000}$ (0.5 h); Lane 4: 5.12 µg arginase-SPA-$PEG_{5000}$ (1h); Lane 5: 5.12 µg arginase-SPA-$PEG_{5000}$ (2 h); Lane 6: 5.12 µg arginase-SPA-$PEG_{5000}$ (3 h); Lane 7: 5.12 µg arginase-SPA-$PEG_{5000}$ (20 h).

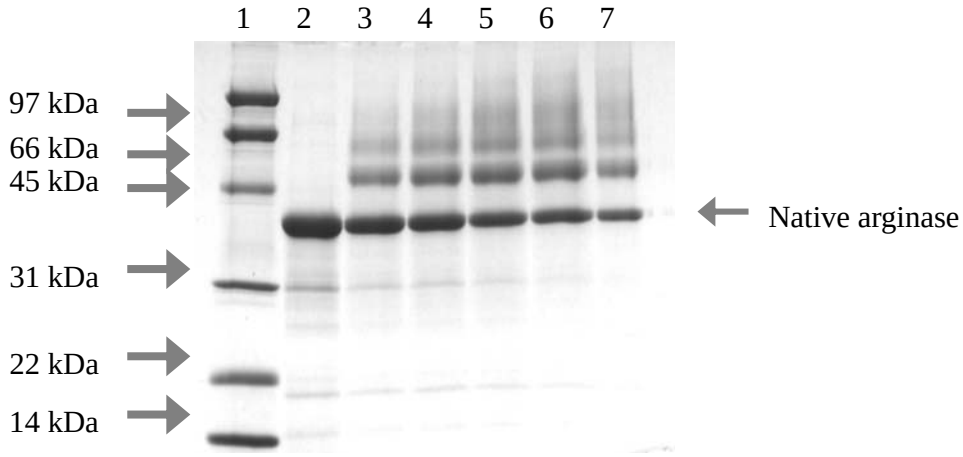



Fig. 3.26: Time course of pegylation of arginase-SPA-$PEG_{5,000}$ (1:20 mole ratio). Lane 1: low-range protein marker; Lane 2: 5.40 µg native arginase (0 h), Lane 3: 5.4 µg arginase-SPA-$PEG_{5,000}$ (0.5 h); Lane 4: 5.4 µg arginase-SPA-$PEG_{5,000}$ (1 h); Lane 5: 5.4 µg arginase-SPA-$PEG_{5,000}$ (2 h); Lane 6: 5.4 µg arginase-SPA-$PEG_{5,000}$ (3 h); Lane 7: 5.4 µg arginase-SPA-$PEG_{5,000}$ (19.5 h).

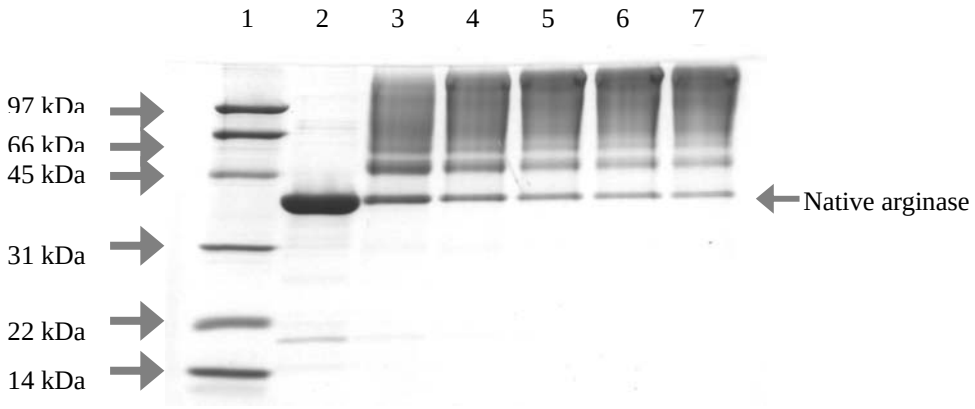



Fig. 3.27: Time course of pegylation of arginase-SPA-$PEG_{5,000}$ (1:30 mole ratio). Lane 1: low-range protein marker; Lane 2: 5.12 µg native arginase; Lane 3: 5.12 µg arginase-SPA-$PEG_{5000}$ (0.5 h); Lane 4: 5.12 µg arginase-SPA-$PEG_{5000}$ (1h); Lane 5: 5.12 µg arginase-SPA-$PEG_{5000}$ (2 h); Lane 6: 5.12 µg arginase-SPA-$PEG_{5000}$ (3 h); Lane 7: 5.12 µg arginase-SPA-$PEG_{5000}$ (20 h).

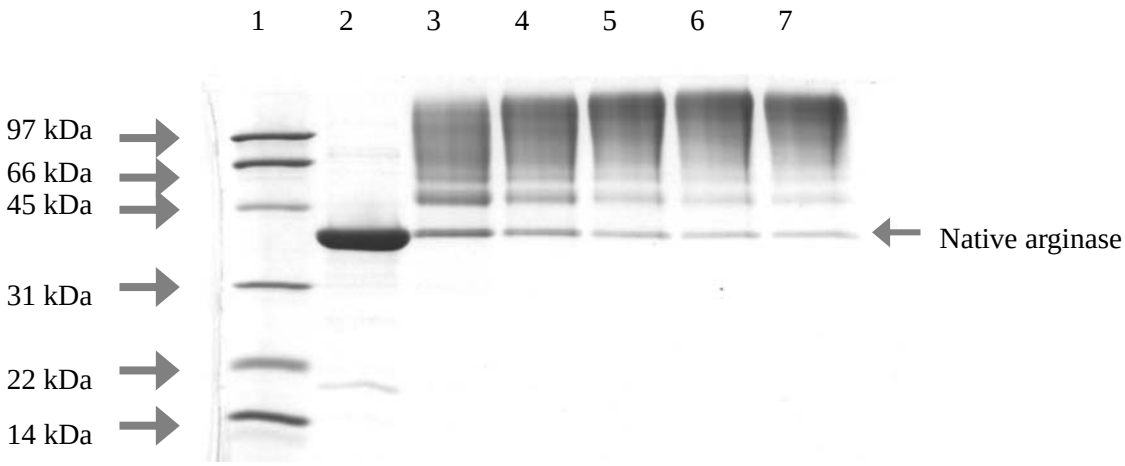



Fig 3.28: Time course of pegylation of arginase-SPA-$PEG_{5,000}$ (1:40 mole ratio). Lane 1: low-range protein marker; Lane 2: 5.12 µg native arginase; Lane 3: 5.12 µg arginase-SPA-$PEG_{5000}$ (0.5 h); Lane 4: 5.12 µg arginase-SPA-$PEG_{5000}$ (1h); Lane 5: 5.12 µg arginase-SPA-$PEG_{5000}$ (2 h); Lane 6: 5.12 µg arginase-SPA-$PEG_{5000}$ (3 h); Lane 7: 5.12 µg arginase-SPA-$PEG_{5000}$ (20 h).

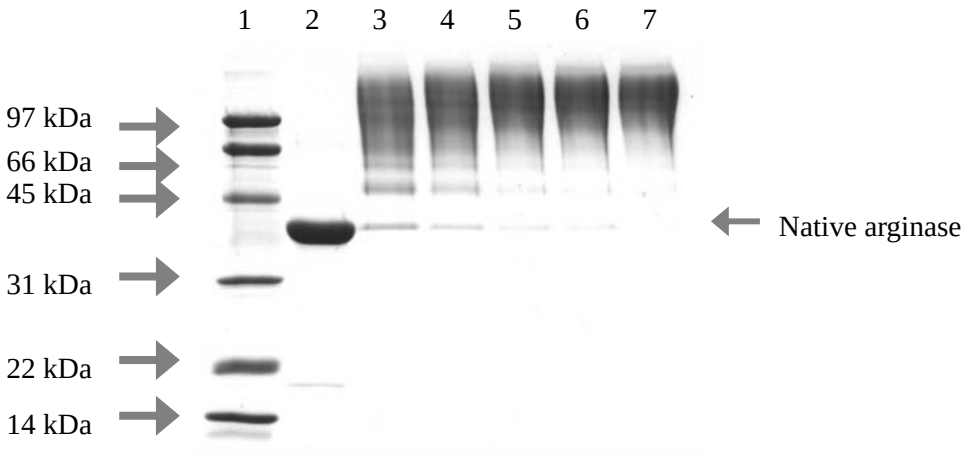



Fig. 3.29: Time course of pegylation of arginase-SPA-$PEG_{5,000}$ (1:50 mole ratio). Lane 1: low-range protein marker; Lane 2: 5.4 µg native arginase; Lane 3: 5.4 µg arginase-SPA-$PEG_{5000}$ (0.5 h); Lane 4: 5.4 µg arginase-SPA-$PEG_{5000}$ (1h); Lane 5: 5.4 µg arginase-SPA-$PEG_{5000}$ (2 h); Lane 6: 5.4 µg arginase-SPA-$PEG_{5000}$ (3 h); Lane 7: 5.4 µg arginase-SPA-$PEG_{5000}$ (20 h).

## 3.4 Characterization of purified arginase

### 3.4.1 Kinetic analysis

The kinetic parameters of the native arginase and arginase-SPA-$PEG_{5000}$ were investigated in this project. Kinetic properties of the native arginase were determined using the $A_{340}$ assay. The steady-state parameters were determined by the initial rate analysis and non-linear regression plots of the results are illustrated in Fig. 3.30 and Fig. 3.31. The $K_m$ value of the native arginase for L-arginine was 1.89 ± 0.70 mM and the $k_{cat}$ value was 1.798 $s^{-1}$. The $K_m$ value of arginase-SPA-$PEG_{5000}$ for L-arginine was 2.91 ± 0.27 mM.

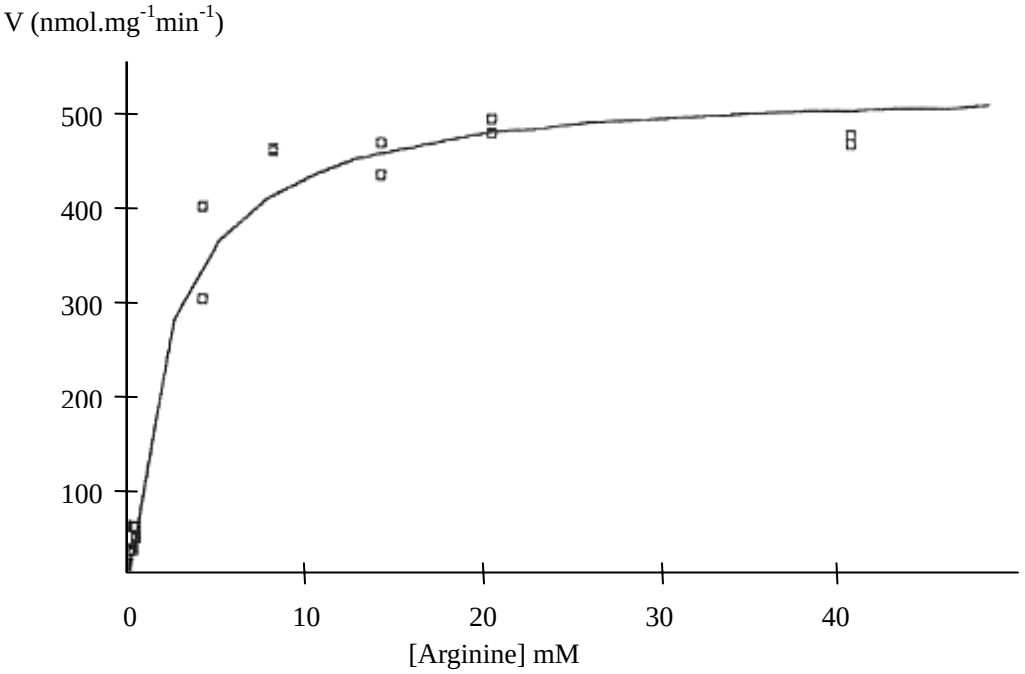



Fig. 3.30: Result of the kinetic analysis of the native arginase with different concentrations of L-arginine. The $K_m$ value of the native arginase for L-arginine was 1.89 ± 0.70 mM and the $k_{cat}$ value was 1.798 $s^{-1}$. The results are illustrated in the form of a non-linear regression plot. Each point represents a single measurement.

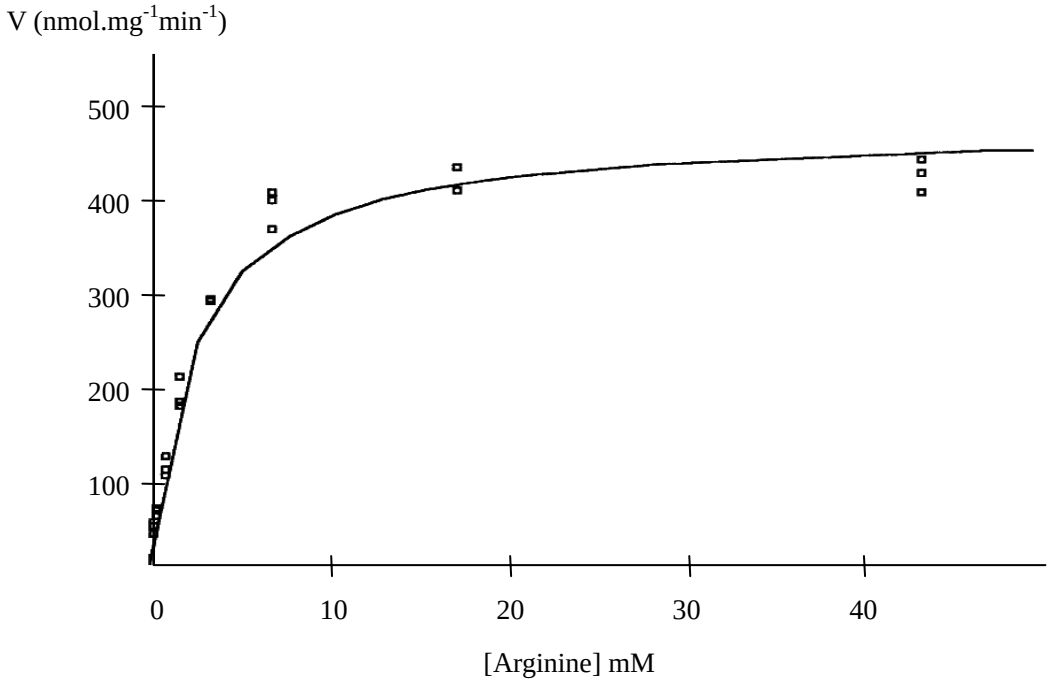



Fig. 3.31: Result of the kinetic analysis of the pegylated arginase with different concentrations of L-arginine. The $K_m$ value of arginase-SPA-$PEG_{5000}$ for L-arginine was 2.91 ± 0.27 mM. The results are illustrated in the form of a non-linear regression plot. Each point represents a single measurement.

### 3.4.2 Optimum $Mn^{2+}$ concentration

Arginase has a specific catalytic and physiological requirement for two manganese ions. Therefore, the requirement for manganese ions on the effect of arginase activity was investigated. The change of absorbance at 340 nm was measured at different manganese ion concentrations from 0-1500 μM. The steady-state parameters were determined by the initial rate analysis and non-linear regression plots of the results are illustrated in Fig. 3.32 and Fig. 3.33. From this experiment, it is concluded that about 1 mM of Mn (II) should be used to achieve maximum activity of both arginase enzymes.

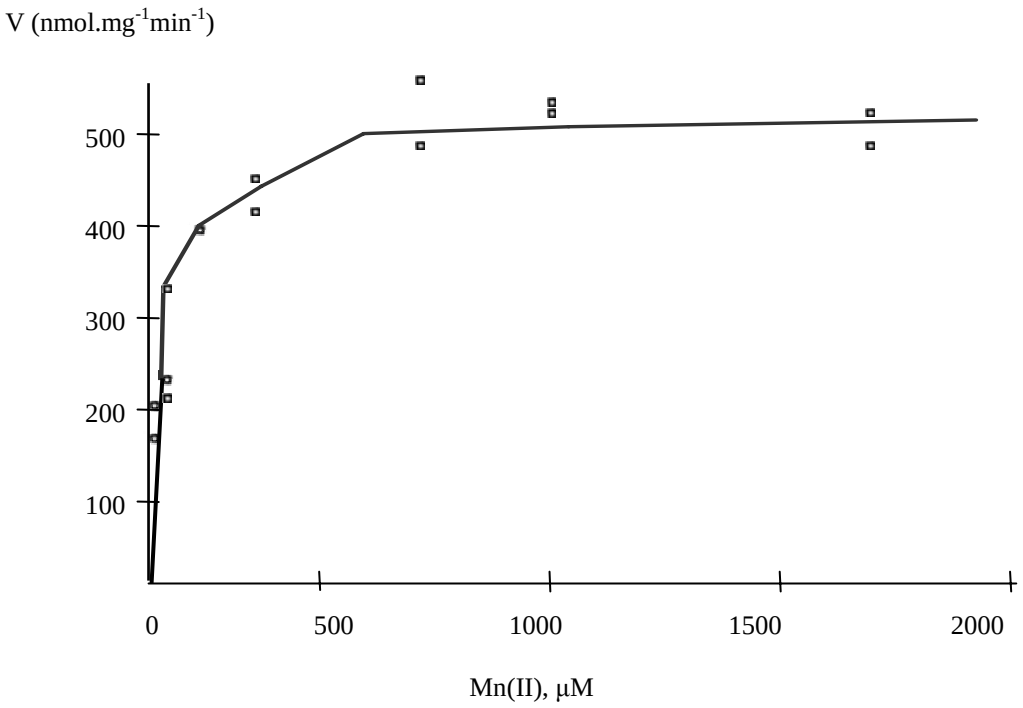



Fig. 3.32: Manganese dependence of the native arginase. Approximately 1 mM of Mn (II) was used to achieve maximum activity of the native arginase. The results are illustrated in the form of a non-linear regression plot. Each point represents a single measurement.

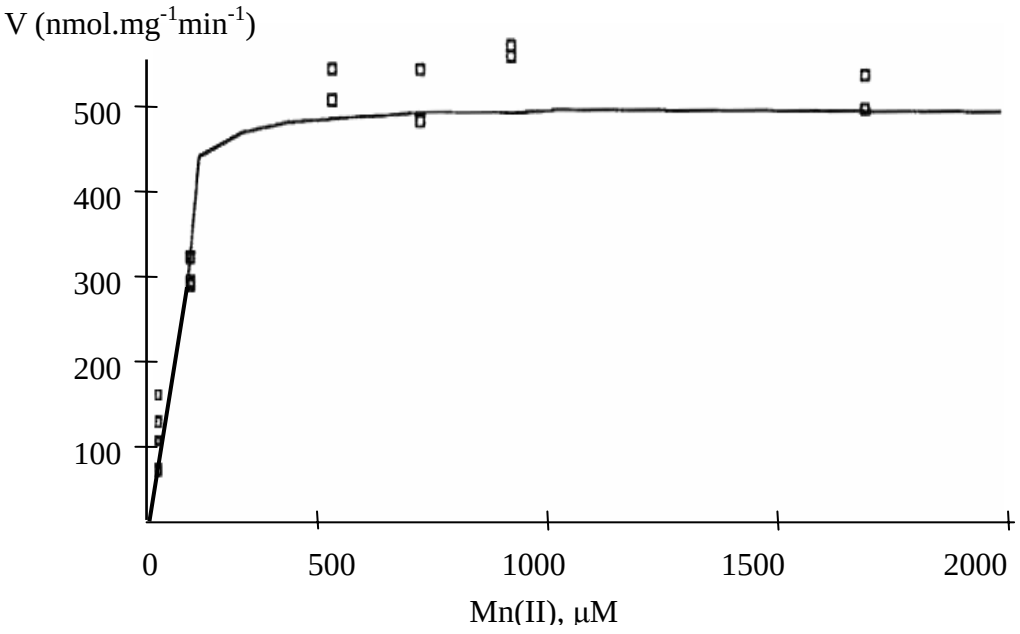
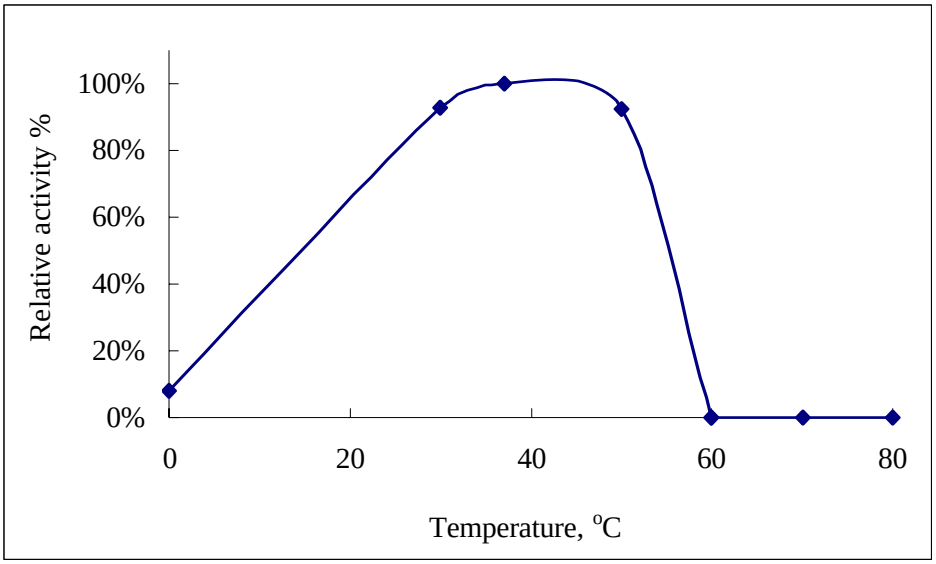



Fig. 3.33: Manganese dependence of arginase-SPA-$PEG_{5000}$. Approximately 1 mM of Mn (II) was used to achieve maximum activity of arginase-SPA-$PEG_{5000}$. The results are illustrated in the form of a non-linear regression plot. Each point represents a single measurement.

### 3.4.3 Optimum temperature and pH

The optimum temperature and pH for activity of the native arginase and arginase-SPA-$PEG_{5000}$ were investigated in this experiment. The native arginase and arginase-SPA-$PEG_{5000}$ were exposed to different pH and temperatures and the enzyme activity was measured by activity assay using an amino acid analyzer. The results are illustrated from Fig. 3.34 to Fig. 3.35. It was found that the native arginase exhibited maximum activity at temperature 30-50$^{o}$C and arginase-SPA-$PEG_{5000}$ exhibited maximum activity at temperature 50$^{o}$C. The optimum pH value for arginase-SPA-$PEG_{5000}$ was pH 10.5 for maximum activity.

(A)




(B)

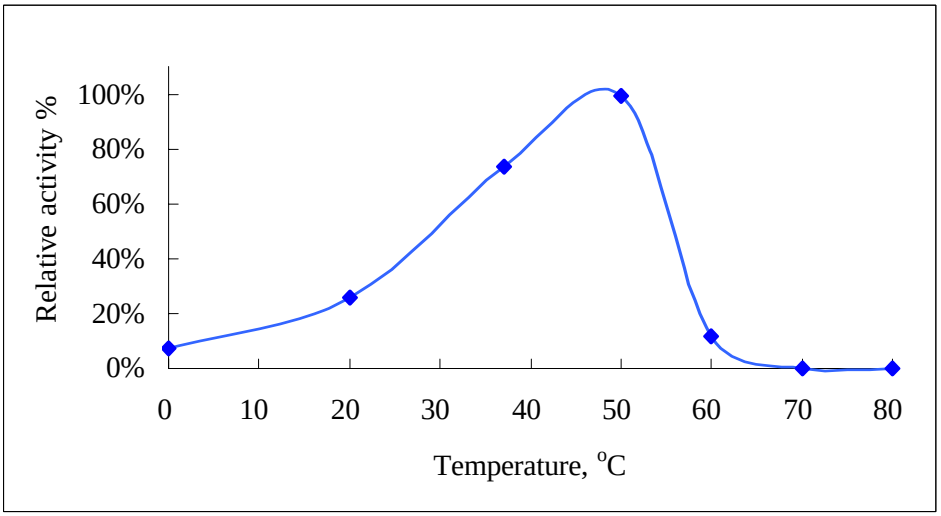



Fig. 3.34: Effect of temperature on the activity of the native arginase (A) and arginase-SPA-$PEG_{5000}$ (B). Samples were exposed in different temperature conditions (0-80$^{o}$C). Each point represents a single measurement.

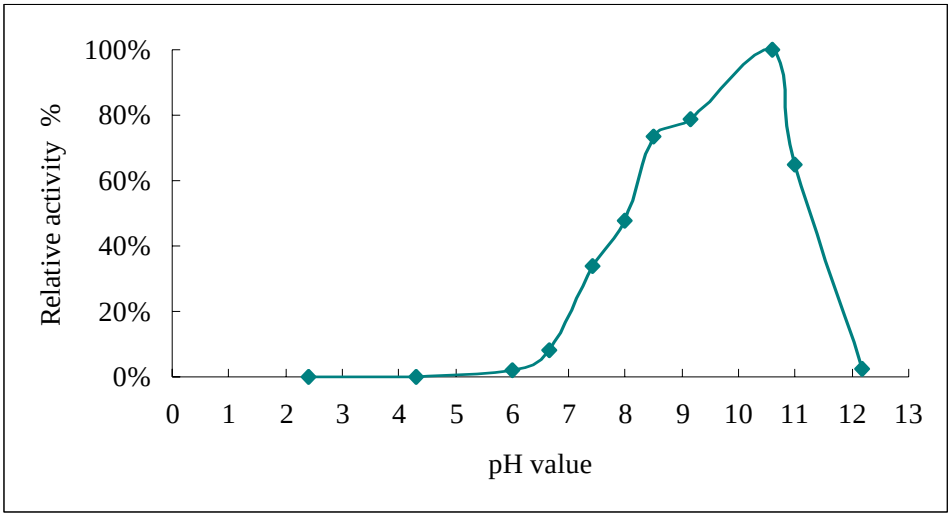



Fig. 3.35: Effect of pH change on the activity of arginase-SPA-$PEG_{5000}$. Pegylated arginase was exposed in different pH conditions (pH 2.5 to pH 12). Each point represents a single measurement.

### 3.4.4 Determination of pI value

Isoelectric focusing (IEF) separates proteins on the basis of surface charge as a function of pH. Isoelectric focusing was used for the determination of the pI value of the native arginase. Polyacrylamide gel was used as the non-sieving medium for pI determination. Broad range calibration kit (pH 9.3- 3.5) was used as the pI marker. The result is shown in Fig. 3.36. The pI value of the native arginase calculated from the broad range calibration kit was 9.01 and the standard deviation (SD) was ±0.045.

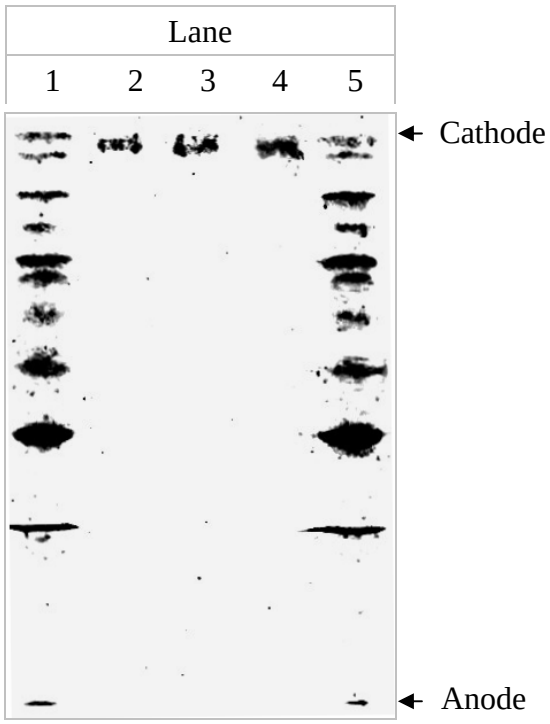



Fig. 3.36: Isoelectric focusing of the native arginase on polyacrylamide gel. Lanes 1 & 5: Broad pI Calibrator; Lanes 2-4: purified arginase. Component of the broad pI Calibration kit :Trypsinogen, pI 9.3; Lentil lectin-basic, pI 8.65; Lentillectin-middle, pI 8.45; Lentillentin-acidic, pI 8.15; Horse myoglobin-basic, pI 7.35; Horse myoglobin-acidic, pI 6.85; Bovine carbonic anhydrase B, pI 5.85; β-lastoglobulin A, pI 5.2.

### 3.4.5 Determination of the molecular weight of arginase

Molecular weight of the native arginase and arginase-SPA-$PEG_{5000}$ were estimated by 12% SDS-PAGE as well as gel filtration chromatography.

#### 3.4.5.1 SDS-PAGE analysis

SDS-PAGE analysis was carried out to estimate the molecular weight of the native arginase and arginase-SPA-$PEG_{5000}$. From Fig. 3.37, the size of the native arginase was about 35 kDa and the size of arginase-SPA-$PEG_{5000}$ was larger than 97 kDa.

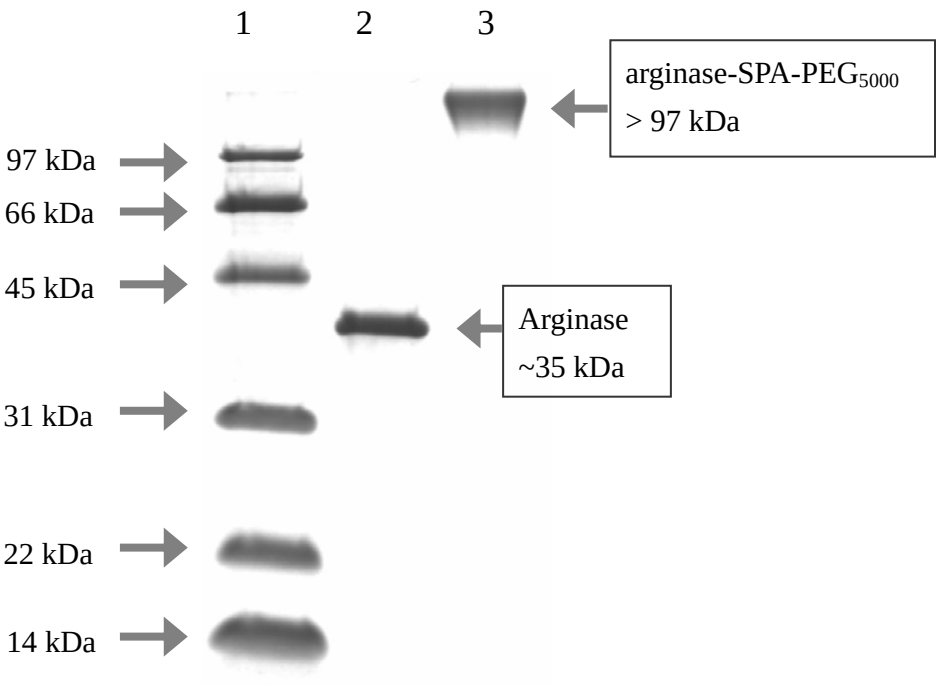



Fig. 3.37: 12% SDS-PAGE of the native arginase and arginase-SPA-$PEG_{5000}$.

Lane 1: low range protein marker; Lane 2: native arginase; MW= ~35 kDa,

Lane 3: arginase-SPA-$PEG_{5000}$, MW = >97 kDa.

### 3.4.5.2 Gel filtration chromatography

Gel filtration chromatography was used to determine the molecular weight of the native arginase and arginase-SPA-$PEG_{5000}$. Both low and high molecular weight gel filtration kit were used as molecular weight standards and HiLoad 16/60 Superdex gel filtration column was used. The result for the molecular weight markers is illustrated in Fig. 3.38. The elution profiles for the native arginase and argainse-SPA-$PEG_{5000}$ are shown in Fig. 3.39 and Fig. 3.40, respectively.

The size of the native arginase determined by gel filtration chromatography was about 82 kDa. It suggested that the native arginase protein existed as a dimer because the size of an arginase subunit determined by 12 % SDS-PAGE was ~35 kDa. The size of argainse-SPA-$PEG_{5000}$ determined by gel filtration chromatography was about 691 kDa, but the oligomeric structure of argainse-SPA-$PEG_{5000}$ cannot be determined directly from the gel filtration results.

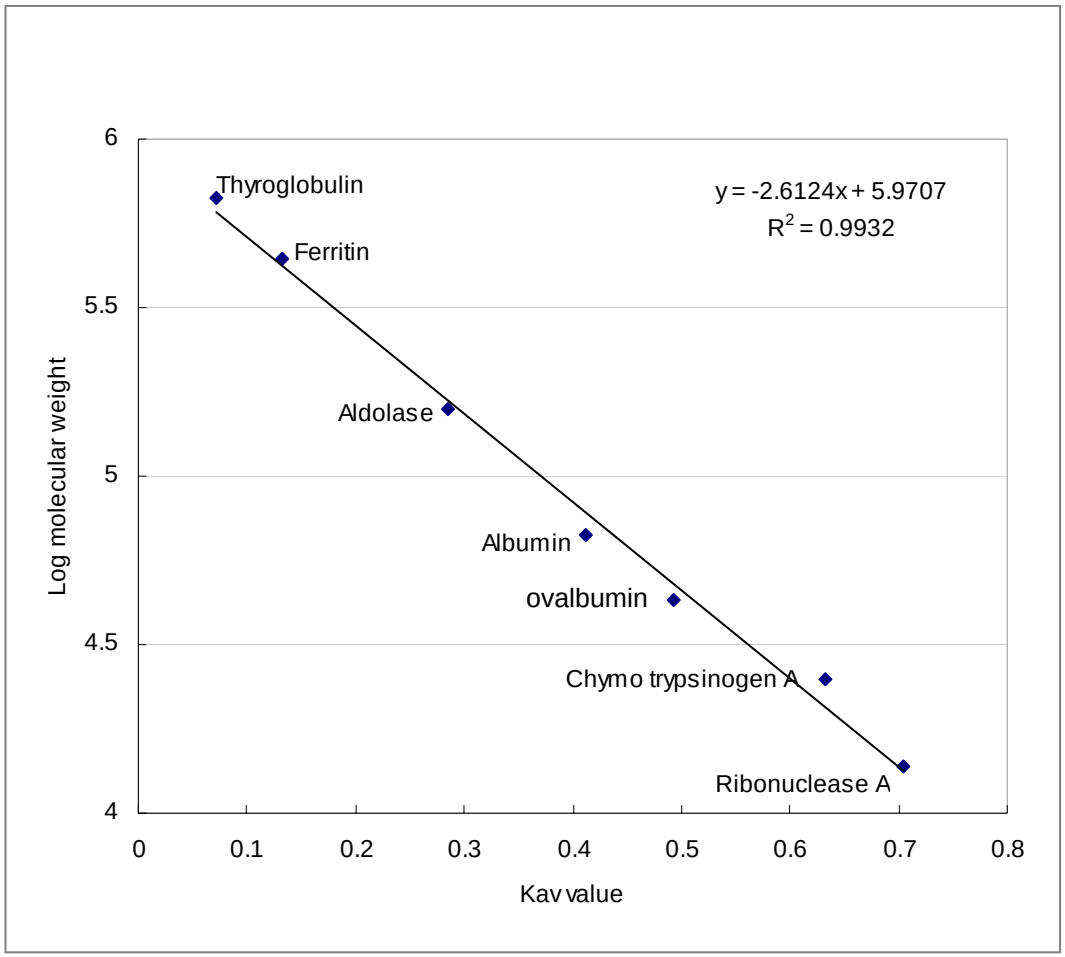



Fig. 3.38: Standard curve of the low and high molecular weight markers. The standard curve was plotted by log MW against the $K_{av}$ value of each marker and seven molecular weight markers were used (Thyroglobulin 669 kDa, Ferritin 440 kDa, Aldolase 158 kDa, Albumin 67 kDa, Ovalbumin 43 kDa, Chymotrypsinogen A 25 kDa and Ribonuclease A 13.7 kDa). The $K_{av}$ value was calculated by: $K_{av}$ value = (elution volume of MW marker – column void volume) / (column total bed volume – column void volume).

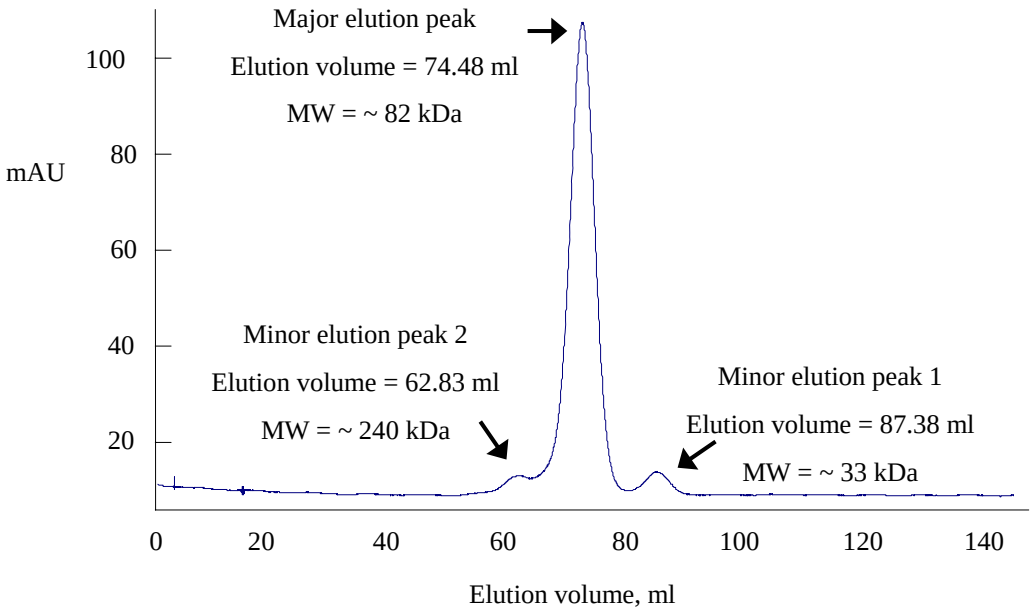



Fig. 3.39: Elution profile of the native arginase (5.7 mg/ml). Three peaks were observed from the elution profile. The major peak indicates the native arginase with a size of 82 kDa (dimer), the minor peak 1 shows arginase with a size of about 33 kDa (monomer) and the minor peak 2 shows arginase with a size of about 240 kDa (hexamer).

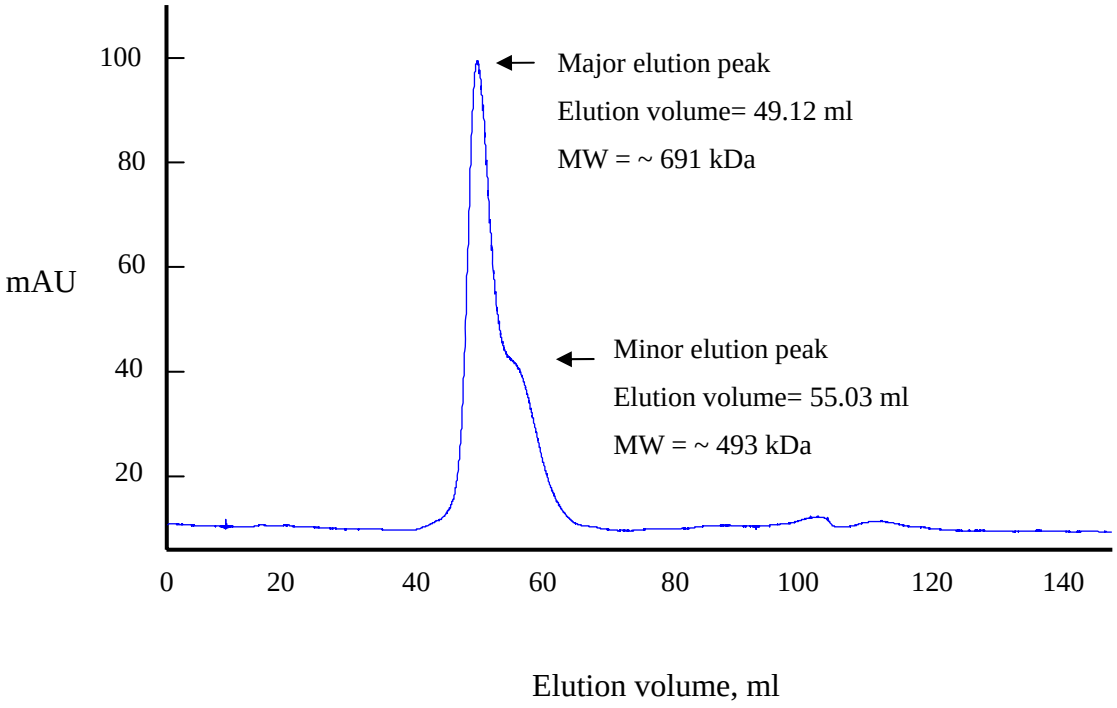


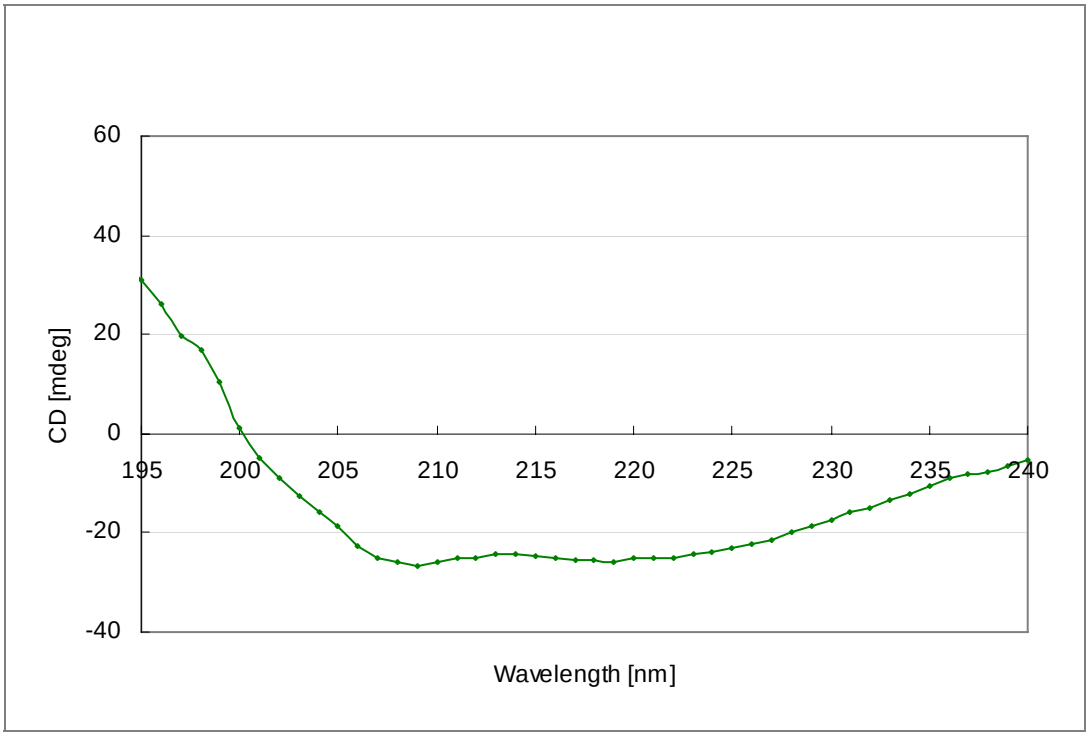

Fig. 3.40: Elution profile of the arginase-SPA-$PEG_{5000}$ (5.4 mg/ml). Two peaks were observed from the elution profile. The major peak shows pegylated arginase with a size of 691 kDa and the minor peak shows pegylated arginase with a size of 493 kDa.

### 3.4.6 Circular dichroism spectroscopic analysis

Circular dichroism (CD) was used to study the secondary structure of arginase. CD spectroscopy is a form of light absorption spectroscopy that measures the difference in absorbance of right- and left-circularly polarized light by a substance. CD spectra between 260 and approximately 180 nm can be analyzed for the different secondary structural types: alpha helix, parallel and antiparallel beta sheet, turn, and others (Sreerama *et al*., 2000).

For CD experiments, high purity of enzyme was required. Both the native arginase and arginase-SPA-$PEG_{5000}$ were studied by CD experiments. The changes in the secondary structures of the arginase-SPA-$PEG_{5000}$ compared to the native arginase were examined by the CD spectroscopy in the far-UV region of the spectra covering the wavelength range 190-250 nm. The CD spectra generated from the native arginase and arginase-SPA-$PEG_{5000}$ are shown in Fig. 3.41. The CD spectra of the two enzymes exhibited a negative band (magnetic dipole) centered at around 200 nm and a positive band (electric dipole) at around 195 nm. This suggested that arginase-SPA-$PEG_{5000}$ preserved a similar form of secondary structure comparable to the native arginase. Secondary structure compositions of the native arginase and arginase-SPA-$PEG_{5000}$ estimated by CD experiments are shown in Table 3.4.

(A)




(B)

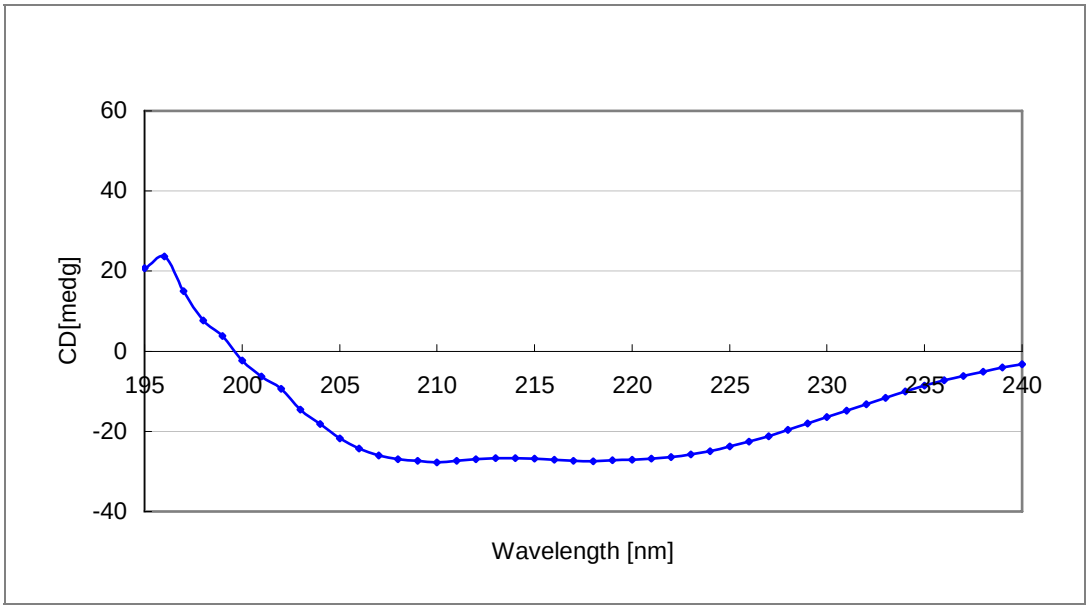



Fig. 3.41: CD spectrum of the native arginase (A) and arginase-SPA-$PEG_{5000}$ (B). All measurements were preformed in 10 mM potassium phosphate buffer at pH 7.0 using a protein concentration of about 0.3 mg/ml. Spectra were generated by the Jasco J-810 spectropolarimeter.

Table 3.4: Secondary structure compositions of the native arginase and arginase-SPA-$PEG_{5000}$ estimated by CD experiments.

| | Native arginase | Arginase-SPA-$PEG_{5000}$ |
|---|---|---|
| α-Helix | 24.9 % | 23.7 % |
| β-Sheet | 27 % | 27.6 % |
| Turns | 12.5 % | 16.5 % |
| Random structure | 35.6 % | 32.2 % |

### 3.4.7 *In vitro* half-life studies

The circulating half-life of arginase-SPA-$PEG_{5000}$ was studied by *in vitro* experiments. Arginase-SPA-$PEG_{5000}$ was incubated in human plasma at 37$^{o}$C for 17 days. The activity of arginase-SPA-$PEG_{5000}$ was determined each day during the incubation. The results are shown in Fig. 3.42. The activity half-life of arginase-SPA-$PEG_{5000}$ was about 3 days, indicating that it took 3 days for its relative activity to be reduced from 100% to 50%.

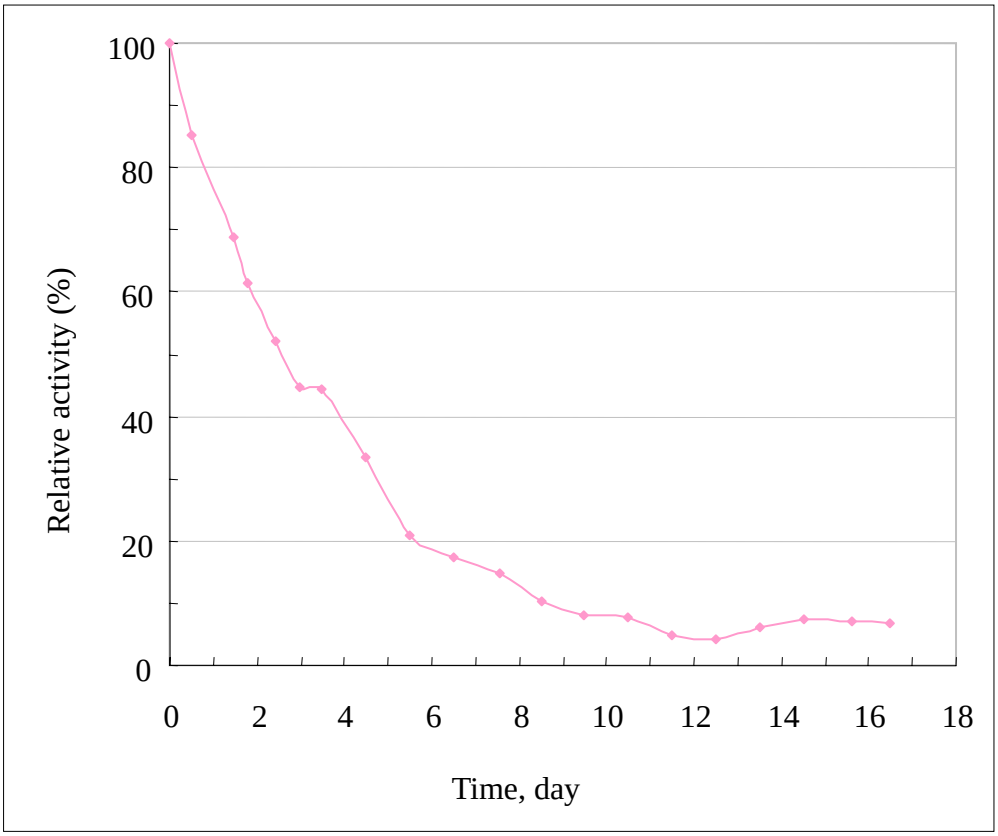


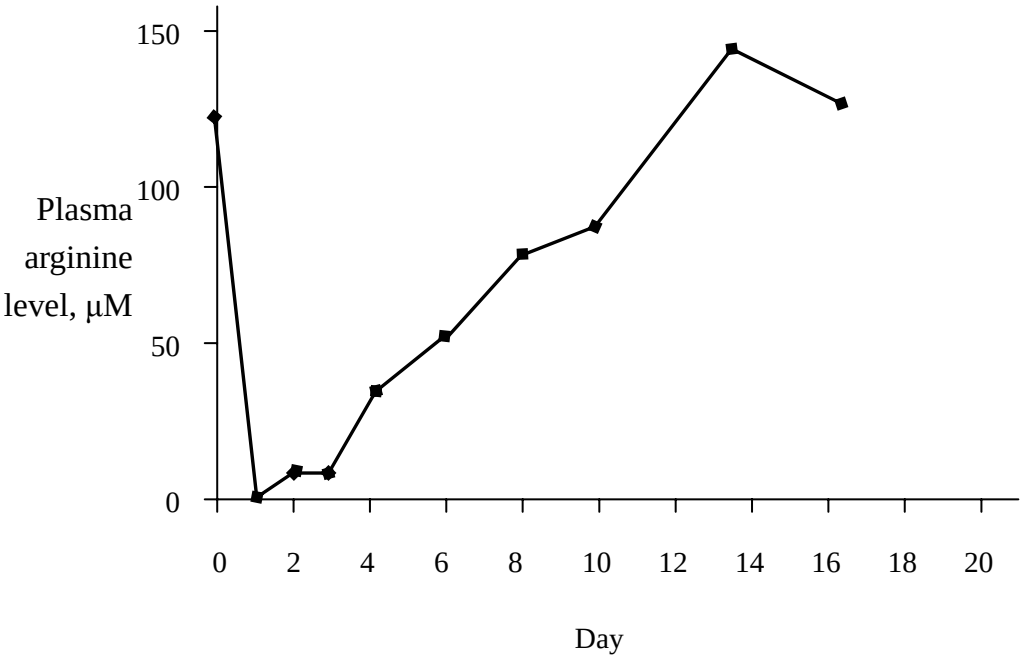

Fig. 3.42: Time course of relative activity of arginase-SPA-$PEG_{5000}$ in blood plasma for 17 days. Activities of arginase-SPA-$PEG_{5000}$ were monitored each day by the $A_{340}$ assay. Each point represents a single measurement.

### 3.4.8 *In vivo* arginine depletion by the pegylated arginase in rat

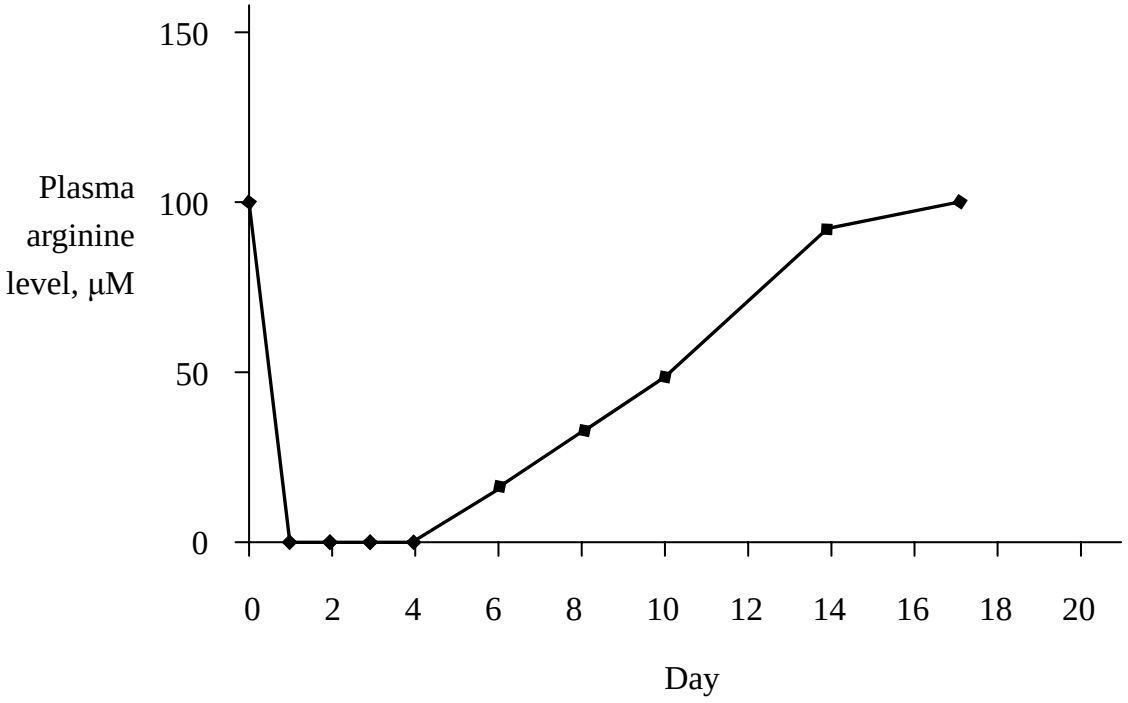
Four different dosages (500 I.U., 1000 I.U., 1500 I.U. and 3000 I.U.) of arginase-SPA-$PEG_{5000}$ were injected into rats. The rat plasma arginine levels after intraperitonal administration of arginase-SPA-$PEG_{5000}$ were detected from day 0 to day 17. The results are illustrated in Fig. 3.43.

As shown in Fig. 3.43, administration with dose 500 I.U. could deplete arginine in the rat plasma for 1 day. For the administration of 1000 I.U., undetectable arginine levels were achieved and a 4 day period with complete arginine depletion was observed. For the administration of 1500 I.U. and 3000 I.U. of arginase-SPA-$PEG_{5000}$, a period of 6 day arginine depletion was observed.

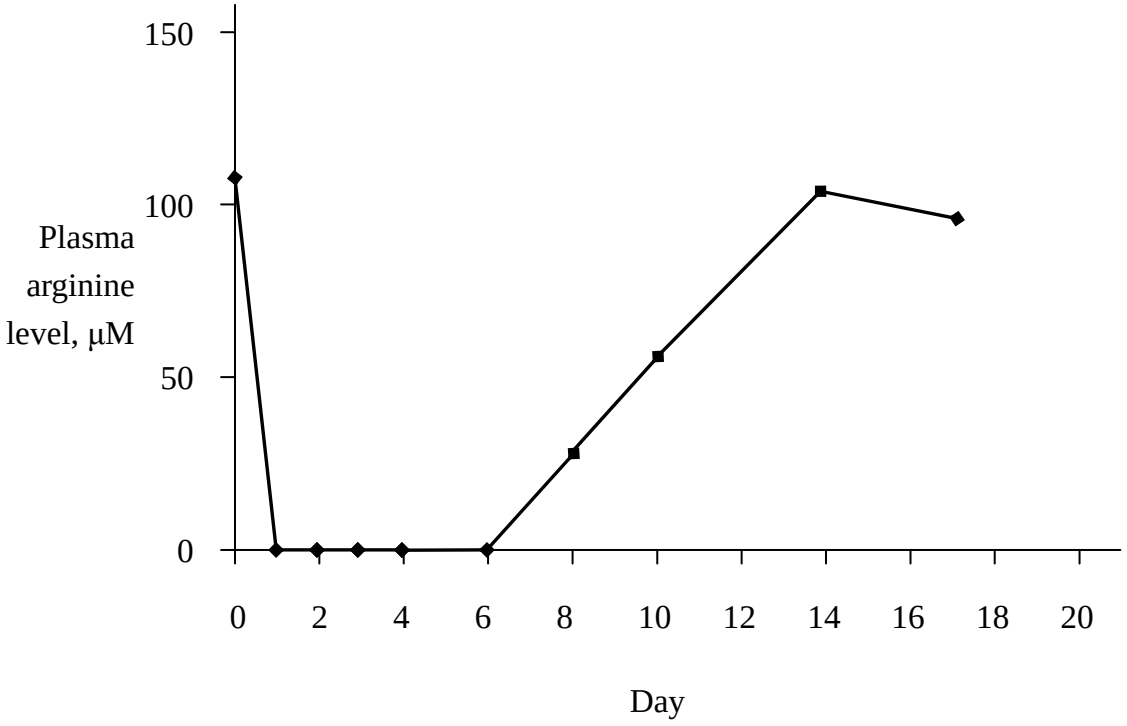
**(A) 500 I.U.**




**(B) 1000 I.U.**



**(C) 1500 I.U.**




**(D) 3000 I.U.**

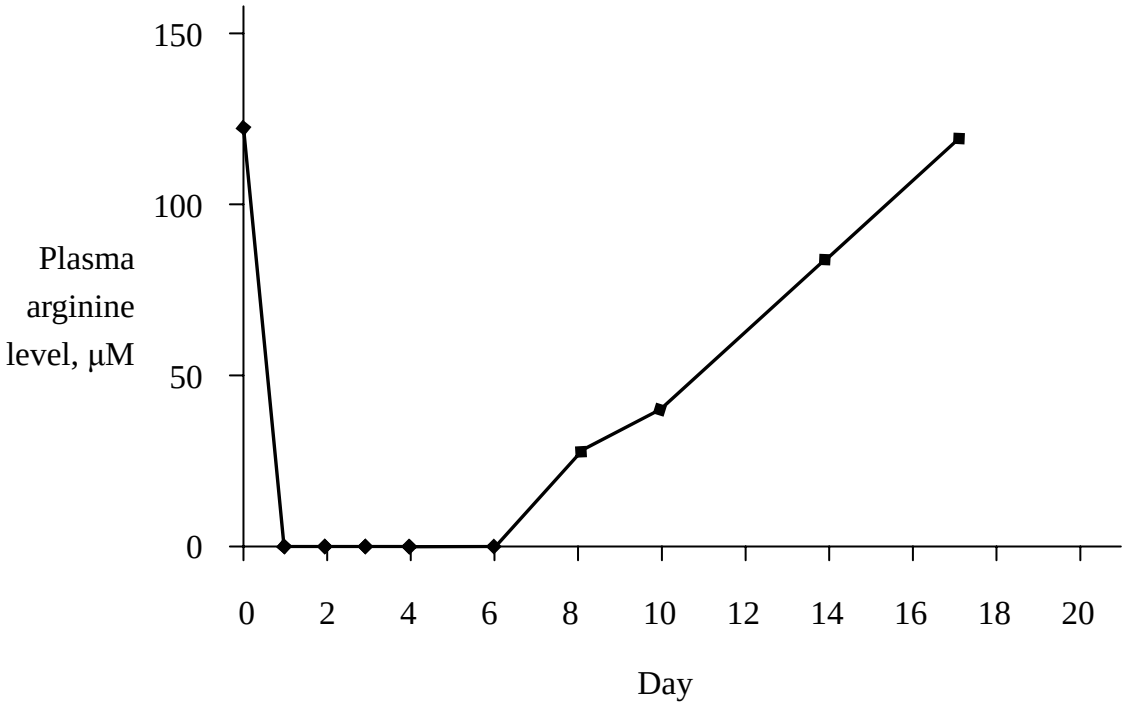



Fig. 3.43: Effect of single dose administration of (A) 500 I.U.; (B) 1000 I.U.; (C)

1500 I.U.; (D) 3000 I.U. of arginase-SPA-$PEG_{5000}$ on rat blood plasma arginine level. One male rat and one female rat were used for each dose in this experiment.

# Chapter Four:
# Discussion

## 4.1 Overexpression of arginase

Arginine deprivation has been demonstrated to have anti-tumour activities *in vitro* (Wheatley *et al*., 2000). The application of enzymatic degradation of arginine by arginase for the treatment of cancer has attracted much interest. In order to produce sufficient amount of arginase for studies, recombinant DNA technology was used.

Recombinant DNA technology has been used for the production of a whole range of possible human therapeutic agents in sufficient quantities for both efficacy testing and eventual human use. A large number of proteins that have the potential to become pharmaceutical agents have been “synthesized” from cloned genes in bacteria. The strategy is first to isolate the target gene from a cDNA library and then subclone the selected target gene into an appropriate expression vector. Finally, a novel and authentic protein product can be produced.

The first recombinant human liver arginase has been successfully produced and purified in *E. coli* (Ikemoto *et al.*, 1990). However, the main drawback of using *E. coli* as the host of expression has been the presence of endotoxins which are unacceptable if present in therapeutically used products.

In this project, the recombinant human liver arginase was expressed in a prophage–based *Bacillus subtilis* expression system. *B. subtilis* has the advantage

of non-pathogenic and produces no endotoxins, has been granted the "generally regarded as safe" (GRAS) status in the U.S. This prophage–based *B. subtilis* expression system has also been used for the high-level overproduction of a cytoplasmic protein β-galactosidase (βGal) and a secreted β-lactamase (BlaI) (Thornewell *et al*., 1993). The human liver arginase gene was put under the control of a heat-inducible promoter in this expression system (Leung and Errington, 1995). The N-terminus of the recombinant arginase was fused to six histidines to facilitate purification and the protein was produced intracellularly.

### 4.1.1 Shake-flask culture

The His-tagged human liver arginase encoding gene was first expressed in *B. subtilis* in shake-flask culture. Shake-flask culture is a convenient method of growing microorganisms in submerged culture. The purpose of shaking is to supply oxygen and nutrients to the growing cells. Since *B. subtilis* requires large amount of oxygen for growth, baffled flasks were used to enhance the dissolved oxygen level in the culture medium. The shaking speed required for the growth of *B. subtilis* was relatively high, which is about 300 rpm (for other bacteria, e.g., *E. coli*, the shaking speed required for growth is about 200 rpm). Arginase expression in our *B. subtilis* system was initiated by heat-induction at $OD_{600}$ of

3.5-4.5. This induction condition was determined empirically; both the cell density and physiological state of cells were considered and optimized. After heat shock, the culture was grown for 6 h and harvested. The method of enzyme lysis was used for disrupting *B. subtilis*. The cell walls are hydrolyzed by the enzyme lysozyme, which is isolated from egg whites. Lysozyme treatments for cell disruption are highly specific and the conditions for lysis are mild. However, cost consideration limits the use of lysozyme in cell lysis. The SDS-PAGE analysis (Fig. 3.2) of expression in *B. subtilis* showed that the protein band corresponding to arginase can be observed but not very obvious. It demonstrated that the expression level of arginase was not high enough. According to the total amount of proteins of crude extract and the purified arginase, it was estimated that the expressed arginase was approximately 5 % of the total cellular proteins.

Some optimizations of the fermentation conditions of *B. subtilis* in shake-flasks were carried out. From Fig. 3.3 and Fig. 3.4, the growth of the *B. subtilis* strain LLC101 was greatly inhibited when the pH was low (pH 5)**.** However, the growth of the bacteria increased significantly when pH value of the medium was increased to 6. Further increase of the pH values of the medium from 6 up to 9 did not have significant effects on the growth of the bacterial cells. From the result of Section 3.1.1.2, we knew that the richer BHY medium, which

contained 3.7% brain heart infusion, could achieve a better cell growth than the 0.5 % glucose medium. However, the BHY medium was a expensive complex medium containing bovine brain heart infusion and it will be difficult to manipulate after scaling up or for further studies. Finally, the glucose medium was selected to be used in the fed-batch fermentation.

### 4.1.2 Purification of arginase from shake-flask culture

Proteins destined for diagnostic or therapeutic applications must be purified to a very high degree. This is particularly true in the case of any protein preparation that is to be administered to the animals. The high level of purity demanded in arginase, which is designed for therapeutic use, is necessary to minimize or eliminate the occurrence of adverse reactions against trace contaminants in the protein products.

On the other hand, for studying the properties of recombinant arginase, the crude protein extracts were also required to be purified to isolate pure arginase. The purification of arginase was carried out by four steps: (1) affinity chromatography, (2) a desalting step, (3) ion-exchange chromatography and (4) another desalting step.

As mentioned before, the N-terminus of the recombinant arginase was fused

with six histidines (the 6xHis tag), HiTrap chelating affinity chromatography (5 ml) charged with $Ni^{2+}$ ions was used at the beginning of the purification. The 6xHis tag incorporated in the arginase facilitated the purification of the protein because the poly-histidine tag can bind a number of metals, thus a purification tag consisting of 6 histidines may be employed to purify proteins by metal chelating chromatography, which is carried out on a suitable matrix to which nickel ions have been immobilized.

Arginase fractions eluted and collected from HiTrap chelating chromatography possessed a high salt content; it was desalted by a desalting step using the HiPrep 26/10 desalting chromatography column (53 ml) and the $AKTA_{FPLC}$ system. The elution buffer used was 10 mM Tris-HCl buffer (pH 7.5). The protein sample was eluted first and the salts were eluted out later from the column. The desalting step finished a complete run in several min, which is faster than the traditional dialysis process.

After that, ion-exchange column chromatography was selected for further purification. A pre-packed SP Sepharose Fast Flow chromatography column was used. The principle of ion-exchange chromatography is based on the attractive forces between molecules carrying opposite charges. A protein has a net charge of zero at pH equal to its iosoelectric point (pI). However, if the pH of the

environment is lower than its pI value, the protein will be positively charged. If the pH of the environment is higher than its pI value, the protein will be negatively charged.

The pI value of the recombinant human liver arginase reported was about 10 (Ikemoto *et al*., 1990), and the pH of the purification condition was 7.5, hence, arginase was positively charged and bound to a cation exchanger. Therefore, SP Sepharose Fast Flow ion exchange chromatography was selected in our studies for arginase purification. After ion exchange chromatography, salts were removed by another desalting column.

After using the purification procedures mentioned above, the purity of the purified arginase was analyzed by 12% SDS-PAGE. From the results (Fig. 3.9), it was observed that the arginase enzyme was approximately 95% pure. Therefore, this purification method is efficient for arginase purification; also, the purification method developed is simple, rapid and convenient. For further analysis of the arginase enzyme, high purity of this enzyme is required. The purification of arginase was started from 1.6 L of culture, and the purified enzyme yield was about 30 mg/ L.

## 4.2 Production of arginase from the fed-batch fermentation

After the shake-flask experiments, it was important to ensure that arginase could be expressed in large quantities after the scale-up of fermentation. Arginase was produced using a 10-L fermentor containing 8 L of culture under fed-batch conditions.

Fermentation can be carried out in batch or fed-batch mode. In batch fermentation (e.g., shake-flask experiment), the sterile growth medium was inoculated with *B. subtilis* and the fermentation was preformed without further additions of fresh growth medium. In fed-batch fermentation, nutrients were added incrementally at various time points during the fermentation reactions, no growth medium was removed until the end of the process. These additions prolong both the log and stationary phases, thereby increasing the biomass and the amount of the metabolites produced. Generally, fed-batch fermentation requires more monitoring and greater control than batch fermentation. However, fed-batch fermentation improved the development of the systems for the production of arginase from *B. subtilis* and finally increased the yield of product.

The results of batch (shake-flask experiments) and fed-batch fermentation are summarized in Table 4.1. The average yield of arginase from fed-batch fermentation was 104.7 mg/ L of cell culture. It is concluded that the use of

fed-batch fermentation yielded about 300 % more arginase than batch fermentation.

Table 4.1: Summary of the yields and specific activities of arginase from different fermentation trials.

| | Yield of arginase (mg of protein / L of cell culture) | Specific activity (I.U./mg) |
|---|---|---|
| Shake-flask fermentation (1.6 L) | ~ 30 mg/L | / |
| Fed-batch fermentation (8 L) 01 | 134 mg/L | / |
| Fed-batch fermentation (8 L) 02 | 94 mg/L | 336 |
| Fed-batch fermentation (8 L) 03 | 102 mg/L | 309 |
| Fed-batch fermentation (8 L) 04 | 94 mg/L | 592 |
| Fed-batch fermentation (8 L) 05 | 87 mg/L | 479 |
| Fed-batch fermentation (8 L) 06 | 104 mg/L | 596 |
| Fed-batch fermentation (8 L) 07 | 117 mg/L | 511 |

### 4.2.1 Purification of arginase from fed-batch fermentation

A nickel-nitrilotriacetic acid (Ni-NTA) metal-affinity chromatography column (130 ml) was used to purify arginase from the 8-L of culture instead of HiTrap chelating chromatography. The Ni-NTA resins will also bind to bio-molecules that are tagged with poly-histidine residues and the advantage of using the Ni-NTA affinity column is to allow a single step purification. After arginase has been eluted from the Ni-NTA chromatography column, desalting was carried out by a tangential flow filtration (TFF) system instead of using the HiPrep 26/10 desalting chromatography column (53 ml).

As shown in Table 4.1, the amount of arginase yielded from fed-batch fermentation was 3 times higher than shake-flask fermentation, and a total of about 1-L of total fractions containing arginase was eluted from the Ni-NTA column. The loading volume for the HiPrep 26/10 desalting column was only 10 ml, therefore, it is not suitable for desalting purpose.

## 4.3 Pegylation of arginase

The disadvantages of using the native arginase as a therapeutic agent are the short circulation half-life and the immunogenicity. Moreover, recombinant arginase produced in this project was tagged with 6 histidines. To eliminate any adverse effect from the 6xHis tag, the arginase was modified with polyethylene glycol (PEG).

Pegylation is a well developed process and can be applied to therapeutic proteins to give a number of potential clinical advantages such as enhanced pharmacologic activity, increased half-life and reduced immunogenicity. Improved properties of pegylated proteins also include better protection against susceptibility to enzymatic degradation, longer *in vivo* circulating half-life and decreased renal clearance (Harris *et al*., 2001).

Pegylated arginase was prepared using mPEG-SPA to produce arginase-SPA-$PEG_{5000}$. Methoxy-PEG molecules are amphiphilic polymers comprised of repeating ethylene oxide subunits with a methoxy group at one end and a terminal hydroxyl group that can be chemically activated at the other end. PEG molecules are available in a variety of configurations and molecular weights.

To achieve the desired pharmacological properties, the process to produce pegylated arginase has to be optimized in terms of the molecular weight of the PEG groups, the configuration of the PEG group and the experimental conditions for the pegylation reactions. Appropriate choice of the mass and configuration of the PEG group was the key to balance the desired prolonged plasma elimination half-life of arginase as well as to maintain the arginine depleting activity.

In this project, arginase was pegylated with five different PEGs. They are mPEG-N-hydroxysuccinimide, MW= 10,000 (mPEG-NHS); mPEG-propionaldehyde, MW= 5,000 (mPEG-ALD); mPEG-maleimide MW= 5,000 (mPEG-MAL); mPEG-succinimidyl propionate MW= 5,000 **(**mPEG-SPA) and cyanuric chloride activated PEG (mPEG-CN).

The most frequently used points for attachment of PEG molecules are lysine and cysteine. For the five PEGs used in this experiment, mPEG-MAL is a cysteine selective PEG and it was attached to arginase via the thiol-containing amino acid cysteine. mPEG-NHS, mPEG-ALD, mPEG-SPA and mPEG-CN are all lysine-active PEGs. Stable amide linkage was formed between the amine groups (such as lysine and other nucleophiles) of arginase and the PEG molecules.

The choice of PEG molecules for arginase is a compromise between the size,

configuration of PEG and the retention of specific activity of arginase. From the results, we found that mPEG-NHS and mPEG-ALD were not suitable for the pegylation of arginase. In the SDS-PAGE analysis of mPEG-NHS and mPEG-ALD, a large band of native arginase was still observed after pegylation (Fig. 3.19 and Fig. 3.21). The results indicated that more than 70% of arginase was remaining native after pegylation. Possible explanation is that mPEG-NHS and mPEG-ALD were not suitable for coupling to arginase under a mild pegylation condition (in PBS buffer, pH 7.4, room temperature). However, the recombinant arginase produced was targeted for therapeutic use; therefore, adverse pegylation condition was avoided.

mPEG-MAL was also not recommended for arginase pegylation because the size of the pegylated arginase produced was relatively small (when compared with mPEG-SPA). Small pegylated proteins may not have enough protection from degradation (Bowen *et al*., 1999). In fact, there is a direct correlation between the molecular size of the pegylated protein and the half-life of a pegylated protein because the radius of the PEG molecules is inversely related to the rate of renal clearance (Baker *et al*., 2001).

mPEG-CN was once the most widely used reagent for the activation of PEG and protein pegylation. Early in 1979, pegylated bovine liver arginase was

generated successfully with mPEG-CN (Savoca *et al.*, 1979). But the coupling condition required for mPEG-CN was a very basic condition (pH 9) and the pegylated arginase produced lost about 60% of activity (Fig. 3.24). mPEG-CN was therefore not selected for the pegylation of arginase. The decrease in specific activity after pegylation may result from either a simple steric hindrance of the protein for its biological reception or conformational changes which may occur upon pegylation and thus directly influence the receptor-ligand interaction of the protein.

Finally, mPEG-SPA was selected for arginase pegylation because mPEG-SPA was readily coupled with arginase under mild conditions (Fig. 3.23) and nearly 90% of enzyme activity was retained (Fig. 3.24). The optimum mole ratio of arginase to mPEG-SPA molecules was 1: 50. With the addition of such mole ratio of PEG, all the arginase can be pegylated and resulted in an increased protein size (Fig. 3.29).

## 4.4 Characterization of the pegylated arginase and its kinetic analysis

Characterizations were carried out to determine if there are enhancement or changes in properties of the pegylated arginase compared with the native arginase.

### 4.4.1 Kinetic analysis

The specific activity of arginase and arginase-SPA-$PEG_{5000}$ are about 500 I.U./mg (Table 4.1) when determined with the $A_{340}$ activity assay. In this project, the kinetic properties of the arginase and arginase-SPA-$PEG_{5000}$ were studied and compared.

The meaning of $K_m$ is evident from Michaelis-Menten equation, which is often associated with the affinity of the enzyme for their substrate. Also, $K_m$ is a measurement of the substrate concentration required for effective catalysis to occur. $K_m$ is equal to the substrate concentration at which the reaction rate was half of its maximal value. On the other hand, $k_{cat}$ is a direct measurement of the catalytic production of product under optimum conditions. The $k_{cat}$ value measures the number of substrate molecules turned over per enzyme molecule per second.

In this experiment, kinetic properties of the purified native arginase and

arginase-SPA-$PEG_{5000}$ were determined. The $K_m$ value of the native arginase for L-arginine was 1.89 ± 0.7 mM and the $k_{cat}$ value of it was 1.798 $s^{-1}$. The $K_m$ value of arginase-SPA-$PEG_{5000}$ for L-arginine was 2.91 ± 0.27 mM. However, the $k_{cat}$ value of arginase-SPA-$PEG_{5000}$ could not be determined since the molecular size of arginase-SPA-$PEG_{5000}$ could not be estimated (arginase-SPA-$PEG_{5000}$ was actually a mixture of pegylated isomers of different sizes). But the average molecular weight of the pegylated arginase might be determined by gel filtration chromatography.

The results suggested that the catalytic properties of arginase were retained after modification with PEG. Also, these results are consistent with the previous studies. For example, the $K_m$ value of the native human liver arginase for L-arginine was determined to be 2.6 mM (Carvajal *et al*., 1999) and the $K_m$ value of the arginase from human erythrocytes for L-arginine was determined as 1.6 mM (Ikemoto *et al*, 1989).

The $K_m$ value of arginase for L-arginine is relatively large, indicating the low affinity of arginase towards arginine. $K_m$ of arginine deiminase (another arginine depleting enzyme) for L-arginine is about 30 μM (Ensor *et al*., 2002). The major drawback for the low substrate affinity of arginase is the requirement of high drug dosage, and it is suggested that the low substrate affinity of arginase

may not be able to deplete arginine in plasma since the normal plasma arginine level is only 100-150 μM. However, our studies on the *in vivo* arginine depletion of pegylated arginase in rats (Fig. 3.43) have demonstrated clearly that arginase-SPA-$PEG_{5000}$ is capable of depleting plasma arginine to an undetectable level.

### 4.4.2 Optimum $Mn^{2+}$ concentration

Arginase has a specific catalytic and physiological requirement for two manganese ions. It is proposed that the function of Mn(II) ions is to bind a water molecule for attacking the guanidino carbon on L-arginine and subsequently as an acid to protonate the production of L-ornithine. The effects of manganese ions on the activity of the native arginase and arginase-SPA-$PEG_{5000}$ were studied. The optimal concentration of manganese ions for activities of both enzymes was 1 mM.

Kanyo reported that the trimeric crystal structure of the rat type I arginase contains 2 Mn(II) ions. It is revealed that both Mn(II) ions are surrounded by negatively charged amino acid residues (Kanyo *et al*., 1996). To address the issue of metal-dependent thermostability and activity, partially purified samples of arginase were subjected to heat activation in the presence of divalent cations,

Mn(II). Thermal denaturation of arginase occurs in the absence of any Mn(II) cations, while all metals examined afforded protection against thermal denaturation. However, only those samples treated with Mn(II) retained enzymatic activity. Activation of arginase in the presence of Mn(II) is intriguing. In our studies, addition of Mn(II) to all purification steps decreased the inactivation effect. However, the presence of Mn(II) may not be favourable to arginase activity since the oxidation of Mn(II) to Mn(IV) which takes place at alkaline pH.

### 4.4.3 Optimum temperature and pH

#### 4.4.3.1 Thermostability

The effects of temperature on the stability of recombinant arginase and arginase-SPA-$PEG_{5000}$ were investigated (Fig. 3.34A & Fig. 3.34B). Both enzymes showed good temperature stability around 50$^{o}$C. All samples were incubated for 10 min, pH 7.4. When the temperature was increased to above 50$^{o}$C, a sharp decrease in enzyme stability was observed. It seems that the activity of arginase will be reduced after pegylation at pH 7.4 (physiological condition).

Although the optimum temperature was changed after pegylation and affecting the drug action of arginase under physiological temperature, the prolonged half-life of arginase by pgeylated still able to compensate for the loss of activity. ADI is another arginine depleting enzyme. ADI would only retaines 50% of enzyme activity after pegylation. However, it is still preferable to pegylate the enzyme because of the significantly advantages were gained in both pharmacodynamic and pharmacokinetic characters of ADI, e.g., prolonged half-life as well as reduced immunogenicity.

#### 4.4.3.2 pH stability

Each enzyme has an optimum pH at which the reaction rate is at its maximum. Small deviations in pH from the optimum value lead to decreased activity due to changes in the ionization of groups at the active site of the enzyme. Larger deviations in pH lead to the denaturation of the enzyme itself due to interference with the many weak noncovalent bonds maintaining its three-dimensional structure. In this project, the catalytic activity of arginase-SPA-$PEG_{5000}$ as a function of pH was determined using the amino acid analyzer. Fig. 3.35 shows the pH optima for arginase-SPA-$PEG_{5000}$; the enzyme activity versus pH dependence exhibited a bell-shaped curve with a pH optimum

around 10.

The intracellular arginine concentrations are in the 0.5–2.0 mM range, given a relatively high *Km* of ~2 mM for arginine at physiological pH has been reported. In addition, arginase has a pH optimum of arginase (pH 9~ 10.5). One question arises: How does arginase catalyze the hydrolysis of arginine inside the cells? Nevertheless, the enzyme is expressed at high levels in mammalian liver, overcoming these catalytic limitations. The alkaline pH optimum of pH 9~ 10.5 for arginase is consistent with previously reported values from studies utilizing crude extracts. The profile is bell shaped, suggesting the presence of two ionizable groups.

pH may affect the result of pegylation, and may generate different positional isomers of pegylated arginase. The effect of pH is based on the pKa values of histidines (6-7), the alpha-amino group of cystine (7-8) and the lysines (9-10). Increasing the pH of the pegylation condition would increase the relative amount of the nucleophilic deprotonated form of lysine which becomes the predominated reactive site. Also, arginase may have structural differences when it is under different pH condition, and produce different isomers of arginase-SPA-$PEG_{5000}$ . Different numbers of PEG molecules could be attached to arginase, like the case reported by.

### 4.4.4 Determination of pI value

Isoelectric focusing separates proteins on the basis of surface charge as a function of pH. Separation was carried out in a non-sieving medium, with the presence of carrier ampholytes to establish a pH gradient. Since protein contains both positively and negatively charge-bearing groups, a particular protein will migrate towards either anode or cathode until it reaches a position in the pH gradient where its net charge is zero (isoelectric point, pI). At this point, migration of the protein will cease and the protein is focused.

As shown in Fig. 3.36, the pI value of the recombinant arginase was 9.0. The pI value of human liver arginase was determined as 9.1 (Christopher *et al.*, 1996) and 10 (Ikemoto *et al.*, 1990) in previous studies. Therefore the calculated pI value of the recombinant arginase is consistent with the results of published literatures. The pI value for arginase-SPA-$PEG_{5000}$ cannot be determined in this experiment. Since the PEG molecules were attached on the surface of the arginase, masking effect from PEG was resulted. The PEG molecules act as an impediment for ionization of surface charge of arginase during isoelectric focusing.

### 4.4.5 Determination of molecular weight of arginase

It is observed that the molecular weight of each subunit of arginase was approximate 35 kDa as compared with a low-range protein marker (Bio-Rad) on a 12% SDS-PAGE (Fig. 3.37). From the studies of the recombinant arginase expressed from *E. coli*, the molecule weight of arginase was estimated as 35 kDa (Ikemoto *et al.*, 1990). Therefore, our result about molecular weight of arginase is consistent with the previous studies.

From the result of SDS-PAGE analysis, the size of arginase-SPA-$PEG_{5000}$ and the quaternary structure of arginase could not be determined, thus gel filtration chromatography was used. In this project, the quaternary structure of arginase in PBS buffer (pH 7.4) was determined using the HiLoad 16/60 Superdex gel filtration column (Amersham Bioscience). From Fig. 3.39, recombinant arginase mainly exhibited a molecular weight of 82 kDa, which suggested that recombinant arginase existed in dimer forms endowed with catalytic activity. In fact, there were some minor forms of arginase detected after gel filtration, which could be due to the presence of monomer and hexamer of arginase. The co-existence of different oligomeric structures of arginase was also reported in previous investigations (Ikemoto *et al*., 1990; Colleluori *et al*., 2000). From Fig. 3.40, the major size of arginase-SPA-$PEG_{5000}$ was determined as ~ 691

kDa. However, the oligomeric structure of arginase-SPA-$PEG_{5000}$ could not be directly determined from the results of gel filtration.

### 4.4.6 Circular dichroism spectroscopic analysis

The far-UV spectra of CD provide an excellent method for following changes in the secondary structure of a protein. Different conformations of the protein have different amide-amide orientations and hence different CD spectra. For CD experiments, the purified arginase and arginase-SPA-$PEG_{5000}$ were studied. The changes in the secondary structure of arginase after pegylation were examined by CD spectroscopy in the far UV region of the spectra covering the wavelength 190-250 nm.

From the results, the CD spectra of native arginase and arginase-SPA-$PEG_{5000}$ showed similar forms. These results further suggested that the PEG modified arginase preserved a similar form of secondary structures comparable to the native enzyme. In Table 4.2 the results of CD experiments are compared with those of the rat liver arginase (Cavalli e*t al.*, 1994). The structure of the recombinant arginase is consistent with the structure proposed in previous studies.

Table 4.2: Determination of secondary structures of arginase, arginase-SPA-$PEG_{5000}$ and rat liver arginase by CD spectroscopy.

| | Native arginase | Arginase-SPA-$PEG_{5000}$ | Rat liver arginase (Cavalli e*t al*., 1994) |
|---|---|---|---|
| α-Helix | 24.9 % | 23.7 % | 22 % |
| β-Sheet | 27 % | 27.6 % | 28 % |
| Turns | 12.5 % | 16.5 % | 17 % |
| Random structure | 35.6 % | 32.2 % | 33 % |

## 4.5 Pharmacological studies of arginase

### 4.5.1 *In vitro* half-life studies

Savoca *et al*. (1979) reported that the blood circulating half-life of native arginase in mice was 12 h. Experiments were carried out by injecting native arginase into mice. The relative arginase activity was found to decrease within 12 h to 10% of its initial value. However, the relative activity of arginase-CN-$PEG_{5000}$ after 12 h was 52 % of its initial value (Savoca *et al*., 1979).

After pegylation, the enzymatic half-life of arginase-SPA-$PEG_{5000}$ may have been enhanced. In order to characterize the prolonged enzymatic half-life of arginase-SPA-$PEG_{5000}$, enzymes were incubated in human plasma (*in vitro*) at 37°C for 17 days. The results indicated that the half-life of the pegylated arginase was about 3 days, showing that arginase-SPA-$PEG_{5000}$ took 3 days to have its relative activity to be reduced from 100% to 50%. When compared with the half-life of the native arginase (about 5 h), the enzymatic half-life of the arginase was increased after PEG modification. However, more experiments should be done in order to demonstrate the *in vivo* circulating half-life of the arginase-SPA-$PEG_{5000}$.

### 4.5.2 *In vivo* arginine depletion of pegylated arginase in rat

In order to demonstrate that the pegylated arginase retained its arginine depleting properties with enhanced pharmacological properties, *in vivo* arginine depletion of arginase-SPA-$PEG_{5000}$ was carried out with rats. As shown in Fig. 3.43, undetectable plasma arginine level was achieved in rats after single administration of arginase-SPA-$PEG_{5000}$ of different doses (500 I.U., 1000 I.U., 1500 I.U. and 3000 I.U.). For administration with dose 500 I.U., the period of arginine depletion was maintained for 1 day. With 1000 I.U., 4 days period of complete arginine depletion was observed. With 1500 I.U. and 3000 I.U., 6 days period of complete arginine depletion was observed. Therefore, 1500 I.U. of arginase-SPA-$PEG_{5000}$ was suggested as the optimal dose for *in vivo* arginine depletion in rats.

The major advantage of the pegylation of arginase is the reduction of immunogenicity, and the effect of repetitive injection of pegylation arginase into mice should be studied. Although such experiment was not carried out in this project, the immunogenicity of another arginine depriving enzyme, ADI (arginine deiminase), was examined after modification with PEG molecules (Frederick *et al*., 2002). Frederick *et al.* (2002) carried out the experiment by injecting both native and pegylated ADI into mice and rabbits. Enzyme was

injected once a week for eight weeks, and the amount of antibodies was measured in week 5 and 9. The result of this experiment showed that pegylated ADI had reduced immunogenicity than the native one. We therefore believe that pegylated arginase ahould also have reduce immunogenicity even if it is repeatedly injected into mice.

## 4.6 Further work

For further study, optimization and production of arginase in larger scale fermentation (e.g., 100 L and 1000 L) can be carried out. Also, molecular engineering can be used to increase the yield of arginase from *B. subtilis*, to direct arginase to be secreted extracellularly or to express arginase from *B. subtilis* using a double-promoter expression system. Since arginase is targeted for pharmacological use, detailed pharmacokinetic and pharmacodynamic studies should be carried out, e.g., the response of the native arginase and arginase-SPA-$PEG_{5000}$ in tumour bearing animals should be investigated.

# Chapter Five:
# Conclusion

In this project, recombinant human liver arginase was successfully produced and purified from *Bacillus subtilis*. The arginase was tagged with six histidines (6xHis) at its N-terminus to facilitate purification. The yield was increased 300% when fed-batch fermentation was used instead of batch (shake-flask) fermentation. Using the method that we have developed, about 100 mg of the arginase was routinely purified to > 95% pure from 1-L of cell culture. The purification protocol for the recombinant arginase comprised of only two steps: a Ni-NTA affinity chromatography step and a desalting step. The developed protocol provides a simple and convenient method for the purification of fully active arginase.

The purified arginase was modified with PEG to enhance its pharmacological activity. mPEG-SPA was used for arginase pegylation to produce arginase-SPA-$PEG_{5000}$. The pegylated arginase has been shown to have an increased molecular size and 90% of its activity retained. Biochemical characterizations of the native arginase and arginase-SPA-$PEG_{5000}$ were carried out to study the changes in enzymatic properties after pegylation. From the enzyme kinetic data, the modification of arginase has not altered its catalytic properties. Moreover, the ability of the arginase to deplete arginine was retained after the PEG modification. Furthermore, the far–UV CD study suggested that

the arginase and arginase-SPA-$PEG_{5000}$ had similar secondary structures. The results were consistent with the published data.

The molecular weight of the recombinant arginase was estimated to be approximate 82 kDa from gel filtration chromatography and about 35 kDa from 12 % SDS-PAGE analysis. Therefore, the data suggested that approximate 90% of the native arginase existed in dimer forms, with co-existence of 10% of monomers and hexamers. The molecular weight of arginase-SPA-$PEG_{5000}$ was estimated to be approximate 691 kDa from gel filtration chromatography.

The pharmacological properties of arginase-SPA-$PEG_{5000}$ were investigated by *in vitro* and *in vivo* studies. The *in vitro* studies demonstrated that the activity half-life of the pegylated arginase was approximate 3 days. The *in vivo* studies using rats have confirmed that the pegylated arginase was able to degrade plasma arginine due to an enhanced circulation half-life. With a single intraperitoneal administration of 1,500 I.U. of arginase-SPA-$PEG_{5000}$, the rat plasma arginine was deprived to an undetectable level for 6 days.

The results obtained from this project have broadened our knowledge on the biological and pharmacological properties of the recombinant human arginase as well as the pegylated arginase. These data will certainly facilitate the development of the recombinant human arginase as pharmacological agents in

the treatment of human cancer.

# References

Ash D. E., Cox J. D., Christianson D. W., “Arginase: a binuclear manganese metalloenzyme”, *Met Ions Biol Syst*, 37:407-28 (2000).

Ash D. E., Scolnick L. R., Kanyo Z. F., Vockley J. G., Cederbaum S. D., Christianson D. W., “Molecular basis of hyperargininemia: structure-function consequences of mutations in human liver arginase”, *Mol Genet Metab*, 64:243-9 (1998).

Baker D. E., “Pegylated interferons”, *Rev Gastroenterol Disord*, 1:87-99, (2001).

Barbul A., “Arginine: biochemistry, physiology, and therapeutic implications”, *J Parenter Enteral Nutr*, 10:227-38 (1986)

Bastone A., Diomede L., Parini R., Carnevale F., Salmona M., “Determination of argininosuccinate lyase and arginase activities with an amino acid analyzer”, *Anal Biochem*, 191:384-9 (1990).

Bell J., Ash D. E., Snyder L. M., Kulathila R., Blackburn N. J., Merkler D. J., “Structural and functional investigations on the role of zinc in bifunctional rat peptidylglycine alpha-amidating enzyme”, *Biochemistry,* 36:16239-46 (1997).

Beloussow K., Wang L., Wu J., Ann D., Shen W. C., “Recombinant arginine deiminase as a potential anti-angiogenic agent”, *Cancer Lett*, 183:155-62 (2002).

Bermudez V. M., Kaplan R., Khan M. A., Kuznia J. N., “Growth of thin Ni films on GaN(0001)-(1 x 1)”, *Phys Rev B Condens Matter*, 48:2436-2444 (1993).

Beruter J., Colombo J. P., Bachmann C., “Purification and properties of arginase from human liver and erythrocytes”, *Biochem J*, 175:449-54 (1978).

Bowen S., Tare N., Inoue T., Yamasaki M., Okabe M., Horii I., Eliason J. F., “Relationship between molecular mass and duration of activity of polyethylene glycol conjugated granulocyte colony-stimulating factor mutein”, *Exp Hematol*, 3:425-32 (1999).

Boyse E. A., Stockert E., Iritani C. A., Old L. J., “Implications of TL phenotype changes in an H-2-loss variant of a transplanted H-2b-H-2a leukemia”, *Proc Natl Acad Sci*, 65:933-8 (1970).

Cama E., Colleluori D. M., Emig F. A., Shin H., Kim S. W., Kim N. N., Traish A. M., Ash D. E., Christianson D. W., “Human arginase II: crystal structure and physiological role in male and female sexual arousal”, *Biochemistry*, 42:8445-51 (2003a).

Cama E., Emig F. A., Ash D. E., Christianson D. W., “Structural and functional importance of first-shell metal ligands in the binuclear manganese cluster of arginase I”, *Biochemistry*, 42:7748-58 (2003b).

Carvajal N., Salas M., Lopez V., Uribe E., Herrera P., Cerpa J., Fuentes M., "Manganese-dependent inhibition of human liver arginase by borate", *J Inorg Biochem*, 77:163-7 (1999).

Carvajal N., Torres C., Uribe E., Salas M., "Interaction of arginase with metal ions: studies of the enzyme from human liver and comparison with other arginases", *Comp Biochem Physiol B Biochem Mol Biol*, 112:153-9 (1995).

Cavalli R. C., Burke C. J., Kawamoto S., Soprano D. R., Ash D. E., "Mutagenesis of rat liver arginase expressed in Escherichia coli: role of conserved histidines", *Biochemistry*, 33:10652-7 (1994).

Colleluori D. M., Morris S. M. Jr., Ash D. E., "Expression, purification, and characterization of human type II arginase", *Arch Biochem Biophys*, 389:135-43 (2001).

Dillon B. J., Holtsberg F. W., Ensor C. M., Bomalaski J. S., Clark M. A., "Biochemical characterization of the arginine degrading enzymes arginase and arginine deiminase and their effect on nitric oxide production", *Med Sci Monit*, 8:248-53 (2002).

Ensor C. M., Holtsberg F. W., Bomalaski J. S., Clark M. A., "Pegylated arginine deiminase (ADI-SS PEG20,000 mw) inhibits human melanomas and hepatocellular carcinomas in vitro and in vivo", *Cancer Res*, 62:5443-50 (2002).

Frederick W. H., Charles M. E., Marion R. S., John S. B., Mike A. C., "Poly(ethylene glycol) (PEG) conjugated arginine deiminase: effects of PEG formulations on its pharmacological properties", *Journal of controlled release* 80: 259-271 (2002).

Gobert G. N., Schatten H., "Improved ultrastructure of the desmosome-intermediate filament complex in MCF-7 breast cancer cells", *Electron Microsc*, 49:539-44 (2000).

Gong H., Zolzer F., von Recklinghausen G., Havers W., Schweigerer L., "Arginine deiminase inhibits proliferation of human leukemia cells more potently than asparaginase by inducing cell cycle arrest and apoptosis", *Leukemia*, 14:826-9 (1999).

Goodson R. J., Katre N. V., "Site-directed pegylation of recombinant interleukin-2 at its glycosylation site", *Biotechnology*, 8:343-6 (1990).

Green S. M., Eisenstein E., McPhie P., Hensley P., "The purification and characterization of arginase from Saccharomyces cerevisiae", *J Biol Chem*, 265:1601-7 (1990).

Haraguchi K., Hata Y., Ibaragi Y., Matsuda K., "A case of geminated primary canine associated with fused teeth and consideration of developmental mechanism", *Higashi Nippon Shigaku Zasshi*, 6:143-53 (1987).

Harris J. M., Chess R. B., "Effect of pegylation on pharmaceuticals", *Nat Rev Drug Discov*, 2:214-21 (2003).

Harris J. M., Martin N. E., Modi M., "Pegylation: a novel process for modifying pharmacokinetics", *Clin Pharmacokinet*, 40:539-51 (2001).

Holtsberg F. W., Ensor C. M., Steiner M. R., Bomalaski J. S., Clark M. A., "Poly(ethylene glycol) (PEG) conjugated arginine deiminase: effects of PEG formulations on its pharmacological properties", *J Control Release*, 80:259-71 (2002).

Hus-Citharel A., Levillain O., "Identification of two arginase isoenzyme activities along the nephron of Meriones shawi", *Pflugers Arch*, 437:423-31 (1999).

Ikemoto M., Tabata M., Miyake T., Kono T., Mori M., Totani M., Murachi T., "Expression of human liver arginase in *Escherichia coli*. Purification and properties of the product", *Biochem J*, 270:697-703 (1990).

Ikemoto M., Tabata M., Murachi T., Totani M., "Purification and properties of human erythrocyte arginase", *Ann Clin Biochem*, 26:547-53 (1989).

Ikemoto M., Tsunekawa S., Awane M., Fukuda Y., Murayama H., Igarashi M., Nagata A., Kasai Y., Totani M., "A useful ELISA system for human liver-type arginase, and its utility in diagnosis of liver diseases", *Clin Chem*, 34:455-61 (2001a).

Ikemoto M., Tsunekawa S., Kasai Y., Tanaka K., Ueda K., Totani M., "Clinical significance of anti-liver-type arginase autoantibody in blood of recipients after partial liver transplantation", *Rinsho Byori,* 49:651-6 (2001b).

Ikemoto M., Tsunekawa S., Toda Y., Totani M., "Liver-type arginase is a highly sensitive marker for hepatocellular damage in rats", *Clin Chem,* 47:946-8 (2001).

Iyer R. K., Bando J. M., Jenkinson C. P., Vockley J. G., Kim P. S., Kern R. M., Cederbaum S. D., Grody W. W., "Cloning and characterization of the mouse and rat type II arginase genes", *Mol Genet Metab*, 63:168-75 (1998).

Jenkinson C. P., Grigor M. R., "Rat mammary arginase: isolation and characterization", *Biochem Med Metab Biol*, 51:156-65 (1994).

Jenkinson C. P., Grody W. W., Cederbaum S. D., "Comparative properties of arginases", *Comp Biochem Physiol B Biochem Mol Biol*, 114:107-32 (1996).

Kanda M., Ohgishi K., Hanawa T., Saito Y., "Arginase of Bacillus brevis Nagano: purification, properties, and implication in gramicidin S biosynthesis", *Arch Biochem Biophys*, 344:37-42 (1997).

Kanyo Z. F., Chen C. Y., Daghigh F., Ash D. E., Christianson D. W., "Crystallization and oligomeric structure of rat liver arginase", *J Mol Biol*, 224:1175-7 (1992).

Kanyo Z. F., Scolnick L. R., Ash D. E., Christianson D. W., "Structure of a unique binuclear manganese cluster in arginase", *Nature*, 383:554-7 (1996).

Kim P. S., Iyer R. K., Lu K. V., Yu H., Karimi A., Kern R. M., Tai D. K., Cederbaum S. D., Grody W. W., "Expression of the liver form of arginase in erythrocytes", *Mol Genet Metab*, 76:100-10 (2002).

Komada Y., Zhang X. L., Zhou Y. W., Ido M., Azuma E., "Apoptotic cell death of human T lymphoblastoid cells induced by arginine deiminase", *Int J Hematol*, 65:129-41 (1997).

Kuhn N. J., Talbot J., Ward S., "pH-sensitive control of arginase by Mn(II) ions at submicromolar concentrations", *Arch Biochem Biophys*, 286:217-21 (1991).

Kuhn N. J., Ward S., Piponski M., Young T. W., "Purification of human hepatic arginase and its manganese (II)-dependent and pH-dependent interconversion between active and inactive forms: a possible pH-sensing function of the enzyme on the ornithine cycle", *Arch Biochem Biophys*, 320:24-34 (1995).

Lamb J., Wheatley D. N., "Single amino acid (arginine) deprivation induces G1 arrest associated with inhibition of cdk4 expression in cultured human diploid fibroblasts", *Exp Cell Res*, 255:238-49 (2000).

Lavulo L. T., Sossong T. M. Jr, Brigham-Burke M. R., Doyle M. L., Cox J. D., Christianson D. W., Ash D. E., "Subunit-subunit interactions in trimeric arginase. Generation of active monomers by mutation of a single amino acid", *J Biol Chem*, 276:14242-8 (2001).

Leung Y. C., Errington J., "Characterization of an insertion in the phage phi 105 genome that blocks host Bacillus subtilis lysis and provides strong expression of heterologous genes", *Gene*, 154:1-6 (1995).

Miyazaki K., Takaku H., Umeda M., Fujita T., Huang W. D., Kimura T., Yamashita J., Horio T., "Potent growth inhibition of human tumor cells in culture by arginine deiminase purified from a culture medium of a Mycoplasma-infected cell line", *Cancer Res*, 50:4522-7 (1990).

Mora A., del Ara Rangel M., Fuentes J. M., Soler G., Centeno F., "Implications of the S-shaped domain in the quaternary structure of human arginase", *Biochim Biophys Acta*, 1476:181-90 (2000).Nieves C. Jr., Langkamp-Henken B., "Arginine and immunity: a unique perspective", *Biomedecine & pharmacotherapie*, 56:471-82 (2002).

Nieves C. Jr., Langkamp-Henken B., "Arginine and immunity: a unique perspective", *Biomed Pharmacother*, 56:471-82 (2002).

Noh E. J., Kang S. W., Shin Y. J., Kim D. C., Park I. S., Kim M. Y., Chun B. G., Min B. H., "Characterization of mycoplasma arginine deiminase expressed in *E. coli* and its inhibitory regulation of nitric oxide synthesis", *Mol Cells*, 13:137-43 (2002).

Ozer N., "A new enzyme-coupled spectrophotometric method for the determination of arginase activity", *Biochem Med*, 33:367-71 (1985).

Park I. S., Kang S. W., Shin Y. J., Chae K. Y., Park M. O., Kim M. Y., Wheatley D. N., Min B. H., "Arginine deiminase: a potential inhibitor of angiogenesis and tumour growth", *Br J Cancer*, 89:907-14 (2003).

Philip R., Campbell E., Wheatley D. N., "Arginine deprivation, growth inhibition and tumour cell death: 2. Enzymatic degradation of arginine in normal and malignant cell cultures", *Br J Cancer*, 88:613-23 (2003).

Que L. G., Samuel E. G., Tomomi G., Mori M., Huang Y. C. T., "Effects of arginase isoforms on NO production by nNOS", *Nitric Oxide*, 6:1–8 (2002).

Roberts M. J., Bentley M. D., Harris J. M., "Chemistry for peptide and protein PEGylation", *Advanced Drug Delivery Reviews*, 54:459-76 (2002).

Roberts M. J., Harris J. M., "Attachment of degradable poly(ethylene glycol) to proteins has the potential to increase therapeutic efficacy", *J Pharm Sci*, 87:1440-5 (1998).

Ronghe M., Burke G. A., Lowis S. P., Estlin E. J., “Remission induction therapy for childhood acute lymphoblastic leukaemia: clinical and cellular pharmacology of vincristine, corticosteroids, L-asparaginase and anthracyclines”, *Cancer Treat Rev*, 27:327-37 (2001).

Savoca K. V., Abuchowski A., van Es T., Davis F. F., Palczuk N. C., “Preparation of a non-immunogenic arginase by the covalent attachment of polyethylene glycol”, *Biochim Biophys Acta*, 578:47-53 (1979).

Savoca K. V., Davis F. F., van Es T., McCoy J. R., Palczuk N. C., “Cancer therapy with chemically modified enzymes. II. The therapeutic effectiveness of arginase, and arginase modified by the covalent attachment of polyethylene glycol, on the taper liver tumor and the L5178Y murine leukemia”, *Cancer Biochem Biophys*, 7:261-8 (1984).

Schuber F., “Influence of polyamines on membrane functions”, *Biochem J*, 260:1-10 (1989).

Scott L., Lamb J., Smith S., Wheatley D. N., “Single amino acid (arginine) deprivation: rapid and selective death of cultured transformed and malignant cells”, *Br J Cancer*, 83:800-10 (2000).

Sreerama N., Venyaminov S. Y., Woody R. W., “Estimation of protein secondary structure from circular dichroism spectra: inclusion of denatured proteins with native proteins in the analysis”, *Anal Biochem*, 287:243-51 (2000).

Sreerama N., Woody R. W., “Estimation of protein secondary structure from circular dichroism spectra: comparison of CONTIN, SELCON, and CDSSTR methods with an expanded reference set”, *Anal Biochem*, 287:252-60 (2000).

Storr J. M., Burton A. F., “The effects of arginine deficiency on lymphoma cells”, *Br J Cancer*, 30:50-9 (1974).

Sugimura K., Ohno T., Kusuyama T., Azuma I., “High sensitivity of human melanoma cell lines to the growth inhibitory activity of mycoplasmal arginine deiminase in vitro”, *Melanoma Res*, 2:191-6 (1992).

Thomas J. B., Holtsberg F. W., Ensor C. M., Bomalaski J. S., Clark M. A., “Enzymic degradation of plasma arginine using arginine deiminase inhibits nitric oxide production and protects mice from the lethal effects of tumour necrosis factor alpha and endotoxin”, *Biochem J*, 363:581-7 (2002).

Thornewell S. J., East A. K., Errington J., “An efficient expression and secretion system based on Bacillus subtilis phage phi 105 and its use for the production of B. cereus beta-lactamase I”, *Gene*, 133:47-53 (1993).

Wang Y. S., Youngster S., Grace M., Bausch J., Bordens R., Wyss D. F., “Structural and biological characterization of pegylated recombinant interferon alpha-2b and its therapeutic implications”, *Adv Drug Deliv Rev*, 54:547-70 (2002).

Wheatley D. N., Campbell E., “Arginine catabolism, liver extracts and cancer”, *Pathol Oncol Res*, 8:18-25 (2002).

Wheatley D. N., Campbell E., “Arginine deprivation, growth inhibition and tumour cell death: 3. Deficient utilisation of citrulline by malignant cells”, *British journal of cancer*, 89:573-6 (2003).

Wheatley D. N., Philip R., Campbell E., “Arginine deprivation and tumour cell death: arginase and its inhibition”, *Mol Cell Biochem*, 244:177-85 (2003).

Wheatley D. N., Scott L., Lamb J., Smith S., “Single amino acid (arginine) restriction: growth and death of cultured HeLa and human diploid fibroblasts”, *Cell Physiol Biochem*, 10:37-55 (2000).

Zor T., Selinger Z., “Linearization of the Bradford protein assay increases its sensitivity: theoretical and experimental studies”, *Anal Biochem*, 236:302-8 (1996).

# Appendix

# Reagents and solutions

## (A) Fermentation Media

### *1. Shake-flask culture*

**BHY Medium (Brain Heart infusion and yeast extract)**

3.7 % bovine brain heart infusion (4.625 g in 125 ml)

0.5 % yeast extract (0.625 g in 125 ml)

pH was adjusted to 7.0.

**Glucose Medium**

Glucose 5 g/L,

Tryptone 10 g/L,

Yeast extract 3 g/L,

Citric acid 1 g/L,

$KH_2PO_4$ 1.5 g/L,

$K_2HPO_4$ 1.5 g/L,

$(NH_4)_2\ SO_4$ 3 g/L

pH was adjusted to 7.0.

### *2. Fed-batch fermentation*

**Basic Medium**

5 g/L Glucose, 10 g/L Tryptone, 3 g/L Yeast Extract, 1 g/L Sodium Citrate ($C_6H_5Na_3O_7 \cdot 2H_2O$), 3 g/L Ammonium sulphate $(NH_4)SO_4$, 1.5 g/L $K_2HPO_4$, 1.5 g/L $KH_2PO_4$

pH was adjusted to 7.0.

**Feeding Medium**

Medium A: 300 g/L glucose, 3.75 g/L $MgSO_4 \cdot 7\ H_2O$

Medium B: 75 g/L tryptone, 11.25 g/L $K_2HPO_4$, 5.625 g/L $KH_2PO_4$

Medium A & B were mixed with equal volume.

**(B) Media for purification of arginase**

***1. Solubilization buffer***

50 mM Tris-HCl (pH 7.4), 0.1 M NaCl

***2. $MnCl_2$ solution***

1.193 g $MnCl_2$ in 10 ml $dH_2O$

***3. Lysozyme (10 mg/ml)***

50 mM Tris-HCl (pH 8), 50 mg lysozyme

***4. Buffers for Ni-NTA column***

Start buffer 1 = 0.02M sodium phosphate buffer (pH 7.4), 0.5M NaCl
Elution buffer 1 = 0.5M imidazole in Start buffer 1 (pH 7.4)
Cleaning buffer 1 = 1M imidazole in Start buffer 1 (pH 7.4)

Where 0.2 M Sodium phosphate buffer (100 ml) was prepared by:
81 ml 0.2 M $Na_2HPO_4$ + 19 ml 0.2 M $NaH_2PO_4$

***5. Buffers for HiLoad$^{TM}$ 26/10 SP Sepharose$^{TM}$ HP column***

Start buffer 2 = 10 mM Tris-HCl (pH 7.5)
Elution buffer 2 = 10 mM Tris-HCl (pH 7.5), 1 M NaCl
Cleaning buffer 2 = 2M NaCl in Start buffer 2 (pH 7.4)

***6. PBS buffer***

8 g NaCl, 0.2 g KCl, 1.44 g $Na_2HPO_4$, 0.24 g $KH_2PO_4$
Volume was adjusted to 1L with MilliQ water and pH was adjusted to 7.4.

## (C) Media for characterization of arginase

### *1. $A_{340}$ activity assay*

**Assay mixture (100 ml)**

0.1 M Tris,
0.25 mM NADPH
5 mM α-ketoglutaric acid
35 I.U./ ml urease
15 I.U./ ml glutamate dehydrogenase
50 mM arginine
pH was adjusted to 8.3

**Washing solution**

1 M HCl

### *2. SDS-PAGE*

**Stain solution**

250 ml $dH_2O$
250 ml methanol
100 ml acetic acid
0.6 g Coomassie Blue R

**Destain solution**

100 ml acetic acid
100 ml methanol
800 ml $dH_2O$

**Running buffer (10 x)**

30 g Tris base
144 g Glycine
10 g SDS
Volume was adjested to 1 L with $dH_2O$
The buffer was diluted to **1 x** before use.

**Sample loading dye (16 ml)**

| Reagent | Volume |
|---|---|
| $dH_2O$ | 0.2 ml |
| 0.5 M Tris-HCl (pH 6.8) | 2 ml |
| glycerol | 3 ml |
| 10 % (w/v) SDS | 3.2 ml |
| 2-b-mercaptoethanol | 0.8 ml |
| 0.05% (w/v) bromophenol blue | 6.8 ml |

***3. Isoelectric focusing (IEF)***

**Components for IEF gel:**

| | Reagent | Volume |
|---|---|---|
| Monomer-ampholyte solution | Milli-Q water | 5.5 ml |
| | Acylamide/Bis (25% T, 3% C) | 2.0 ml |
| | 25% (w/v) glycerol | 2.0 ml |
| | Ampholyte (40%) | 0.5 ml |
| Catalyst solutions | 10 % (w/v) APS | 22 ul |
| | 0.1 % (w/v) FMN | 50 ul |
| | TEMED | 5 ul |

APS = ammonium persulfate

FMN = riboflavin-5'-phosphate

**Fixing and Staining Reagents for IEF**

**Fixative:**

4% sulfosalicylic acid

12.5% trichloroacetic acid (TCA)

30% methanol

100% sulfosalicylic acid (4 ml) + 100% TCA (12.5 ml) + methanol (30 ml), adjust to a final volume 100 ml

**IEF Staining solution Coomassie brilliant blue R-250:**

27% ethanol

10% acetic acid

0.04% Coomassie brilliant blue R-250

0.5% $CuSO_4$

Volume was adjusted to 50 ml with $dH_2O$.

**IEF Destaining solution:**

***1st destaining solution***

12% ethanol

7% acetic acid

0.5% CuSO4

Dissolve 3.9g $CuSO_4.5H_2O$ in ~ 100 ml $dH_2O$ and then add 35 ml acetic acid to this solution. Add 60 ml ethanol to this mixture and then adjust the final volume to 500 ml with $dH_2O$.

***2nd destaining solution***

25% ethanol

7% acetic acid

ethanol (6.25 ml)

acetic acid (1.75 ml)

adjust to a final volume 50 ml