NOVEL STRUCTURES AND PROPERTIES OF POLYSACCHARIDES DERIVED FROM EXOPOLYSACCHARIDES PRODUCED BY A *CORDYCEPS SINENSIS* FUNGUS IN LIQUID FERMENTATION

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Novel Structures and Properties of Polysaccharides Derived from Exopolysaccharides Produced by a *Cordyceps sinensis* Fungus in Liquid Fermentation

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CERTIFICATE OF ORIGINALITY

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Chen Xia

13th May, 2015

Abstract

Polysaccharides (PS) from edible and medicinal fungi are valuable nutraceuticals because of their functional properties and bioactivities. *Cordyceps* (*Ophiocordyceps*) *sinensis*, generally known as the Chinese caterpillar fungus, is a precious medicinal fungus and widely used as a favorable tonic in China. Because natural caterpillar fungi are very rare and expensive, mycelial fermentation of a *C. sinensis* fungus Cs-HK1 has been established in our lab, and applied to liquid fermentation for production of mycelial biomass and exopolysaccharides (EPS). This research project aims to discover and characterize novel PS structures from the EPS produced by Cs-HK1 mycelial fermentation and their functional properties and bioactivities.

The crude EPS from Cs-HK1 mycelial fermentation was a mixture of PS and PS-protein (PSP) complexes in a wide molecular weight (MW) range. Two-step ethanol precipitation was applied to separate the EPS into a high-MW and a low-MW EPS fractions. The high-MW EPS, EPS1, was obtained in the first step with 40% ethanol and the low-MW EPS, EPS2, was obtained in the next step with 70% ethanol.

The high MW EPS1 had poor solubility and high viscosity in water. To improve its solution properties, EPS1 was partially degraded by high-intensity ultrasound (US), yielding a water-soluble polysaccharide EPS1U. EPS1U exhibited a single, symmetrical peak on HPGPC with an average MW about 730 kDa by light scattering analysis. EPS1U had a much lower intrinsic viscosity (1.7 versus 15.6 dL/g) but a much higher solubility in water (77.5 versus 5.1 g/L) than EPS1. Methylation analysis and NMR spectrometry were performed to investigate the structural feature of EPS1U. The structure of EPS1U was elucidated as a (1→3)-β-D-glucan with glucose side chains attached to *O*-6 position at the branching points. This is the first report on a high-MW (1→3)-β-D-glucan isolated from the EPS produced by Cs-HK1. EPS1U showed a high moisture absorption capability comparable to chitosan and urea, suggesting its potential use as moisturizing agent for food and cosmetic applications. In RAW264.7 murine macrophage cell cultures, EPS1U showed significant immunomodulatory activities by stimulating the production of nitric oxide, and release of tumor necrosis factor α and interleukin 6.

A protein-containing PSP complex, EPS2BW, was isolated and purified from the low-MW EPS fraction EPS2. EPS2BW was mainly composed of galactomannan with about 16% (w/w) protein and 50 kDa average MW. The galactomannan part consisted of mannose and galactose at a molar ratio of

1.7:1.0, and the protein segments were composed of sixteen amino acids with 12.5% proline and 16.6% threonine (mol%) being the most abundant. Based on the analytical results from NMR, methylation analysis, partial acid hydrolysis and GC-MS, the galactomannan structure was elucidated as a (1→2)-α-D-mannopyranosyl (Man*p*) backbone with *O*-6-linked galactopyranosyl (Gal*p*) branches. EPS2BW exhibited high antioxidant capacity in both chemical and cell culture assays, with a Trolox equivalent radical scavenging activity of 44.7 μmol Trolox/mg, a Fe^{3+} reducing power of 38.9 μmol Fe^{2+}/mg, and significant cytoprotective effect against H_2O_2-induced PC12 cell death at 50-250 μg/mL.

Silver nanoparticles (AgNPs) have attracted much attention because of their unique functional properties and potent antimicrobial activity. Natural PS have been widely exploited as biocompatible carriers for green synthesis of AgNPs. As a novel application of EPS produced by Cs-HK1, EPS1 was used for the synthesis of AgNPs in water. The formation and properties of AgNPs were evaluated at various temperatures, time periods, and silver nitrate/EPS1 concentrations in water. At suitable conditions (100 ^{o}C, 60 min and 10 mM $AgNO_3$ with 1.0 mg/mL EPS1), AgNPs were formed with an average diameter of 50 nm and a narrow size distribution, and they had stable dispersion that lasted for at least 2 months. EPS1 might have acted as a reducing and stabilizing agent in the formation of AgNPs, which were

attached to the hydroxyl groups of EPS1. The AgNPs formed in EPS1 solution exhibited concentration-dependent inhibition of both Gram-negative and -positive bacteria but very low cytotoxicity on the RAW264.7 murine macrophage cells. The results demonstrated the potential of EPS produced by Cs-HK1 for green synthesis of AgNPs.

The production of PS in microbial fermentation can be affected by the major nutrients. Therefore we further evaluated the effects of alternative carbon sources on the productivity and properties of EPS in the Cs-HK1 mycelial culture. Galactose or mannose was used (at 5 g/L) as a secondary carbon source together with glucose (35 g/L) at the mass ratio of 1:7. The yield and composition of EPS produced by Cs-HK1 varied as the component of carbon source changed. The addition of galactose improved the total yield of EPS in culture while mannose increased the glucan content of EPS.

In summary, the results from this project showed that the mycelial fermentation of Cs-HK1 fungus produced a variety of EPS structures with interesting and useful properties, and bioactivities for nutraceutical applications and green synthesis of AgNPs. The major findings from this project has laid the foundation for further development and application of EPS from the Cs-HK1 in the pharmaceutical, food and cosmetic industries.

List of publications

Journal papers

1. **Chen, X.**, Wu, J. Y., & Gui, X. T. (Under Review). Production and characterization of exopolysaccharides in mycelial culture of *Cordyceps sinensis* fungus Cs-HK1 with different carbon source. *Chinese Journal of Chemical Engineering*.
2. **Chen, X.**, Yan, J. K., & Wu, J. Y. (2016). Characterization and antibacterial activity of silver nanoparticles prepared with fungal exopolysaccharide in water. *Food Hydrocolloids, 53*, 69-74.
3. Siu, K. C., **Chen, X.**, & Wu, J. Y. (2014). Constituents actually responsible for the antioxidant activities of crude polysaccharides isolated from mushrooms. *Journal of Functional Foods, 11*, 548-556.
4. Wu, J. Y., **Chen X.**, & Siu, K. C. (2014). Isolation and structure characterization of an antioxidative glycopeptide from mycelial culture broth of a medicinal fungus. *International Journal of Molecular Sciences*, *15*, 17318-17332.
5. **Chen, X.**, Ding, Z. Y., Wang, W. Q., Siu, K. C., & Wu, J. Y. (2014). An antioxidative galactomannan-protein complex isolated from fermentation broth of a medicinal fungus Cs-HK1. *Carbohydrate Polymers*, *112*, 469-474.

6. **Chen, X.**, Siu, K. C., Cheung, Y. C., & Wu, J. Y. (2014). Structure and properties of a (1→3)-β-d-glucan from ultrasound-degraded exopolysaccharides of a medicinal fungus. *Carbohydrate Polymers*, *106*, 270-275.

Conference presentations and papers (International)

1. **Chen, X.**, Cheung, Y. C., Siu, K. C. (2013). Study of a galactomannan-protein from medium culture broth of *Cordyceps sinensis* (Cs-HK1) with antioxidant activities. The 9th Hong Kong-Macau Postgraduate Symposium on Chinese Medicine, Hong Kong.
2. **Chen, X.**, Yan, J. K., & Wu, J. Y. (2014). Green synthesis of silver nanoparticles using exopolysaccharides extracted from fermentation broth of *Cordyceps sinensis* with antimicrobial and immunological activity. 12th International Hydrocolloids Conference, Taiwan.
3. **Chen, X.**, Ding, Z. Y., Wang, W. Q., & Wu, J. Y. (2014). An antioxidative galactomannan-protein from medium culture broth of *Cordyceps sinensis* (Cs-HK1). Symposium on Glycobiology & Glycobiotechnology, Xi'an, China.
4. **Chen, X.**, Yan, J. K., & Wu, J. Y. (2014). Silver nanoparticles made with exopolysaccharides from a medicinal fungus Cs-HK1 and antibacterial activity. The Sixth Global Chinese Symposium of Chemical Engineering,

Hong Kong.

Conference presentations and papers (Local)

1. **Chen, X.**, Cheung, Y. C., Siu, K. C., & Wu, J. Y. (2013). Improving the solubility and antioxidant activities of polysaccharides from medicinal fungus by ultrasonic degradation and chemical modifications. The 20th Symposium of Chemistry Postgraduate Research in Hong Kong.
2. **Chen, X.**, Yuan, J., & Wu, J. Y. (2014). Preparation, characterization and antimicrobial of silver nanoparticles using exopolysaccharides extracted from fermentation broth of *Cordyceps sinensis*. The 21th Symposium of Chemistry Postgraduate Research in Hong Kong.

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on $ABTS^{+}$ radicals; (b) FRAP activity for reducing Fe^{3+} ions; (c) Cytoprotective effects against H_2O_2-induced PC12 cell injury at various EPS2BW concentrations. The TEAC or FRAP activity value was the average (slope) at various concentrations from 100 to 1000 mg/L. For the cell culture tests, cells were treated with 80 μM H_2O_2 and EPS2BW or with an equal volume of PBS for the negative control group for 24 h. Each value was expressed as mean ±SD (n=3).

Figure 6-8 PC12 cells were treated with different concentration of EPS2BW for 72 h followed by cell viability assay using MTT method.

Figure 7-1 UV-Vis light absorbance spectra of $AgNO_3$ and EPS1 mixture reaction solution at various conditions: (A) 10 mM $AgNO_3$ with various concentrations of EPS1; (B) 1.0 mg/mL EPS1 with various concentrations of $AgNO_3$; (C) 10 mM $AgNO_3$ and 1.0 mg/mL EPS1 at various temperatures; (D) 10 mM $AgNO_3$ and 1.0 mg/mL EPS1 for various periods of reaction time.

Figure 7-2 Analysis of AgNPs formed in EPS1 solution: (A) FT-IR spectra of EPS1, $AgNO_3$ and AgNPs; (B) particle size distribution of EPS1 and AgNPs (1.0 mg/mL) in water (by DLS performed at 25 ^{o}C and 90 ° scattering angle). (AgNPs prepared with 10 mM $AgNO_3$ and 1.0 mg/mL EPS1 at 100 ^{o}C for 60 min).

Figure 7-3 TEM images: (A) freshly prepared AgNPs; (B) freshly prepared AgNPs after filtration through 0.22 μM filter; (C) AgNPs stored for 2

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List of abbreviations and symbols

EPS	Exopolysaccharides
PS	Polysaccharides
PSP	Polysaccharide-protein
US	Ultrasound
MW	Molecular weight
FT-IR	Fourier transform infrared spectoscopy
NMR	Nuclear magnetic resonance
DEPT	Distortionless enhancement by polarization transfer
HSQC	Heteronuclear singular quantum correlation
HMBC	Heteronuclear multiple bond correlation
TEM	Transmitted electron microscopy
HPGPC	High performance gel permeation chromatograph
AgNPs	Silver nanoparticles
SEM.	Standard error of the mean

Chapter 1 Introduction

Edible and medicinal fungi (or mushrooms) provide an abundant and attractive source of natural polysaccharides (PS) and polysaccharide-protein (PSP) complexes with notable antitumor, immunomodulation and other bioactivities (Fung & Tan, 2015; Ren, Perera & Hemar, 2012; Stachowiak & Regula, 2012; Wang et al., 2014; Wu, 2014). *Cordyceps sinensis* (syn. *Ophiocordyceps sinensis*), the Chinese caterpillar fungus or Cordyceps in brief, is a special mushroom with a fruiting body formed on a caterpillar (Fig. 1-1), which is called Dong-chong-xia-cao in Chinese. *C. sinensis* is one of the most famous and highly valued medicinal fungi in China (Zhang, Li, Wang, Li & Liu, 2012), and has attracted worldwide attention (Paterson, 2008; Winkler, 2010). *C. sinensis* has long been used in traditional Chinese medicine for a number of health promoting functions such as promoting longevity, relieving exhaustion and increasing athletic prowess (Chioza & Ohga, 2014; Shashidhar & Parvatam, 2015; Zhou, Gong, Su, Lin & Tang, 2009). It has also been used in herbal medicine to treat numerous illnesses including chronic kidney disease, liver and heart injuries and cancer (Liu et al., 2014a; Yan, Wang & Wu, 2014; Zhang et al., 2014).

Figure 1-1 *Cordyceps sinensis* (syn. *Ophiocordyceps sinensis*) fruiting body-caterpillar complexes: morphology and natural habitat (Paterson, 2008; Winkler, 2010).

A number of bioactive constituents from *C. sinensis* have been reported to show various activities (Chen, Wang, Nie & Marcone, 2013). PS including the PSPs represent a class of the most abundant bioactive constituents of *C. sinensis*. More than 35 years ago, a galactomannan was isolated from the fruit bodies of *C. sinensis* and its structure was elucidated as a (1→2)-α-D-Man*p* main chain (Miyazaki, Oikawa & Yamada, 1977). Another galactomannan containing a minor protein content was later isolated from the fruiting bodies of *C. sinensis*, and it had a (1→2)-α-D and (1→6)-α-D-Man*p* main chain (Kiho, Tabata, Ukai & Hara, 1986). Other

types of PS, like mannoglucan, glucan, acid polysaccharide, and heteropolysaccharide, have been reported in recent years (Akaki, Matsui, Kojima, Nakajima, Kamei & Tamesada, 2009; Kiho, Ookubo, Usui, Ukai & Hirano, 1999; Wang, Peng, Lee, Tang, Cheung & Wu, 2011; Zhang, Cui, Cheung & Wang, 2006).

As natural *C. sinensis* is very rare and not sufficient to meet the increasing demand in recent years, mycelial fermentation has become a dependable measure for production of the fungal materials as the major source of PS and other useful components. A number of PS structures have been reported, which were mostly isolated and purified from the water or alkaline extracts of mycelial biomass produced by solid or liquid fermentation (Yan & Wu, 2014; Yan, Wang & Wu, 2014; Zhou, Gong, Su, Lin & Tang, 2009). In addition to extraction of (intracellular) PS from the mycelium biomass, exopolysaccharides (EPS) can be produced by some fungal species and isolated from the liquid fermentation broth. Culture conditions during the fermentation process including nutrients, aeration and other factors can have a significant influence on the quality and quantity of EPS and biomass. Cs-HK1 is a fungal species isolated from the natural *C. sinensis* fruiting body in our lab and Cs-HK1 mycelial culture has been established, and applied to liquid fermentation for production of fungal mycelia and EPS (Yan, Wang & Wu, 2014). The factors affecting Cs-HK1 mycelial growth and EPS production have been optimized in our previous

study (Leung, Zhang & Wu, 2006), though their influence on the composition of the EPS has not been assessed.

The crude and partially purified EPS fractions isolated from the Cs-HK1 showed notable antioxidant, cytoprotective and radioprotective activities in our previous studies (Huang, Siu, Wang, Cheung & Wu, 2013; Leung, Zhao, Ho & Wu, 2009; Wong, Wu & Benzie, 2011). However, the EPS produced by the Cs-HK1 mycelial fermentation had a high molecular weight (MW) over 10,000 kDa with poor solubility and high viscosity in water (Huang, Siu, Wang, Cheung & Wu, 2013). Partial depolymerization of the high-MW EPS may be an effective approach to improve the solution properties and functional activities. In a previous study conducted in our lab, high-intensity ultrasound (US) was employed for partial and controlled degradation of the high-MW EPS produced by Cs-HK1 to improve the water solubility and reduce the solution viscosity of EPS (Wang, Cheung, Leung & Wu, 2010).

This research project is aimed at further studying and better understanding of the structures, bioactivities and potential applications of PS fractions derived from EPS by fraction and partial degradation, and the discovering of novel PS structures from the EPS produced by Cs-HK1 mycelial fermentation.

Chapter 2 Objectives and significance

2.1 Objectives

This research project aims to investigate novel polysaccharides (PS) structures derived from the crude exopolysaccharides (EPS) produced by the Cs-HK1 fungus and the functional properties of the novel PS, and to evaluate the effects of alternative carbon sources on the composition and molecular properties of EPS. In particular, we will carry out the following experimental studies:

1) Fractionation of EPS with different molecular weights (MW) by a two-step ethanol precipitation.

2) Modification, purification and characterization of novel homogenous PS structures from the EPS fractions.

3) Evaluation of the properties and bioactivities of the novel homogenous PS, including moisture absorption, immunomodulatory activities and antioxidant activities.

4) Application of EPS fraction in preparation of silver nanoparticles

(AgNPs) and evaluation of the antibacterial activities of the prepared AgNPs.

5) Investigation of the effect of alternative carbon sources on the chemical composition and molecular structure of EPS in various treatment conditions.

2.2 Significance

PS and polysaccharide-proteins (PSPs) represent a class of the most abundant bioactive constituents of *Cordyceps sinensis.* Cs-HK1 is a fungus isolated from the fruiting body of *C. sinensis* and has been used for EPS and biomass production. However, the EPS produced by Cs-HK1 mycelial fermentation contained high MW PS and PSPs with poor solubility and high viscosity in water. Power ultrasound (US) has been applied as an effective means for partial degradation of the high-MW EPS to derive lower-MW EPS with improved solution properties. Novel homogenous PS/PSPs were retained from the US-degraded EPS.

Mycelial fermentation is an efficient and sustainable process for the production of EPS and other biomass from medicinal fungi, especially those rare natural species such as *C. sinensis*. The PS and PSPs from the Cs-HK1 mycelial fermentation provide a source of natural biopolymers with potential applications in health foods and pharmaceutical products. The

function properties and applications of the US-degraded EPS fractions have been evaluated for moisture retention, immunomodulatory activities, antioxidant activities and formulation of AgNPs. Moreover, alternative carbon sources are applied to modify the EPS properties. All the results may be useful for further development of the Cs-HK1 mycelial fermentation process, and novel functions and applications of the EPS from Cs-HK1.

Chapter 3 Literature Review

3.1 *Cordyceps sinensis* fungus

Cordyceps sinensis (syn. *Ophiocordyceps sinensis*), also called Cordyceps or Dong-Chong-Xia-Cao in Chinese, is a complex of a fungal fruiting body on the larva of caterpillar (Fig. 1-1) in the family Hepialidae. The parasitic complex of the fungus and the caterpillar are found in the soil of a prairie at an elevation of 3,500 to 5,000 meters on the Qinghai-Tibetan plateaus that extend from the western China, the mountain range of Nepal, northern Bhutan and the northern states of India (Chen, Wang, Nie & Marcone, 2013; Li et al., 2003; Shrestha, Zhang, Zhang & Liu, 2010). *C. sinensis* has an uncommon life cycle (Zhang, Li, Wang, Li & Liu, 2012). The fungus infects the larvae of ghost moths of the family Hepialidae underground in late autumn. How the fungus infects the caterpillar is still unknown. After entering the caterpillar's body, the fungus grows vegetatively and fills the caterpillar with threadlike hyphae. After infected by the fungus, the larvae can still survive for a period of time before dying with the head upward. Then the fungus grows out from the dead host (usually from the head) and forms a small stroma bud before the soil gets frozen in winter.

C. sinensis has been known and used in China as a medicinal herb and health food for over 300 years. It was first recorded in “Ben Cao Bei Yao” by Wang Ang in 1694 A.D. and became known to the Western society during the 17th century. In 1878, Italian scholar Saccardo named Cordyceps derived from China officially as *Cordyceps sinensis* (Berk.) Sacc., and this nomenclature had been adopted until 2007 (Li, Yang & Tsim, 2006). Based on the findings of a molecular phylogenetic study, *Cordyceps sinensis* was recategorized to *Ophiocordyceps*, hence renamed *O. sinensis* (Shrestha, Zhang, Zhang & Liu, 2010). However, *C. sinensis* is still used as the common name together with *O. sinensis*. *C. sinensis* first gained worldwide attention when it was revealed that several Chinese runners who broke world records in 1993 had used this fungus as part of their training program (Buenz, Bauer, Osmundson & Motley, 2005). Then *C. sinensis* is being a famous medicinal mushroom in traditional Chinese medicine. The growth cycle of natural *C. sinensis* is rather long and complex as described in the previous paragraph. The mimic of natural *C. sinensis* is still unsuccessful. However, the demand of *C. sinensis* is increasing and the natural sources cannot meet the rising demand. Under such circumstances, fungal fermentation has become a major source of *C. sinensis* products. Mycelial fermentation products of *C. sinensis* have been demonstrated to have chemical composition and pharmacological efficacy similar to the natural *C. sinensis* (Li, Su, Dong & Tsim, 2002). Therefore, mycelial fermentation can

be a viable means for mass production of the bioactive components from *C. sinensis* such as exopolysaccharides (EPS) (Shashidhar & Parvatam, 2015; Yan, Wang & Wu, 2014; Yan & Wu, 2014).

3.2 Polysaccharides from *Cordyceps sinensis*

C. sinensis contains various bioactivity compounds, such as proteins, PS and nucleosides (Chen, Wang, Nie & Marcone, 2013; Li, Yang & Tsim, 2006; Shashidhar, Giridhar, Sankar & Manohar, 2013). A number of therapeutic effects of *C. sinensis* such as antitumor, antioxidant, anti-inflammatory, and hypoglycemic activity have been reported (Chen, Wang, Nie & Marcone, 2013; Leung, Zhao, Ho & Wu, 2009; Rao, Fang & Tzeng, 2007; Yan, Li, Wang & Wu, 2010; Zhang, Huang, Bian, Wong, Ng & Wang, 2006). Although the bioactive constitutes of *C. sinensis* are still not fully understood, PS have been widely characterized and recognized as a major class of the bioactive molecules (Chen, Wang, Nie & Marcone, 2013; Chen, Shiao, Lee & Wang, 1997; Kiho, Hui, Yamane & Ukai, 1993; Yan, Wang & Wu, 2014). In this part we would focus on the structural features of the PS from *C. sinensis* and some related activities of these PS.

Natural *C. sinensis* is highly valued as a medicinal and edible mushroom. However, the natural products are not enough to meet the increasing demand and artificial cultivation of *C. sinensis* is still not successful. Therefore, mycelial fermentation for *C. sinensis* has been

applied to produce bioactive compounds similar to those from fruiting bodies (Shashidhar, Giridhar, Sankar & Manohar, 2013; Zhao, Xie, Wang & Li, 2014). Many recent studies have been focused on the PS from mycelial fermentation (Yan, Wang & Wu, 2014). PS from *C. sinensis* consisted of diverse structures with various bioactivities as summarized below.

3.2.1 Glucans

Fungal cell walls usually contain glucans with (1→3)-β-, (1→6)-β- and (1→4)-β-glucose-linkage (Fullerton et al., 2000; Rout, Mondal, Chakraborty, Pramanik & Islam, 2005; Ukawa, Ito & Hisamatsu, 2000). These glucans are proved to have various applications in the pharmaceutical and healthcare industry (Manzi & Pizzoferrato, 2000; Ruthes, Smiderle & Iacomini, 2015). A number of β-glucans were found in the mycelia of *C. sinensis*. Wu et al. (Wu, Sun & Pan, 2005) obtained a β-glucan from the hot water extract of the mycelia of a *C. sinensis* fungus and another β-glucan composed of (1→3)-β-D linkage . CS-Pp is a (1→3)-β-glucan from the mycelia of *C. sinensis* with TNF-α production enhancement activity (Akaki, Matsui, Kojima, Nakajima, Kamei & Tamesada, 2009).

Some α-glucans have been isolated from the fruiting body of mushrooms (Hong, Xun & Wutong, 2007; Hoshi, Yagi, Iijima, Matsunaga, Ishihara & Yasuhara, 2005; Lavi, Friesem, Geresh, Hadar & Schwartz, 2006), and from the mycelia of *C. sinensis* (Wu, Sun & Pan, 2006). Yan et al.

found two similar (1→4)-α-D-glucans in the extract of Cs-HK1 fungal mycelia (Yan, Wang, Li & Wu, 2011), and exhibiting antitumor activities in melanoma tumor-bearing rats.

3.2.2 Galactomannans

Galactomannan was the first type of polysaccharide found in *C. sinensis* reported by a Japanese group almost 40 years ago, which was isolated from the fruiting bodies of natural *C. sinensis*. It was elucidated as a highly branched galactomannan composed of a mannan main chain and a galactan branch (Miyazaki, Oikawa & Yamada, 1977). Kiho et al. (Kiho, Tabata, Ukai & Hara, 1986) found another galactomannan containing a small ratio of protein. Fermentation has become a popular method to produce PS from *C. sinensis* since 1990. However, there are still no reports about galactomannan from mycelia fermentation of *C. sinensis* fungus.

3.2.3 Mannoglucans

Mannoglucans have been found in *Ganoderma lucidum*, *Lentinus edodes* and other edible and medicinal mushrooms (Zhang, Cui, Cheung & Wang, 2006) and also from *C. sinensis*. A neutral mannoglucan was obtained from the extract of mycelia of *C. sinensis* fungus, which had a backbone of (1→3) and (1→4)-glucose-linkage. This mannoglucan exhibited weak cytotoxicity against SPC-I cancer line (Wu, Hu, Pan, Zhou

& Zhou, 2007).

3.2.4 Acidic polysaccharides

Although most reports about PS in *C. sinensis* were neutral PS, acidic PS were also isolated and characterized. An acidic polysaccharide named AEPS-1 was purified from the mycelia broth of *C. sinensis*. It was composed of glucose and glucuronide in the ratio of 8:1 plus a little mannose. AEPS-1 was proved to have significant immune-stimulatory and anti-inflammatory activities (Wang, Peng, Lee, Tang, Cheung & Wu, 2011).

3.2.5 Heteropolysaccharides

A number of heteropolysaccharides have been isolated from the fermentation broth and mycelia of *C. sinensis*. Most of these PS contain glucose, mannose and galactose. A PSP named HW consisting of arabinose, galactose, mannose and glucose in a ratio of 0.1:0.5:0.8:1.0 was extracted from the mycelia of *C. sinensis*. HW decreased the plasma cholesterol level in rats (Koh, Kim, Chang & Suh, 2003). CS-F10 was purified from the culture mycelia of *C. sinensis* which was composed of mannose, glucose and galactose at the ratio of 0:1.38:1.79. A bioactivity test showed that this polysaccharide had hypoglycemic activity (Kiho, Ookubo, Usui, Ukai & Hirano, 1999). A polysaccharide with a molecular weight (MW) of 210 kDa was purified from the mycelia of *C. sinensis* and designated as CSP-1.

CSP-1 consisted of galactose, mannose and glucose in a ratio of 1.5:1.2:2.0. This polysaccharide had antioxidant activity in the cell protection assays (Li et al., 2003). CSP-1 could stimulate pancreatic release of insulin and decrease insulin metabolism (Li et al., 2006). EPS-1A was purified from the fermentation broth of Cs-HK1 consisting of galactose, mannose and glucose at the ratio of 1.0:3.6:15.2. The backbone of this polysaccharide was composed of (1→6)-α-glucose-linkage and (1→6)-α-mannose-linkage (Yan, Li, Wang & Wu, 2010). CPS1, another heteropolysaccharide, was composed of galactose, mannose and glucose in the ratio of 1.0:2.9:2.8. The backbone of CPS1 was confirmed to be (1→2) and (1→4)-mannose-linkage, (1→3)-galactose-linkage and (1→3,6)-glucose-linkage. This heteropolysaccharide had antioxidant activity (Wang, Wang, Ling, Fan & Yin, 2009) *in vitro*. Wang et al. (Wang, Yin, Lv, Wang, Gao & Wang, 2010) found a water-soluble polysaccharide, CPS-2, also composed of galactose, mannose and glucose. They found CPS-2 could significantly relieve renal failure.

In summary, the PS in the fruiting bodies of *C. sinensis* were isolated and characterized in 1970-1990 as galactomannan (or galactomannan-protein). More recently since 1990, the polysaccharide research on *C. sinensis* has focused on the PS from mycelial fermentation. Most of the reported glucans reported have antitumor or immune activities.

Heteropolysaccharide is another common type of polysaccharide in the fermentation of *C. sinensis* which were mostly composed of glucose, mannose and galactose with various activities.

3.3 Modification of polysaccharides

PS have been reported to have various activities, and one of the most important activities is their immune regulatory function, such as β-glucans widely as immunomodulatory agents (Ooi & Liu, 2000; Kernodle, Gates & Kaiser, 1998; Olafsdottir, Omarsdotti, Smestad Paulsen & Wagner, 2003; Savelkoul, Chanput, Wichers, Calder & Yaqoob, 2013). PS with special functional groups have been confirmed to have special bioactivities (Table 3-1).

Table 3-1 Activities of PS with special functional groups.

Polysaccharide	Activity	References
algal fucan	Anticoagulant	(Mulloy, Mourao & Gray, 2000)
curdlan	macrophage-stimulating	(Kataoka, Muta, Yamazaki & Takeshige, 2002)
PSK (*Coriolus versicolor*)	Immunopotentiation	(Sakagami, Aoki, Simpson & Tanuma, 1991)
chitosan oligomer	Tumoricidal	(Seo et al., 2000)
Bupleuran	anti-ulcer	(Yamada, Hirano & Kiyohara, 1991)
pullulan	Antimetastasis	(Kimura, Sumiyoshi, Suzuki & Sakanaka, 2006)
WSS25 (*Gastrodia elata*)	Antiangiogenesis	(Qiu, Yang, Pei, Zhang & Ding, 2010)
letinan	anti-cancer	(Higashi et al., 2012)
TAP (*Tremella aurantia*)	Hypoglycemic	(Kiho et al., 2000)
polysaccharide from *Ophiopogon japonicus*	anti-myocardial ischemia	(Zheng, Feng, Xu, Lin & Chen, 2009)
RRPs (*Radix Rehmanniae*)	Antioxidation	(Sui et al., 2013)
PG-F2 (*Panax ginseng*)	Antiadhesive	(Lee, Shim, Lee, Kim, Chung & Kim, 2006)
APG (*Panax ginseng*)	Radioprotective	(Kim, Kim, Byon, Park, Jee & Joo, 2007)

Different derivation methods have been used to modify PS to produce or enhance their activities. Some common methods are stated in Fig. 3-1. More useful products could be obtained by carbohydrates modification. The modified carbohydrates not only have better activities but also are more easily absorbed. The following is a summary of the different methods for carbohydrate modification.

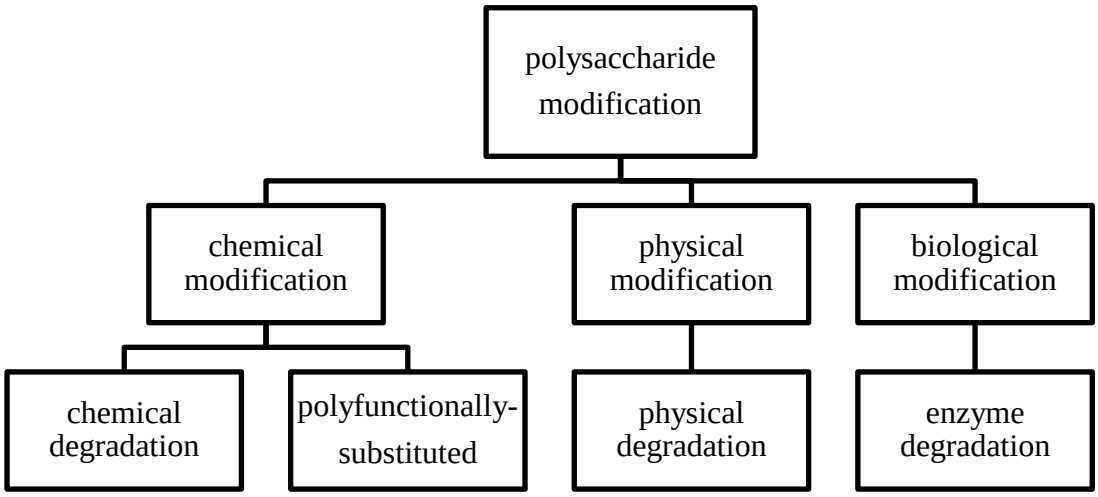


Figure 3-1 The degradation and substitution methods for PS modification.

3.3.1 Chemical modification

The activities of PS are related to functional groups. Various approaches have been applied for structural modification to achieve better properties and activities.

Acid degradation Acid degradation is usually achieved by using

inorganic acid in the food, textile and paper industries. Starch from different plants was used for comparing different modification methods that used different acids. The result showed H_3PO_4 causing the least degradation while HCl and HNO_3 causing the highest (Singh & Ali, 2000). Yan *et al*. used sulfuric acid to hydrolyze EPS from *C. sinensis* (Yan, Li, Wang, Leung, Wang & Wu, 2009). The hypothesized mechanism was achieved by cleaving the glycosidic linkage while the primary molecular structure remained unchanged (Fig. 3-2). Their study revealed that low-MW fraction had much higher antioxidant activity perhaps to an increase of the number of hydroxyl groups available for the reduce reaction.

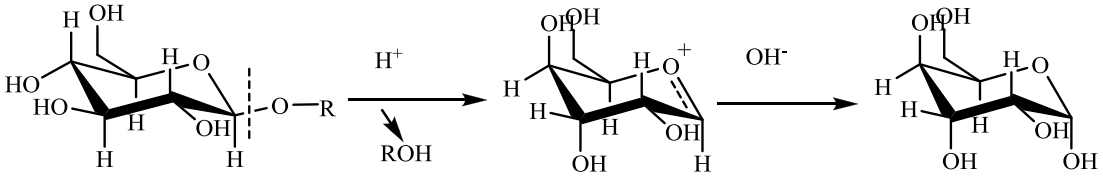


Figure 3-2 A hypothesized reaction mechanism for acidic hydrolysis of EPS (Yan, Li, Wang, Leung, Wang & Wu, 2009).

Organic acids are often used to perform complete acid hydrolysis (Mankarios, Jones, Jarvis, Threlfall & Friend, 1979). Hu *et al.* used trifluoroacetic acid to obtain oligosaccharides from *Nerium indicum*. Two of these oligosaccharides had significant anti-angiogenesis activities (Hu, Liu, Wang & Ding, 2009). In general, acid degradation is easy to achieve, but its safety is still a major concern in the food and medicine industry.

Sulfation Natural sulfated PS have been shown to have anti-human immunodeficiency virus activities (Hayashi, Hayashi & Kojima, 1996), antioxidant activities (Xue et al., 2001), anti-herpetic activities (Ghosh et al., 2004), anticoagulant activities (Alban, Schauerte & Franz, 2002), prevent human leukocyte elastase (HLE)-induced lung injury (Rao, Kennedy, Rao, Ky & Hoidal, 1990) and other activities (Costa et al., 2010; Tao, Zhang & Cheung, 2006). For the better application of PS, sulfation of PS is performed to improve their bioactivities (Fig. 3-3). For pyranose-type PS, pyridine-chlorosulfonic acid was usually used to obtain sulfated PS (Suwan et al., 2009). For furan-type PS, concentrated piperdine-N-sulfonic acid was used to get sulfated products (Nagasawa & Inoue, 1973). Sulfated PS were widely used in anti-HIV virus research. In 1988, scientists found sulfated lentinan could be a novel strategy as antivirals to HIV (Yoshida et al., 1988). Nishimura *et al.* regioselectively synthesized sulfated analogs of chitin and chitosan and these compounds were found to inhibit HIV-1 replication *in vitro* and inhibit blood coagulation (Nishimura et al., 1998). Moreover, research in the application of sulfated PS in antitumor and anticoagulant was very hot (Chaidedgumjorn et al., 2002; Doane & Whistler, 1963). Attention was also paid to other activities, like antiangiogenesis activity (Qiu, Yang, Pei, Zhang & Ding, 2010), antioxidant activity (Wu, Zheng, Ning & Yang, 2007) and antithrombotic activity (Franz & Alban, 1995).

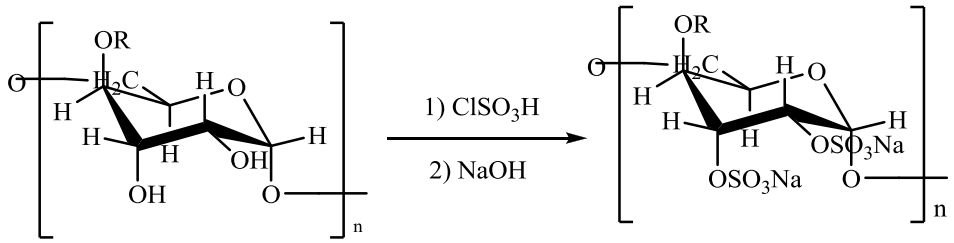


Figure 3-3 Mechanism for saccharides sulfation (Li, Wang & He, 2007).

In generally, sulfated PS have various activities but toxicity has also been reported. How to decrease the toxicity of sulfated PS is a major problem and needs further study.

Acetylation Acetylation (Fig. 3-4) can increase bioactivity of PS by changing their structures. The stretch of the chain could be altered by exposing the hydroxyl group after acetylation, which in turn would lead the changes in solubility (Wang, Zhang, Zhang & Li, 2008). Wang *et al.* had synthesized oversulphated, acetylated and benzoylated fucoidan derivatives and all the derivatives exhibited antioxidant activity stronger than that of fucoidan in certain *in vitro* model. However, the mechanism of modification on influence the antioxidant activity was different (Wang, Liu, Zhang, Zhang, Qi & Li, 2009).

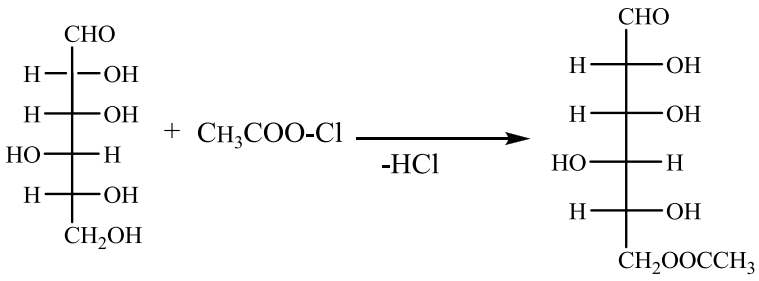


Figure 3-4 Mechanism for saccharides acetylation.

For some PS, the solubility of the product obtained after acetylation is lower than that of the parent polysaccharide (Chen et al., 2011). Other modifications can be carried out to improve the solubility.

For some natural acetylated PS, de-acetylation can also be a way to obtain more potent PS. De-acetylation has been widely used (Suh & Matthew, 2000) to improve the solubility of chitosan. Chitin and chitosan are known to possess multiple functional properties. Chitin is insoluble in any common solvents that contain organic or mineral acid as well as water. Chitosan is water-insoluble and highly viscous in dilute acidic solutions. De-acetylation is often first applied to obtain chitosan from chitin before further modification for the commercial use.

Carboxymethylation Carboxymethylation (Fig. 3-5) has often been applied to obtain PS with better solubility. When hydroxyl groups of saccharides are etherified with carboxymethyl groups, the hydrophilicity of the saccharides is enhanced. Wang *et al.* found that carboxymethylated

polysaccharide could cause the enhancement of *in vitro* bile acid binding capacity by improving water solubility and modifying structure (Wang, Yu & Mao, 2009). Wang *et al.* prepared carboxymethylated polysaccharide of *Ganoderma lucidum* and found it could inhibit the *in vitro* proliferation of Sarcoma 180 tumor cells in a dose-dependent manner with IC_{50} of 38 μg/mL (Wang, Zhang, Yu & Cheung, 2009).

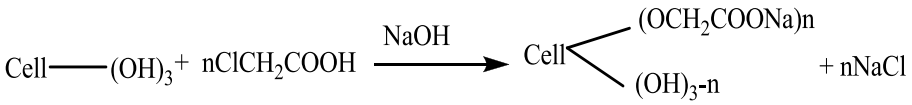


Figure 3-5 Mechanism for cellulose carboxymethylation (Li, Wang & He, 2007).

The introduction of carboxymethylated group usually could improve water solubility and increase chain stiffness, leading to the enhancement of activities. A possible mechanism is that the charged groups and relatively expanded flexible chains of PS have more opportunities to bind with receptors on the cell than the compact conformation of parent PS (Li, Wang & He, 2007).

Phosphorylation Phosphorylated derivatives of PS have exhibited attenuates cardiac dysfunction, anti-inflammatory, antioxidant activity, antimicrobial, immunity and anti-tumor activity (Chen, Xu, Zhang & Zeng, 2009). Suflet *et al.* summarized different ways to obtain phosphorylated

dextran, and they found that the polysaccharide chain was not degraded during phosphorylation (Suflet, Chitanu & Desbrieres, 2010). Phosphorylated *Achyranthes bidentata* polysaccharide was obtained when phosphorus oxychloride was used as a phosphorylating agent and trimethyl phosphate-pyridine or dimethyl formamide was used as solvent. Pharmacology assay showed that the derivative possessed antitumor activity against sarcoma 180 and Lewis lung cancer in mice (Chen, Zhang & Tian, 2002).

Dace *et al.* (Dace, McBride, Brooks, Gander, Buszko & Doctor, 1997) found that the anticoagulant effect of phosphorylated PS appeared to be related to t presence of phosphodiester or diphosphodiester bonds instead of the presence of phosphate groups. Thus, the degree of substitution should be controlled strictly.

Alkylation Usually, PS are hardly dissolved in general organic solvents and have good solubility in water. Therefore, the incursion of alkylation group to PS could improve their solubility in organic solvents (Lin, Luo, Ma & Chen, 2007). Sulfated alkyl oligosaccharides with medium relative molecular masses active against human immunodeficiency virus (HIV), such as sulfated octadecyl maltohexaoside, sulfated dodecyl lamin aripentaoside and sulfated dodecyl laminari-oligomer, were synthesized. These oligosaccharides can cause 50% inhibition of virus infection in the

EC_{50} range of 0.4–0.7 μg/mL *in vitro* using the MT-4 cell line and HIV-1HTLV-IIIB virus isolate, though sulfated oligosaccharides without alkyl groups showed low anti-HIV activities. These compounds were also active against HIV-2 and a clinically isolated HIV-1 with reduced AZT sensitivity. For such sulfated alkyl oligosaccharides, the mechanism of inhibition of HIV infection was assumed to be the inhibition of HIV binding to the cell and to some extent the interaction of the alkyl portion with the lipid bilayer of the virus (Uryu et al., 1992).

Alkylated polysaccharides are usually water insoluble but can form sols in water and several organic solvents.

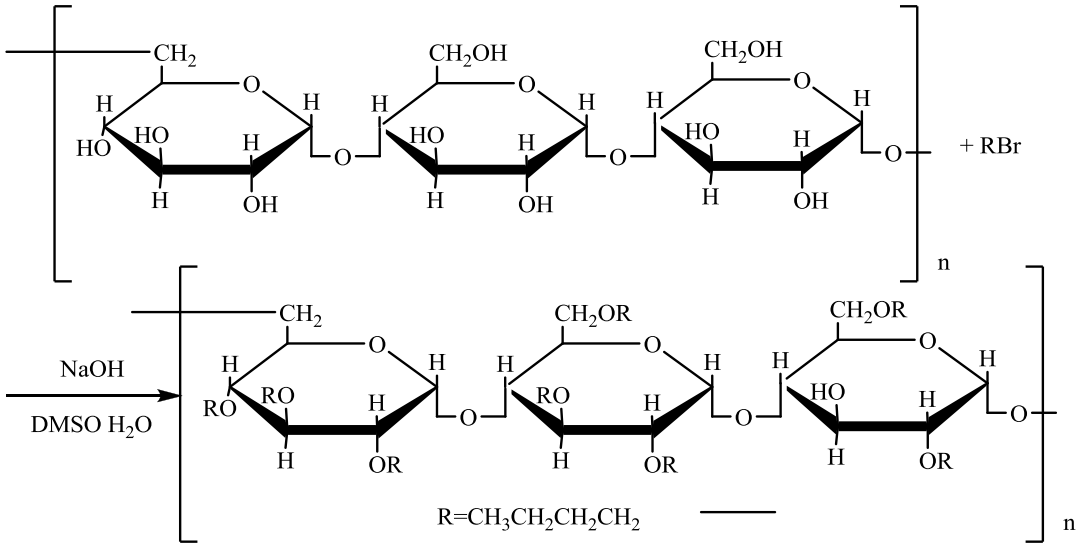


Figure 3-6 The mechanism for alkylation of pullulan (Lin, Luo, Ma & Chen, 2007).

Other chemical modifications, like sulfonylation (Fig. 3-7), could

achieve a new functionalization pattern (Dicke, Rahn, Haack & Heinze, 2001).

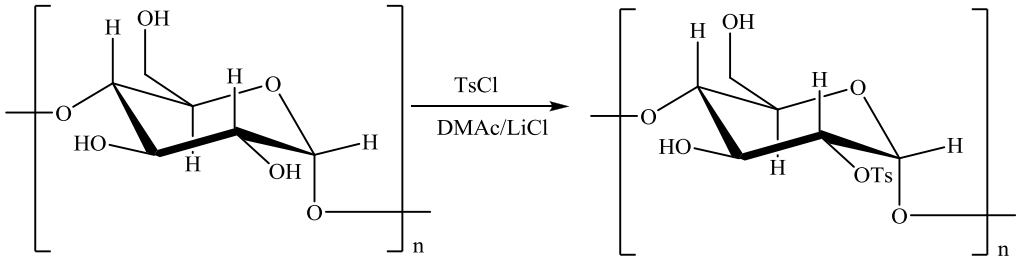


Figure 3-7 The mechanism of sulfonylation (Li, Wang & He, 2007).

The structure-activity relationship is related to the functional groups of PS. Thus, the modification of PS is important to improve the activities of PS.

3.3.2 Biochemical modification

Most biochemical modification is enzyme reaction. The reaction is often specific and easily controlled. But the price of enzyme is expensive and specific enzymes are difficult to obtain. Research on properties of the PS involved has been impeded due to the complex nature of PS and the lack of commercially available polysaccharide components. Many of the enzymes have already been characterized, enabling their existing and

potential roles in biotechnology to be evaluated. Processes for enzymatic conversion can be systematically optimized if suitable enzymes of known degraded activity are available. This approach has been adopted for some PS such as starch and cellulose, which possess small variety of bond types needed to hydrolysis. The process for pectin and hemicellulose is more complex as their carbohydrate structures and sugar composition are more varied, leading to a requirement for a complex mixture of degraded enzymes to substantially break down the polysaccharide structures (Ward & Moo-Young, 1989).

Chen *et al.* found after enzyme degradation for debranching, WGE, a polysaccharide from *Gastrodia elata*, lost its antitumor activity (Chen et al., 2011). However, enzymes are most used for structure analysis (Ohya, Ihara, Murata, Sugitou & Ouchi, 1994). Pan used enzyme to degrade polysaccharide from *Lycium barbarum* (LBP) and found LBP has the immunologic enhancement in mice (Amagase & Farnsworth, 2011).

Although the reaction condition should be strictly controlled during enzyme degradation, the use of enzymes is still a good choice because of their high selectivity and safety for human consumption.

3.3.3 Physical modification

Physical degradation methods are green and efficient methods. They are easy to operate and control. Physical modification usually contains

ultrasonic assisted method, microwave method and radical assisted method. The former two are more commonly used. Compared with other methods, physical degradation shows the advantages of economy in energy and time, streamlining procedures and reduced use in organic solvent. All these properties make its industrial application desirable.

Ultrasound (US)-assisted degradation US-assisted extraction is well established in the processing of PS extracts (Lai, Wen, Li, Wu & Li, 2010; Wang, Cheng, Mao, Fan & Wu, 2009). In the case of polysaccharide degradation, US have preliminarily acted on cellulose derivatives, starches, dextrans, and chitosans. Usually, these macromolecules exposed to high-energy US show permanent reductions in solution viscosity or gel strength attributed to decreases in MW and distribution. The degradation mechanism of macromolecules by such US was frequently attributed to cavitation (mechanical) effects and partially to the stress concentration on the segment of macromolecules (Sun, Sun & Xu, 2002).

Schizophyllan (SPG), a water-soluble β-glucan obtained from *Schizophyllum*, was found to have antitumor activities against Sarcoma-180. The degraded polysaccharide exhibited the same activities while significant differences were observed in physical properties such as MW and intrinsic viscosity. Researchers suggested the hydrolysis was caused in random (Tabata, Ito, Kojima, Kawabata & Misaki, 1981). Higher ultrasonic intensity

and longer treatment time could be used to obtain lower MW of SPG (Zhong, Zhang, Tong, Liu, Zhou & Zhou, 2015). A rice bran polysaccharide designated RON was subjected to partial hydrolysis with partial degradation by ultrasonic irradiation. A significant change in the molecular size was observed during simple chromatography of RON on a strongly acidic ion exchange resin. Degradation products with average MW retained the following activities of RON, *in vivo* antitumor activity against Meth-A fibrosarcoma in mice by oral administration, and *in vitro* macrophage stimulatory effects to induce tumoricidal activity and interleukin 1 production. A structure-activity relationship study found that the degradation product inhibited higher activity and RON was not essential for its antitumor activity (Tanigami et al., 1991).

US is regarded as an innovative, versatile and promising means for processing of medicine and food products (Cheung, 2014). Ultrasonic treatment is a viable modification technology for polymer materials with high-MW.

Microwave-assisted degradation Like the US-assisted method, microwave-assisted method is usually used in polymer extract (Xie, Xie, Shen, Nie, Li & Wang, 2010; Yang et al., 2010). Microwave was used to degrade a polysaccharide in a study about λ-Carrageenan from *Chondrus ocellatus*, and five products with different MW (650, 240, 140, 15, 9.3 kDa)

were obtained. Analytical results confirmed that microwave degradation might not change the chemical components and structure of PS under certain conditions and the results indicated that the five λ-carrageenan samples all showed antitumor and immunomodulation activities in different degrees. MW of PS had notable effect on the activities. In addition, relevance was found between their antitumor and immunomodulation activities and the five λ-carrageenans probably inhibited tumor by means of activating the immunocompetence of the body (Zhou, Sun, Xin, Zhang, Li & Xu, 2004). κ-carra-oligosaccharides can be prepared with microwave-assisted acid hydrolysis and the structures of the obtained oligosaccharides were consistent with those generated by traditional mild acid hydrolysis (Li, Zhao, Lv, Li & Yu, 2015). Drimalova et al. (Drimalova, Velebny, Sasinkova, Hromadkova & Ebringerova, 2005) compared degradation of hyaluronan between ultrasonication and microwave. They found that in contrast to the US method, the course of depolymerisation by microwave was strongly pH-dependent, and the degradation rate increased with decreasing pH. However, the US-assisted method was more appropriate to reduce the MW of HA to ~ 100 kDa without modification of the polysaccharide.

Radiation-assisted degradation PS have attracted tremendous attention due to their abundance with a variety of species and quantities in

nature and extensive applications in wide ranging fields. To obtain more valuable poly-/oligosaccharides, radiation technology is applied for polysaccharide molecular modification. Czechowska-Biskup et al. found that irradiation in dry state, irradiation in aqueous solution and sonication in aqueous solution were all efficient tools for controlled reduction of the average MW of chitosan. The radiation-chemical yields of degradation in the solid state and those in N_2O-saturated aqueous solution were similar. Moreover, the extent of side effects was similar for the doses leading to comparable reduction in MW. One should stress that the exact values of the degradation yields would depend on the properties of the starting material and the irradiation conditions (Czechowska-Biskup, Rokita, Ulanski & Rosiak, 2005). Choi et al. used irradiation to degrade chitosan. They found that the viscosity decrease was slowed down gradually with increase in irradiation dosage (Choi, Ahn, Lee, Byun & Park, 2002). Zhang et al. synthesized novel pH/temperature sensitive hydrogels by radiation-induced copolymerization and cross-linking of dimethylaminoethyl methacrylate and diallyldimethyl ammonium chloride. These hydrogels could be applied as a carrier for notoginsenoside delivery (Zhang, Xu, Yi, Zhai, Wang & Ha, 2006).

Radiation technology has been applied in molecular modification of PS to degrade chitosan, starch, carrageenan, sodium alginate and cellulose (Şen, Toprak & Güven, 2014; Taşkın, Canısağ & Şen, 2014). Compared with the

US-assisted method and microwave-assisted method, the reaction can also cause grafting and crosslinking (Wach, Rokita, Bartoszek, Katsumura, Ulanski & Rosiak, 2014).

Chapter 4 General materials and methods

This chapter only covers the common materials and methods applied in most parts of the research project. Other materials and methods will be described in the chapter where they are first used.

4.1 Cs-HK1 mycelial fermentation and exopolysaccharides (EPS) recovery

The Cs-HK1 fungus was originally isolated from the fruiting body of a wild *Cordyceps sinensis* and was identified as a *Tolypocladium* fungus (Leung, Zhang & Wu, 2006). The mycelial culture conditions of Cs-HK1 have been established in our lab in a liquid medium consisting of (per liter) 40 g glucose, 10 g yeast extract, 5 g peptone, 1 g KH_2PO_4 and 0.5 g $MgSO_4 \cdot 7H_2O$. The mycelial culture was maintained in shake-flasks with Erlenmeyer flasks placed on a shaking incubator at 150 rpm and 20 ^oC (Leung, Zhang & Wu, 2006). For the production of EPS used in this study, Cs-HK1 mycelial fermentation was carried out in 1-L Erlenmeyer flasks, each containing 200 mL of the liquid medium. After shaking incubation for 7 days, the mycelial fermentation broth in the flasks was centrifuged at 14,000 rpm for 15 min and the supernatant was collected for EPS isolation by ethanol precipitation.

The EPS produced by Cs-HK1 fungus in liquid fermentation was composed of polysaccharides (PS) and PS-protein complexes (PSP) in a wide range of molecular weight (MW) (Leung, Zhao, Ho & Wu, 2009). The EPS in the fermentation broth can usually be precipitated as a whole in a single step using 4:1 to 5:1 ethanol to aqueous volume ratio (equivalent to 80-85% v/v). Alternatively, EPS can be roughly fractionated into different MW fractions by step-wise or gradient precipitation from low to high ethanol volume ratio (Huang, Siu, Wang, Cheung & Wu, 2013). In this study, a two-step ethanol precipitation was applied to separate the EPS into a high-MW at a lower ethanol concentration (40%) and a low-MW fraction at a higher ethanol concentration (70%) (Fig. 4-1). Ethanol (95% grade) was added to the broth supernatant with stirring to a final concentration of 40% (v/v). The mixture was kept in stationary condition at room temperature for 12 h, followed by centrifugation at 10,000 rpm for 20 min. The precipitate was collected and lyophilized to give fraction EPS1 (high-MW fraction, 2.9 g/L). The second step (after the removal of EPS1) was carried out in the remaining supernatant using an ethanol volume ratio of 1:1 (~50% v/v) to precipitate the lower-MW EPS fraction, named EPS2 with a yield of 0.2 g/L.

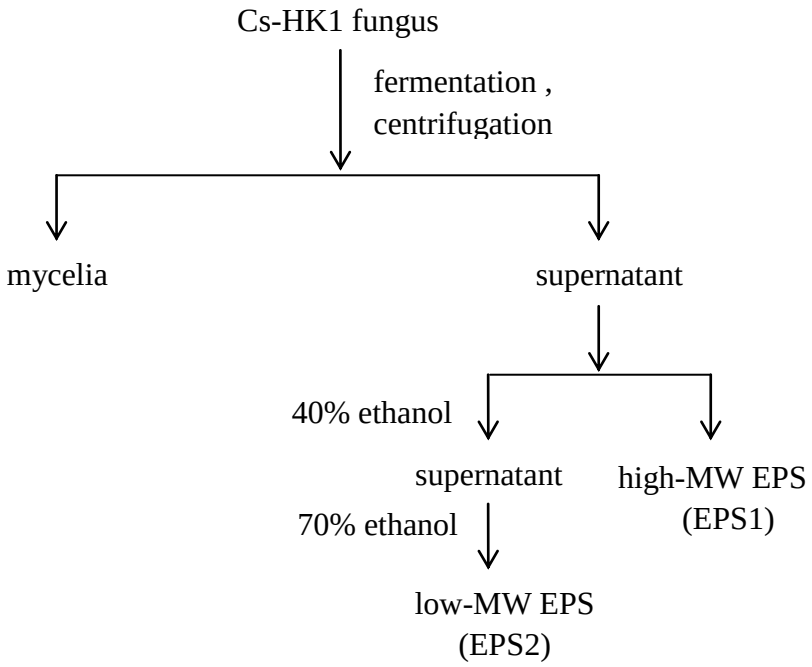


Figure 4-1 The procedures for isolating EPS1 and EPS2. Ethanol was added to the concentration of 40% to collect the high-MW fraction (EPS1). After removing EPS1, the concentration of ethanol was increased to 70% to precipitate the low-MW fraction (EPS2).

The formation of EPS1 and EPS2 was much different. EPS1 can aggregate and float in the solution while EPS2 can form particles and was easily obtained after centrifugation (Fig. 4-2).

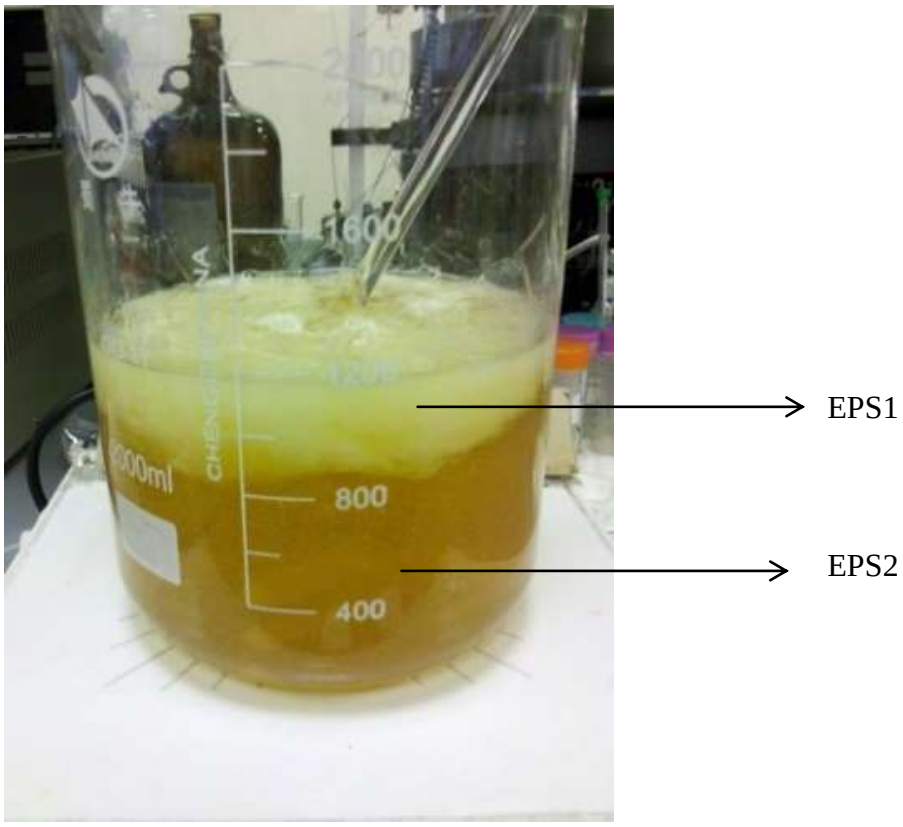


Figure 4-2 The formation of EPS1 and EPS2.

The MW of EPS1, which was about 3000 kDa, was extremely high, while the MW of EPS2 was about 40 kDa (Fig. 4-3). The physical properties and chemical composition of EPS1 and EPS2 will be further determined in the following chapters.

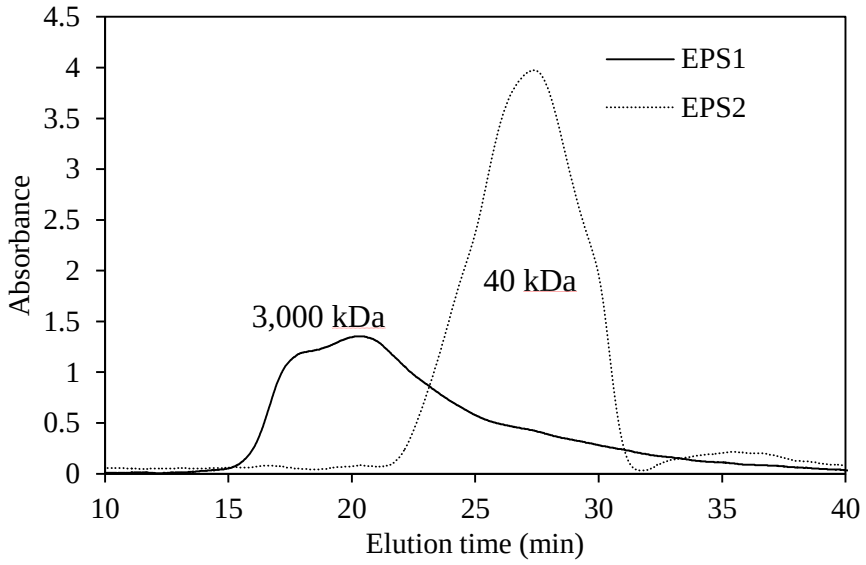


Figure 4-3 The MW distribution of EPS1 and EPS2.

4.2 Analysis of EPS content

The total carbohydrate content of EPS samples was determined by the Anthrone test through sulfuric acid hydrolysis of the samples in the presence of an anthrone agent (Sigma-Aldrich) at 100 ^{o}C. The absorbance of the sample solution was measured at 620 nm and calibrated to total carbohydrate content using glucose as a standard. The protein content of the EPS samples was determined by the Lowry method using bovine serum albumin (BSA) as a standard and absorbance was measured at 750 nm (Leung, Zhao, Ho & Wu, 2009b).

Chapter 5 Structure, properties and immunomodulatory activity of a β-D-glucan from ultrasound-degraded exopolysaccharides of Cs-HK1

5.1 Introduction

Polysaccharides (PS) from edible mushrooms possess various bioactivities that can be utilized not only as health products but also as pharmaceutical drugs (Wu, 2014). Most of the reported bioactive mushroom PS are extracted from the cell wall of fungus. Fungal cell wall is composed of two major types of PS: one is a rigid fibrillar of cellulose (or chitin), the other is a matrix-like β-glucan (Ruiz-Herrera, 1991). β-glucan is one of the key components of the fungal cell wall. The immunological and anticancer bioactivities of β-glucan from mushrooms have been studied for many years.

Microbial PS, especially the exopolysaccharides (EPS) produced by liquid fermentation, have been widely used in food, pharmaceutical and cosmetic products because of their special biological functions compared with synthetic polymers (Rehm, 2009). In addition to their bioactive functions, PS such as hyaluronic acid are frequently used as moisturizing agents (Sutherland, 1998; Zhang et al., 2013). For example, EPS from *Streptococcus thermophilus* bacteria has been used to maintain the moisture

content in cheese (Petersen, Dave, McMahon, Oberg & Broadbent, 2000). Compared with the low molecular weight (MW) organic compounds, polymeric moisturizing agents are more stable with various environmental conditions (Osada & Kajiwara, 2000). Nwodo et al. (Nwodo, Green & Okoh, 2012) have recently reviewed the various functions of bacterial EPS including adhesion, nutrition and water retention. For moisture retention (1→3)-β-D-glucans are of special interest because of their strong nature to form a helix structure to give high viscosity and gelling properties (Zhang, Li, Wang, Zhang & Cheung, 2011).

There is still not yet any reported work on the moisture absorption and retention capacity of PS from the *Cordyceps sinensis* fungus. In this chapter, the higher EPS fraction EPS1 isolated from the Cs-HK1 fermentation broth was exposed to high-intensity ultrasound (US) for partial degradation. A homogenous PS, EPS1U, was isolated from the US-degraded EPS1 and purified for structural analysis. Experimental studies were carried out to characterize the molecular structure and properties of EPS1U, and to evaluate its moisture absorption and retention properties. Its immunomodulatory activity was also tested in macrophage cell culture on NO production and cytokine release.

5.2 Materials and methods

5.2.1 Ultrasonic treatment of EPS1

Power US was generated with a Model VCX 750 processor of 20 kHz frequency and 750 W maximum output power (Sonics & Materials Inc., Newton, USA). A probe horn with 13-mm tip diameter was used in this work. The high-MW EPS fraction EPS1 was dissolved (dispersed) in de-ionized (DI) water at 10 mg/mL by vigorous stirring at room temperature. For ultrasonic treatment, 100 mL of the EPS1 solution was filled in a 200 mL plastic bottle, and the US probe was inserted into the liquid at a fixed depth of 2 cm. The sample bottle was surrounded with ice-water during the treatment to avoid overheating. The US power was fixed at 70% amplitude (corresponding to intensity of 4.3 W/cm^2 tip surface and power density of 0.31 W/cm^3 liquid). After the ultrasonic treatment for a selected period of time, the EPS solution was dialyzed (MWCO 3,000 Da) against DI water at room temperature for 48 hours. The retentate in the dialysis bag was freeze-dried to give US-degraded EPS1.

5.2.2 Purification of EPS1U from EPS1

The freeze-dried US-degraded EPS1 from above was re-dissolved in DI water at 50 mg/mL and purified by size exclusion chromatography (SEC) on a Sephacryl S-300 HR column (100 × 2.6 cm) (Sigma-Aldrich). The column was eluted with 0.1 M NaCl aqueous solution at 0.6 mL/min and detected by the Anthrone test, yielding a major fraction designated EPS1U. The MW distribution and homogeneity of EPS1 and EPS1U were analyzed by size

exclusion chromatography (SEC). Composition content of EPS1 and EPS1U was analyzed by the Anthrone test and Lowery method for the total carbohydrate and protein contents, respectively.

5.2.3 Determination of intrinsic viscosity and water solubility

The intrinsic viscosity [η] of EPS samples was determined by the serial dilution method. The viscosity of dilute EPS sample solution in water was measured with an Ubbelohde viscometer (0.5 – 0.6 mm capillary diameter) at 30 ± 0.1 ^{o}C. The [η] value of each sample was the intercept of line η_{sp}/c versus c (η_{sp} for specific viscosity and c for concentration in g/L) estimated by linear regression (Huang, Siu, Wang, Cheung & Wu, 2013).

The water solubility of EPS1 and EPS1U was determined as described by Wang et al. (Wang, Cheung, Leung & Wu, 2010). In brief, each sample (~100 mg) was suspended in 1 mL of DI water in a micro-centrifuge tube and agitated vigorously with a mini magnetic stir bar at room temperature for 24 h. The liquid was centrifuged at 18,000 rpm for 30 min, and the un-dissolved residue was collected, freeze-dried, and its weight was deducted from the original sample weight to attain the solubility.

5.2.4 Analysis of EPS1U structure and conformation

EPS1 and EPS1U were subjected to nuclear magnetic resonance (NMR) spectroscopy, infrared spectroscopy and methylation analysis to find out the

linkage information.

Congo red test was performed to examine the chain conformation of EPS1 and EPS1U in a procedure as reported by Vilares (Villares, 2013) with minor modifications. The EPS samples were dissolved in 2.0 mL NaOH solution of various concentrations from 0 to 0.5 M. Congo red was added to the solution at a final concentration of 24.4 μM and maintained at room temperature for 1 h, and the maximum absorbance λ_{max} values was recorded from 200 to 700 nm on a UV-Vis spectrophotometer.

5.2.5 Evaluation of moisture absorption and retention

The moisture absorption and retention properties of EPS1 and EPS1U were examined gravimetrically and compared with those of chitosan and urea, which are frequently used as hygroscopic and moisturizing agents (Li et al., 2011). All samples (EPS1, EPS1U, chitosan and urea) were ground into fine powder and oven-dried at 100 ℃ for 4 h. For measurement of moisture absorption, the samples (100 mg each) were placed in a closed humidity chamber maintained by saturated K_2CO_3 at 43% relative humidity (RH) or saturated $(NH_4)_2SO_4$ at 81% RH at 25 ℃ for a selected time intervals. The moisture absorption rate (Xa) was represented by the weight gain: X_a (%) = 100 × $(W_t - W_0)/W_0$, where W_0 was the weight of an oven-dried sample and W_t the weight of the sample after moisture absorption over a specific time period in the humidity chamber (Li et al.,

2011; Onoda, Yamaguchi & Takenaka, 2012).

For measurement of moisture retention, the samples (100 mg each) were first placed in a humidification chamber containing DI water at 25 ℃ for 24 h and then transferred to a humidity chamber containing saturated K_2CO_3 (43% RH) to dehydrate at 25 ℃ over a period of 24 h. The moisture retention rate (X_r) was evaluated by the weight loss of the sample: Xr (%) = 100 × W_t/W_0, where W_0 was the weight of DI water absorbed in the humidification chamber and W_t the weight of the water remaining after a specific time in the K_2CO_3 saturated chamber (Zhang, Wang, Han, Zhao & Yin, 2012).

5.2.6 Endotoxin removal

Endotoxins (lipopolysaccharides) are potent elicitors of immune responses in animal cells (David & Ryan, 1979). To avoid the influence of endotoxins possibly present in the EPS smaples, endotoxin removal was performed on the EPS samples before the immunoactivity assays (Talmadge & Siebert, 1989). In breif, the EPS samples were applied to a column containing Affi-Prep Polymyxin Matrix gel (Bio-Rad, USA) and eluted with distilled water. Both treated and untreated samples were analyzed for their biological activity as described below.

5.2.7 Immunoactivity assays

Immunoactivity of EPS fractions was evaluated by assessing the production of nitric oxide (NO) and selected cytokines of RAW264.7 cells in response to the EPS treatment. NO concentration in the culture medium was determined by Griess reaction method (Rao, 1997). RAW 264.7 macrophages (from the ATCC) were grown in DMEM supplemented with 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA), 100 units/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere (5% CO_2) at 37 ℃. After cultured for two days, RAW264.7 cells were seeded at a density of 5×10^5 cells/well (180 μL) into a sterile 96-well plate and stimulated with samples (250 μg/mL) for 48 h. LPS (final concentration at 1 μg/mL) was used as a positive control. Supernatants were then collected and mixed with 50 μL of Griess reagent {1 % (w/v) sulfanilamide, 0.1 % (w/v) naphthylethylenediamine dihydrochloride, 2% (v/v) phosphoric acid} and incubated at room temperature for 10 min. Nitrite production was determined by comparing the absorbance at 540 nm against a standard curve generated using $NaNO_2$.

The cytokine contents in the medium were determined using ELISA kits (R&D, USA) for TNF-α (DY410) and IL-6 (DY406), following the manufacturer's instructions. Briefly, cells were treated with various concentrations of EPS1U or 1 μg/mL of LPS for 12 h. The supernatant was subjected to ELISA assay.

5.2.8 Statistical analysis

All data are expressed as mean ± SEM. Statistical analysis was performed with Graphpad Prism 5 (Graphpad Software, San Diego, CA, USA) using one-way ANOVA. A *p* value of less than 0.05 was taken as statistically significant.

5.3 Results and discussion

5.3.1 Molecular properties and chemical composition of EPS1 and EPS1U

The EPS1 attained by 40% ethanol precipitation of the Cs-HK1 mycelial broth was poorly soluble in water and had high intrinsic viscosity. During the US treatment, the intrinsic viscosity of EPS1 dropped rapidly from about 16.0 dL/g to about 3.0 dL/g within 10 min and decreased slowly in the remaining period to about 1.0 dL/g after 60 min treatment (Fig. 5-1). Based on this trend in the changes in intrinsic viscosity, EPS1 was treated by US at the same intensity for 20 min to attain an US-degraded product. The US-degraded EPS1 was further purified by gel filtration to obtain the homogeneous polysaccharide EPS1U. Table 5-1 provides a summary of the molecular properties and chemical composition of EPS1 and EPS1U. Compared with the original EPS1, EPS1U had a much lower average MW (730 versus 2,700 kDa, roughly), lower intrinsic viscosity (1.7 versus 15.6 dL/g) but much higher solubility in water (77.5 g/L versus 5.1 g/L).

Table 5-1 Properties and composition of EPS1 and EPS1U

Sample	Yield (g/L)	[η] (dL/g)	MW (kDa)	Solubility (g/L)	Carbohydrate (%)	Protein (%)	Composition (mol %)		
							Glc	Man	Gal
EPS1	2.9	15.6±1.3	2,700	5.1	77.8±3.2	15.6±0.3	81.3	13.8	4.9
EPS1U	2.1	1.7±0.1	730	77.5	95.2±2.3	0.6±2.0	98.5	1.3	0.2

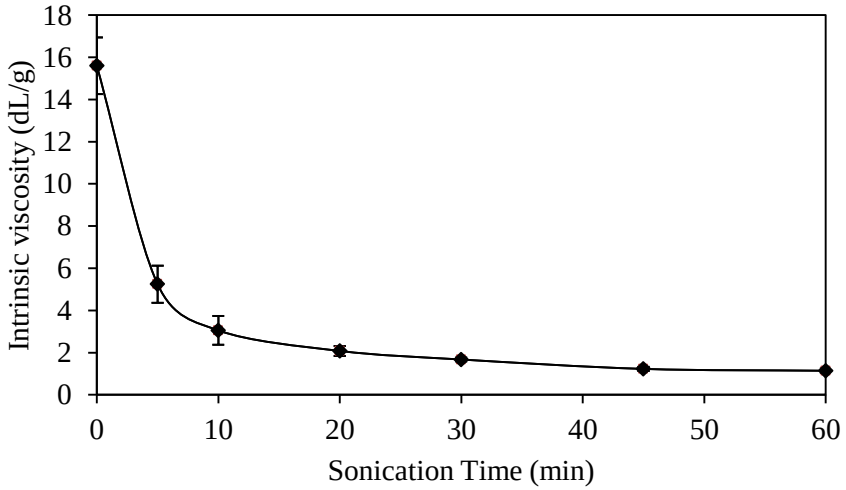


Figure 5-1 Intrinsic viscosity of EPS1 solution during ultrasonic treatment at 70% amplitudes (Error bars for standard deviations at n=3).

On the HPSEC spectrum (Fig. 5-2), EPS1U exhibited a higher and more symmetric peak with a lower average MW (longer elution time) than EPS1. Although the same concentration of EPS1 and EPS1U was applied to the HPSEC analysis, there was a much higher peak of EPS1U which might be attributed to its much lower MW and higher solubility. The more uniform MW distribution of EPS resulting from the US treatment is a common outcome of ultrasonic polymer degradation that occurs mainly to the

higher-MW components (Wang, Cheung, Leung & Wu, 2010). Associated with the dramatic reduction of average MW was a significant drop in the intrinsic viscosity of EPS1U. The solubility of EPS1U in water also increased dramatically compared with that of EPS1. After 24 h of vigorous mixing in water, EPS1 (5 mg/mL) formed a milky dispersion (Fig. 5-3A), while EPS1U (5 mg/mL) formed a clear solution (Fig. 5-3B).

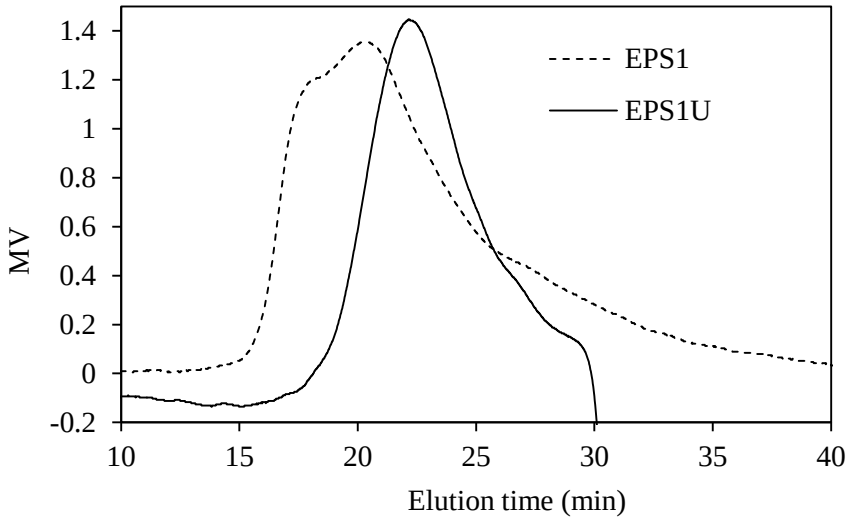


Figure 5-2 HPGPC chromatogram of EPS1 and EPS1U.

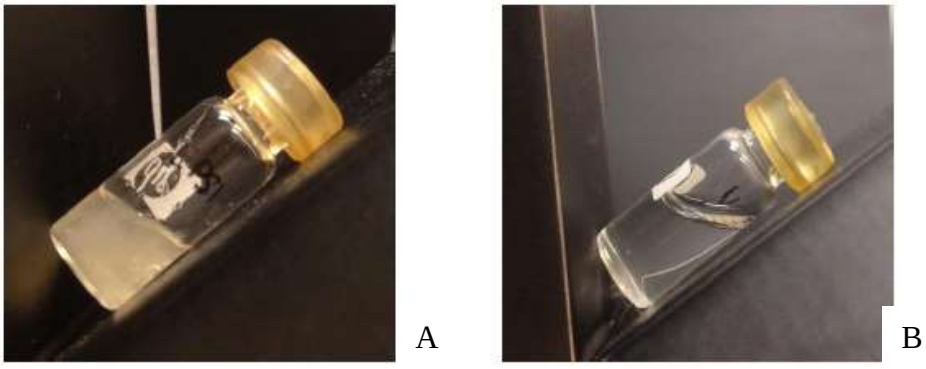


Figure 5-3 Appearance of EPS1 (A) and EPS1U (B) dissolved in water at 5

mg/mL and 20 ^{o}C.

In addition to the physical and molecular properties, the chemical compositions of EPS1 and EPS1U were quite different. EPS1 had relatively high protein content and was either a mixture or a complex of PS and proteins, while EPS1U was nearly pure PS with negligible protein content. As for the carbohydrates constituents, EPS1 was composed of glucose, mannose and galactose at the molar ratio of 81.3:13.8:4.9; while EPS1U was composed of glucose (> 95 %) with minor or negligible mannose and galactose (Table 5-1). The mannose and galactose constituents were probably weakly bound to EPS1 and removed during the US treatment and the following purification steps.

It is noteworthy that EPS1U had good water solubility with such a high MW of 730 kDa. Previous studies on the EPS produced by Cs-HK1 mycelial fermentation have been concerned mainly with either the crude EPS precipitated with 3-5 volumes of ethanol (Huang, Siu, Wang, Cheung & Wu, 2013; Leung, Zhao, Ho & Wu, 2009b; Wang, Cheung, Leung & Wu, 2010) or purified homogenous PS at relatively low MW below 50 kDa (Wang, Peng, Lee, Tang, Cheung & Wu, 2011b; Yan, Li, Wang & Wu, 2010). Even for the PS with known structures such as β-glucans from edible and medicinal fungi, the MW ranges were mostly below 200-500 kDa (Ahmad, Anjum, Zahoor, Nawaz & Dilshad, 2012; El Enshasy & Hatti-Kaul, 2013).

By applying a low ethanol concentration of (2/5 or ~2/3 volume ratio), we were able to isolate the higher MW fraction of crude EPS (EPS1) with an average MW of about 2,700 kDa. Upon ethanol precipitation, EPS1 formed a large clump floating on top of the liquid (Fig. 4-2) because of the highly hydrated nature. After the US treatment of EPS1, the resultant EPS1U had a lower viscosity and higher solubility, and could be readily purified through gel-filtration. Similarly, Liu et al. (Liu et al., 2014b) recently isolated a high MW (1→3)-β-D-glucan of about 3,750 kDa from the hot water extract of the *Ganoderma luciduma* fruiting bodies by precipitation with a low ethanol concentration of 20% (v/v).

5.3.2 Structure elucidation of EPS1U

The methylation analysis results of EPS1U (Table 5-2) showed the linkages of terminal glucopyranose (T-Glc), 1,3- and 1,3,6-linked glucopyranose at a molar ratio of about 1:1:1. Because of the very very low contents of manose (1.3%) and galactose (0.2%) in EPS1U (Table 5-1), the peaks of the two residues were barely visible (Fig. 5-4). It can be deduced that EPS1U had a 1,3- glucopyranose backbone with a glucose side chain at *O*-6 in every two repeating units.

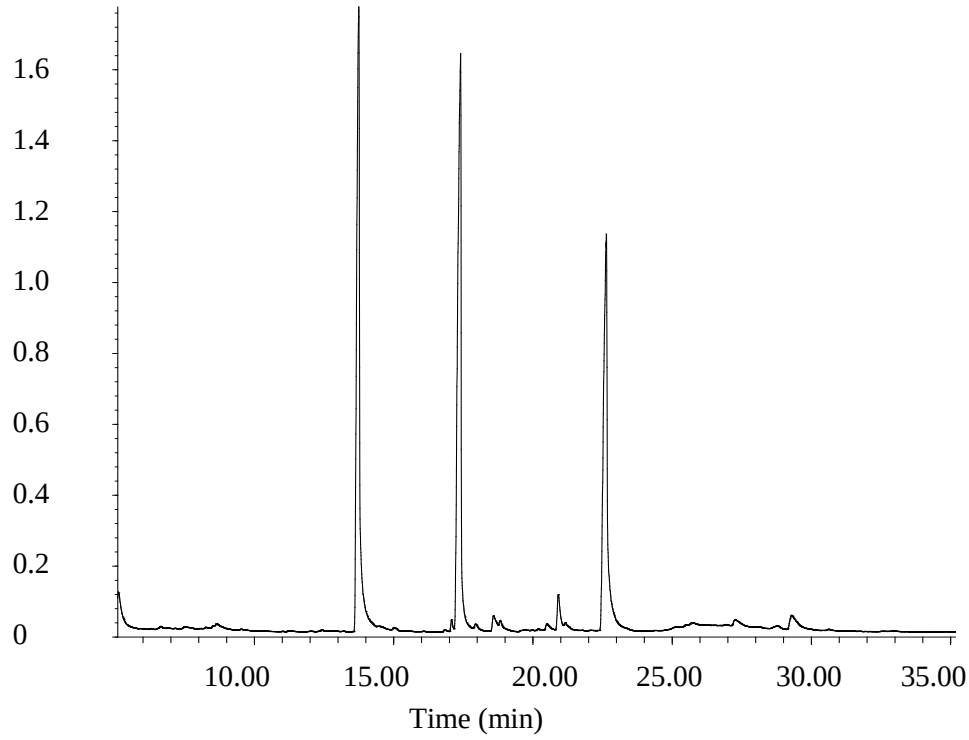


Figure 5-4 GC pattern of alditol acetates from the methylation product of EPS1U. Three fragments with the retention time of 13.7, 17.4 and 22.6 min were terminal glucopyranose (T-Glc), 1,3- and 1,3,6-linked glucopyranose, respectively.

Table 5-2 Linkage analysis of EPS1U

Methylated sugars	Linkages	Molar ratios (%)
2,3,4,6-Me_4-Glc*p*	T-Glc*p*	34.9
2,4,6-Me_3-Glc*p*	1,3-Glc*p*	35.4
2,4-Me_2-Glc*p*	1,3,6-Glc	29.7

Fig. 5-5A shows the DEPT NMR spectrum of EPS1U. Usually, the shift of anomeric carbon in (1→3)-β-D-glucan is about 103 ppm while the shift of C-1 in (1→3)-β-D-glucan is around100 ppm (Lehtovaara & Gu,

2011b). Thus, the stronger signals at 105.9, 102.2 and 99.3 ppm correlated to C-1 of (1→3,1→6)-β-D- glucopyranosyl, (1→3)-β-D-glucopyranosyl and terminal D-glucopyranosyl, respectively. The signal at 65.8 ppm was assigned to the substituted C-6 of (1→3, 1→6) -β-D-glucopyranosyl. The weak anomeric signals appearing in the NMR spectrum of EPS1U could be attributed to impure sugar residues such as galactomannan (Fig. 5-6). DEPT spectrometry of EPS1U in D_2O was difficult to perform due to its high MW and viscosity even though warming and accumulation time had been applied to improve the resolution. Improved ^{13}C NMR spectrum was attained with the EPS1U sample dissolved in the solvent mixture of DMSO-*d*6/D_2O (Fig. 5-5B), in what the signal at 103.4 ppm also indicated a β configuration for glucopyranosyl units.

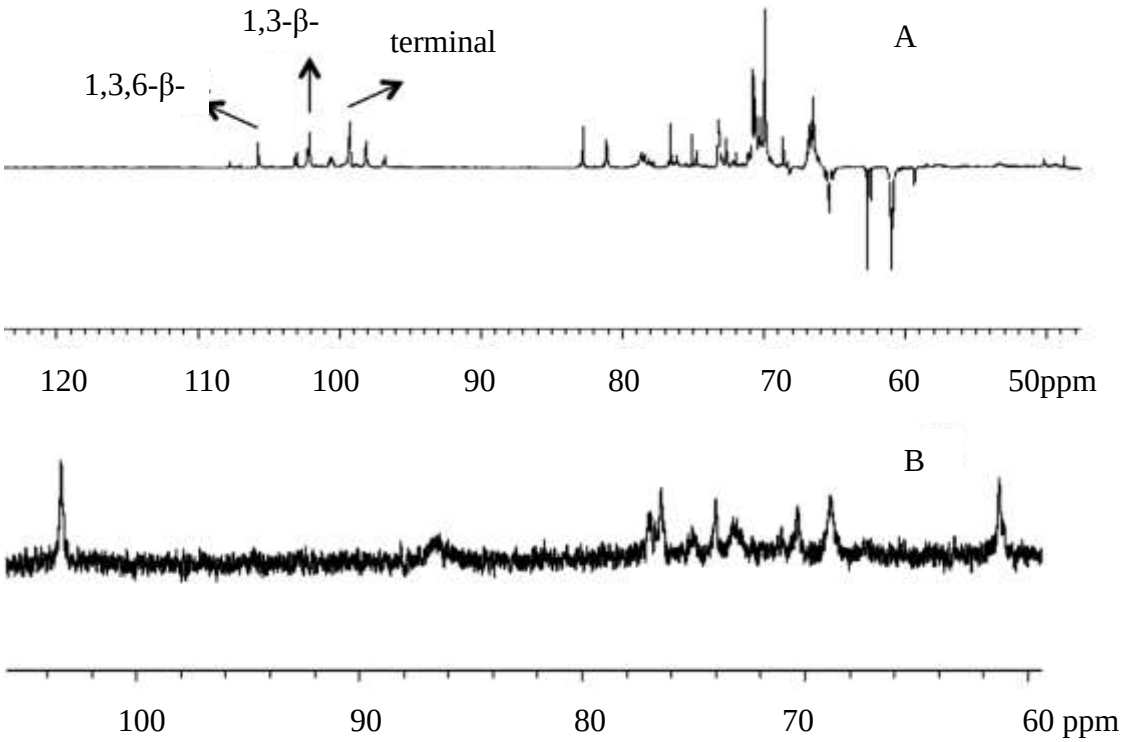


Figure 5-5 ^{13}C NMR spectra of EPS1U: (A) distortionless enhancement by polarization transfer (DEPT) spectrum of EPS1U in D_2O; (B) spectrum of EPS1U dissolved in DMSO-*d*6/D_2O (6:1).

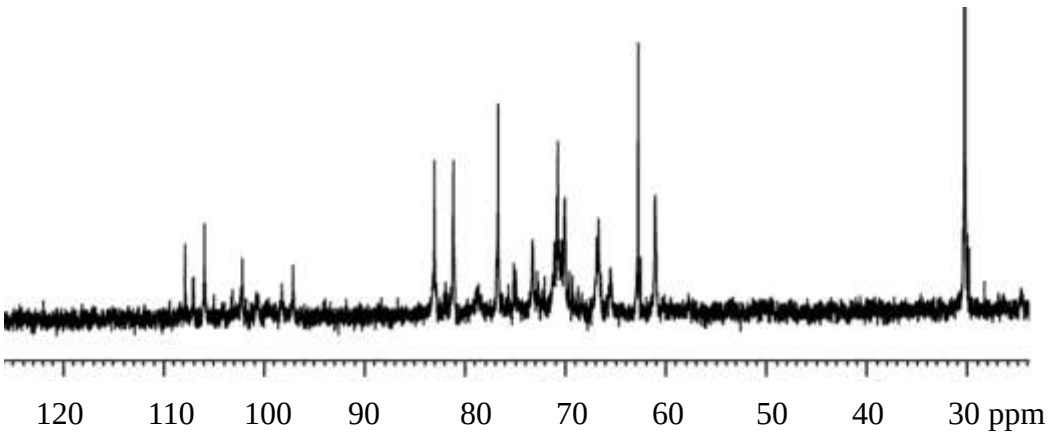
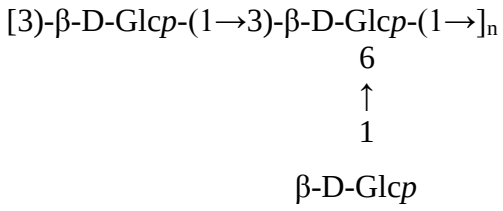


Figure 5-6 ^{13}C-NMR spectrum of a homogeneous galactomannan from the fermentation broth of Cs-HK1.

Based on the methylation analysis and NMR results, the repeating unit of EPS1U is represented as follows,



5.3.3 Chain conformation of EPS1 and EPS1U

Congo red test was performed to study the chain conformation of EPS1 and EPS1U. It has been estalished that polysaccharide chains in single helix but not other confomation in solution can form a complex with Congo red, resulting in a red shift (Wang, Cheung, Leung & Wu, 2010). Fig. 5-7 shows changes in the maximum absorbance (λ_{max}) of EPS-Congo red solutions with various alkaline concentrations. The changes in λ_{max} of Congo red complexes with EPS1 and EPS1U followed a simliar trend. All the Congo red and EPS complex solutions at 0 M NaOH had the same λ_{max}, indicating there was no single helix conformation. With 0.2 M or a lower concentration of NaOH, both EPS1-Congo red and EPS1U-Congo red solutions exhibited a red shift solution, indicating the exsistence of single helixes. The EPS1 and EPS1U in water probably formed multiple helixes as observed with another high-MW EPS isolated from the Cs-HK1 fermentation broth (Wang, Cheung, Leung & Wu, 2010). With NaOH in the low concentration range, the multiple complexes were split into single helixes by alkaline disruption

of the hydrogen bonds. The λ_{max} red shift of EPS1U was more than that of EPS1, indicating that EPS1U formed more single helixes than EPS1 which was probably due to the much lower MW of EPS1U. At much higher NaOH concentrations (> 0.2 M), the λ_{max} red shifts of EPS1 and EPS1U all disappeared, which was probably due to the complete disruption of the helical chain conformation into random coils.

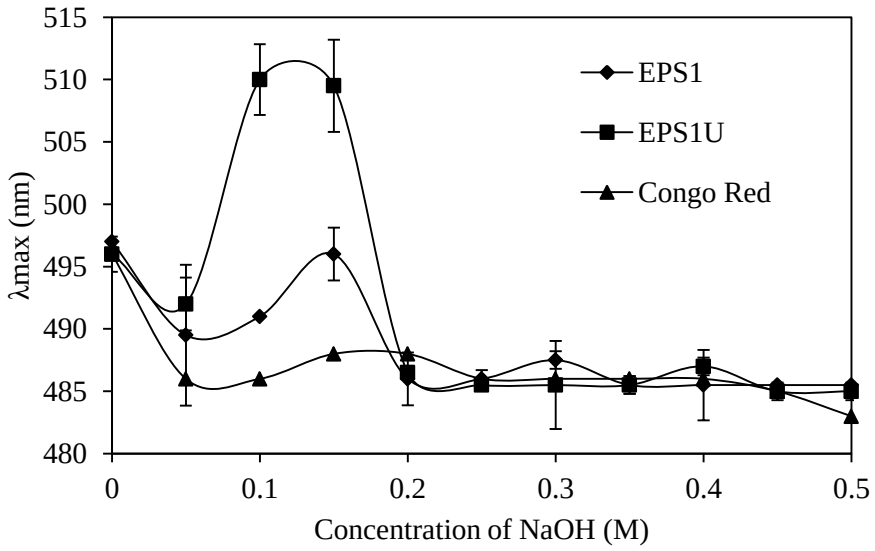


Figure 5-7 Changes in the maximum absorption λ_{max} in solution of Congo red alone, Congo red plus EPS1 and Congo red plus EPS1U at various NaOH concentrations. (Error bars for standard deviations from three triplicate tests).

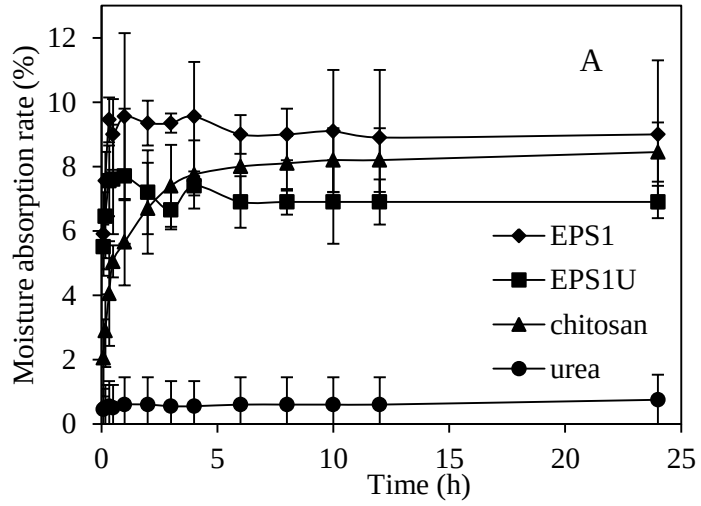
The results of the Congo red test sugegsted that high intensity US treatment of EPS1 did not cause any conformation changes of the polysaccharide chains from multiple to single helixes. It has been suggested

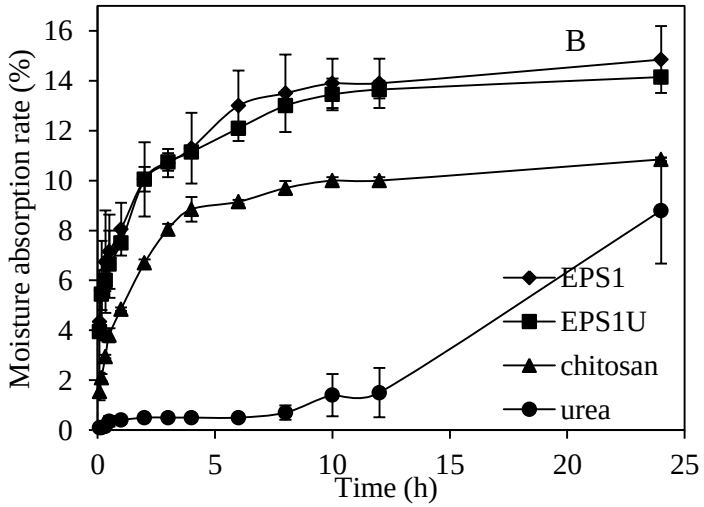
that the gel state of polysaccharides such as β-glucans in water is mostly associated with the triple-helix conformation (Zhang, Li, Wang, Zhang & Cheung, 2011). As aforementioned, EPS1 formed gels with a milky appearance in water (Fig. 5-3A). β-1,3-glucans such as lentinan and curdlan have a strong tendency to form helical structures via the hydrogen bonds, giving rise to high viscosity and gel formation in water. Aqueous alkali can disrupt the hydrogen bonds, leading to the transition from multiple to single helix conformation, and eventaully to random coils (Wang, Cheung, Leung & Wu, 2010).

5.3.4 Moisture absorption and rentention properties

As shown in Fig. 5-8A, the moisture absorption rate (X_a) of all samples at 43 % relative humidity (RH) increased rapidly in the first 4 h and that of EPS1 increased faster than all other samples. The maximum or stauration moisture absoprion (at 24 h) of EPS1 was also higher (9.0 %) than those of EPS1U (6.9 %), chitosan (8.5 %) and urea (1.3 %). At 81% RH (Fig. 5-8B), the moisture absorption rates of EPS1 and EPS1U were very close throughtout the test period, both being higher than those of chitosan and urea. After exposure to 81% RH for 24 h, the X_a of EPS1, EPS1U, urea and chitosan were 13.9%, 14.0%, 7.3% and 10.8%, respectively. Fig. 5-8C shows the moisture-retention rates of the samples after the moistrue absorption for 24 h at 43 % RH. The moisture absorbed in the EPS1 and

EPS1U samples dropped rapidly to below 10 % in the first 5 h, while that of chitosan and urea dropped more slowly. After 24 h, the moisture retention rates X_r of EPS1, EPS1U, chitosan and urea were 5.7 %, 5.8 %, 8.1 % and 15.1 %, respectively. The results showed that EPS1 and EPS1U had a lower moisture retention capacity than chitosan and urea.





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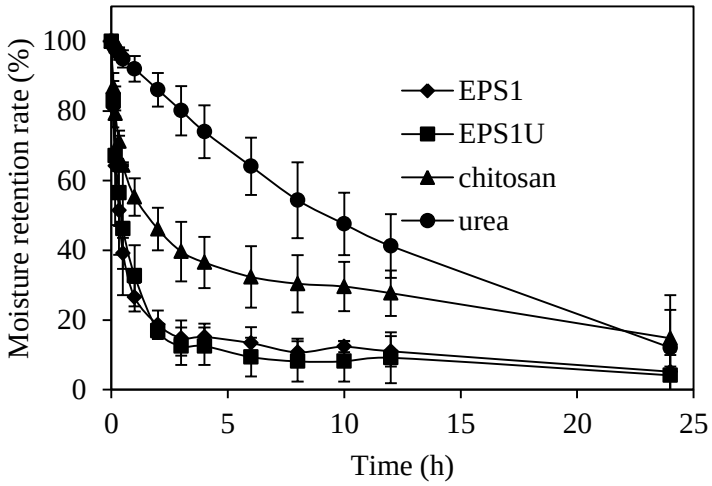


Figure 5-8 Moisture absorption by samples at RH=43 % (A) and 81 % (B) and moisture retention by samples at RH=43 % (C). (Errot bars for SD from three replicate tests.)

The intermolecular hydrogen bonding of polysacchairde chains is regarded as a major factor for the moisture absorption and retention ability of PS (Chen, Du & Zeng, 2003). The multiple-helix conformation is favorable for water retention as water molecules can be retained within the chain networks. According to the results of Congo red test, EPS1U still maintained the multiple-helix conformation, having similar moisture absorption and retention capability to EPS1. With much higher solubility in water, EPS1U should be more desirable than EPS1 for used as a moisturizing agent. However, the absorbed water molecules by EPS1 or EPS1U easily volatilized in dry environment, leading to low moisture rentention abilities.

5.3.5 Immunomodulatory activity

Endotoxin consists of LPS and outer membrane components of gram-negative bacteria. It is an important pathogenic substance to trigger the production of a variety of mediators that induce systemic inflammation (Shoji, Tani, Hanasawa & Kodama, 1998). Endotoxin contamination usually happens during PS extraction. The presence of endotoxin affects the effect of PS on many mediators such as NO and cytokines. In this test, Affi-Prep polymyxin Matrix gel was used to remove endotoxins in EPS1U. As shown in Fig. 5-9, the NO stimulation ability of EPS1U decreased by about 40% after the first removal of endotoxins but the decrease was not significant after the second removal.

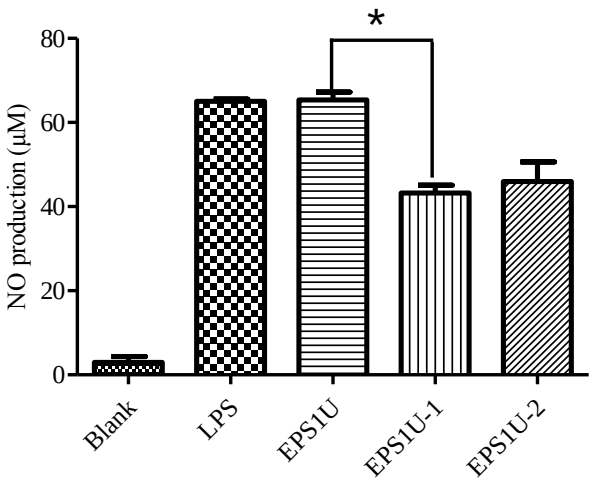


Figure 5-9 The Effect of EPS1U on nitrite production by macrophages. RAW264.7 cells were treated with EPS1U with different treatment (250 μg/mL) or LPS (1 μg/mL) for 48 h and culture supernatants were analyzed for nitrite production. Values are the means ± SEM. of triplicate determinations. * indicates significant differences between EPS1U with

different treatment. EPS1U-1 and EPS1U-2 were EPS1U after first and second endotoxin removal, respectively.

The cell inhibition of EPS1U to RAW264.7 also decreased after the removal of endotoxins (Fig. 5-10) from over 40% before the removal to below 20% afterwards.

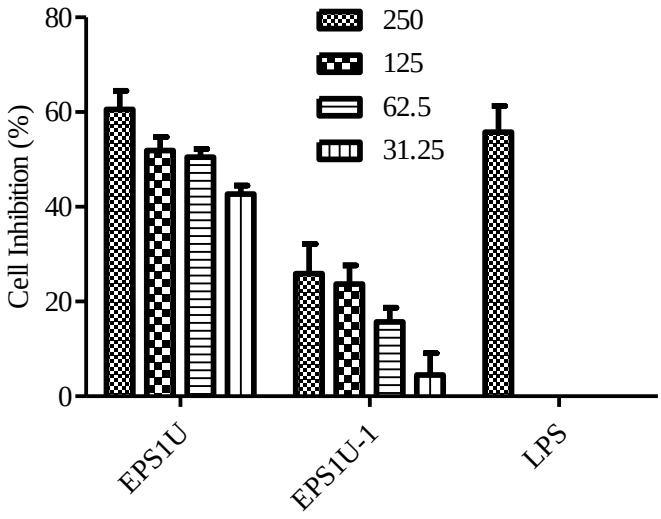


Figure 5-10 Cell inhibition of EPS1U, its endotoxin removal products and LPS (1 μg/mL) to RAW264.7 cells at different concentrations. Samples were added to the media after cells were maintained in the fresh medium for 2 h. Cell viability was evaluated by MTT assay after 48 h. Values are the means ± SEM. of triplicate treatments.

The NO production of RAW264.7 cells treated with different concentrations of EPS1U for 48 h increased significantly compared with the

untreated controls (Fig. 5-11A). This indicated that EPS1U was effective in stimulating macrophages to release NO.

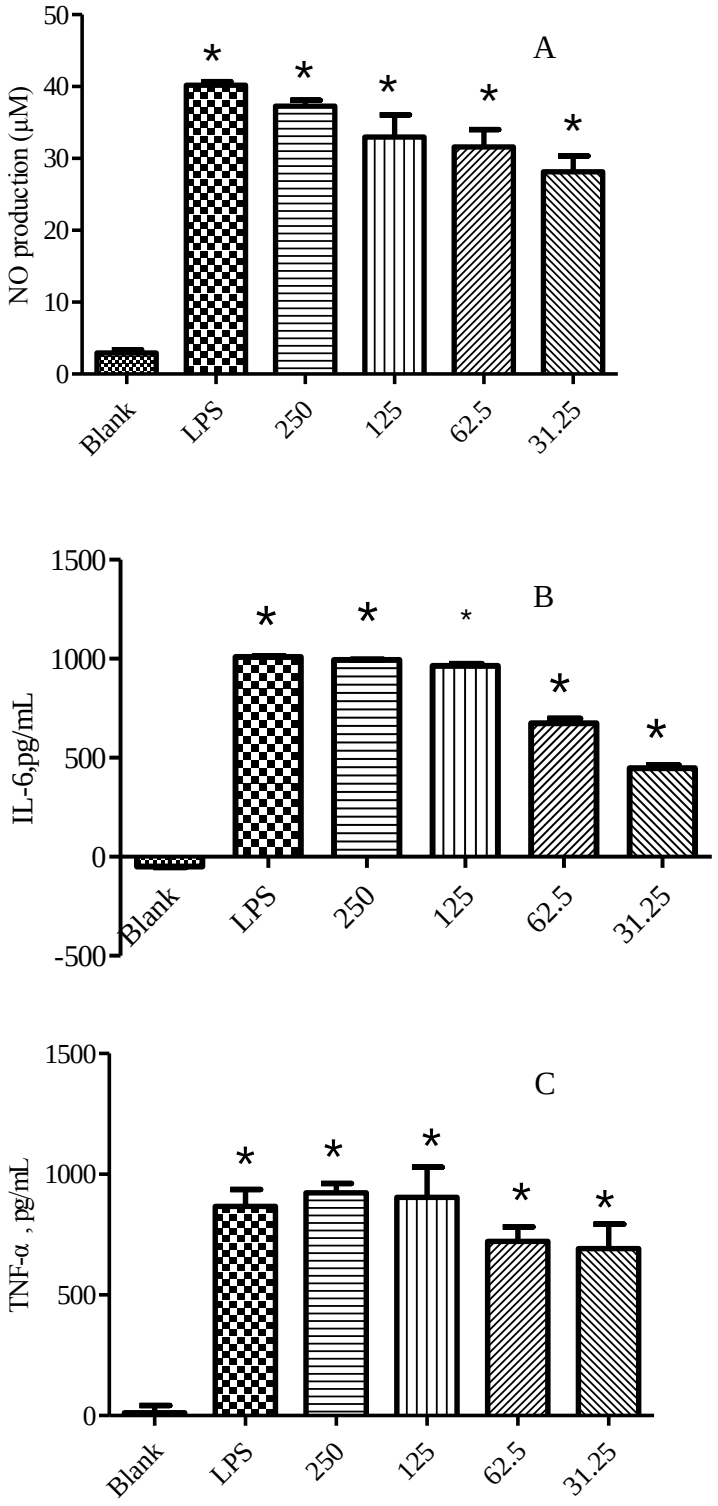


Figure 5-11 The Effect of EPS1U on NO production by macrophage

RAW264.7 cells (A) and release of cytokines by macrophage RAW264.7 cells IL-6 (B) and TNF-α (C). Cells were treated with different concentrations of EPS1U or 1 μg/mL of LPS for 48 h. Values are the means ± SEM. of triplicate treatments. * indicates significant differences from control.

A number of immunomodulatory compounds can regulate cytokine production. In this test, we analyzed the effect of EPS1U on macrophage IL-6 (Fig. 5-11B) and TNF-α (Fig. 5-11C) production.

Untreated RAW264.7 cells produced very little IL-6 and TNF-α while incubating these cells with EPS1U would enhance the production of IL-6 and TNF-α in a concentration-dependent manner. The result on cytokine production in general was in agreement with the results of the above NO production test.

A number of PS from edible and medicinal mushroom have been reported to have immune activity (Ramberg, Nelson & Sinnott, 2010; Schepetkin, Xie, Kirpotina, Klein, Jutila & Quinn, 2008). Fucose-containing glycoprotein fraction from the water-soluble extract of *Ganoderma lucidum* can stimulate spleen cell proliferation and cytokine expression (Wang, Khoo, Chen, Lin, Wong & Lin, 2002). Sclerotial PS from *Pleurotus tuber-regium* and *Polyporus rhinoceros* increase the weight of spleen weight of both

healthy BALB/c mice and healthy athymic nude mice (Wong, Lai & Cheung, 2011). As potential immune receptors, a polysaccharide from *Coriolus versicolor* can bind and induce activation of B cells using membrane Ig and TLR-4 (Yang, Zhuang, Si, Qi & Zhao, 2015). A heteropolysaccharide from *Pleurotus eryngii* has potential to be an immunomodulatory adjuvant for chemotheraphy (Liu, Wang, Zhang, Wang, Zhang & Li, 2015). AEPS-1, a homogenous acidic PS, was isolated from the fermentation broth of Cs-HK1 (Wang, Peng, Lee, Tang, Cheung & Wu, 2011). However, the yield of AEPS-1 was low. EPS1U can be obtained easily with a high yield of 2.2 g/L, making it more suitable for industrial application.

5.4 Summary

In conclusion, the high-MW EPS fraction EPS1 was isolated by ethanol precipitation from Cs-HK1 mycelial fermentation broth and was degraded with power ultrasound. A (1→3)-β-D-glucan named EPS1U with a high MW of about 730 kDa was purified from the US-degraded EPS1 by gel-filtration chromatography. The EPS1U polymer chain exhibited multiple helix conformation when dissolved in DI water. Compared with EPS1, EPS1U had significantly higher water solubility and lower viscosity. EPS1U as well as EPS1 showed strong moisture absorption capability. With its high water solubility, high viscosity and moisture absorption capacity, EPS1U has great potential as a thickening and moisturizing agent for food and

cosmetic applications. EPS1U has also shown potent immunomodulatory properties which can enhance macrophage functions of cells. Further studies are needed to determine which receptor is essential to the expression of various immunomodulatory effects ascribed to EPS1U.

Chapter 6 An antioxidative galactomannan-protein complex separated from lower-MW exopolysaccharide fraction of Cs-HK1

6.1 Introduction

Polysaccharides (PS) and their complexes with proteins (PSPs) represent a class of the most abundant bioactive constituents of *Cordyceps sinensis* and other medicinal fungi. A galactomannan was isolated from the fruiting bodies of *C. sinensis* more than 35 years ago and its structure was elucidated as having a (1→2)-α-D-Man*p* main chain (Miyazaki, Oikawa & Yamada, 1977). Another galactomannan containing a minor protein content was later isolated from the fruiting bodies of *C. sinensis*, and it had a (1→2)-α-D and (1→6)-α-D-Man*p* main chain (Kiho, Tabata, Ukai & Hara, 1986). Galactomannans have been used as food stabilizers (Cerqueira et al., 2011) and diagnostic agents for invasive aspergillosis in immunocompromised patients (Pfeiffer, Fine & Safdar, 2006). Very recently, a galactomannan from guar gum has been shown to be safe as a clinical grade galectin antagonist for cancer patients (Demotte et al., 2014).

Up to date, no galactomannan has been retained from the fungal mycelia or exopolysaccharides (EPS) produced by *C. sinensis* fermentation. This study was to analyze the chemical composition and elucidate the

molecular structure of a novel galactomannan-protein complex fractionated and purified from the EPS produced by the Cs-HK1 fungus in liquid fermentation. Its antioxidant capacity was evaluated by chemical and cell culture assays.

6.2 Materials and methods

6.2.1 Isolation and fractionation of EPS from fermentation broth

The lower-MW fraction EPS2 was obtained after a two-step ethanol precipitation as described in Chapter 4. EPS2 (~400 mg) was dissolved in 2 mL distilled water and loaded to a DEAE-Sephadex A-25 column (2.6 × 100 cm) (Pharmacia), and eluted stepwise with distilled water and 0.1 M NaCl at a flow rate of 0.5 mL/min. Polysaccharide in the elute was detected by the anthrone agent (Sigma). A major EPS fraction was collected during elution with distilled water and treated with 15% trichloroacetic acid (Douek et al., 2001) at 4 ^{o}C to remove protein and to give the EPS fraction EPS2BW (~80 mg).

6.2.2 Partially acid hydrolysis and methylation analysis

EPS2BW (50 mg) was partially hydrolyzed with 0.1 M TFA at 100 ^{o}C for 1 h. The acid was removed under reduced pressure by repeated evaporation with methanol followed by dialysis (3,500 Da MWCO). Both the retentate (designated EPS2BW-I: 27 mg) and the dialysate (EPS2BW-O:

22 mg) were collected, concentrated and lyophilized, and subjected to composition, MW distribution, NMR spectrum, IR spectrum and methylation analyses.

6.2.3 Antioxidant activity assays

The antioxidant capacity of EPS2BW was evaluated with two chemical assays, the Trolox equivalent antioxidant capacity (TEAC) and the ferric reducing ability of plasma (FRAP), and a cell culture assay on cytoprotective activity against oxidative stress. The TEAC and FRAP assays were performed with the procedures and conditions as reported in detail by Leung et al. (Leung, Zhao, Ho & Wu, 2009). In brief, the EPS sample was predissolved in distilled water and added to the assay reagent solution at a suitable final concentration, and incubated at a set temperature over 24 h, during which the sample was taken at 2 h, 12 h and 24 h for measurement of absorbance at 734 nm for TEAC or 593 nm for FRAP assay. The radical scavenging activity value at each time point was calculated by $(1-A/A_0) \times 100\%$, where A and A_o are the absorbance values of $ABTS^{\bullet+}$ or Fe^{3+} solution in the presence and absence of the PS sample, respectively or by the antioxidant reference (Trolox for TEAC assay or Fe^{2+} for FRAP assay). The antioxidant activities were converted to TEAC (μmol Trolox/mg sample) by the calibration with Trolox from 0 to 60 μmol or to FRAP (μmol Fe^{2+}/mg sample) by calibration with Fe^{2+} from 0 to 50 μmol.

The cytoprotective activity test was performed in rat pheochromocytoma PC12 cell culture and the cells were subjected to peroxide H_2O_2 treatment as described by Chen et al. (Chen, Siu, Wang, Liu & Wu, 2013). The PC12 cell culture was routinely propagated in RPMI1640 medium, which was supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin and 2 mM L-glutamine, and incubated in humidified, 5% CO_2 atmosphere at 37 ^oC. Cell viability was determined by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. For the activity test, the EPS2BW sample was pre-dissolved in phosphate buffered saline (PBS) at 10 mg/mL and the H_2O_2 solution was freshly prepared at 100 mM in PBS. In brief, 5,000 PC12 cells were seeded into a 96-well microplate in phenol red free RPMI 1640 medium. After 24 h, H_2O_2 (80 μM) was added to the wells and incubated for 4 h, followed by the addition of the EPS solution at 3.9- 500 μg/mL final concentrations and incubation for another 24 h. The cell viability was determined by MTT (Mosmann, 1983). The procedure for determining the cytotoxicity of EPS2BW to PC12 cells was the same as that of the cytoprotective activity test except that PC12 cells were not injured by H_2O_2.

6.3 Results and discussion

6.3.1 MW distribution and chemical composition of EPS2BW

EPS2 was eluted with distilled water by DEAE-Sephadex A-25 column

(Figure 6-1). The major peak was collected and deprotainized (by TCA) to give EPS2BW.

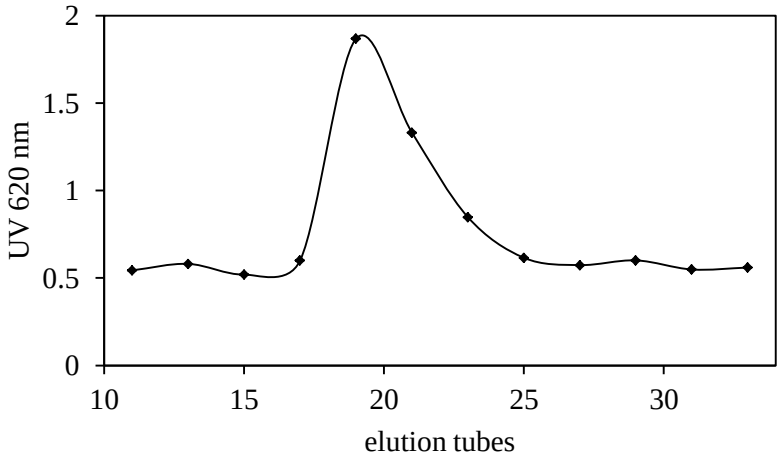


Figure 6-1 Fractionation of EPS2 on a DEAE-Sephadex A-25 column (2.6 cm × 100 cm) detected by anthrone agent. The main fraction from tube 17-23 was further treated with TCA to remove protein to give the EPS fraction EPS2BW.

EPS2BW exhibited a symmetrical peak on the HPGPC detected by RI and UV detector (Fig. 6-2), indicating it is a homogeneous polysaccharide with protein. The average MW of EPS2BW was estimated at 50 kDa.

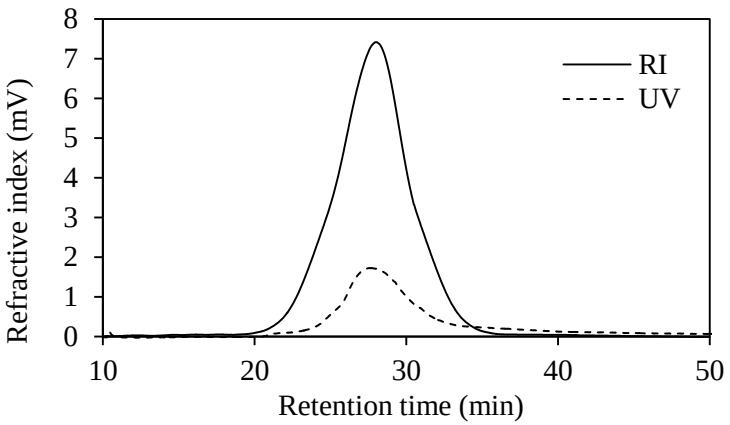


Figure 6-2 HPGPC spectrum of EPS2BW.

EPS2BW had 74.3% total carbohydrate content and 15.8% total protein content (carbohydrate to protein ratio 4.7:1.0). HPLC analysis of the completely hydrolysed products with 2 M TFA showed that EPS2BW consisted of mannose and galactose at a molar ratio of 1.7:1.0 (Table 6-1). As for the two partially hydrolyzed products with 0.1 M TFA, EPS2BW-I (the retentate of dialysis) consisted of mannose and galactose at a molar ratio of 7.2:1.0 as detected by HPLC; EPS2BW-O (the dialysate) was composed of ~34% total carbohydrate and ~46% protein and its carbohydrate part consisted of mannose and galactose in a ratio of 1.0:8.9 (Table 6-1). It was deduced from GPC and composition analysis that EPS2BW was a polysaccharide-protein complex. The protein in EPS2BW was bonded to the branch or the end point of the backbone.

Table 6-1 Composition analysis of EPS2BW and its partial acid hydrolysis products

Fractions	Content (% w/w)		
	Mannose	Galactose	Protein
EPS2BW	46.8	27.5	15.8
EPS2BW-I	11.3	81.7	-
EPS2BW-O	30.6	3.4	46.0

Table 6-2 showed the amino acid composition of the protein part in EPS2BW and a protein-galactomannan (CT-4N) isolated from the fruiting body of *C. sinensis* by Kiho et al. (Kiho, Tabata, Ukai & Hara, 1986). The two had very similar amino acid composition in the amino acid constituents and relative contents.

Table 6-2 Amino acid composition of the protein segments in EPS2BW (mol%).

Amino acid	EPS2BW	CT-4N [a]
aspartic acid	7.9	6.3
glutamic acid	6.8	13.3
serine	8.0	11.8
histidine	1.8	0.8
glycine	5.5	12.9
threonine	16.6	10.7
arginine	4.7	1.2
alanine	8.3	14.2
tyrosine	1.3	0.5
cysteine	0.6	1.5
valine	8.3	8.4
phenylalanine	2.5	1.4
isoleucine	4.0	2.5
leucine	3.5	2.6
lysine	7.7	1.4
proline	12.5	10.5

[a] Kiho et al. (1986)

6.3.2 Glycosidic linkages of EPS2BW from methylation analysis

Table 6-3 showed the results from methylation analysis of EPS2BW and its two hydrolysates. The intra-chain residues contained (1→2)-linked Man*p* (28.2%) and (1→3)-linked Gal*p* (3.4%). The non-reducing terminals consisted of Gal*p* (30.3%) and a small amount of Man*p* (3.6%), indicating that EPS2BW was highly branched, with the main branching points situated

at (1→2,6)-linked Man*p* (31.3%) and (1→2,3)-linked Gal*p* (3.2%).

Table 6-3 Linkage analysis of EPS2BW and its partial acid hydrolysis products

Methylated sugars	Linkages	Molar ratios (%)		
		EPS2B	EPS2B-I	EPS2B-O
2,3,4,6-Me_4-Man*p*	T-Man*p*	3.6	20.4	7.5
2,3,4,6-Me_4-Gal*p*	T-Gal*p*	30.3	7.1	78.4
3,4,6-Me_3-Man*p*	1,2-Man*p*	28.2	52.8	1.1
2,4,6-Me_3-Gal*p*	1,3-Gal*p*	3.4	5.9	4.4
4,6-Me_2-Gal*p*	1,2,3-Gal*p*	3.2	-	-
3,4-Me_2-Man*p*	1,2,6-Man*p*	31.3	13.8	1.0
hexitol hexaacetate	-	-	-	7.6

With one of the partially hydrolyzed fraction, EPS2BW-I, the proportion of (1→2)-linked Man*p* significantly increased with the decrease in T-Gal*p*, suggesting that EPS2BW had a backbone consisting mainly of a (1→2)-linked Man*p* residue. The ratios between (1→2)- and (1→2,6)-linked Man*p* in EPS2BW and EPS2BW-I were 0.9 and 3.8, respectively, indicating that the increase in (1→2)-linked Man*p* was at the expense of the (1→2,6)-linked Man*p* and EPS2BW contained (1→2,6)-linked Man*p* in the backbone. Therefore, the backbone of EPS2BW consisted both of (1→2,6)-linked Man*p* (31.3%) and (1→2)-linked Man*p* (28.2%) residues in a ratio of 1.1:1.0. The other partially hydrolyzed fraction, EPS2BW-O (Table 6-3), contained 78.4%

T-Gal and 7.6% hexitol hexaacetate (by mole), suggesting the presence of galactose and galactobiose residues in the oligosaccharides.

6.3.3 IR and NMR spectral characteristics of EPS2BW

As shown in Fig. 6-3, the IR peak at 3455 cm^{-1} was characteristic of O-H stretching vibration, the peak at 2937 cm^{-1} was characteristic of C-H stretching, and the peak at 1633 cm^{-1} was ascribed to C=O in protein. In addition, no peak appeared at 1250 cm^{-1} or 850 cm^{-1}, indicating there was no sulfation in EPS2BW.

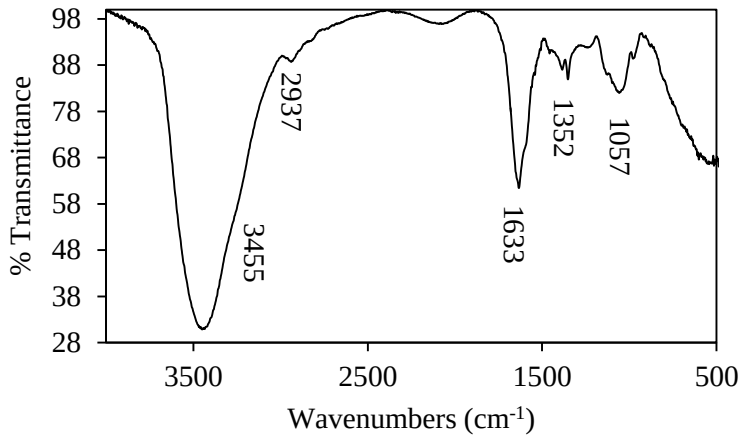


Figure 6-3 IR spectrum of EPS2BW.

The ^{13}C-NMR spectrum of EPS2BW (Fig. 6-4A) showed anomeric carbon signals at 107.8, 107.0, 106.0, 102.2, 98.2 and 97.1 ppm, which were labeled A-F, respectively. The signals at 174.8 and 21.8 ppm were attributed to the C=O and alkyl groups of protein. Compared with the ^{13}C-NMR

spectrum of EPS2BW, some signals disappeared in the ^{13}C-NMR spectrum of EPS2BW-I (Fig. 6-4B). The disappearance of protein signals suggested that protein was lost after partial acid hydrolysis. This result was consistent with that of total protein content analysis, indicating that EPS2BW-I consisted purely of carbohydrates. The signals at 107.8, 107.0 and 105.9 ppm also disappeared after partial acid hydrolysis. Together with the linkage analysis, these three signals were attributed to the anomeric carbon of galactose residues which were removed by partial acid hydrolysis. It was deduced that EPS2BW was a protein-containing polysaccharide with a backbone consisting of Man*p* residues and side chains of Gal*p* residues.

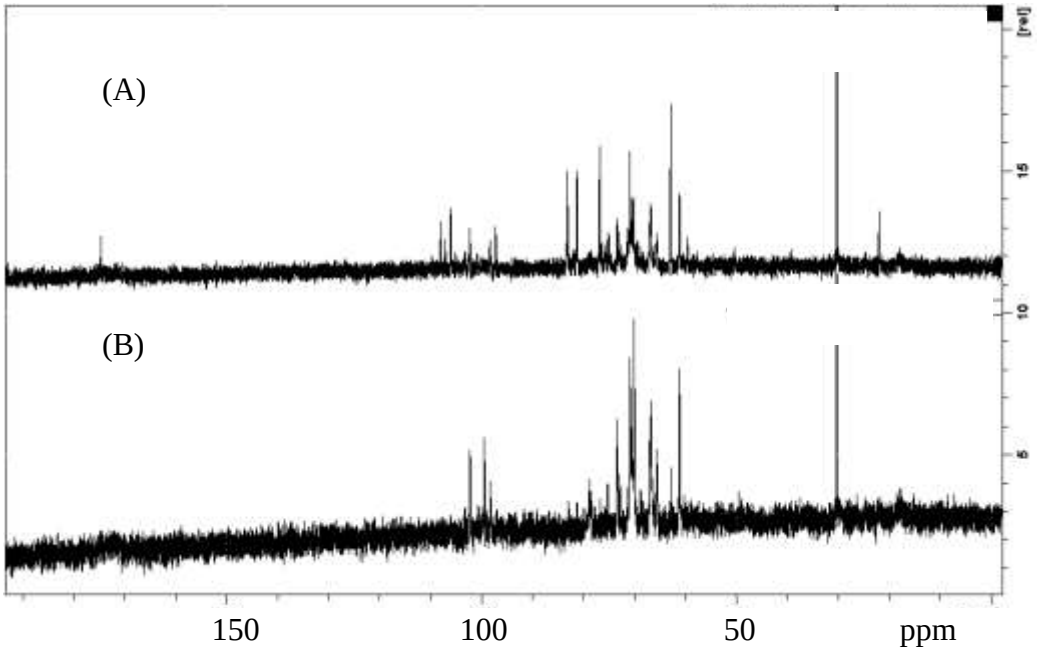


Figure 6-4 ^{13}C-NMR spectrum of EPS2BW (A) and its hydrolysis product EPS2BW-I (B).

Fig. 6-5 shows the heteronuclear single- and multiple-quantum coherence spectrum (HSQC: Fig. 6-5A; HMQC: Fig. 6-5B) of EPS2BW, from which the relevant hydrogen and carbon signals were assigned (Table 6-4). The shift of anomeric carbon in the non-reducing terminal of β-D-Gal*p* residues was slightly higher than that in (1→3)-β-D-linked Gal*p* residues (Bilan, Vinogradova, Shashkov & Usov, 2007) but lower than that in (1→2)-β-D-linked Gal*p* residues (Zhao et al., 2007). Therefore, the carbon signals at 107.8, 107.0 and 105.9 ppm were attributed to the anomeric carbon signals A, B and C (Table 6-4), respectively. The ratio of (1→2)-α-D-Man*p* residues increased while the ratio of (1→2,6)-α-D-Man*p* residues decreased after partial acid hydrolysis. Based on these changes in the signal strength of Man*p* residues before and after acid hydrolysis, the major anomeric carbon signals at 102.2, 98.2, 97.1 ppm were assigned to the anomeric carbon signals D, E and F (Table 6-4), respectively. Other than the major signals A-F assigned from the HSQC and HMBC spectrum, it is not possible to assign all signals of the saccharide units because of signal overlapping. The reversed resonance at 66.66 ppm in distortionless enhancement by polarization transfer (DEPT) spectrum (Fig. 6-6) undoubtedly arose from C6 of (1→2,6)-α-D-Man*p* residues. In Fig. 6-5B, the cross peak at 4.98 and 66.7 ppm suggested the coupling of H1 of (1→2,3)-β-D-Gal*p* residues with C6 of (1→2,6)-α-D-Man*p* residues, and (1→2,3)-β-D-Gal*p* residues was attached at O-6 of (1→2,6)-α-D-Man*p*

residues. The cross peak at 5.17 and 107.84 ppm suggested that the coupling of C1 of (1→2,3)-β-D-Gal*p* residues with H1 of β-D-Gal(1→ and Gal*p* terminals was attached to (1→2,3)-β-D-Gal*p* residues.

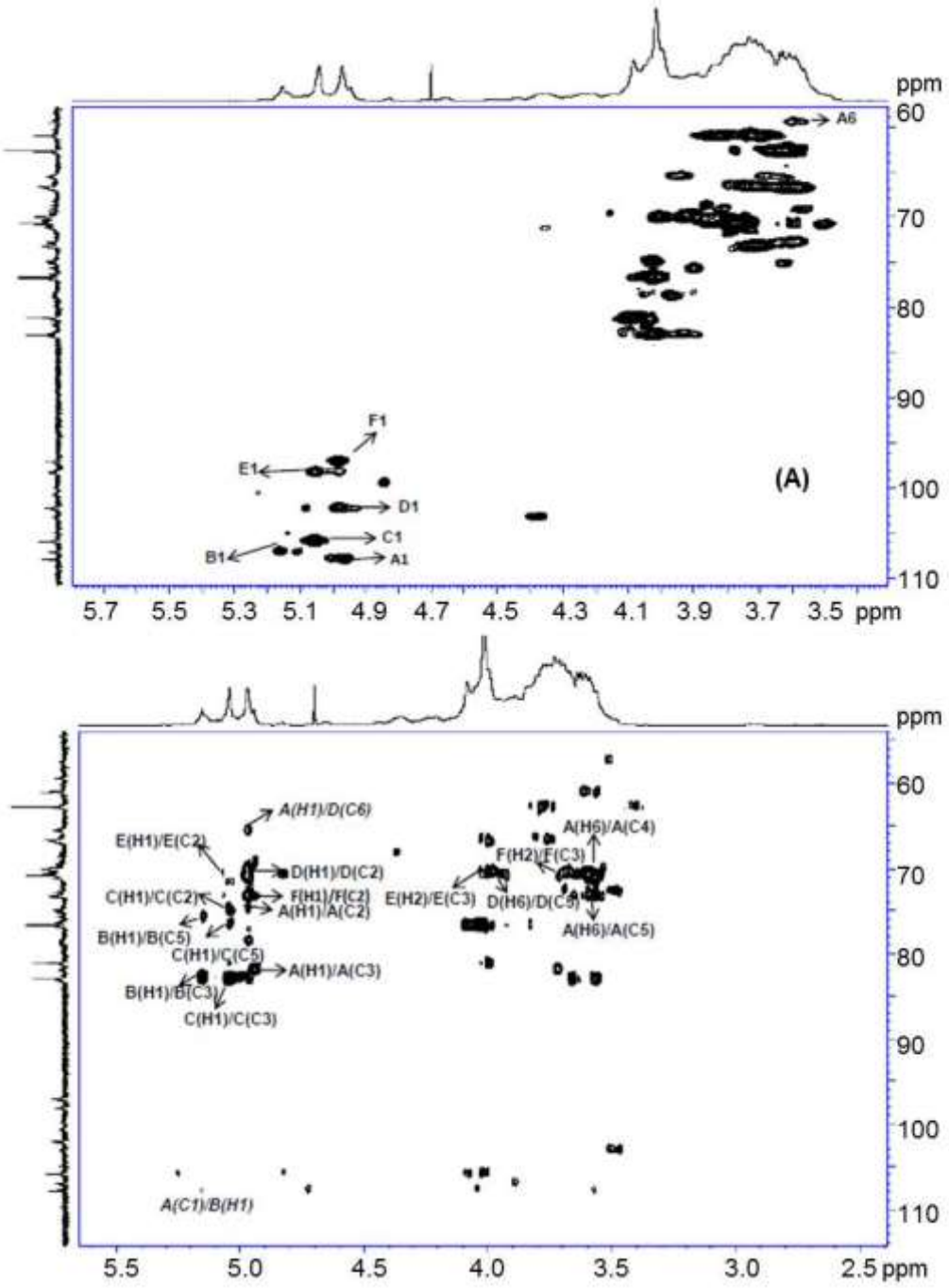


Figure 6-5 HSQC spectrum (A) and HMBC spectrum of EPS2BW (B).

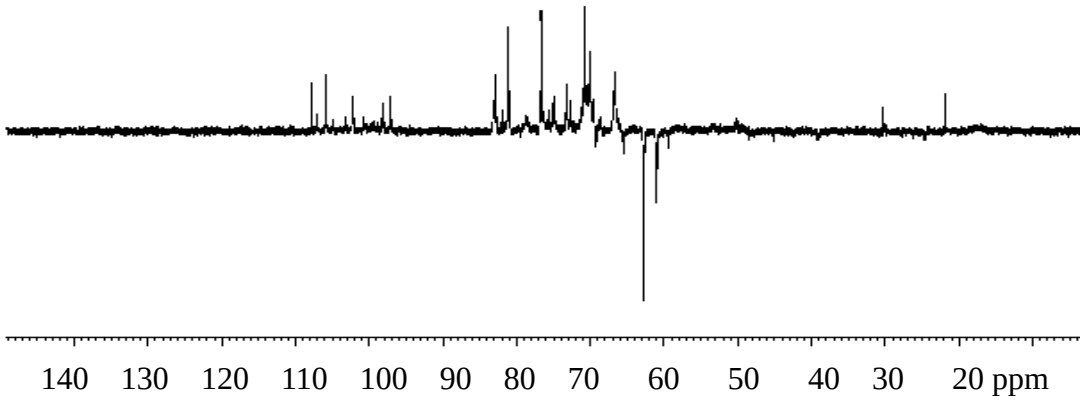


Figure 6-6 The DEPT spectrumof EPS2BW.

Table 6-4 ^{1}H-NMR and ^{13}C-NMR shifts of EPS2BW (δ, ppm)

Section	Sugar residues	H1/C1	H2/C2	H3/C3	H4/C4	H5/C5	H6/C6
E	→2) -α-D-Man(1→	5.04	3.99	3.83	n.m	n.m	3.63
		98.21	70.02	70.46	n.m.	n.m.	62.73
C	→3)-β-D-Gal(1→	5.09	3.72	4.03	n.m	4.03	3.83
		105.89	74.32	82.73	n.m.	76.91	61.17
F	α-D-Man(1→	4.96	3.71	3.73	n.m	n.m	3.60
		97.08	73.42	70.89	n.m.	n.m.	62.73
A	→3)-β-D-Gal(1,2→	4.96	3.72	4.07	3.87	3.61	3.62
		107.84	74.23	81.98	70.81	72.25	59.53
B	β-D-Gal(1→	5.14	n.m	3.90	n.m.	3.97	3.56
		106.98	n.m.	82.73	n.m.	75.23	59.53
D	→6) -α-D-Man(1,2→	4.93	3.80	n.m.	n.m.	3.70	3.93
		102.15	70.80	n.m.	n.m.	72.91	66.66

n.m.: not marked

From all above analytical results, the structure of EPS2BW was

deduced as,

$[\rightarrow 2)$-α-D-Man*p*-$(1]_a \rightarrow [2)$-α-D-Man*p*-$(1]_b$
6
↑
R

R = β-D-Gal*p*(1→, or $[\rightarrow 1)$-β-D-Gal*p*-$(3]_n \rightarrow 1)$-β-D-Gal*p*/protein,

or $[\rightarrow 3)$-β-D-Gal*p*-$(1] \rightarrow [1)$-β-D-Gal*p*-$(3]_n \rightarrow 1)$-β-D-Gal*p*/protein
2
↑
β-D-Gal*p*

a/b=1.0:1.1

6.3.4 Antioxidant capacity of EPS2BW

Fig. 6-7A and Fig. 6-7B show the TEAC $ABTS^+$ radical scavenging activity and the FRAP activity, respectively. In both figures, the antioxidant activity levels increased with the reaction time, significantly from 2 h to 12 h and slightly from 12 to 24 h. The TEAC and FRAP scavenging capability of EPS2BW was also dose-independent in the preliminary test. In a recent report (Chen, Siu, Wang, Liu & Wu, 2013), a few protein-containing EPS fractions with average MW from 6-30 kDa isolated from the Cs-HK1 mycelial fermentation broth had shown TEAC and FRAP activity in the ranges of 0.155-1.12 μmol Trolox/mg and 0.0095-0.611 μmol Fe^{2+}/mg, respectively. In comparison, EPS2BW had shown much higher TEAC and FRAP activity (23.0-45.0 μmol Trolox/mg and 15.0-38.9 μmol Fe^{2+}/mg, respectively), and had a stronger antioxidant capacity according to these two assays. Free radical scavenging by carbohydrates occurred by the hydroxyl

radical ($ABTS^{+}$ in TEAC assay) reacting with a hydroxyl group bonded to a carbon (Hernandez-Marin & Martinez, 2012). The FRAP assay was based on reduction of ferric ions to ferrous ions by the single electronic of oxygen in hydroxyl group (Huang, Ou & Prior, 2005). Both the two assays probably involved reactions with the hydroxyl groups in PS. Because of the large size and complex conformation of PS, the antioxidative reaction by electron-transfer reaction may be slower and lasts longer (Morris, Rees, Thom & Welsh, 1977; Yanaki & Norisuye, 1983). Therefore, EPS2BW may be a potential long-action antioxidant to be used in combination with fast-action small molecule antioxidants.

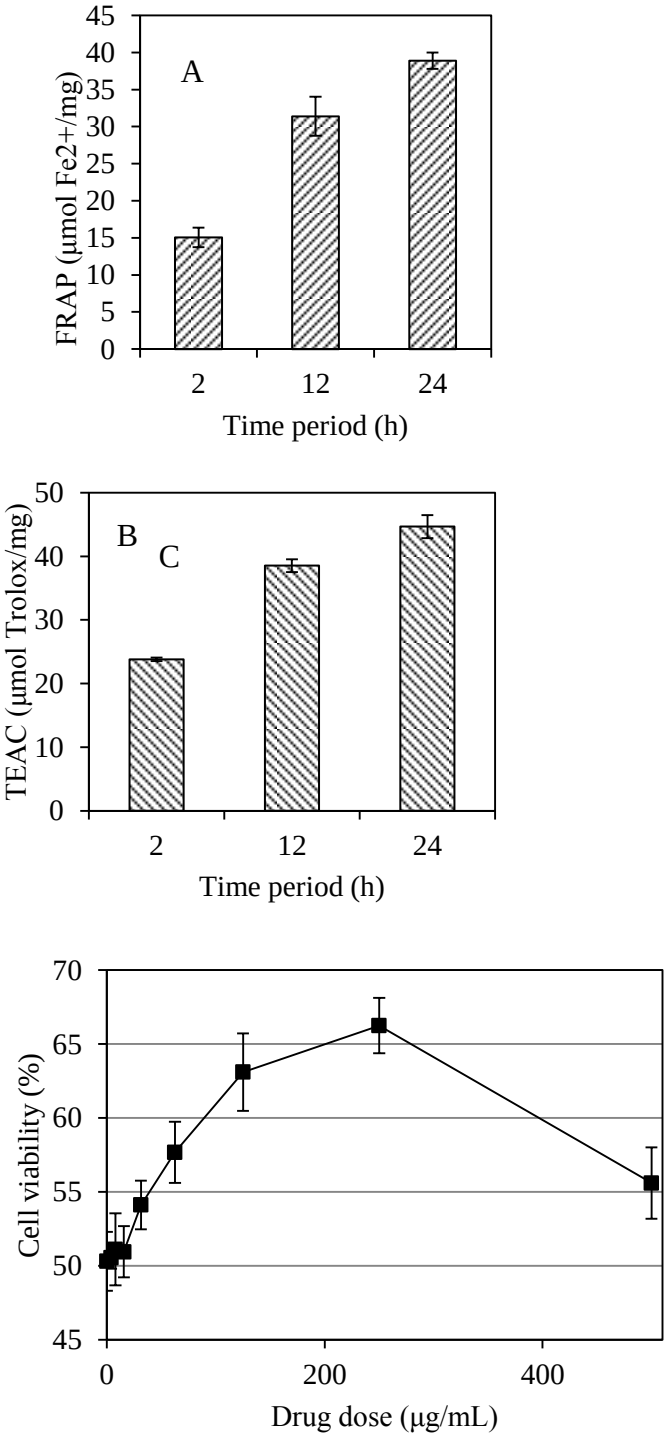


Figure 6-7 Antioxidant activities of EPS2BW: (a) TEAC scavenging activity on $ABTS^{+}$ radicals; (b) FRAP activity for reducing Fe^{3+} ions; (c) Cytoprotective effects against H_2O_2-induced PC12 cell injury at various EPS2BW concentrations. The TEAC or FRAP activity value was the

average (slope) at various concentrations from 100 to 1000 mg/L. For the cell culture tests, cells were treated with 80 μM H_2O_2 and EPS2BW or with an equal volume of PBS for the negative control group for 24 h. Each value was expressed as mean ±SD (n=3).

EPS2BW did not show significant effect inhibition or stimulation on the growth of normal PC12 cells (Fig. 6-8). Fig. 6-7C shows the cytoprotective activity of EPS2BW against H_2O_2-induced PC12 cell death (decrease of viability) at different concentrations. Pretreatment of the PC12 cells with EPS2BW at suitable concentrations (31-250 μg/mL) protected the PC12 cells from H_2O_2 (80 μM) damage in a dose-dependent manner, retaining a higher cell viability from 54% to 66.2% than the control of 50% viability. The protective effect of EPS2BW dropped when the treatment dosage increased from 250 to 500 μg/mL (55.6% viability), due probably to the adverse effect on cells at a high concentration.

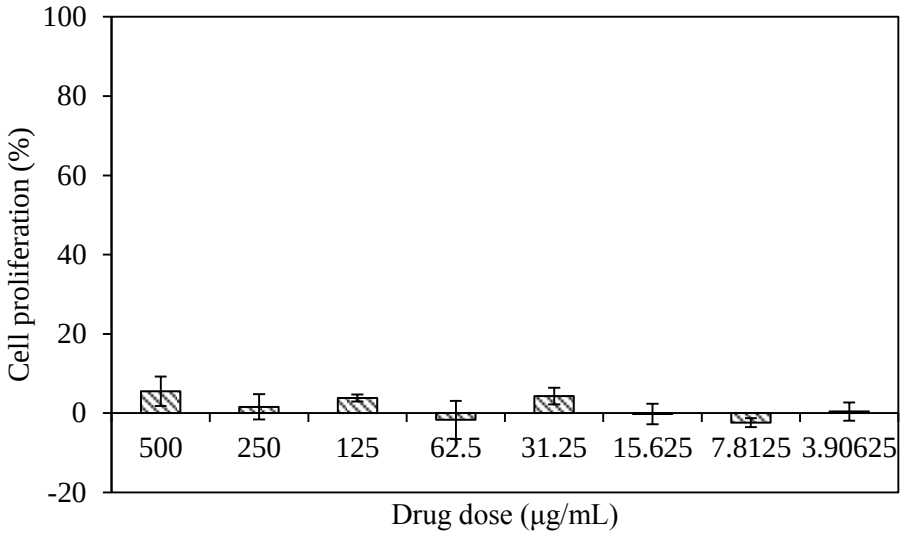


Figure 6-8 PC12 cells were treated with different concentrations of EPS2BW for 72 h followed by cell viability assay using MTT method.

In the early years before 1990, polysaccharides from the *C. sinensis* species were mostly isolated from the fruiting body of natural caterpillar fungi Dong-chong-xia-cao such as a galactomannan and galactomannan-protein complexes (Kiho, Tabata, Ukai & Hara, 1986; Miyazaki, Oikawa & Yamada, 1977). In recent years with the rising demand for and price of natural *C. sinensis*, mycelial fermentation has become the major source of *C. sinensis* materials for research and commercial uses. Most PS have been extracted from the mycelial biomass such as the glucans isolated from the mycelia with immunomoldulatory and antitumor activities (Akaki, Matsui, Kojima, Nakajima, Kamei & Tamesada, 2009; Wu, Omar, Sun & Pan, 2005) and heteropolysaccharides with hypoglycemic and antioxidant activities (Kiho, Ookubo, Usui, Ukai & Hirano, 1999; Li et al.,

2003). A few PS and PSPs have been isolated from the crude EPS produced by liquid fermentation of Cs-HK1 fungus previously in our lab and they were mostly heteropolysaccharides composed of glucose (major portion), mannose and galactose (Yan, Wang & Wu, 2014). Acidic polysaccharide was also found in the fermentation broth of Cs-HK1 and its immune-stimulatory and anti-inflammatory activities were proven (Wang, Peng, Lee, Tang, Cheung & Wu, 2011). A (1→3)-β-D-glucan was isolated from the ultrasound product of EPS of Cs-HK1 (Chapter 5).This is the first report about a galactomannan-protein from the fermentation broth of *C. sinensis*. The composition and linkage of EPS2BW were similar to those of CT-4N, a galactomannan-protein from the fruiting body of *C. sinensis* (Kiho, Tabata, Ukai & Hara, 1986). The structural feature of EPS2BW is an evidence to prove that liquid fermentation can be a way to obtain effective compounds from *C. sinensis*.

6.4 Summary

EPS2BW, a galactomannan-protein complex of about 50 kD, has been fractionated from the lower-MW EPS produced by liquid fermentation of Cs-HK1 fungal mycelia. EPS2BW has shown a strong antioxidant capacity in both chemical and cell culture assays. The composition of carbohydrates and amino acids in EPS2BW as well as its galactomannan backbone showed a high similarity to a protein-galactomannan isolated many years ago from

the fruiting bodies of *C. sinensis*. Our present study is the first to isolate and elucidate the structure of a galactomannan-protein complex from the EPS produced by a *C. sinensis* fungus in liquid fermentation. This study has again demonstrated that mycelial fermentation is a feasible process for production of bioactive PS and their protein complexes with structures and biological functions similar to those from the natural *C. sinensis* fruiting bodies.

Chapter 7 Characterization and antibacterial activity of silver nanoparticles prepared with an exopolysaccharide fraction from Cs-HK1

7.1 Introduction

Silver nanoparticle (AgNP) has attracted enormous interest because of its great potential for wide applications in food, cosmetic, clothing and pharmaceutical industries (Ahamed, Alsalhi & Siddiqui, 2010; Marambio-Jones & Hoek, 2010; Rai, Kon, Ingle, Duran, Galdiero & Galdiero, 2014). Many physical (Breitwieser et al., 2013; Pal, Shah & Devi, 2009) and chemical methods (Guzman, Dille & Godet, 2012; Raveendran, Fu & Wallen, 2003) have been exploited to produce AgNPs. Chemical reduction of Ag^+ ions is one of the most common approaches for AgNP synthesis, in which sodium borohydride, sodium citrate and dimethylformamide have been the highly reactive reducing agents (Leung, Wong & Xie, 2010). However, some of the AgNPs formed with the small molecule reducing agents have been found to cause potent cytotoxicity (AshaRani, Low Kah Mun, Hande & Valiyaveettil, 2009). A possible cause for the strong cytotoxicity of AgNPs is their rapid diffusion into cells (Travan et al., 2009). Alternatively, biopolymers especially natural polysaccharides (PS) such as cellulose and chitosan have been applied to produce non-toxic and biocompatible AgNPs (Li, Zhang, Xu & Zhang, 2011). Modified chitosan has been used to form well-dispersed non-toxic AgNPs in water (Travan, et al., 2009).

Microbial exopolysaccharides (EPS), which are usually industrially produced by submerged fermentation, have found wide applications in the food and pharmaceutical inductries (Lehtovaara & Gu, 2011; Schmid, Meyer & Sieber, 2011). AgNPs have been synthesized with an EPS extracted from a lactic acid bacterium and showed notable antimicrobial activity (Kanmani & Lim, 2013). *Schizophyllan*, a linear (1→3)-β-D-glucan with strong anticancer and immunomodulatory activities produced by the medicinal fungus *Schizophyllum commune*, has been used to form non-toxic AgNPs with a diameter of 6 nm (Abdel-Mohsen et al., 2014).

EPS1 was a partially purified EPS fraction (~2,700 kD) isolated from Cs-HK1 mycelial fermentation broth, which was mainly composed of (1→3)-β-D-glucan with glucose side chains (see Chapter 5) and about 27% (w/w) galactomannan-protein complexes (~50 kDa) (see Chapter 6). This study was carried out to evaluate the preparation and properties of AgNPs with EPS1 in an aqueous solution. The AgNPs prepared at various conditions were characterized using several instrumental methods and further evaluated for cytotoxicity and antibacterial activities.

7.2 Materials and methods

7.2.1 Synthesis of AgNPs

The synthesis of AgNPs with EPS1 followed a reported procedure with minor modifications (Leung, Wong & Xie, 2010). All the $AgNO_3$ and EPS1 solutions were prepared in distilled water. $AgNO_3$ was purchased from Sigma-Aldrich (ACS reagent, #209139). The $AgNO_3$ solution (2 mL) at various concentrations was mixed with an equal volume of EPS1 solution at a selected concentration. The reaction mixture was incubated with constant stirring for 10 to 60 min at 25, 40, 60 or 100 ^{o}C in the dark. After completion of the reaction, the solution was stored at room temperature (~25 ^{o}C) in the dark before use.

7.2.2 Characterization of AgNPs

The UV-Vis spectra of AgNPs and EPS1 in water were measured from 300-600 nm on a HEWLETT Packard 8453 spectrophotometer against pure EPS1 solution as the blank. Dynamic light scattering (DLS) measurement was performed on AgNPs at 25 ^{o}C on a Malvern Zetasizer Nano (Malvern Instruments Ltd., UK) at 632.8 nm and 90^{o} scattering angle. The particle size, morphology and selected-area electron diffraction (SAED) of AgNPs were observed by field emission electron microscope (JEOL model JEM-2100F). TEM samples were prepared by placing a drop of sample solution on the carbon coated copper grids and vacuum dried. Silver content was determined by ICP-OES (Agilent Technologies 700 Series). Standard solutions were prepared by 0-2.0 mM silver nitrate solution. Infrared (IR)

spectra were measured at room temperature from 4000-500 cm^{-1} at 4 cm^{-1} resolution with baseline corrected on a Perkin-Elmer 1600 instrument.

7.2.3 Cytotoxicity assay

The murine macrophage cell line RAW264.7 was employed for the cytotoxicity assay. The RAW264.7 cells were maintained in DMEM medium containing 10% FBS (v/v), 2 mM L-glutamine and antibiotics (100 U/mL penicillin, 100 μg/mL streptomycin) (Gibco BRL, U.S.A.). Cells were cultured in a humidified incubator at 37 ^{o}C with 5% CO_2. For the assay, 0.5 $\times 10^4$ of RAW264.7 cells were grown in DMEM medium containing the AgNPs/EPS1 samples at chosen concentrations. Cell viability was determined by the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) method (Mosmann, 1983). The morphology of cells was observed by a microscope (Olympus IX51, Japan).

7.2.4 Antibacterial assay

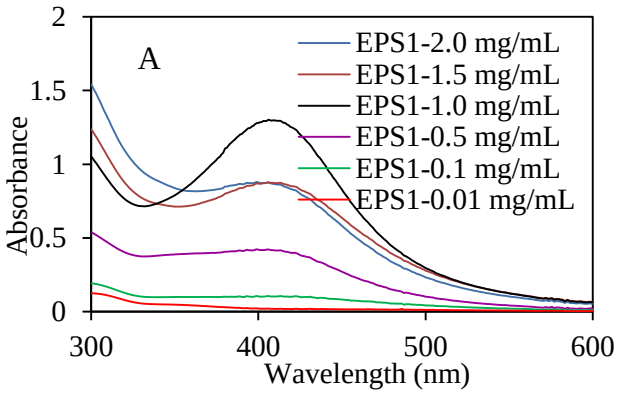
Escherichia coli (Gram-negative) and *Staphylococcus aureus* (Gram-positive) were used in the antibacterial tests. The *in vitro* antibacterial activity was conducted in 96-well microplates using the broth micro-dilution procedure according to the Clinical and Laboratory Standards Institute guidelines (Jorgensen & Hindler, 2007). Four to five colonies from overnight cultures of the test bacteria on a tryptone (Fluka,

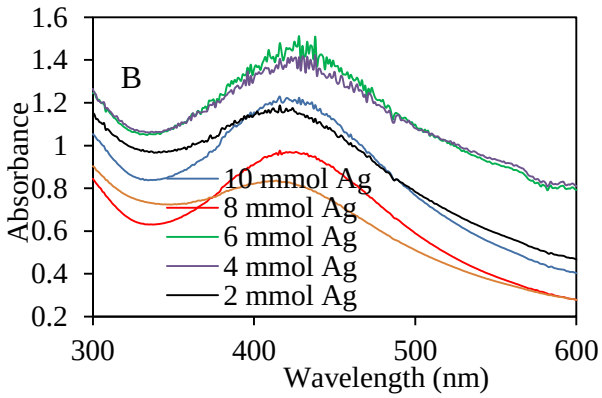
Analytical, Sigma-Aldrich Co., USA) agar plate were inoculated in 10 mL of Luria-Bertani broth at 37 ^{o}C for 4-6 h. The bacterial suspension was inoculated into a 96-well microplate (final concentration of ~10^5 CFU/mL) containing 100 μL of serial dilutions of the tested samples. After incubation for 12 h at 37 ^{o}C, the absorbance at 600 nm (A_{600}) was recorded to calculate the percentage of bacteria cell inhibition with respect to the untreated control, using a microplate reader (Chan et al., 2013). The assay of each sample was performed in triplicate.

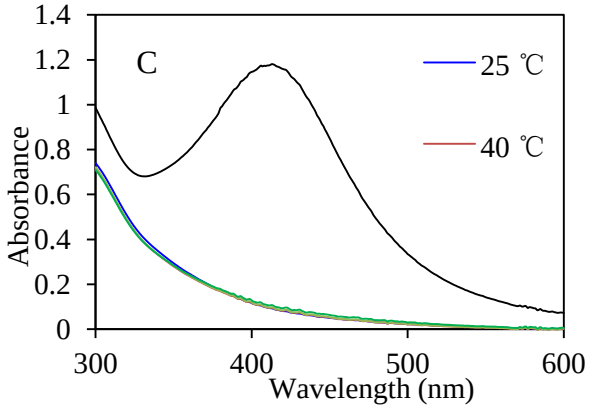
7.3 Results and discussion

7.3.1 Optical properties of AgNPs

Fig. 7-1 shows the UV-Vis spectra of $AgNO_3$ and EPS1 mixture solutions in water prepared at various concentrations, temperatures and time periods. When Ag ion is reduced to Ag atom in a solution, the colorless solution is turned into yellowish brown. An absorption band is an indication of AgNP formation in the solution (Xu et al., 2014), which can be detected by the surface plasmon resonance (SPR) band using UV-Vis spectroscopy (Kanmani & Lim, 2013). In this study, the AgNPs formed in EPS1 solution showed a strong SPR peak at about 410 nm (Fig. 7-1). The formation of AgNPs was due probably to the reduction of Ag ions into Ag atoms by the EPS1 added to the $AgNO_3$ solution.


A
EPS1-2.0 mg/mL
EPS1-1.5 mg/mL
EPS1-1.0 mg/mL
EPS1-0.5 mg/mL
EPS1-0.1 mg/mL
EPS1-0.01 mg/mL
Absorbance
Wavelength (nm)


B
10 mmol Ag
8 mmol Ag
6 mmol Ag
4 mmol Ag
2 mmol Ag
Absorbance
Wavelength (nm)


C
25 ℃
40 ℃
Absorbance
Wavelength (nm)

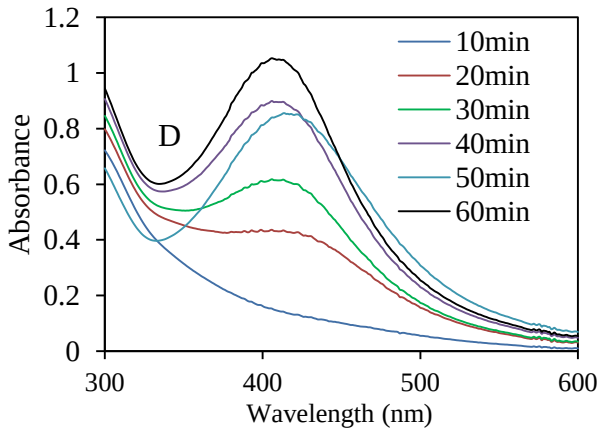

D
10min
20min
30min
40min
50min
60min
Absorbance
Wavelength (nm)

Figure 7-1 UV-Vis light absorbance spectra of $AgNO_3$ and EPS1 mixture reaction solution at various conditions: (A) 10 mM $AgNO_3$ with various concentrations of EPS1; (B) 1.0 mg/mL EPS1 with various concentrations of $AgNO_3$; (C) 10 mM $AgNO_3$ and 1.0 mg/mL EPS1 at various temperatures; (D) 10 mM $AgNO_3$ and 1.0 mg/mL EPS1 for various periods of reaction time.

The absorption intensity of the $AgNO_3$-EPS1 mixture solution initially increased and then decreased with the increase of EPS1 concentration from 0.01 mg/mL to 2.0 mg/mL (Fig. 7-1A), indicating the increase or decrease in the concentration of the AgNPs formed. The same trend of UV-Vis absorption change has also been reported previously (Wu, Zhang & Zhang, 2012), due probably to the formation of large particles or clusters by the prepared AgNPs. Fig. 7-1B shows the UV-Vis absorption of AgNPs prepared with 1.0 mg/mL EPS1 and various concentrations of $AgNO_3$. Relatively high absorption intensities were attained at 4 mM and 6 mM $AgNO_3$ but with notable noise, which was probably due to the poor stability of the AgNPs formed (Wei, Sun, Qian, Ye & Ma, 2009). The most symmetrical absorption peak was observed with 10 mM $AgNO_3$, indicating more uniform AgNPs (Wu, Zhang & Zhang, 2012). SPR absorption of AgNPs is sensitive to variations in physical properties and aggregation of particles in solution (Liu, Lee, Kim & Kim, 2007). Chemical modification

of the particle surface may be effective to attain uniform particle size and avoid aggregation. Fig. 7-1C shows the UV-Vis absorption spectra of AgNPs prepared at various temperatures (and a fixed reaction time of 20 min); there was no absorption band until the temperature increased to 100 ^{o}C. In our preliminary experiments, absorption band was also observed at 80 ^{o}C with a longer reaction time of 40 min. The results suggested that the effect of temperature on the formation of AgNPs in the EPS1 solution was mainly attributed to its influence on the reaction rate of Ag ions reduction. Similarly, formation of AgNPs using sophorolipids as reducing and capping agent was not observed until the temperature increased to 90 ^{o}C (Kasture, et al., 2008). Fig. 7-1D shows the UV-Vis spectra of AgNPs prepared in EPS1 solution for different periods of time; stronger absorbance was exhibited (more AgNPs formed) with longer time of heat treatment. The experimental results altogether suggested that the most favorable reaction conditions for formation of uniform AgNPs were 10 mM $AgNO_3$, 1.0 mg/mL EPS1 at 100 ^{o}C for 60 min. AgNPs formed at this set of conditions were used in the following morphology analysis and bioactivity tests.

7.3.2 FT-IR spectra of AgNPs

FT-IR measurements were carried out to detect possible reactions for the reduction of Ag ions and stabilization of Ag atoms in EPS1 (Fig. 7-2A). The peaks at 3000-3600 cm^{-1} corresponded to the stretching vibrations of

hydroxyl groups and amine groups. Usually the wave numbers of N-H are lower than those of O-H (Pawlak & Mucha, 2003). The peak at 3407 cm^{-1} in the spectrum of EPS1 could be attributed to the N-H in the proteins and the shift of N-H was still 3407 cm^{-1} in the spectrum of AgNPs. The absorption peak at 3482 cm^{-1} in the spectrum of EPS1 was attributed to the stretching vibration of O-H. The peak shifted to lower wavenumbers (3458 cm^{-1}) in the spectrum of AgNPs, suggesting interactions between hydroxyl groups of EPS1 and the AgNPs (Yan et al., 2013). This observation can be further verified by the shift of the deformation vibration of O-H at about 1100 cm^{-1}. The slightly red shift from 1078 cm^{-1} to 1074 cm^{-1} was also indicative of the interaction between hydroxyl groups of EPS1 and the AgNPs (Li, Zhang, Xu & Zhang, 2011). The lack of obvious differences at about 1380 cm^{-1} revealed that any excess of $AgNO_3$ had been removed by dialysis (Sedenkova, Trchova, Stejskal & Prokes, 2009). The peak at 889 cm^{-1} was indicative of β-configuration in the polysaccharide structures of EPS1 (Cael, Koenig & Blackwel.J, 1974).

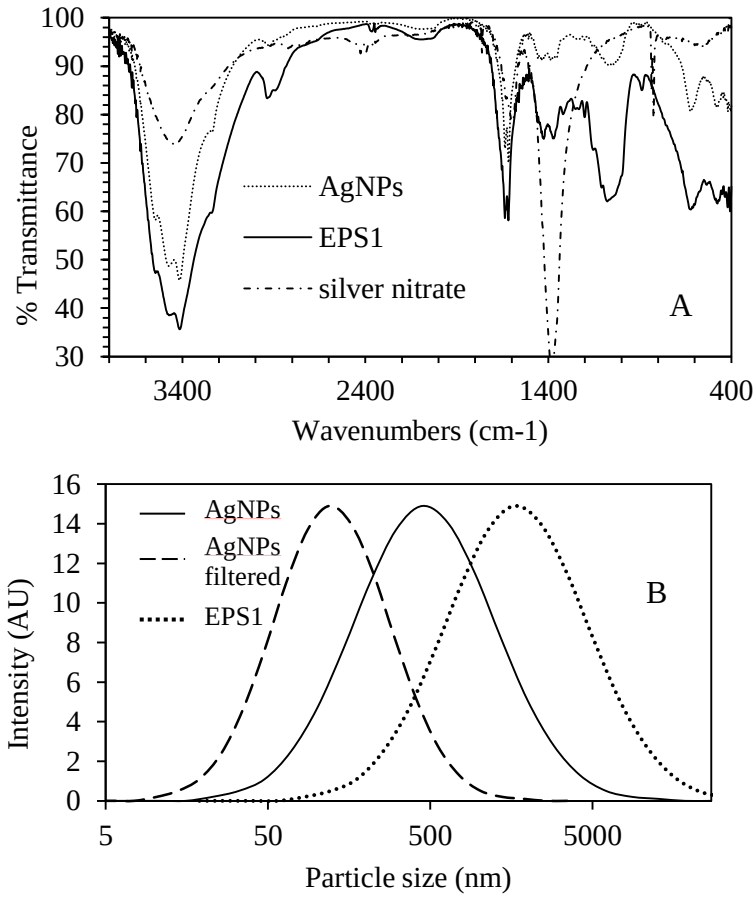


Figure 7-2 Analysis of AgNPs formed in EPS1 solution: (A) FT-IR spectra of EPS1, $AgNO_3$ and AgNPs; (B) particle size distribution of EPS1 and AgNPs (1.0 mg/mL) in water (by DLS performed at 25 ^{o}C and 90 ° scattering angle). (AgNPs prepared with 10 mM $AgNO_3$ and 1.0 mg/mL EPS1 at 100 ^{o}C for 60 min)

7.3.3 Element composition and morphology properties of AgNPs

The content of atomic silver in the prepared nanoparticles was detected by ICP-OES. Excess silver ions were removed by dialysis with MWCO 3000 dialysis bag. After this, the content of silver was about 6.7% (w/w) in

the prepared AgNPs. After filtration through a 0.22 μm membrane, 2.3% (w/w) of silver was found in the AgNPs sample and the color of the AgNPs solution was much lighter. About 2/3 of the silver atoms was removed by micro-filtration.

As shown in Fig. 7-2B, the particle size of EPS1 was about 1650 nm. After the reaction, the particle size of the AgNPs was 450 nm, which was much larger than the filtrated AgNPs solution (120 nm). Degradation and conformation changes of the polysaccharide chains are the two most possible causes for the size reduction of polysaccharide aggregates in water. However, in our preliminary test, the MW of EPS1 did not change after it placed in hot water (100 ^{o}C) for several hours. Therefore, the size reduction during the formation of AgNPs was most probably attributed to conformation changes in the polysaccharide chains. The chain of the excess PS in EPS1 was closer after the reduction due to the attached silver atoms. Although the particle size of AgNPs determined by DLS was inaccurate owing to the large particle size of EPS1, only a symmetrical peak of AgNPs was observed in Fig. 7-2B, indicating the synthesized AgNPs were attached to the chains of the excess PS.

TEM provided further insight into the morphology and size details of the formed AgNPs (Fig. 7-3). Histograms of particle size distribution were

obtained by counting more than 100 silver nanoparticles in several TEM images (Fig. 7-4). Most particles were around 40 nm in the newly prepared AgNPs (Fig. 7-3A & Fig. 7-4A) although some larger particles (>70 nm) were also observed. After filtration with 0.22 μm membrane, some large particles were removed and most particles were around 30-40 nm (Fig. 7-3B & Fig. 7-4B). The larger particle size of AgNPs detected by DLS was probably attributed to the aggregation of excess PS in the solution according to the TEM result reported previously (Li, Wang, Cui, Huang & Kakuda, 2006). Denaturation had been observed during the formation of AgNPs in xanthan solution and after storage for 1 week, the renaturation of xanthan due to intra- and inter-molecular hydrogen bonds induced the aggregation of the formed nanoparticles (Xu et al., 2014). In contrast, AgNPs formed in EPS1 solution in this study remained well dispersed in the solution after 2 months without any significant changes in particle size distribution (Fig. 7-3A & Fig. 7-3C; Fig. 7-4A & Fig. 7-4C). As suggested by Li et al. (Li, Zhang, Xu & Zhang, 2011), the stable AgNPs formed in lentinan [a (1→3)-β-D-glucan] solution were attributed to the provision of a matrix for the formation of AgNPs and a stabilizer for good dispersion by the PS. Similarly, the main component of EPS1 was a (1→3)-β-D-glucan (Chen, Siu, Cheung & Wu, 2014), which may present a triple-helix conformation for formation and stabilization of AgNPs in an aqueous solution (Li, Zhang, Xu & Zhang, 2011). The SAED pattern of AgNPs showed concentric rings

resulting from the random orientation of crystal planes (Fig. 7-3A, inset), suggesting that AgNPs existed as polycrystalline metallic rather than non-crystalline NPs.

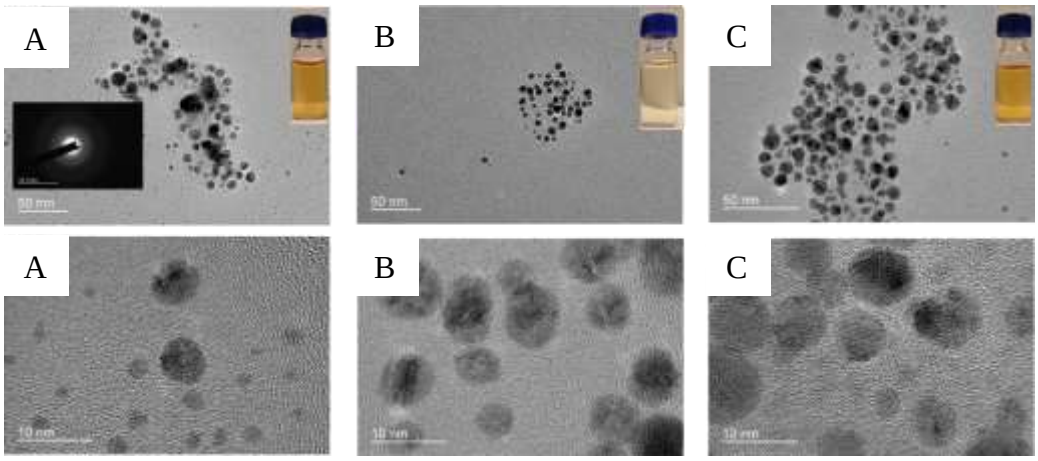


Figure 7-3 TEM images: (A) freshly prepared AgNPs; (B) freshly prepared AgNPs after filtration through 0.22 μM filter; (C) AgNPs stored for 2 months at 4 ^{o}C. (AgNPs prepared with 1.0 mg/mL EPS1 and 10 mM $AgNO_3$ at 100 ^{o}C and 60 min; inset: the electron diffraction pattern of particles)

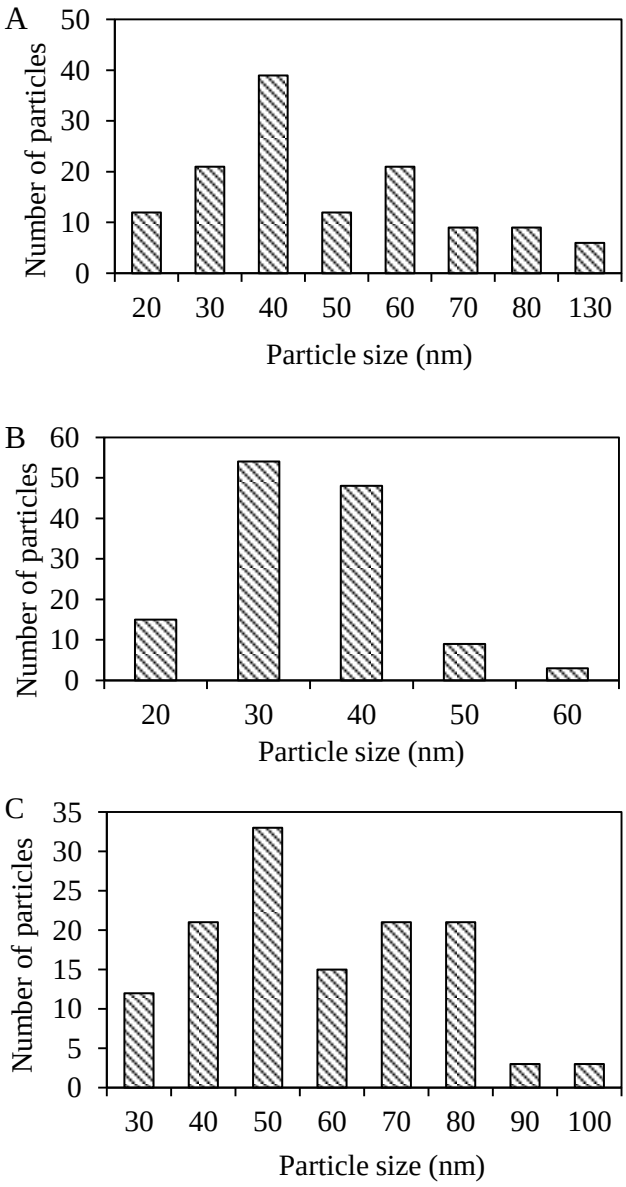


Figure 7-4 Particles size distribution histogram determined from the TEM micrograph: (A) freshly prepared AgNPs; (B) AgNPs after filtration through 0.22 μM filter; (C) AgNPs stored for 2 months at 4 ^{o}C. (AgNPs prepared with 1.0 mg/mL EPS1 and 10 mM $AgNO_3$ at 100 ^{o}C and 60 min)

Based on the above results from FT-IR, DLS and TEM, we proposed a schematic model to describe the binding of AgNPs to EPS1 in Fig. 7-5. As EPS1 was composed of (1→3)-β-D-glucans (Chen, Siu, Cheung & Wu,

2014) and galactomannan-protein complexs (Chen, Ding, Wang, Siu & Wu, 2014), (1→3)-β-D-glucans usually form triple helixes and the galactomannan-protein complexes can form random coils in an aqueous solution (Andrade, Azero, Luciano & Goncalves, 1999). A purified, ultrasound-degraded fraction of EPS1 (called EPS1U, ~730 kD) consisting of (1→3)-β-D-glucan was also tested for the synthesis of AgNPs, though the reduction rate of silver ions was much lower than that using EPS1 (Fig. 7-6). This suggests that the other constituents of EPS1, namely the polysaccharide-protein complexes, improved the reduction of silver ions to form AgNPs. These lower MW polysaccharide-protein complexes may be attached to the triple-helix formed by the β-glucan chains and ion charges on the proteins can attract the silver ions. As reported previously (Travan et al., 2009), AgNPs can interact with nitrogen atoms in the modified chitosan (Chitlac) and the exterior amino groups in Chitlac can contribute to prevent the aggregation of metal nanoparticles. In this study, only interactions between O-H and AgNPs were observed.

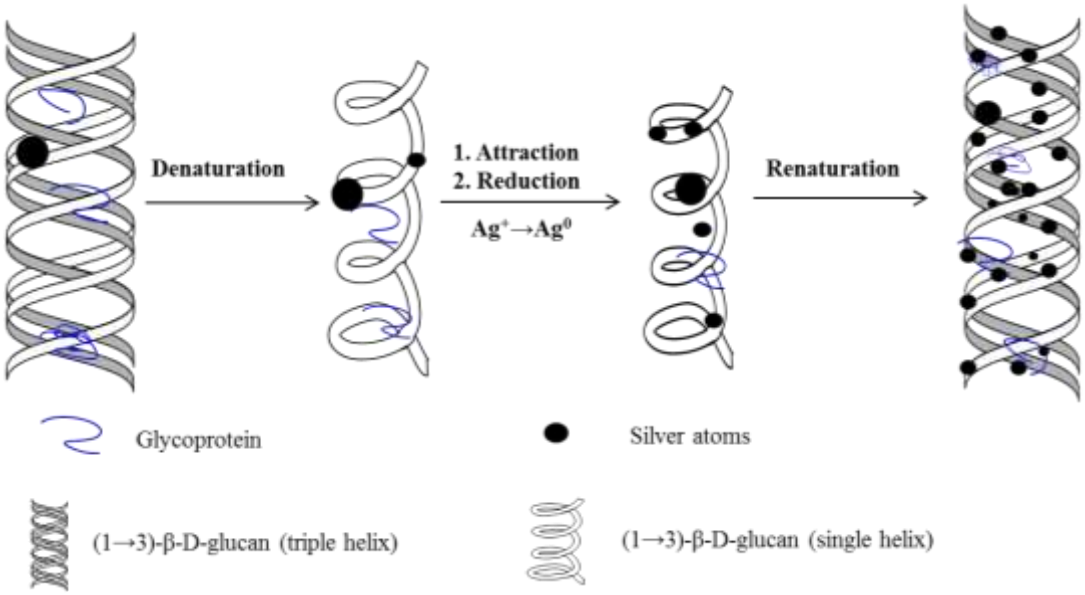


Figure 7-5 Schematic model for the reaction between $AgNO_3$ and EPS1 leading to the formation of AgNPs and their interactions with the EPS1 chains and constituents.

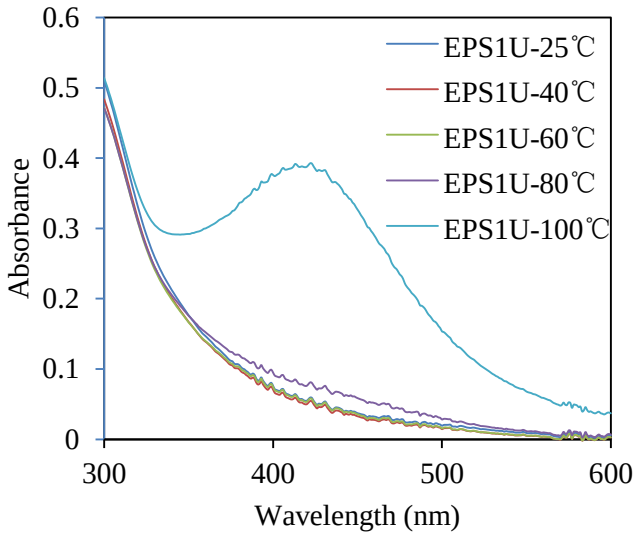


Figure 7-6 UV-vis spectra of silver nanoparticles formed in EPS1U solution under the same condition as EPS1 in different time.

7.3.4 Cytotoxicity of AgNPs

On the basis of the aforementioned results, both the content of silver and the particle size of AgNnPs changed after filtration. Therefore, samples used in the cytotoxicity study and antibacterial test were eliminated bacteria and germs by autoclave sterilization instead of membrane filtration. The cytotoxicity of EPS1 was also detected to eliminate the effect of excess EPS1 in AgNPs. As shown in Table 7-1, treatment with AgNPs for 24 h showed no significant cytotoxicity to RAW264.7 cells. However, when the treatment period was extended to 48 h, the growth of RAW264.7 cells was inhibited significantly. EPS1 (5 mg/mL) alone did not cause any significant cytotoxicity to RAW264.7 cells. The effect of AgNPs and EPS1 treatment on the morphology was also observed (Fig. 7-7). The morphology of cells was not significantly influenced by treatment with AgNPs at lower concentration (0.5 mg/mL) or shorter period (24 h) in comparison with the healthy control cells. The cells were notably deformed with AgNPs at the higher concentration (5 mg/mL) and longer treatment period (48 h). Treatment with EPS1 did not affect the growth of RAW264.7 cells even at high concentration (5 mg/mL). The microscopic observation was in general agreement with the above results of cytotoxicity test by MTT assay.

Table 7-1 Inhibition and antimicrobial activity of EPS1 and AgNPs

Sample	CC_{50} [a] (mg/mL)		MIC [b] (mg/mL)	
	24 h	48 h	*E. coli*	*S. aureus*
EPS1	> 5.0	> 5.0	> 5.0	> 5.0
AgNPs	> 5.0	0.5 ± 0.1	1.6 ± 0.3	0.8 ± 0.1

[a] The 50% cytotoxic concentration of samples on RAW264.7 cells.

[b] Minimum inhibitory concentration of samples.

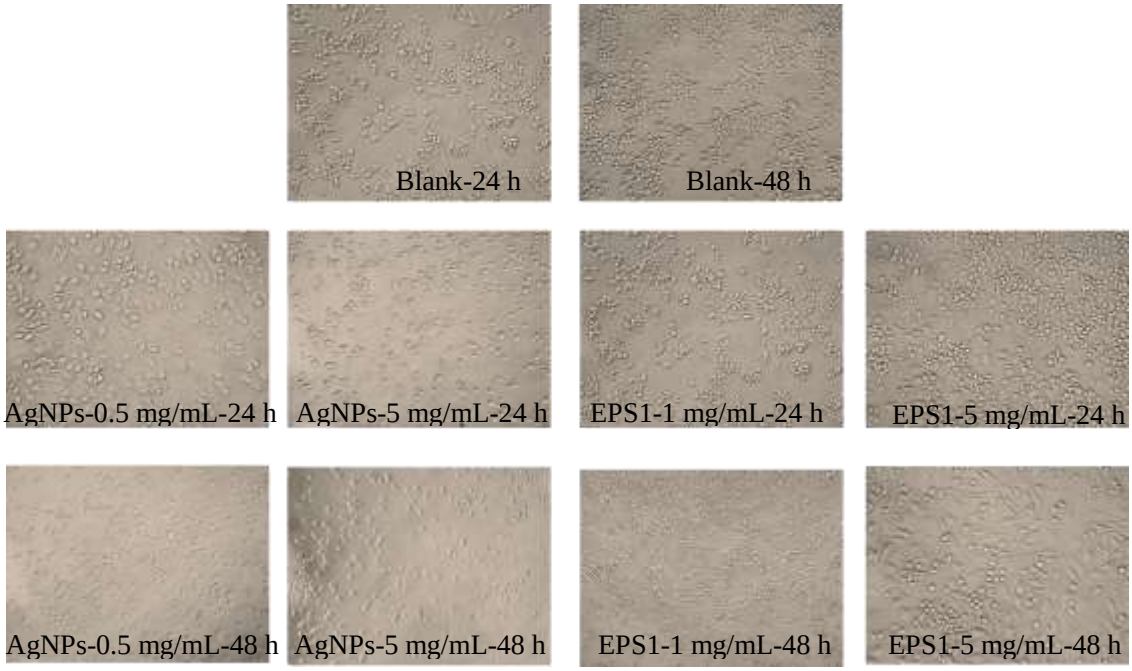


Figure 7-7 Morphology of RAW264.7 cells.

7.3.5 Antibacterial activities of AgNPs

AgNPs completely inhibited the growth of *E. coli* at 1.6 mg/mL and *S. aureus* at 0.8 mg/mL (Table 7-1). The results showed that AgNPs can inhibit Gram-positive bacteria more than Gram-negative bacteria. EPS1 alone did not inhibit any of the bacteria (Table 7-1).

Although various EPS have been used to produce AgNPs with antimicrobial activities (Rai, Kon, Ingle, Duran, Galdiero & Galdiero, 2014), the strong cytotoxicity of AgNPs hindered their application (AshaRani, et al., 2009). Although non-cytotoxic AgNPs have been reported, the content of silver atoms in the prepared silver nanoparticles was unknown (Abdel-Mohsen et al., 2014; Travan et al., 2009). In this study, we used ICP-OES to determine the concentration of silver in the prepared AgNPs. AgNPs at 0.8 mg/mL have been shown to have notable antibacterial activity but low cytotoxicity in 24 h treatment. Further study is needed to gain a better understanding of the properties of AgNPs, improve their antibacterial activity and reduce their toxicity.

7.4 Summary

In this study, AgNPs were synthesized using an EPS fraction produced by a medicinal fungus Cs-HK1 in liquid fermentation. Fairly uniform, stable and well-dispersed AgNPs could be formed with suitable conditions including $AgNO_3$ and EPS1 concentrations, reaction time and temperature. The synthesis procedure was simple and green; water was used as the sole reaction solvent without any other chemical agents or organic solvents. Moreover, AgNPs formed with EPS1 exhibited significant antibacterial activity against both Gram-positive and Gram-negative pathogenic bacteria with very low cytotoxicity, suggesting the great potential for application in

the food and pharmaceutical industries. There is still a need to investigate the reaction mechanisms and physical interactions between Ag ions/Ag atoms and EPS1 constituents responsible for the formation of AgNPs in EPS1 solution and to optimize the yield and quality of AgNPs.

Chapter 8 Effects of carbon sources on exopolysaccharides production in Cs-HK1 mycelial fermentation

8.1 Introduction

Cordyceps sinensis is a precious medicinal fungus in Chinese herbal medicine with a wide range of health benefits and bioactivities (Chen, Wang, Nie & Marcone, 2013; Shashidhar, Giridhar, Sankar & Manohar, 2013). Since natural *C. sinensis* in the form of a caterpillar-fruiting body complex is very rare in nature and difficult to cultivate artificially, mycelial fermentation has become the main source of *C. sinensis* fungal materials. The mycelial culture of Cs-HK1 has been established and optimized for mycelial growth with a liquid medium containing glucose as the major carbon source and a few other components (Leung, Zhang & Wu, 2006). In addition to mycelial biomass, significant amounts of exopolysaccharides (EPS) have been produced by the Cs-HK1 mycelial culture in the liquid medium. Microbial EPS produced by liquid fermentation have found wide industrial applications especially in the food and biomedical fields such as the use of xanthan and gellan as food additives, scleroglucan as laxative tablet coating, and dextran as plasma expanders (Kocharin, Rachathewee, Sanglier & Prathumpai, 2010). More recently there has been increasing

interest in the pharmaceutical applications of microbial EPS (Smelcerovic, Knezevic-Jugovic & Petronijevic, 2008).

The crude EPS isolated from Cs-HK1 mycelial culture medium by ethanol precipitation was composed of polysaccharides (PS) and polysaccharide-protein complexes (PSPs) in a wide range of molecular weight (MW). As described in Chapters 5 and 6, the main components of EPS of Cs-HK1 are (1→3)-β-D-glucan (Chapter 5) and galactomannan-protein (Chapter 6) while the yield of EPS was about 3.2 g/L. However, (1→3)-β-D-glucan in EPS of Cs-HK1 was hardly dissolvable in water and even after dissolved in aqueous, the viscosity of EPS was very high and difficult to handle. The galactomannan-protein complex fractionated from EPS of Cs-HK1 showed stronger antioxidant activity with high water solubility, though its yield in the culture was much lower than that of (1→3)-β-D-glucan. Carbon source is one of the most important nutrients and glucose is a common and favorable carbon source for biomass growth and EPS production in most microbial fermentations (Kim, Hwang, Xu, Na, Song & Yun, 2002; Wu, Ding & Zhang, 2006). The addition of other monosaccharide sugars may be utilized by the mycelial cells and converted into uridine-diphosphate (UDP)-monosaccharides for EPS synthesis (Mozzi, Rollan, de Giori & Font de Valdez, 2001). Galactose and mannose may be transferred to UDP-galactose and UDP-mannose during the fermentation process and further synthesize EPS. In this study, galactose

and mannose were each added as a secondary carbon source for production of EPS in the Cs-HK1 mycelial culture.

8.2 Materials and Methods

8.2.1 Cs-HK1 mycelial culture conditions

The mycelial culture conditions have been described in details in Chapter 4. In brief, the Cs-HK1 mycelial culture was routinely maintained in a liquid medium consisting of 40 g/L glucose, 10 g/L yeast extract, 5 g/L peptone, 1 g/L KH_2PO_4 and 0.5 g/L $MgSO_4 \cdot 7H_2O$ in shake-flasks at 150 rpm and 20 ^oC. In the experiments on the effects of a secondary monosaccharide carbon source, 5 g/L of galactose or mannose was added to the liquid medium containing 35 g/L of glucose. To ensure a good yield of EPS, sufficient glucose was added together with galactose or mannose in the ratio of 7:1. The Cs-HK1 mycelial liquid fermentation was carried out in 250 mL Erlenmeyer flasks each containing 50 mL of the liquid medium for an overall period of 8 days. Three flasks were removed every day from the shaking incubator for determination of biomass, EPS and carbohydrate concentrations.

8.2.2 Determination of biomass and EPS in fermentation liquid

The fermentation liquid in the culture flasks was centrifuged at 10,000 rpm for 20 min to separate the biomass from the liquid medium. The

precipitated biomass was washed twice with distilled water and freeze-dried to give the biomass dry weight. For isolation of crude EPS, the supernatant liquid collected from the centrifuge was subject to ethanol precipitation by adding three volumes of 96% (v/v) ethanol to each volume of the liquid medium. The precipitated EPS was collected by centrifugation at 10,000 rpm for 20 min, and then freeze-dried for MW distribution, composition and structure analysis.

8.2.3 Determination of sugar consumption in fermentation medium

Concentration of glucose in the liquid medium was determined with a Biochemistry Analyzer (YSI Inc., Yellow Springs, OH, USA). Concentrations of galactose and mannose were determined by the 1-phenyl-3-methyl-5-pyrazolone (PMP)-HPLC method as reported by Honda et al. (Honda, Akao, Suzuki, Okuda, Kakehi & Nakamura, 1989). In brief, 450 μL of fermentation broth was derivatized with 450 μL PMP solution (0.5 M in methanol) and 450 μL of 0.3 M NaOH at 70 ^{o}C for 30 min. The reaction was stopped by neutralization with 450 μL of 0.3 M HCl, followed by extraction with chloroform (1 mL, 3 times). The extract solution was then applied to HPLC analysis.

8.3 Results and discussion

8.3.1 Consumption of carbon sources during mycelia fermentation

The consumption of the monosaccharide nutrients was shown in Fig. 8-1. Glucose, galactose and mannose may be produced during the fermentation process. However, they exist as the form of derivatives, which cannot be detected by HPLC. Glucose was consumed day by day and about 32 g/L glucose was utilized (Fig. 8-1A) after cultured for 8 days. The absorption of mannose and galactose was much different as mannose was taken since the first day while galactose was not consumed even after 8 days (Fig. 8-1B). Carbon source is a major limiting nutrient factor for microbial growth. Although glucose is a common carbon source to achieve high production of biomass and EPS in mycelium fermentation (Kim et al., 2005; Lee et al., 2004), other monosaccharaides such as fructose, galactose and xylose have also been used in mycelial fermentation (Mao, Eksriwong, Chauvatcharin & Zhong, 2005; Pokhrel & Ohga, 2007; Tang, Zhu, Li, Mi & Li, 2008). Glucose can be directly transferred to UDP-glucose and to further synthesize EPS in mycelia fermentation, while mannose or galactose may be converted to UDP-mannose or UDP-galactose directly or transferred to UDP-glucose first by enzyme before the uptake (Mozzi, Rollan, de Giori & Font de Valdez, 2001). However, galactose was not utilized due probably to the lack of related enzymes in the fungus.

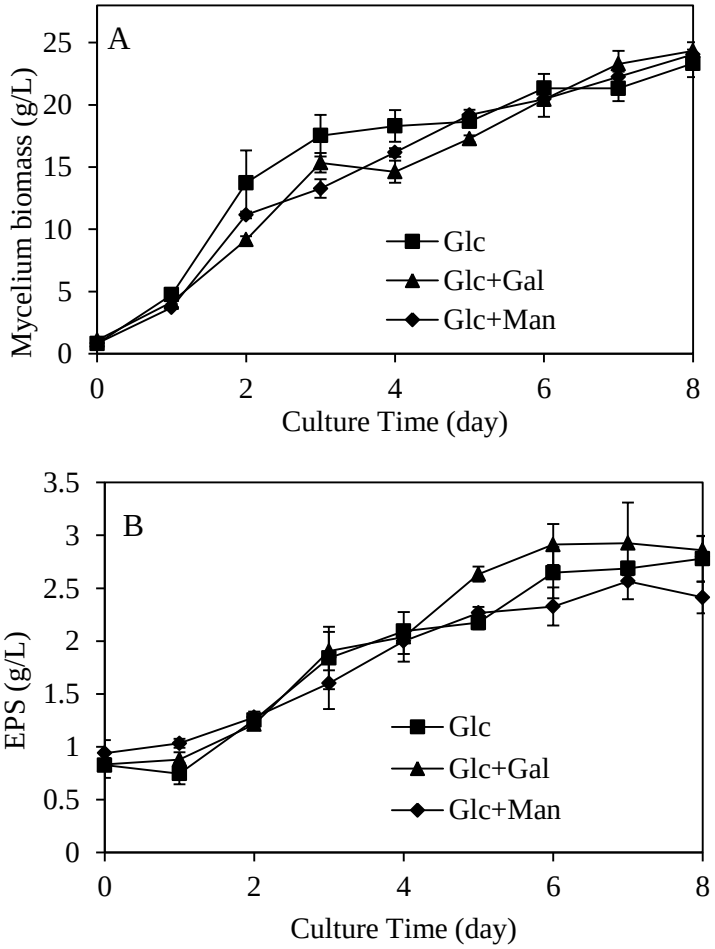


Figure 8-1 Time courses of biomass growth (A) and EPS production (B) in Cs-HK1 mycelial culture with Glc (40 g/L), Glc (35 g/L)+Gal (5 g/L) and Glc (35 g/L)+Man (5 g/L) as the carbon sources.

8.3.2 Effect of carbon sources on biomass and EPS production

Fig. 8-2 shows the biomass growth and EPS production by Cs-HK1 fungus on different carbon sources in liquid fermentation. Galactose could not be utilized in Cs-HK1 mycelial fermentation as discussed above. However, the production of mycelium and EPS in the presence of galactose was almost equal to the other two and the yield of EPS was also slightly

higher than the other two from day 5 to day 7. A previous study has shown that galactose was consumed to promote the growth of mycelia during fermentation process (Mao, Eksriwong, Chauvatcharin & Zhong, 2005). In this study, galactose also affected the biosynthesis of EPS although it was not seen utilized. At day 8, the EPS production from the three types (Glc+Gal, Glc, Glc+Man) were 2.9 g/L, 2.8 g/L and 2.4 g/L, respectively. As observed in our preliminary experiments, galactose or mannose supplied as the sole carbon source in the Cs-HK1 mycelial culture could not support the biomass growth and EPS production.

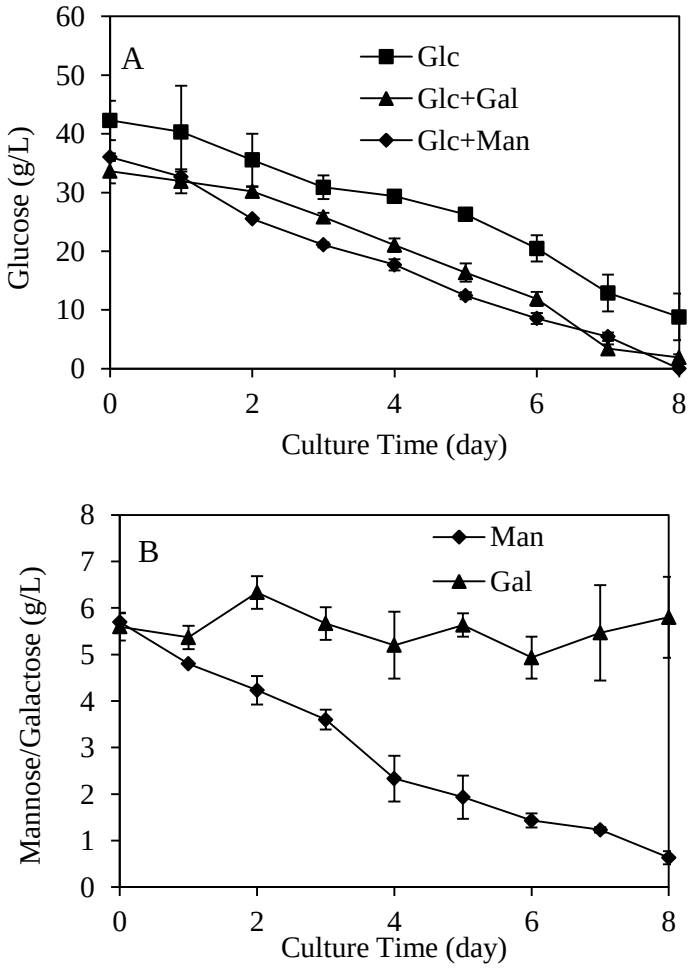


Figure 8-2 Time courses of residual carbon sources in Cs-HK1 mycelial cultures: (A) Glucose; (B) Galactose and mannose.

Table 8-1 shows the total carbohydrate and protein contents of EPS from Cs-HK1 mycelial culture with three different carbon sources on various days from days 3-8. Overall the total carbohydrate contents varied from 14-36% and the protein contents from 15-31%, which were higher in the later days with all carbon sources except for the occasional fluctuations.

Table 8-1 Total carbohydrate and protein contents of EPS from Cs-HK1 mycelial culture with different carbon sources (% by weight).

Culture day		3	4	5	6	7	8
Carbohydrate content (%)	Glc	18.6±3.2	23.5±4.3	26.6±2.5	31.8±0.9	32.5±2.1	36.5±1.9
	Glc+Gal	16.9±2.5	27.5±1.5	25.5±3.4	23.9±2.0	29.0±2.0	36.2±1.6
	Glc+Man	14.0±1.1	23.0±3.4	21.1±2.1	26.2±1.2	19.2±2.0	27.6±2.3
Protein content (%)	Glc	15.3±3.0	24.1±2.4	22.9±3.4	29.0±2.4	30.6±2.5	31.1±3.2
	Glc+Gal	22.9±1.0	28.4±2.0	30.7±2.2	27.9±1.4	27.7±2.6	27.8±1.7
	Glc+Man	21.7±0.1	27.4±1.5	26.0±1.4	30.1±1.0	32.2±2.5	28.5±2.1

As shown in Table 8-2, the monosaccharide composition of EPS isolated from the Cs-HK mycelia cultures on various days varied with the carbon sources. Three monosaccharides, Glc, Gal and Man, were found in all EPS samples and Glc represented the major and most abundant monosaccharide constituent, accounting for 50-75% molar. The addition of galactose and mannose to the culture did not lead to a notable increase in

their content in the EPS or even to a lower content.

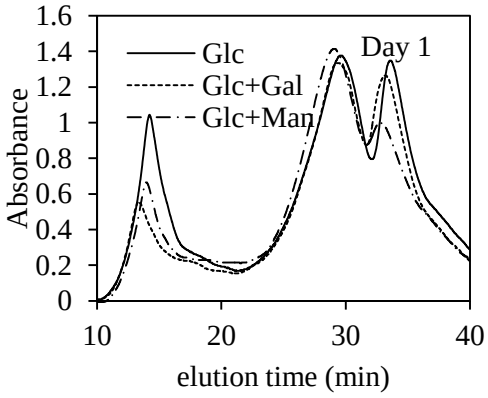
Table 8-2 Monosaccharide composition of EPS from Cs-HK1 mycelial culture with different carbon sources (% by mole).

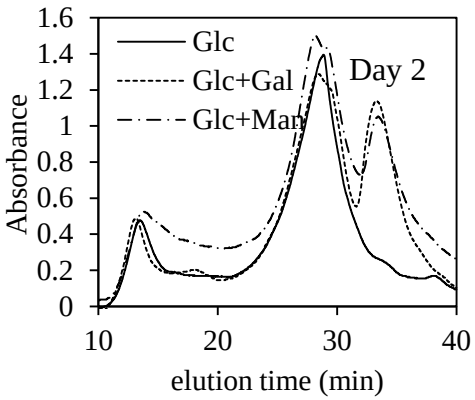
Culture day	Carbon source	Composition			Molar ratio
		Glc (%)	Gal (%)	Man (%)	Glc:Gal:Man
day 3	Glc	71.3	8.2	19.2	8.7:1.0:2.3
	Glc+Gal	68.4	10.2	17.4	6.7:1.0:3.9
	Glc+Man	70.2	9.8	16.2	7.2:1.0:1.7
day 4	Glc	63.7	10.6	23.7	6.0:1.0:2.2
	Glc+Gal	68.0	10.0	17.0	6.8:1.0:1.7
	Glc+Man	67.3	12.7	15.6	5.3:1.0:1.2
day 5	Glc	67.8	8.6	18.7	7.9:1.0:2.2
	Glc+Gal	62.8	12.1	18.1	5.2:1.0:1.5
	Glc+Man	59.4	13.8	19.8	4.3:1.0:1.4
day 6	Glc	59.7	17.7	20.6	3.4:1.0:1.2
	Glc+Gal	66.2	12.8	19.1	5.2:1.0:1.5
	Glc+Man	72.3	8.2	11.3	8.8:1.0:1.4
day 7	Glc	65.0	10.3	19.1	6.3:1.0:1.9
	Glc+Gal	54.0	20.0	20.0	2.7:1.0:1.0
	Glc+Man	71.7	12.6	9.8	5.7:1.0:0.8
day 8	Glc	62.1	11.6	21.9	5.4:1.0:1.9
	Glc+Gal	59.1	15.2	22.7	3.9:1.0:1.5
	Glc+Man	75.5	11.3	10.7	6.7:1.0:0.9

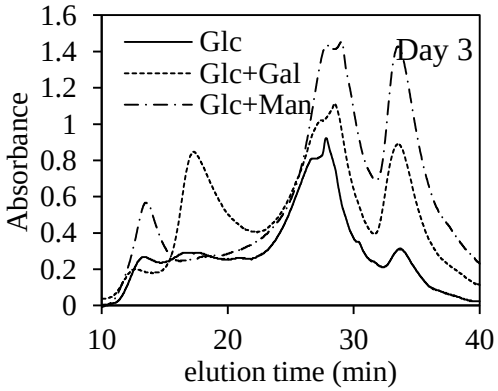
8.3.3 Effect of carbon sources on EPS molecular properties

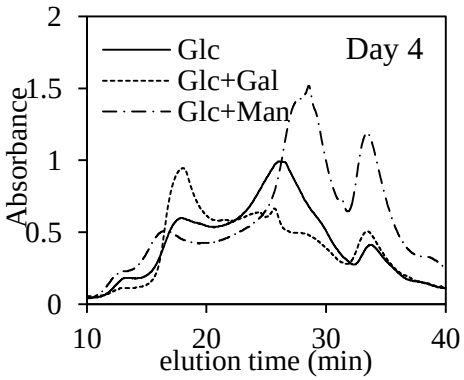
Fig. 8-3 shows the HPGPC MW profiles of EPS collected on all 8 days of culture. In the first two days, the production of EPS was slow with three

carbon sources and no significant differences were observed among the three EPS. After day 3, the MW profiles of EPS produced with three different carbon sources showed notable differences. In comparison with the peak area ratios (Table 8-3), which represent the relative amounts of different MW fractions of EPS, the amounts of the highest MW (with the shortest elution time of 10-15 min) and the lowest MW EPS (eluted out at 22-32 min) decreased, and the amounts of the middle MW EPS (eluted out at 12-22 min) increased with the culture duration. Different monosaccharides as carbon source have different pathway in the microbial fermentation process (Mozzi, Rollan, de Giori & Font de Valdez, 2001). The variation of EPS structural composition could be examined by NMR analysis. The ^{1}H-NMR of EPS from the Cs-HK1 mycelial culture showed different proportions of β-glucan in the EPS produced with three different carbon sources (Fig. 8-4). The proportion of β-glucan formed with glactose and glucose as the carbon source was higher (the shift around 4.8 ppm & 4.2 ppm) than that in the EPS produced with glucose as the carbon source. Overall the results indicated that the EPS composition and MW distribution were affected significantly by the carbon sources and also varied with the culture duration.


Day 1
Glc
Glc+Gal
Glc+Man
Absorbance
elution time (min)


Day 2
Glc
Glc+Gal
Glc+Man
Absorbance
elution time (min)


Day 3
Glc
Glc+Gal
Glc+Man
Absorbance
elution time (min)


Day 4
Glc
Glc+Gal
Glc+Man
Absorbance
elution time (min)

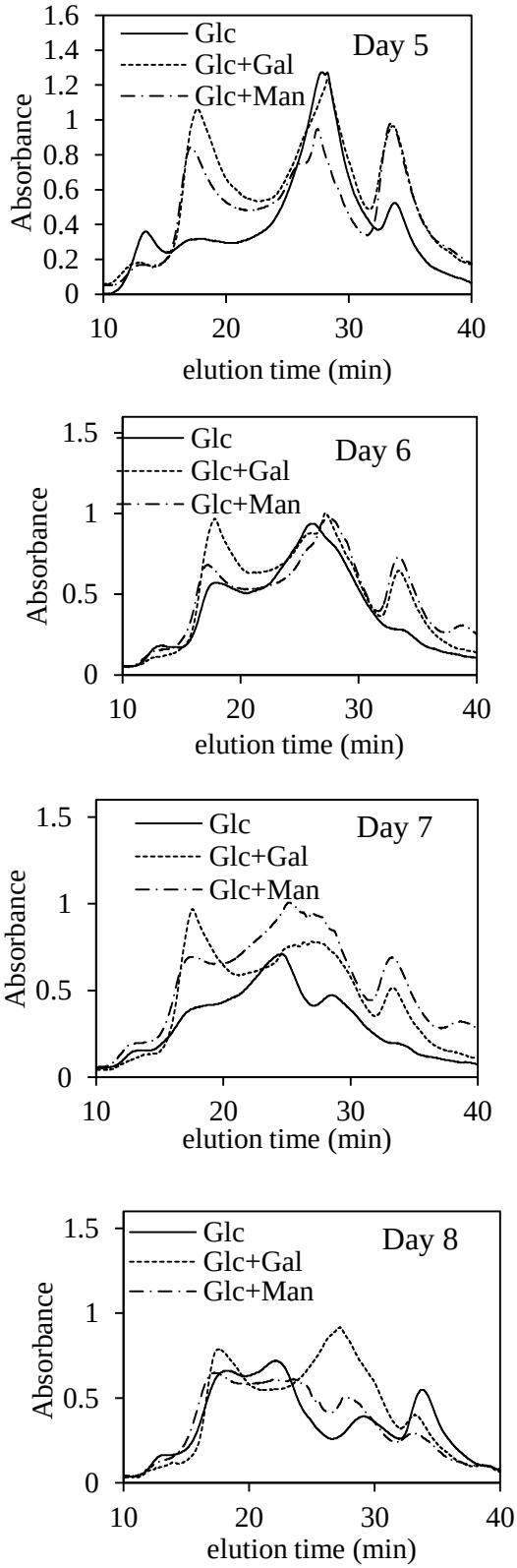


Figure 8-3 HPGGPC MW distributions of EPS from Cs-HK mycelial culture on day 1-8 with different carbon sources. The peak area ratios are summarized in Table 8-3.

Table 8-3 The MW distributions of EPS from Cs-HK1 with three carbon sources over 8 days of culture.

Culture day	Carbon source	Area %		
		10-15 min	15-22 min	22-32 min
Day 1	Glc	34.5	-	65.5
	Glc+Gal	26.8	-	73.2
	Glc+Man	25.8	-	74.2
Day 2	Glc	20.0	-	80.0
	Glc+Gal	12.1	4.3	83.6
	Glc+Man	26.4	-	73.6
Day 3	Glc	10.2	30.8	59.0
	Glc+Gal	3.9	37.8	58.3
	Glc+Man	12.3	18.9	68.8
Day 4	Glc	10.4	16.0	73.6
	Glc+Gal	2.8	47.4	49.8
	Glc+Man	3.0	44.1	53.9
Day 5	Glc	2.8	20.6	76.6
	Glc+Gal	1.0	79.2	19.8
	Glc+Man	1.8	13.7	84.5
Day 6	Glc	3.9	25.3	70.8
	Glc+Gal	1.1	56.9	42.0
	Glc+Man	1.9	29.7	68.4
Day 7	Glc	3.9	18.0	78.1
	Glc+Gal	1.0	48.5	50.5
	Glc+Man	2.5	24.1	73.4
Day 8	Glc	5.7	73.9	20.4
	Glc+Gal	0.7	50.6	48.7
	Glc+Man	1.2	36.7	62.1

Glucose and sucrose have been used as the favorable carbon sources for most of microbial fermentations (Radchenkova, Tomova & Kambourova, 2011; Rusinova-Videva, Pavlova & Georgieva, 2011; Wu et al., 2014; Yoon et al., 2012). Although previous studies have also evaluated the use of galactose, mannose and some other mono- and oligo-saccharides for the production of biomass and EPS (Prasertsan, Wichienchot, Doelle & Kennedy, 2008; Raza, Makeen, Wang, Xu & Qirong, 2011), few have assessed the composition and structure changes of EPS with these alternative carbon sources. In this study, the HPGPC and NMR analysis of EPS produced with alternative carbon sources showed the changes in the molecular composition of EPS affected by galactose and mannose. When galactose and mannose were added as a secondary carbon source, mannose was consumed but galactose was not during the culture process. Galactose has been reported previously with low utilization in the fermentation of *Listeria monocytogenes* (Datta & Kothary, 1993). Although galactose was not used by the mycelial culture, it affected the yield and composition of EPS. The mannose consumed did not increase the EPS yield and may be involve in the production of pyruvate (Kleerebezem & Hugenholtz, 2003).

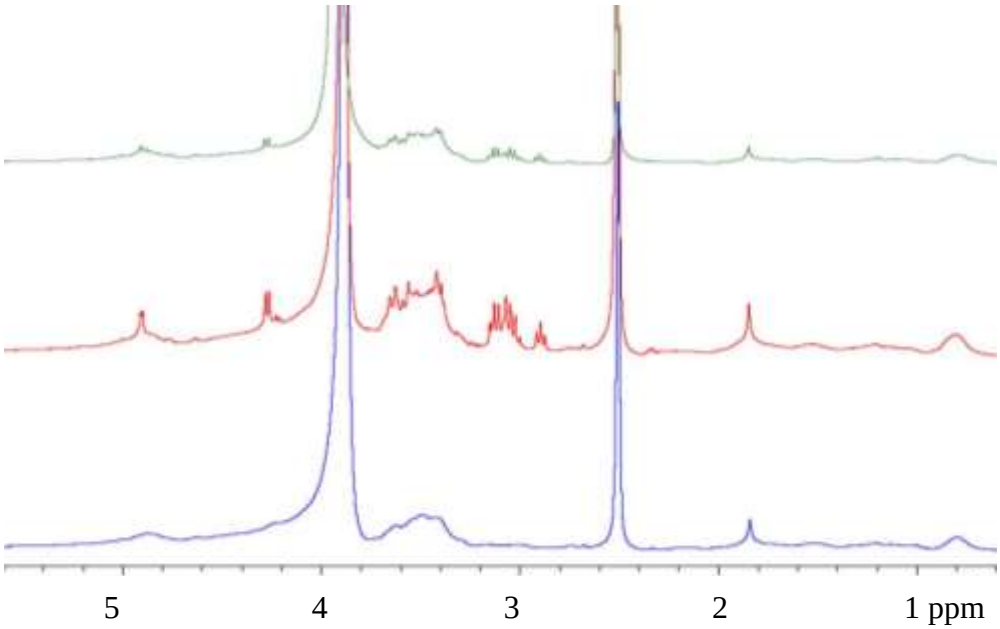


Figure 8-4 ^{1}H-NMR of EPS collected on the day 3 from the Cs-HK1 fermentation broth on three different carbon sources: mannose+glucose (top), galactose+glucose (middle), glucose (bottom).

8.4 Summary

The application of galactose or mannose as a secondary carbon source may influence the yield and composition of EPS in fungal mycelia culture. However, the influence appeared to be irrelevant to their utilization or consumption during the mycelial fermentation process. The results suggest that galactose and mannose can be applied to vary the molecular properties of EPS composed of glucose, galactose or mannose. Varying the carbon sources may be effective to generate new polysaccharide structures. Further studies should investigate the physiological mechanisms such as the metabolism and biosynthetic pathways for the EPS with various carbon sources.

Chapter 9 General conclusions and future work

9.1 General conclusions

This research project has accomplished an experimental study on the isolation and characterization of two homogenous polysaccharides (PS), their applications and other new properties of exopolysaccharide (EPS) from Cs-HK1 mycelial fermentation. The chief findings from this project are summarized below.

1. A two-step ethanol precipitation method was applied to separate EPS into high-molecular weight (MW) and low-MW fractions. The chemical composition and the physical properties of the two fractions differed greatly.

2. A more water-soluble and lower-MW polysaccharide named EPS1U was attained by partial degradation of the high-MW EPS using high-intensity ultrasound (US). EPS1U had an average MW about 730 kDa, much lower than EPS1 (2700 kD), and a much higher solubility in water than EPS1 (77.5 versus 5.1 g/L). EPS1U was elucidated as a (1→3)-β-D-glucan with glucose side chains attached to *O*-6 position at

the branching points. EPS1U showed high moisture absorption capability and significant immunomodulatory properties.

3. A protein-containing polysaccharide named EPS2BW was purified from the lower-MW fraction EPS. EPS2BW was mainly composed of galactomannan with about 16% (w/w) protein and the MW of EPS2BW was about 50 kDa. The carbohydrate part consisted of mannose and galactose at a molar ratio of 1.7:1.0, and the protein segments were composed of sixteen amino acids. The galactomannan structure was elucidated as a (1→2)-α-D-mannopyranosyl (Man*p*) backbone with *O*-6-linked galactopyranosyl (Gal*p*) branches. EPS2BW exhibited a high antioxidant capacity in both chemical and cell culture assays.

4. Silver nanoparticles (AgNPs) were synthesized with EPS1, the high-MW EPS fraction in water. The reaction conditions including temperature, time period and concentration of reactants were optimized to produce more uniform AgNPs. AgNPs with an average diameter of 50 nm were formed and remained as a stable dispersion in aqueous for at least 2 months. EPS1 might be acting as a reducing and stabilizing agent for the formation of AgNPs, which were attached to the hydroxyl groups of EPS1. The AgNPs formed in EPS1 solution exhibited a concentration-dependent inhibition of both Gram-negative and -positive

bacteria but a very low cytotoxicity on the RAW264.7 murine macrophage cells.

5. The yield and composition of EPS produced by Cs-HK1 were affected by feeding of a secondary carbon source galactose or mannose.

9.2 Future studies

The results from this project provide foundation and useful references for further research, development, and application of PS from mycelial fermentation of Cs-HK1. Specifically the following areas are suggested for future work.

1. Chemical modification of EPS can be applied to improve the physical properties and bioactivities of EPS, such as sulfation, carboxymethylation and phosphorylation.

2. The immunomodulatory activity of EPS1U *in vitro* was been investigated in this project. Further research should be focused on the *in vivo* test in animal models for better application of EPS1U.

3. Investigation and understanding the effects of carbon sources and other culture nutrients on EPS structure and properties.

4. Investigation of the formation, properties and antibacterial activities of AgNPs by using a series of EPS fractions with different MW and protein content, their relationships to the molecular properties of EPS and the experimental conditions, the interactions between AgNPs and EPS, and the synthesis process and reaction mechanisms.

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