Utilization of Soybean Curd Residue for Polysaccharides Production by *Morchella esculenta* and Evaluation of the Biological Activity

A Dissertation Submitted to

the Graduate School of Life and Environmental Sciences,

the University of Tsukuba

in Partial Fulfillment of the Requirements

for the Degree of Doctor of Environmental Studies

(Doctoral Program in Sustainable Environmental Studies)

Shuhong LI

Abstract

Soybean curd residue (SCR) is the main byproduct from soybean manufactring and it is often regarded as waste. The disposal of SCR has become a big problem to the environment. However, it is a good supporter and carrier for fungi to ferment because of its diverse source, porosity, nutrition and cheapness.

In recent years, polysaccharides have attracted much attention due to their biological activities. Accumulated evidences suggest that polysaccharides from fungi have immune regulation and antitumor effect.

Morchella esculenta (*M. esculenta*) is a precious edible and medicinal fungi. It is reported that the *M. esculenta* had biological activities such as antioxidant activity, antimicrobial properties, hepatoprotective activity and antitumor activity.

In order to reuse SCR, a new method is put forward to increase their economic efficiency. SCR was used as nutrient media to ferment polysaccharides by *M. esculenta*, and the fermentation conditions were optimized after comparing the physicochemical properties of SCR before and after fermentation. Furthermore, the characteristics, purification and biological activity including antioxidant, immunomodulatory and antitumor activity of produced polysaccharides were investigated. Compared with the polysaccharides obtained from fruit body and mycelia, the polysaccharides from SCR fermentation possess the advantages of waste minimization, time efficiency and high production at low cost.

The following results could be concluded in this research.

(1) Orthogonal experimental design and response surface methodology were employed to optimize the fermentation conditions of SCR for crude polysaccharides production from the strain *M. esculenta, namely* MPS. The MPS yield varied depending on the nutrition contents added to SCR and fermentation time, fermentation temperature and inoculum size during solid-state fermentation. The optimal fermentation conditions with the highest MPS production of 95.82±1.37 mg/g were glucose 4%, $(NH_4)_2SO_4$ 1.5%, water content 75% and $MgSO_4 \cdot 7H_2O$ 0.2%, fermentation temperature 22.6°C, fermentation time 21 days and inoculum size 2.67%.

(2) Morphological changes and slight increases in powder hygroscopicity were found for SCR after the solid state fermentation for fermented SCR. Thermal analysis of the fermented SCR showed a high decomposition temperature (Td) trend of 210°C. Increase in the free amino acids, polysaccharides (from 25.22±2.23 to 95.82±1.37 mg/g) and total polyphenol (from 5.99±0.27 to 7.70±0.18 mg/g) were also detected in the fermented SCR. The fermentation process presents a possible way of changing or improving the physicochemical properties of SCR.

(3) Compared with the crude polysaccharides from unfermented SCR (PS), MPS has different micrographs and higher hygroscopicity (from 108.60±0.30 to 112.73±0.26 g water/100 g solids) as well as dark color (total color different value from 89.25±0.71 to 63.39±0.49) after fermentation. More diverse monosaccharide was found in MPS than PS. Furthermore, the antioxidant activity of crude polysaccharides before and after fermentation was evaluated. Based on $IC_{50,}$ the antioxidant activity of crude polysaccharides in a descending order was: hydroxyl

radical scavenging activity (IC_{50}: 1.39 mg/mL), ABTS scavenging activity (IC_{50}: 2.64 mg/mL), DPPH radical scavenging activity (IC_{50}: 3.13 mg/mL), ferrous metal ions chelating activity (IC_{50}: 3.53mg/mL). Meanwhile, the obtained MPS demonstrated higher positive antioxidant activity than PS.

(4) Macrophage cells (RAW 264.7) were used as experimental objects to evaluate immunomodulatory activity. The proliferation effect of MPS on cells was 317.8% at 50 μg/mL and MPS induce 14.0 μM NO at the concentration 6.25 μg/mL. At the same time, MPS showed a phagocytosis activity (O.D. 540 nm) which was 0.58 at doses of 100μg/mL and a proliferation effect of 96.0% at 50μg/mL on the macrophages when exposed to Doxorubicin (DOX). In addition, the antitumor experiment indicated that MPS had higher inhibition effect on human cervical carcinoma cells (Hela), human colon cancer cell line (DLD- 1) and human hepatocarcinoma cell line (HepG2) than PS, with inhibition ratios being 32.60%, 37.70% and 77.39% respectively.

(5) The crude polysaccharides were purified by DEAE-SephadexA-50 chromatography and subsequently Sephadex LH-20 size-exclusion chromatography. Three main fractions, MP-1, MP-3 and MP-4 were obtained through purification steps and the extraction ratios were 26.2%, 29.1% and 18.7% respectively. The characteristics, such as monosaccharide composition, ultraviolet spectrum and infrared spectrum of the three fractions were analyzed in this study. The three purified polysaccharides were found to have a similar monosaccharide composition but at different mol ratios. Furthermore, the influence of purified polysaccharides fractions

upon activation of macrophage cell RAW 264.7 and antitumor activities to HepG2 in vitro were evaluated. The results indicated that the proliferation of MP-3 on macrophages RAW 264.7 was 313.57% at 100 μg/mL which was the highest while MP-1 had the highest growth inhibition on HepG2 cells, 68.01% at a concentration of 50 μg/mL.

The results obtained in this study will provide references for the large-scale production of polysaccharides by *M. esculenta* and point to a new direction for the utilization of SCR. The present work can also afford a theoretical foundation for low-cost production of polysaccharides in industrial scale. Furthermore, animal models in vivo of the polysaccharides should be validated to confirm and thus to extend the findings from this study.

Contents

List of tables

List of figures

Abbreviations

ABTS	2, 2'-Azinobis (3-ethylbenzothiazoline-6-sulfonic acid)
CCK-8	Cell Counting Kit-8
DOX	Doxorubicin
DPPH	2, 2-Diphenyl-1-picrylhydrazyl
DTA	Differential thermal analysis
FAA	Free amino acids
FSCR	Fermented soybean curd residue
FTIR	Fourier transform infrared
LPS	Lipopolysaccharide
M. esculenta	*Morchella esculenta*
MPS	Polysaccharides from fermented soybean curd residue
O.D.	Optical density
PS	Polysaccharides from unfermented soybean curd residue
RSM	Response surface methodology
SCR	Soybean curd residue
SSF	Solid state fermentation
TA	Titratable acidity
TFA	Trifluoro acetic acid
TG	Thermogravimetric
USCR	Unfermented soybean curd residue
Vc	Vitamin C

Chapter 1 Introduction

1.1 Background

Soybean is one of the most important legumes in the world, particularly in Asian countries like Japan. The production of soybean products has been increasing throughout the world, and there has been a corresponding increase in the quantity of soybean curd residue (SCR), being discarded. The disposal of SCR has become a problem to be solved due to its contamination of environment. However, SCR is rich in fiber, fat, protein, vitamins and trace elements. It has potential for value added processing and utilization.

Polysaccharides isolated from the plants, fungi, yeasts, mushrooms, and alga have been increasingly concerned about in the field of biochemistry and pharmacology. They exhibit a wide range of biological functions, such as anti-inflammatory (Kang et al., 2011), antioxidant, antitumor (Song and Du, 2012), immunostimulatory (Dai, et al., 2009), anticancer activity (Xie et al., 2013). Current polysaccharides production from medicinal fungi is mainly from submerged culture and fruit body. Submerged fermentation not only has the problem of more energy-consumption during extraction, but also water-consumption and low yield (Hölker and Juörgen, 2005; Meng et al., 2010). Extracting polysaccharides from the fruit body takes more than 3 months, which is of high cost and time-consuming. It is very urgent to find an easy, time saving and efficient way to produce polysaccharides with high biological activity.

For centuries, *M. esculenta* has been consumed and appreciated for its nutritional value as well as medicinal properties (Wahid et al., 1988). The crude polysaccharides

isolated from *M.esculenta* mycelia have been proven to possess potential antioxidant properties (Elmastas et al., 2006). Currently, *M. esculenta* is highly valued in China, partially due to its biological activity, rareness and cultivation difficulty via traditional methods.

In this study, SCR as the nutrient media, it was investigated to ferment polysaccharides with *M. esculenta*. The fermentation conditions were optimized and physicochemical properties of SCR before and after fermentation were compared. Furthermore, the characteristics, purification and biological activity of polysaccharides were investigated, including the antioxidant, immunomodulatory and antitumor activity.

Soy milk and tofu have maintained wide popularity as food source for thousands of years and large quantities of their byproducts are generated during the manufacturing process (Hsieh and Yang, 2004). In Asian countries, soybean is made into various foods such as tofu, soymilk, soymilk powder, bean sprouts, dried tofu, soy sauce, soy flour and tempeh soybean oil. SCR, namely okara in Japanese, is the main surplus material of soybean products and it is often regarded as waste. About 1.1 kg of fresh SCR is produced from every kilogram of soybeans processed into soymilk or tofu (Khare et al., 1995).

In 2010, the annual output of soybean exceeded 261 million tons. As far as Japan is concerned, imported soybean amounted to 3.5 million tons in 2009 according to FAO report (FAO, 2010). About 800,000 tons of SCR are disposed of annually as by-

products of tofu production in Japan. The expense for SCR disposal costs around 16 billion yen per annum (Muroyama et al., 2006).

Currently, SCR is used as stock feed, fertilizer or dumped in landfill. Particularly in Japan, most of the SCR is burnt which will create carbon dioxide (Muroyama et al., 2001). Meanwhile, discarding of SCR as waste is a potential environmental problem because it is highly susceptible to putrefaction (Almaraz et al., 2009). The environmental problems arising from the massive generation of SCR has been attracting considerable attention.

1.2 Soybean curd residue

SCR is a loose material consisting of a good source of nutrient, including protein, oil, dietary fibre, minerals. Several reports could be found on the reuse of SCR for fermentation products, such as β-fructofuranosidase, *ganoderma lucidum*, *bacillus subtilis* B_2 and polysaccharides (Redondo-Cuenca et al., 2008; Cheng et al., 2005).

1.2.1 Soybean curd residue composition

The main components of SCR are ruptured cotyledon cells and the soybean seed coat. Characterization of this byproduct, including the protein, oil, dietary fibre, mineral composition, along with un-specified monosaccharide and oligosaccharides can be found in the literature (Surel and Couplet, 2005). The lyophilized SCR gives 6.99% of H, 46.34% of C, 3.99% of N, 0.25% of S and 3.59% of mental oxides as ash. The composition of SCR is different based on different regions and processing method. The composition of SCR is shown in Table 1-1 and Table 1-2.

(1) Carbohydrates and its function

SCR is rich in cellulose which accounts for approximately 50% of the dry weight in soybean with very few calories. It contains lots of dietary fiber that can't be digested in the small intestine but can be fermented by microbes in the large intestine. Especially, dietary fiber is known to provide important health benefits to the body, with regard to the maintenance of normal bowel function (Periago et al., 1997). Studies have shown that combining SCR with soft wheat flour resulted in increased protein, dietary fiber and isoflavone contents compared with the use of soft wheat flour alone (Rinaldi et al., 2000). Several positive effects also have been attributed to the lipid profile in blood plasma, liver and in faecal lipid excretion in Syrian golden hamsters fed with SCR (Villanueva et al., 2011). The monosaccharide units in the polysaccharides include glucose, arabinose, and galactose when it is enzymatically digested. SCR also contains rhamnose, mannose and xylose according to the study of Redondo-Cuenca et al (Redondo-Cuenca et al, 2008).

In addition, there is some soluble dietary fiber in SCR. Previous research indicated that the amount of soluble dietary fiber in SCR treated by high hydrostatic pressure increased more than 8-folds (Mateos-Aparicio et al., 2010b). Furthermore, soluble dietary fiber plays an important role in the reduction of cholesterol levels in some hyperlipidemic individuals, and it can also be used to improve glucose tolerance in diabetics (Jenkins et al., 2003). The soluble fiber in SCR treated by high hydrostatic pressure has anti-inflammatory and anti-carcinogenic effects on the digestive tract (Mateos-Aparicio et al., 2010a). On the other hand, insoluble dietary fiber increases faecal bulk and reduces gastrointestinal transit time. Moreover, it seems to have a

positive effect on diarrhea, constipation and as a treatment for irritable bowel (Bosaeus, 2004).

(2) Protein and amino acids

SCR has high quality of protein, especially essential amino acids. It is well documented that SCR contains about 27% of protein (dry basis) with good nutritional quality and a superior protein efficiency ratio, which shows a potential source of low cost vegetable protein for human consumption. The protein in SCR is of better quality than from other soy products, e.g., the protein efficiency ratio of SCR is 2.71 compared with 2.11 for soymilk, but the ratio of essential amino acids to total amino acids is similar to tofu and soymilk. It is clear that fermented thua nao, a Thai fermented soybean has much higher concentrations of amino acids than its unfermented counterparts, which increases the flavor (Dajanta et al., 2011). However the low solubility in water of protein in SCR is detrimental which affects its extraction. The functional properties of protein from SCR, including emulsification, water, fat binding and foaming properties, were found to be comparable to the commercial soy isolate. Moreover, the protein in SCR after fermentation produces free amino acid and soy peptides. Therefore the development of protein resources from SCR has great potential.

1.2.2 Utilization of soybean curd residue

In the past, SCR had limited uses because of drying cost, storage and shipment. Recently, the utilization of by-products from the food industry has become widespread (Schieber et al., 2001). As a nutritious food byproduct, SCR is no

exception. Some countries, such as Japan and America pay more attention to reusing SCR. Previous articles focused on the following aspects: Firstly, the ingredients are directly extracted from water dry SCR. Some useful materials, such as pectic polysaccharides, protein, dietary fiber can be produced from SCR. Secondly, the useful components are extracted from SCR after processing or fermentation with microorganisms. Polysaccharides (Shi et al., 2012), β-fructofuranosidase (Hayashi et al., 1992), iturin A, ethanol and methane (Muroyama, et al., 2001) can also be produced from SCR under different fermentation conditions. It is reported that some constituents extracted have anticancer functions and antioxidants (Zhu et al., 2008). In the food and drug processing industry, SCR is used in some process to make biscuits, biodegradable edible paper, fiber beverages and lactic acid.

(1) Unfermented products

The SCR is rich in nutrients. Accordingly, the unfermented products are the most extractive of the products which are made directly from SCR. Great efforts have been undertaken to study the fiber, polysaccharides and protein in SCR. At the same time, it is considered to be attractive due to its low cost and availability in large quantities as a raw material. Recent research on cell wall polysaccharides and the swelling and water retention capacity of SCR indicated its potential application as a texturing additive (Mateos-Aparicio et al., 2010c). The use of SCR in food and feed is a natural way of promoting reasonable usage. A little of SCR can be added into food as rough are resulting in many advantages: (1) low-calorie, (2) providing a full belly as food, (3)

the carbohydrates are digested and enter the bloodstream more slowly to balance nutrition.

In the field of SCR extraction, firstly, SCR is a good source of dietary fiber to meet consumers growing needs as mentioned previously. Dietary fibre can also impart some functional properties to foods, e.g., increase water holding capacity, oil holding capacity, emulsification or gel formation. The amount of soluble dietary fiber went up from 38.1% to 64.8% after high hydrostatic pressure (Mateos-Aparicio et al., 2010a). Syrian hamsters fed by SCR tested the functional effects of SCR and results suggest that the main components of SCR, dietary fibre and protein could be related to decreases in the total lipids and cholesterol (Villanueva et al., 2011). Soybean polysaccharides are another main extractive from SCR, which can be used for water retention or as a food dispersing agent. Moreover, antioxidants are also important products of SCR. Processed SCR products, extrusion food, convenience food, bakery products, pet food were all chosen for implementation. The use of SCR has received wide attention to date, and various large-scale procedures have been developed. In industrialized production, SCR has been used to produce wrapping paper for food, plastics, cookies, cakes and pies.

(2) Fermented products

As we know, SCR is a loose material consisting of a good source of nutrients. Consequently, it is most suitable for microorganism to ferment. For this reason, a number of authors carried out researches on the fermentation products using SCR. Table 1-3 lists some of the fermented products from SCR. During fermentation, a

series of biochemical reactions occur, such as the decomposition of insoluble polymer material into soluble lower molecular weight compounds. Also SCR contains anti-trypsin, saponin and anti-nutritional factors such as hemagglutin which can't be easily digested. The fermentation process is not only conducive to digestion and absorption of nutrients, but also further improves the nutritional value of SCR.

1.2.3 Restrictive factors for the development of soybean curd residue

The production of SCR throughout the world is large but its utilization ratio is low, which results in poor returns from rich resources. More problematic, some wrong handling is posing a big environmental pollution. The main factors limiting the efficient use of SCR are summarized as follows.

(1) Moisture

The moisture content of SCR is between 70%-80% which is too high to preserve well. However, the nutrition components are rich. Consequently, SCR will decompose rapidly after produced. To overcome these limitations, fresh SCR must be dried as quickly as possible under appropriate drying conditions (Wachiraphansakul and Devahastin, 2007). The natural drying process is not adaptable for drying SCR because of the long duration required and weather influences. That's the reason that SCR will rot before being naturally drying. The general methods to preserve SCR include freezing of SCR under low temperatures (from $0^{\circ}C$ to $-18^{\circ}C$ or lower) to inhibit growth of microbial growth; oven drying can remove water from SCR to make it suitable for preservation and vacuum freeze drying is another method to handle SCR. However, the common drying methods are energy intensive. Nevertheless,

significant variations in flavor, color and aroma always take place. Previous studies suggest some advanced drying technology should be developed for SCR to improve its drying quality.

(2) Anti-nutrients

Raw soybeans have high anti-nutritional factors, especially soybean trypsin inhibitors which are partially inactivated during the solvent extraction and toasting process. Trypsin inhibitor is one of the most important factors that limit the application of SCR in animal feed. That's the reason why SCR can't be fed directly for livestock. Livestock served with SCR will have digestion problem because of the antitrypsin, a further negative effect on its use to facilitate animal growth. The experimental results show that fresh SCR has an impact on growth, behavior and physiological activity used as direct feed (Hinks and Hupka, 1995). The inactivation methods for soybean trypsin inhibitors include processes utilizing physics, chemistry, bio-reduction and fermentation as well as some complex methods using natural compounds (Ao et al., 2010).

(3) Diversify in production

In Asia, SCR is mass produced everyday but most tofu and soy milk plants are small-scale workshops, actually hand-craft workshops. While the decentralized SCR is difficult to collect, so is the arrangement of the storage, handling, transportation and application of this product. Small businesses can't afford expensive disposal equipments for SCR. One method to solve the problems is the implementation of shared resources. Producers can share machines for drying and transportation.

Another way of overcoming this problem is to facilitate tofu and soy milk plant to form the centralized production. At the same time, the plant which facilitates extensive processing of SCR products near the production area saves transportation costs and decreases the SCR deterioration rate as well.

1.2.4 Prospects for the utilization of soybean curd residue

At present, the rational development and utilization of SCR has aroused public concern. A considerable number of studies reported the reutilization and development of SCR in the literature. For some reason, such as a lack of funding and guidance, some countries are at obvious disadvantages. In addition, the storage and transportation process of SCR has not reached normal technical standards.

(1) Food additive

Finding convenient ways to incorporate SCR into food could eliminate a possible source of pollution and add economic value to this currently valueless product. The use of SCR, as a food additive, is one of the developing trends which have an important economic function and social meaning. Adding SCR to bakery products, without further processing or as an ingredient in consumer ready products, can reduce production cost and upgrade quality. Recently, food packaging must not just look good and be practical but the environmental protection design has become an important consideration for the packaging industry. As the food packaging and medical packaging market will continue to show strong growth momentum, green food packaging designs using SCR will become one of the most important developments in this field.

(2) Microbiological medium

Microbial fermentation with SCR is the beginning of a new way forward. As a cheap culture with good characteristics using different fungi, the product has functional components and there have been some trials including the production of sauce, juice and edible fungi (Chung, et al., 2011). To develop functional food by utilizing the resources of SCR is an important way for SCR industries to go into the future markets. Some of the benefits of producing ethanol from agricultural residues are the reduction of the potential for air, water and soil contamination associated with the land application of organic residuals (Champagne, 2007). As a food product residue SCR is an even more promising source to produce biogas. The biogas residue of SCR can be widely used in agriculture and stockbreeding for its rich nutrient content and microelements.

(3) Other products

It's more and more important to expand the industrial chain of SCR. Increasing the value added step by step and reducing the harmful effect to the environment are becoming the key points. Therefore, other high value-added products derived from SCR, including protein, polysaccharide, isoflavones, antioxidant substance (Zhu et al., 2008) and so on. The Japanese have patented a process in which SCR is used to make a reinforced ceramic via a pozzolanic reaction. The carbonized SCR can react with SiO_2 to form silicon carbide that strengthens the ceramic product. Also, a kind of pet product which is used to collect cat wastes at home has started selling in Japan.

1.3 Polysaccharides

1.3.1 The structure of polysaccharides

Polysaccharides are polymeric carbohydrates, made up of repeating monosaccharide joined by glycosidic bonds. Recently polysaccharides extracted from plants and fungi have drawn more attention of researchers and consumers due to their relatively low toxicity and obvious antitumor activities. They can be expressed by a general formula $(C_6H_{10}O_5)_n$, where n is usually a large number between 40 and 3000. The polysaccharides were composed of the same monosaccharide are called homopolysaccharide or homoglycan, such as starch, cellulose and glycogen; while when polysaccharides are composed of different type of monosaccharides, they are called heteropolysaccharides or heteroglycans, such as arabinose, pentose, galactose.

Importantly, polysaccharides are widespread in nature, which is very important. The relative molecular mass of polysaccharides is from tens of thousands to tens of millions. The structure units are connected by glycosidic bond, and the types of bonding are α-1, 4-, β-1, 4- and α-1, 6- glucosides keys. The physiochemical and structural features of a polysaccharide are usually defined by molecular weight, monosaccharide composition, sequences of monosaccharide, configuration and position of glycosidic linkages, type and polymerization degree of branch, spatial configuration, particle size, solubility and rheological properties. (Jin, et al., 2011; Nie and Xie, 2011).

1.3.2 The function of polysaccharides

Polysaccharides exhibit a wide range of biological functions, such as immunostimulatory (Dai et al., 2009), anticancer activity (Xie et al., 2003), anti-inflammatory (Kang et al., 2011), antioxidant, antitumor and so on.

Accumulated evidences suggest that the polysaccharides from fungi have immune regulation effect. The polysaccharides from *Hericiumerinaceus*, which are larger than 1×10^5 kDa, have been shown to increase the levels of T cells and macrophages in mice. The research from Sheu and his coworkers demonstrated that *Hericiumerinaceus* polysaccharides have an immune potentiating effect on dendritic cells (Sheu et al., 2013). A polysaccharides fraction from *Ganoderma (G.) lucidum* had strong stimulatory effects on both macrophages and T-lymphocytes in vitro (Wang et al., 1997).

The involvement and importance of polysaccharides in tumor and cancer treatment were first recognized 100 years ago. It was found that certain polysaccharides could induce complete remission in patients with cancer (Nauts, et al., 1946). The role of polysaccharides as antitumor agent is studied in recent years. Numerous studies have suggested that polysaccharides can inhibit tumor growth through the following common mechanisms: (1) the prevention of genes of tumor is by oral consumption of active preparations; (2) direct anti-cancer activity, such as induction of tumor cell apoptosis; (3) immune potentiation activity in combination with chemotherapy; and (4) inhibition of tumor metastasis (Zong et al., 2012).

1.3.3 Polysaccharides from mushrooms

Mushrooms have been valued as edible and medicinal resources, and antitumor substances have been identified in many mushroom species, especially in traditional oriental therapies in Asian countries. A large amount of bioactive components isolated from mushroom are utilized in modern clinical experiences. Over the last half-century, a growing number of countries have increasingly demonstrated the potential of mushroom extracted compounds in the prevention and treatment of cancer. Polysaccharides are the best known and most potent mushroom-derived substances with antitumor and immunomodulating properties, which commonly act as immunomodulators or as biological response modifiers.

Polysaccharides or extracts mainly containing polysaccharides from dozens of mushrooms have shown anti-cancer activity in animal models. Of these, chemically α- or β-glucans or peptide-bound glucans work. Five polysaccharides constituents from mushrooms have shown significant anti-cancer efficacy against several human cancers in clinical trials as BRMs, including lentinan from *Lentinus edodes*, D-fraction from *Grifola frondosa*, schizophyllan from *Schizophyllum commune*, polysaccharide-K (PSK) from *Trametes versicolor*, and polysaccharide-peptide (PSP) (Wasser, 2002). It has been shown that *β-glucans* can induce biological responses by binding to a membrane receptor, complement receptor type 3. More recently, another receptor, dectin-1, was characterized as a *β-glucan* receptor that mediated this activity (Zaidman et al., 2005).

As for the identification of anti-cancer activity of mushroom extracts, searching for new anti-tumor substances from mushrooms has become a matter of great

significance. Besides the glucans mentioned before, a wide range of anti-cancer polysaccharides or polysaccharide-protein/peptide complexes have been identified.

1.4 *Morchella esculenta*

M. esculenta is a species of fungus in the *Morchellaceae* family of the *Ascomycota*. It is a precious edible and medicinal fungi, containing many biological active materials, such as protein, fat, mineral contents, ash, fiber, and carbohydrates (Litchfteld et al., 2006; Dursun et al., 2006).

M. esculenta is commonly known by various names: morel, common morel, true morel, morel mushroom, yellow morel, sponge morel, Molly Moocher, haystack, and dry land fish. Wahid et al. (1988) conducted one study in which the main nutritional components were determined to be as follows (on a dry weight basis): protein 32.7%, fat 2.0%, fiber 17.6%, ash 9.7%, and carbohydrates 38.0%. It is reported that the *M. esculenta* has biological activities such as antioxidant activity, antimicrobial properties, hepatoprotective activity and antitumor activity (Mau et al., 2004; Heleno et al., 2013; Nitha et al., 2013).

Cancer is one of the major causes of human death worldwide. There are many anticancer therapies available, including chemotherapy and anticancer drugs. The great majorities of them are known to be not only cytotoxic to cancer cells, but also toxic to normal cells and harmful to the immune system (Yang et al., 2007). Polysaccharides from *M. esculenta* fruiting bodies and cultured mycelia have antioxidation, anticancer, and antivirus properties and increase fatigue resistance

(Alves et al., 2012; Elmastas et al., 2006; Nitha and Janardhanan, 2008; Rotaoll et al., 2005; Xu et al., 2008).

1.5 Originality and structure of the thesis

The aim of this study was to produce polysaccharides from SCR fermented with *M. esculenta*. Meanwhile, the biological activities of polysaccharides were evaluated, including antioxidant activity, antitumor activity and immunologic function.

The originality of this research is as follows. Firstly, SCR was firstly used to ferment polysaccharides by *M. esculenta*. Secondly, the physicochemical properties of SCR before and after fermentation were studied. Meanwhile, the biological activity of polysaccharides from fermented and unfermented SCR was firstly compared. Finally, physicochemical properties of polysaccharides were studied.

The structure of this thesis is described below.

In Chapter 1, the background of this research is introduced. The nutrient and utilization of SCR are summarized. Then, the function of polysaccharides and the *M. esculenta* are illustrated in detail.

In Chapter 2, according to previous experiments, the accumulation of polysaccharides by *M. esculenta* using SCR is strongly influenced by fermentation conditions, including fermentation temperature, fermentation time and inoculum size. Therefore, further study on the optimal fermentation conditions for polysaccharides is worthwhile. The aim of this chapter is to apply statistical methods to optimize the fermentation conditions for polysaccharides production. In this chapter, orthogonal experimental design and response surface methodology were employed to optimize

the fermentation conditions for crude polysaccharides (MPS) production from the strain *M. esculenta* by SCR.

In Chapter 3, the effects of fermentation by *M. esculenta* on physicochemical properties of SCR were investigated in terms of physical properties (morphological change, hygroscopicity, differential thermal analysis (DTA), CIELAB color and chemical characteristics (proximate composition, free amino acids (FAA), pH, titratable acidity (TA), polysaccharides and total polyphenol). Fourier transform infrared (FTIR) spectrometric property also provided further structural information on the SCR.

In Chapter 4, the characteristics of crude polysaccharides and their antioxidant activity were studied. Micrographs, hygroscopicity as well as color of polysaccharides before and after fermentation were compared. Besides, the monosaccharide compositions of crude polysaccharides were investigated. Furthermore, the antioxidant activity of crude polysaccharides before and after fermentation were evaluated, including ABTS assay, DPPH test, hydroxyl radical scavenging activities and ferrous metal ions chelating activity.

In Chapter 5, enhancing immunity and antitumor activity is the important biological activity of polysaccharides. Immunity activity included proliferation ratio, NO production, phagocytosis and protection effect on the macrophages. In addition, the inhibition ratio of crude polysaccharides on human colon cancer cell line (DLD- 1), human cervical carcinoma cells (Hela) and human hepatocarcinoma cell line (HepG2) were tested.

In Chapter 6, purification process, immunomodulatory and antitumor activity of polysaccharides were carried out. The crude polysaccharides were purified by filtration, DEAE-SephadexA-50 chromatography and Sephadex LH-20 size-exclusion chromatography in sequence. The characterizations of purified polysaccharides, such as monosaccharide composition, ultraviolet spectrum and infrared spectrum of three main fractions were analyzed in this study. Furthermore, the influence of polysaccharides fractions upon activation of macrophage cell (RAW 264.7) and antitumor activities to human hepatocarcinoma cell line (HepG2) in vitro were evaluated.

In Chapter 7, the major conclusions of this research are summarized. Moreover, the outlook of SCR utilization is put forward. In particular, the future research direction of polysaccharides fermented by SCR is forecasted.

Table 1- 1 Reported values for the percentage of protein, fat/oil, crude fiber, carbohydrates and ash contained in SCR (dry matter basis)

Protein	Crude fat	Crude fiber	Carbohydrates	Ash	Reference
25.4-28	9.3-10.9	52.8-58.1	3.8-5.3	-	Van der Riet et al.,1989
26.8±0.1	22.3±1.5	-	-	-	Guermani et al.,1992
16.1	2.2	-	52.6	5.3	Hsieh et al., 2004
29.3	0.8	-	53.6	4.0	Muroyama et al., 2006
25.0	20.0	33.0	-	-	Suruga et al.,2007
28.5	9.8	55.5	2.6	3.6	Redondo-Cuenca et al., 2008
33.4±0.3	8.5±0.3	54.3±2.3	-	3.7±0.2	Mateos-Aparicio et al., 2010c
25.5	12.0	12.2	32.6	4.0	Rashad et al.,2011

Table 1- 2 Reported values for the percentage of moisture, protein, fat/oil, crude fiber, carbohydrates and ash contained in SCR (wet matter basis)

Moisture	Protein	Fat	Fibre	Carbohydrate	Ash	Reference
84.5	4.7	1.5	1.5	7.0	0.4	O'Toole, 1999
83.9	4.5	2.6	-	8.3	0.7	Turhan et al., 2007
85.0	3.6	1.4	9.2	-	-	Zhu et al., 2008
84.5	4.2	1.5	3.52	5.78	0.55	Fafaungwithayakul et al., 2011

Table 1- 3 SCR products obtained by fermentation with various microorganisms

NO.	Strain	Products	Reference
1	*Aspergillus japonicus MU-2*	β-Fructofuranosidase	Hayashi et al., 1992
2	*NRRL 330+NCIM 653*	Citric Acid	Khare et al., 1995
3	*Bacillus natto*	Antioxidant preparation	Hattori et al., 1995
4	*Methanogens I and II*	Methane	Katsuhiko et al., 2001
5	*Lactobacillus paracasei*	Hydrogen	Tatsuya et al., 2002
6	*Ganoderma lucidum*	Fruiting bodies	Hsieh et al., 2004
7	*Lactobacillus paracasei*	Lactic acid	Katsuhiko et al., 2006
8	*Bacillus subtilis B2*	Antioxidant	Zhu et al., 2008
9	*Digestion sludge*	Methane	Zhou et al., 2011
10	*Bacillus subtilis*	Phenolic compounds	Chung et al., 2011
11	*Flammulina velutipes*	Polysaccharides	Shi et al., 2012

Chapter 2 Optimization of fermentation conditions for polysaccharides

2.1 Introduction

Research efforts are made to reduce the cost of polysaccharides production and improve the polysaccharides yield. Fermentation was found to be a most attractive way for polysaccharides production. However, the method suffers from the high cost of media, long time and low yield. Large quantities of industrial residues generate every year which result in not only the deterioration of the environment but also the loss of potentially valuable material which can be processed to yield a number of value added products such as food, fuel, feed and a variety of chemicals (Bisaria, 1998). Solid state fermentation offers advantages over liquid cultivation, especially for the fungal cultures, because there is higher productivity per unit volume, reduced energy requirements, lower capital investment, less wastewater production, higher concentrations of metabolites obtained and lower downstream processing cost. Response surface methodology (RSM) is a useful statistical method for optimization of complex reaction process. It can overcome the disadvantages of the traditional "one factor at a time" methodology which can't interpret and analyse the combined influence of the parameters affecting the fermentation efficiency.

So far, there are no literature reports on the polysaccharides from *M. esculenta*, which use SCR as the main nutrient media. In this study, SCR is used as the main substrate in order to reduce the cost of polysaccharides production as well as the pollution brought about by it. The objective of this chapter was to maximize MPS

production by optimizing the fermentation conditions including the culture media, fermentation time, fermentation temperature and inoculum size.

2.2 Materials and methods

2.2.1 Chemicals and reagents

Ethanol, glucose, potato extract, agar, monopotassium phosphate and magnesium sulfate heptahydrate were purchased from Wako Pure Chemical (Osaka, Japan). All the other chemical reagents were of analytical grade.

2.2.2 Pre-treatment of SCR

Fresh SCR (80% of moisture content) was obtained from Inamoto Co., Ltd., Tsukuba, Japan. The fresh SCR was dried at 60°C, powdered and sieved through a NO. 60 mesh. In this study all SCR samples were obtained from the same batch in the manufacturing process.

2.2.3 Strain and culture media

The strain of *M. esculenta (ACCC 50764)* was obtained from the Agricultural Culture Collection of China, Beijing, China. The stock culture was maintained on potato dextrose agar (PDA) slants. The slants were incubated at 25°C for 10 days and then stored at 4°C, and sub-cultured every 3 months. The culture was prepared with distilled water containing (g/L): glucose 20.0, potato extract 4.0, agar 20.0, KH_2PO_4 3.0, and $MgSO_4 \cdot 7H_2O$ 1.5.

2.2.4 Inoculum preparation

For preparation of the inoculum, the mycelia of *M. esculenta* were transferred from a slant into a sterile Petri dish (diameter: 100 mm) containing 20 mL of PDA. It was

incubated at 25°C until mycelium permeated the culture dish. The 100 mL liquid culture was undertaken in a 300 mL flask containing ten units of mycelial agar 5 mm×5 mm square obtained using a self-designed cutter. The submerged cultivation was the same as PDA in the absence of agar. Then it was put in a rotary shaker at 120 rpm and 25°C for 7 days and used as a seed for solid-state fermentation. SCR added different nutrition it was autoclaved at 121°C for 15 min, the solid-state culture experiment was performed in a 200 mL flask. Three replications for all investigated factors were used.

2.2.5 Determination of crude polysaccharides

The fermented SCR from different cultured conditions was harvested and dried in a convection oven at 60°C. MPS was assayed using phenol-sulfuric acid method (Shi et al., 2012). The result was expressed as mg of glucose equivalent per g of fermented SCR.

2.2.6 Experimental design

The ingredients contained in the culture media including glucose, $(NH_4)_2SO_4$, water and $MgSO_4 \cdot 7H_2O$ were optimized to improve MPS yield using orthogonal design. Every factor was matched with three levels and the orthogonal experiments design is shown in Table 2-1. During the culture media optimization process, fermentation temperature of 25°C, fermentation time of 18 days and inoculum size of 4% were used. Meanwhile the Box-Behnken design was applied to investigate the influence of fermentation temperature, fermentation time and inoculum size on the yield of MPS. Levels and codes of variables in the Box-Behnken design are shown in Table 2-2.

2.2.7 Statistical analysis

All experiments were carried out in triplicate. Data were processed and analyzed using Design Expert Software (version 8.0.6, Stat-Ease. Inc., Minneapolis, USA) and Data Processing System (version 7.05 Fujitsu Ltd). P-values below 0.05 were regarded as statistically significant.

2.3 Results and discussion

2.3.1 Culture media optimization

Based on the results of single-factor experiment, glucose, $(NH_4)_2SO_4$, water and $MgSO_4 \cdot 7H_2O$ added in SCR were selected and applied to optimize the culture media composition using orthogonal experimental design. The design of the four-factor-three-level orthogonal experiment and the results are described in Table 2-1. As shown in Table 2-1, all these substrates showed significant influence on MPS content ($P < 0.01$), and the four factors affecting MPS content in a descending order are: water> glucose> $(NH_4)_2SO_4$ >$MgSO_4 \cdot 7H_2O$. The optimal culture media added in SCR was obtained as follows: glucose 4.0%, $(NH_4)_2SO_4$ 1.5%, water 75.0% and $MgSO_4 \cdot 7H_2O$ 0.2%. Using the optimal culture media, the verifying experiment indicated that the yield of MPS was 87.36±1.73 mg/g under the following conditions, fermentation temperature of 25°C, fermentation time 18 days and inoculum size of 4% (w/w).

2.3.2 Optimization of the culture conditions

Response surface methodology was used to establish the relationship between the variables with the obtained responses. According to the single factor analysis, MPS

yield varied depending on the fermentation conditions, including fermentation temperature, inoculum size and fermentation time. The MPS yield was taken as the response value and a Box-Behnken design with factors of the fermentation temperature (X_1), fermentation time (X_2) and inoculum size (X_3) at three levels were considered. The experimental design including name, symbol code, and actual level of the variables are shown in Tables 2-2 and Table 2-3. The test factors were coded according to the following equation (2-1):

$$x_i = \frac{X_i - X_0}{\Delta X_i} \qquad (2\text{-}1)$$

where x_i is the coded value of the ith independent variable, X_i is the uncode value of the ith independent variable, X_0 is the uncoded value of the ith independent variable at the centre point and ΔX_i is the step change value.

According to multiple regression analysis of the experimental data, the independent variables and the dependent variables were related by the following second-order polynomial equation (2-2):

$$Y=-1647.98+26.49X_1+6.97X_2+1026.36X_3+0.14X_1X_2+5.76X_2X_3-0.66X_1^2-0.59X_2^2 -215.55X_3^2 \qquad (2\text{-}2)$$

where Y is the predicted response, namely the polysaccharides yield (mg/g) and X_1, X_2 and X_3 are the uncoded values of the test variables, fermentation temperature (°C), time (days) and inoculum size (%), respectively. The statistical significance of Eq. (2-2) was checked by F test, and the analysis of variance for response surface quadratic model is summarized in Table 2-4. The adjusted determination coefficients (R^2Adj) were measured for testing the goodness-of-fit of the regression equations. The value of

(R^2Adj) for this equation was 0.973 as shown in Table 2-4, indicating a high degree of correlation between the experimental and predicted values.

The 3D response surface plots are employed to determine the interaction of the fermentation conditions and the optimum levels that have the most significant effect on MPS production. The response surfaces plots based on the model are depicted in Fig.2-1, which shows the interactions between two variables by keeping the other variable at zero level for MPS production. It is clear from Fig. 2-1 that the yield of MPS increased and later decreased with the increase in time and temperature. When inoculum size was fixed at 2.60% level, fermentation time and fermentation temperature displayed a quadratic effect on MPS yield. Fig. 2-2 demonstrates the effects of temperature and inoculum size on MPS production. It was observed that the MPS production varied significantly with the variation of temperature. It is evident that MPS production significantly increased with increasing temperature up to about 22°C but decreased sharply beyond this, reaching its maximum yield at 22°C - 23°C. However, the effect of inoculum size to the production of MPS was not sensitive within the tested range. MPS yield increased gradually when inoculums size increased. As seen from Fig. 2-3, the MPS yield was significantly affected by fermentation time. It increased when time increased up to 21 days and decreased sharply beyond this. This observation can be attributed to the autolysis of mycelia as time increases. However, the response of inoculum size was also insensitive compared with time. The optimum ranges of fermentation time and inoculum size for the maximum yield of MPS lies between 20 days - 22 days, and 2.60% - 2.70%, respectively.

By solving the inverse matrix using Design Expert software, the optimum values of the test variables in uncoded units were obtained, i.e. fermentation temperature of 22.6°C, fermentation time of 21 days, and inoculum size of 2.67%, respectively. The predicted optimal MPS production corresponding to these values was 96.10 mg/g. In order to validate the suitability of the model equations for predicting optimum response values, a group of verification experiments were carried out under the optimum conditions obtained above. The triplicate experiments indicated that the MPS yield was 95.82±1.37 mg/g, confirming the good fit of the predicted values to the experimental data and also the validity of the model. As a result, the models developed were considered to be accurate and reliable for predicting the production of MPS using SCR as main nutrient medium.

2.4 Summary

In this study, the effects of culture media and fermentation conditions on the yield of MPS were investigated for the first time. The optimal fermentation conditions achieved for MPS production of 95.82±1.37 mg/g were glucose 4.0%, $(NH_4)_2SO_4$ 1.5%, water 75.0% and $MgSO_4 \cdot 7H_2O$ 0.2%, fermentation temperature 22.6°C, fermentation time 21 days, and inoculum size 2.67%. The results will provide references for the large-scale production of polysaccharides by *M.esculenta* and point to a new direction for the utilization of SCR.

Table 2- 1 Results of orthogonal experiments for media optimization

NO.	Glucose (%)	$(NH_4)_2SO_4$ (%)	Water (%)	$MgSO_4 \cdot 7H_2O$ (%)	MPS content (mg/g)
1	2	0.05	65	0.1	64.94±2.05
2	2	0.10	70	0.2	76.48±1.28
3	2	0.15	75	0.3	83.64±0.71
4	4	0.05	70	0.3	78.30±1.74
5	4	0.10	75	0.1	88.99±0.69
6	4	0.15	65	0.2	81.84±1.59
7	6	0.05	75	0.2	84.89±1.08
8	6	0.10	65	0.3	76.42±0.87
9	6	0.15	70	0.1	83.00±0.56
K_1	225.06	228.13	223.20	236.93	
K_2	249.13	241.88	237.78	243.21	
K_3	244.31	248.48	257.52	238.36	
R	24.07	20.35	34.33	6.28	
P	0.0001^{**}	0.0001^{**}	0.0001^{**}	0.0032^{**}	

** indicates statistically significant difference ($P<0.01$).

Table 2- 2 Levels and codes of variables in the Box-Behnken design

Variables	Symbol		Coded and uncode levels		
	Uncoded	Coded	-1	0	1
Fermentation temperature (°C)	X_1	x_1	19	22	25
Fermentation time (d)	X_2	x_2	18	21	24
Inoculum size (%)	X_3	x_3	2.5	2.6	2.7

Table 2- 3 Experimental and predicted values of polysaccharides based on Box-Behnken design

Runs	X_1: Fermentation temperature (°C)	X_2: Fermentation time (d)	X_3: Inoculum size (%)	Polysaccharides content (mg/g)	
				Experimental	Predict
1	22	18	2.5	86.55±0.79	85.76
2	19	24	2.6	82.19±1.50	81.31
3	22	21	2.6	94.23±0.66	94.91
4	22	24	2.5	83.28±1.04	83.81
5	25	18	2.6	82.66±1.99	83.54
6	25	24	2.6	88.03±0.68	87.59
7	22	24	2.7	91.79±0.68	92.58
8	22	18	2.7	88.14±0.79	87.61
9	22	21	2.6	95.23±0.57	94.91
10	22	21	2.6	94.99±1.65	94.91
11	19	21	2.5	82.43±1.76	82.34
12	25	21	2.5	85.72±0.29	86.07
13	22	21	2.6	94.57±2.13	94.91
14	19	21	2.7	87.12±0.29	87.65
15	19	18	2.6	81.90±2.41	82.35
16	22	21	2.6	95.54±1.42	94.91
17	25	21	2.7	92.17±2.00	91.38

The experimental results were means ± standard deviation (SD) of triple determinations.

Table 2- 4 The result of analysis of variance (ANOVA) for the selected model

Source	Sum of squares	Degree of freedom	Mean square	F- value	Probability＞F
Model	420.11	8	52.51	71.99	< 0.0001
X_1	27.87	1	27.87	38.20	0.0003
X_2	4.57	1	4.57	6.26	0.0368
X_3	56.38	1	56.38	77.29	< 0.0001
X_1X_2	6.46	1	6.46	8.86	0.0177
X_2X_3	11.96	1	11.96	16.39	0.0037
X_1^2	146.46	1	146.46	200.78	< 0.0001
X_2^2	119.04	1	119.04	163.19	< 0.0001
X_3^2	19.56	1	19.56	26.82	0.0008
Residual	5.84	8	0.73		
Lack of fit	4.76	4	1.19	4.41	0.0899
Pure error	1.08	4	0.27		
Corrected total	425.94	16			
R=0.9863	R^2Adj= 0.973				

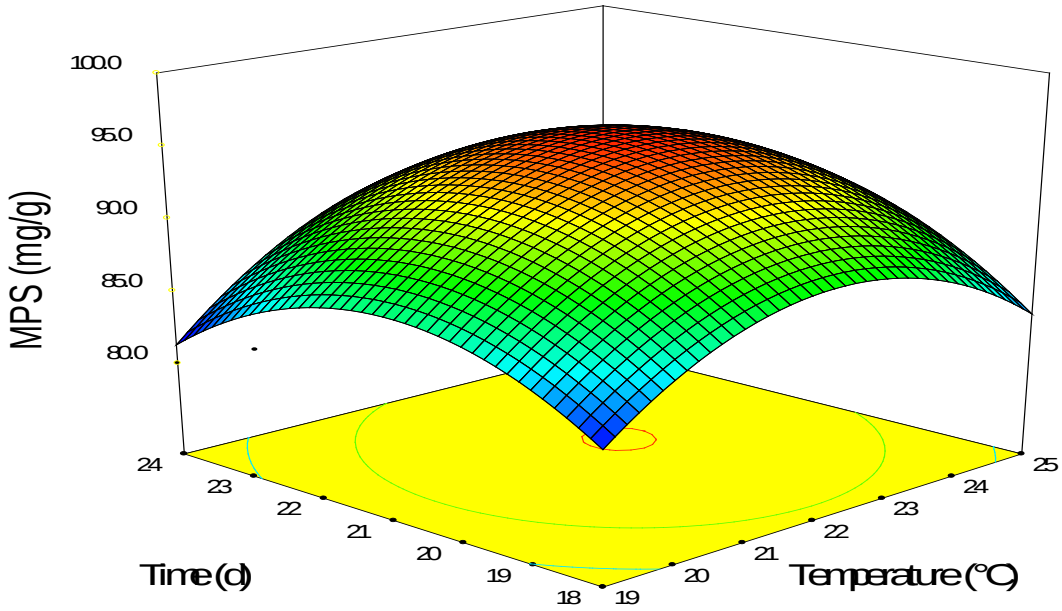

Fig. 2- 1 Response surface plot for the MPS yield in terms of the effects of time and temperature.

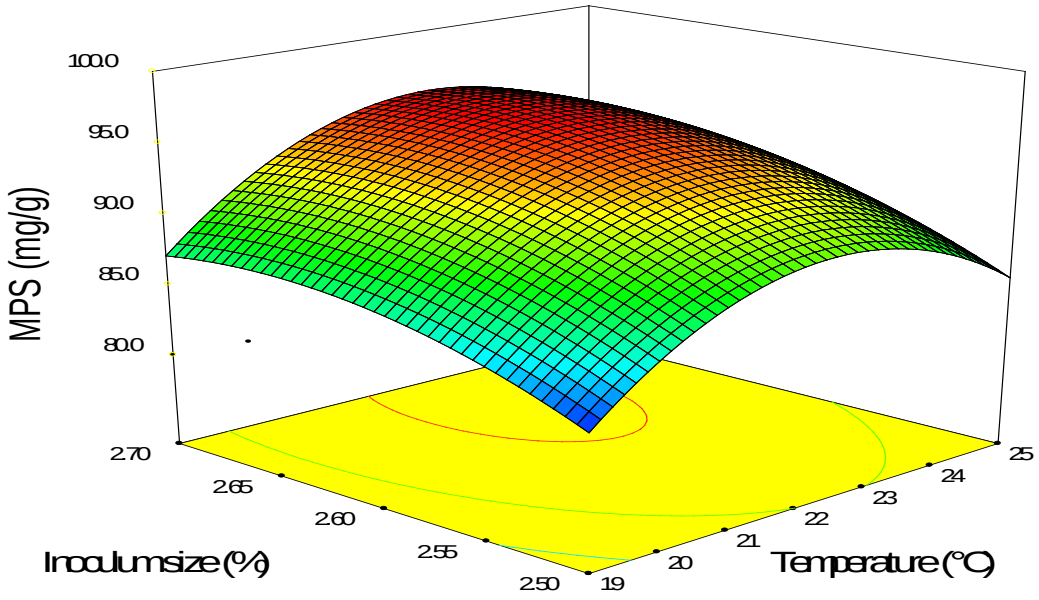

Fig. 2- 2 Response surface plot for the MPS yield in terms of the effects of temperature and inoculum size.

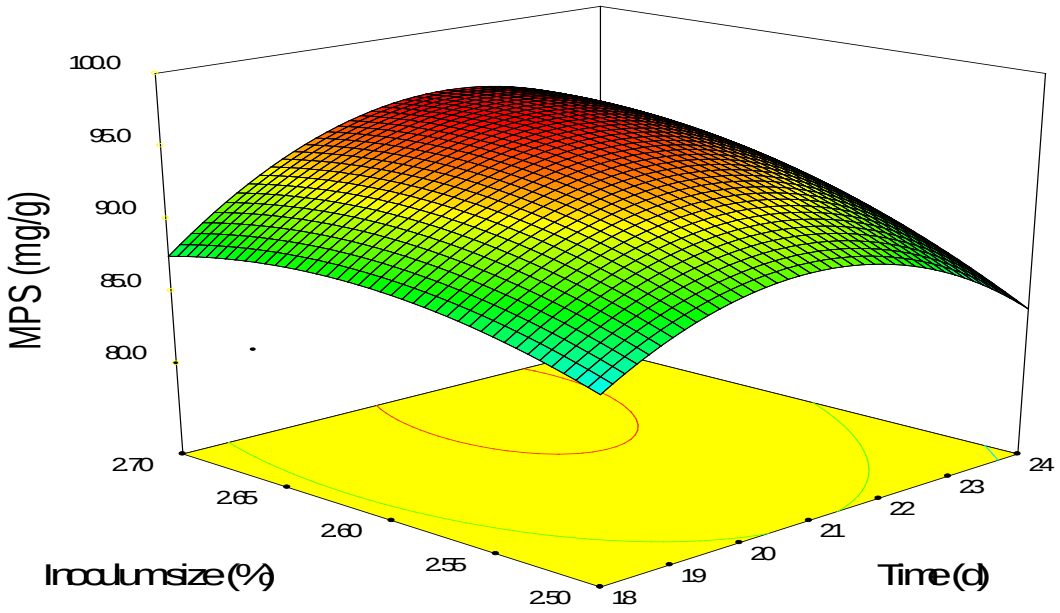

Fig. 2- 3 Response surface plot for the MPS yield in terms of the effects of inoculum size and time.

Chapter 3 Effect of fermentation on the physicochemical properties of soybean curd residue

3.1 Introduction

The food industry is experiencing a growing demand for more new ingredients from natural sources. This demand has therefore drawn researchers to these ingredients obtained from agricultural and industrial waste. At least in part due to their nutritional relevance, efforts have been made to modify physicochemical properties of various insoluble dietary fibers using different techniques, of which fermentation techniques are probably most noteworthy. For an efficient utilization and consumer acceptance of the fermented SCR, a study of their physicochemical properties is necessary.

To the best of our knowledge, very little information has been reported on the influences of fermentation on the physicochemical properties of SCR. Therefore, the main objective of this study is to evaluate the physicochemical properties of SCR before and after fermentation by *M. esculenta* so as to identify the functional compounds of SCR. Furthermore, the physicochemical properties are studied in order to determine the potential of SCR as a source of functional ingredients and provide a theoretical foundation for its application.

3.2 Materials and methods

3.2.1 Chemicals and reagents

Ethanol, glucose, potato extract, agar, sodium hydroxide, hydrochloric acid, trichloroacetic acid, Protein Quantification Kit-Rapid and Folin-Ciocalteau phenol

reagent were purchased from Wako Pure Chemical (Osaka, Japan). Sulfuric acid, gallic acid and sodium chloride were purchased from Sigma Aldrich, Inc. (Saint Louis, MO, USA). All the other chemical reagents were of analytical grade.

3.2.2 Pre-treatment of SCR

Fresh SCR (80% of moisture content) was obtained from Inamoto Co., Ltd., Tsukuba, Japan. The fresh SCR was dried at 60°C, powdered and sieved through a NO. 60 mesh. Then the SCR was fermented according to conditions obtained in Chapter 2. After fermentation, the SCR was dried at 60°C, powdered and sieved through a NO. 60 mesh for analysis. In this study all SCR samples were obtained from the same batch in the manufacturing process.

3.2.3 Strain and culture media

The strain of *M. esculenta (ACCC 50764)* used in this study was obtained from the Agricultural Culture Collection of China (Beijing, China). The stock culture was maintained on potato dextrose agar (PDA) slants and sub-cultured every three months.

3.2.4 Physical properties

(1) Morphological analysis

Scanning electron micrographs (SEM) of the unfermented soybean curd residue (USCR) and fermented soybean curd residue (FSCR) were obtained with a scanning electron microscope (JSM-6330F, JEOL, Tokyo, Japan). Each sample was observed with magnification of 1000 fold at an accelerating potential of 5.0 kV during micrography.

(2) Powder hygroscopicity

Hygroscopicity was determined according to the method proposed by Cai and Corke (2000) with some modifications. Samples of each powder (approximately 1.0 g) were placed at 25°C in a container with a NaCl saturated solution (75.29% RH). After one week, samples were weighed with hygroscopicity expressed as g of adsorbed moisture per 100 g dry solids (g/100 g).

(3) Thermal analysis

Thermal analysis of SCR samples was examined using a TG/DTA 7300 thermal analyzer under nitrogen atmosphere with a flow rate of 20 mL/min (Seiko Instruments Inc. Chiba, Japan), according to the procedure of Tian et al. (2011) with some modifications. An aluminium volatile pan is a sample pan used with volatile solid or liquid samples which exert significant vapor pressure at the temperature of interest. Sample weights ranged from 5 to 10 mg and were subjected to the following temperature program: the sample was placed in an aluminium volatile pan and the heating temperature was increased from 40 to 300°C at a rate of 10°C /min, held for 5 min. An empty pan was used as a reference.

(4) Color determination

Unfermented soybean curd residue (USCR) and fermented soybean curd residue (FSCR) were placed on glass petri dish and L*, a*, and b* parameters were determined by color difference meter (NR 11, Nippon Denshoku, Tokyo, Japan). The L*, a*, and b* values are the three dimensions of the measured color giving the specific color value of the test material. Lightness value, L*, indicates how dark/light the sample is (varying from 0: black to 100: white), a* is a measure of

greenness/redness (varying from -60 to +60) and b* is the grade of blueness/yellowness (also varying from −60 to +60). The colorimeter was calibrated against a standard white plate and a standard black plate before carrying out color measurements. ΔE was defined as the color difference between sample and standard plate and it was calculated as indicated in Eq. (3-1) (Falade and Okafor, 2013):

$$\Delta E=\sqrt{(\Delta L)^2+(\Delta a)^2+(\Delta b)^2} \quad (3\text{-}1)$$

3.2.5 Chemical properties

(1) Proximate composition

Ash, fat and fibre contents were determined by AOAC methods (1995). The protein content of the SCR was determined using the Protein Quantification Kit-Rapid. Briefly, 200 mg of SCR was mixed with 4 mL of phosphate buffer (pH 7.6) and homogenized with a pulp refiner for 2 min. After 10 min, 6 μL of supernatant and 300 μL of Coomassie Brilliant Blue (CBB) were added into a 96-well plate separately, then the optical density (OD) of the mixture was read at 595 nm and the protein content was calculated using the albumin solution from bovine serum as the standard. The results were expressed as mg of protein per g of SCR. Total carbohydrates excluding crude fiber were calculated by difference.

(2) Free amino acids (FAA) analyses

The SCR was extracted by using 80% ethanol in an 80°C water bath for 20 min. The supernatant was recovered and the previous steps were repeated twice. After washing the solid matter with 80% ethanol, all the recovered supernatant was filtered and centrifuged. Next the supernatant was dried in an oven at 40°C and dissolved

again in distilled water incubated at 4°C for 12 h. The solution was mixed with trichloroacetic acid at the ratio of 4:1 and incubated at 4°C for 10 min followed by centrifugation to remove the protein sediment. The pH value of the mixture was adjusted to 2-3 using NaOH and HCl (1 M), then the sample was filtered through a 0.45 μm diameter filter and determined by an auto amino acid analyzer (JLC-500/V2, Jeol Ltd., Tokyo, Japan).

(3) Polysaccharides and total polyphenol

Polysaccharides content was determined according to the method of phenol-sulfuric acid. Total polyphenol content was measured using the modified Folin–Ciocalteau method reported by Quettier-Deleu, et al. (2000) with minor modification. 200 mg sample was mixed with volume of 7.5 mL (80% V/ V) ethanol and put in a rotary shaker at 100 rpm at 25°C for 24 h. Then, the supernatant was collected by centrifugation at 8000×g for 20 min. The supernatant (0.125 mL) was mixed with distilled water (0.375 mL), 0.50 mL of the Folin–Ciocalteu reagent, respectively. After 3 minutes later, 0.5 mL Na_2CO_3 (20%) was added, and the mixture was made up to 5 mL with distilled water. After being kept in the dark for 90 min, the OD of the mixture was read at 725 nm. The quantification was determined based on a standard curve of gallic acid (50-250 μg/mL). Results were expressed as mg of gallic acid.

(4) Fourier-transform infrared spectrum analysis

FTIR spectrum was recorded on a Jasco FTIR 3000 spectrometer (Jasco,Wakayama, Japan). The dried sample was ground with potassium bromide (KBr) powder and

pressed into pellets for spectrometric measurement at a frequency range of 4000-400 cm^{-1}.

(5) Determination of pH and titratable acidity (TA)

Determination of pH was according to the procedure of Xu et al. (2010). Each 5.0 g sample was homogenized with 95 mL of boiled distilled water for 30 min. The homogenate was filtered through a filter paper and the filtrate was measured by a micro-pH meter (Mettler Toledo FE20, Tokyo, Japan). TA was determined according to the method of AOAC (1998). The homogenate was filtered through a filter paper (Whatman No. 4) and the filtrate was titrated with standard 0.1 M NaOH, using 1.0% phenolphthalein as an indicator. The TA in the sample was expressed as % lactic acid.

3.2.6 Statistical analysis

All experiments and measurements were replicated three times. Statistical analysis of data was performed by one-way ANOVA followed by a Duncan's test. Differences were considered statistically significant at $P < 0.05$. All statistical analyses were performed using SPSS 11.5 for Windows.

3.3 Results and discussion

3.3.1 Morphological properties

The SEM was used to study the morphological structure change of SCR before and after fermentation with *M. esculenta*. Fig.3-1 shows the microscopy photographs of the microstructure of the USCR and FSCR, respectively. SEM micrograph (Fig. 3-1 a) of USCR shows that it is composed of aggregates of fibroid, rough and loose shape with inhomogeneous bores. This observation indicates that the SCR is suitable for the

fungi to ferment because of its loose texture. The surface of FSCR is illustrated in Fig. 3-1 b. For the image at 1,000 fold augmentation, the surface of FSCR was an irregular micro-squama texture which showed significant variations in size and shape compared with USCR. The porosity structure of FSCR may increase specific surface area, which can enhance extraction yield of bioactive substances such as polysaccharides and polyphenol. Moreover, porous material has long been used as insulator, structural material, catalyst carrier and absorbent. The morphological characteristic of difference between USCR and FSCR provides solid foundation for its application. The differences can be attributable to the changes in material, intermolecular distance and internal connection caused by fermentation.

3.3.2 Powder hygroscopicity

The hygroscopicity of a powder is its equilibrium moisture content after being exposed to air humidity under given conditions. It reflects the ability to attract and hold water from the surrounding environment. Materials and compounds exhibit different hygroscopic property, and these differences are key factors used to determine their application areas, actual effect and storage method. The hygroscopicity of USCR and FSCR are 108.60±0.30 and 112.73±0.26 g water/100 g solids. According to Cai and Corke (2000), as the molecular weight decreased, the hygroscopicity increased. The FSCR was more hygroscopic than the USCR. This difference can be attributed to that SCR was degraded and transformed into low molecule weight matter during fermentation, making it more hygroscopic.

3.3.3 Thermal analysis

A similar trend in the range of 40-300°C could be observed after investigating the TG of USCR and FSCR (Fig. 3-2). From the TG curve, it can be seen that USCR and FSCR were characterized by their high thermal stability and did not begin to decompose until 190 and 210°C, respectively. The TG curve also indicated that the decomposition process can be divided into two stages. The first stage occurred at 40-120°C. The weight loss in percentage of USCR and FSCR were 5.9% and 4.8%, respectively, which was mainly contributed by water loss and volatile constituents. The second stage was 120-300°C, in which the SCR at first exhibited a slow weight loss and then declined sharply. The total weight loss of USCR and FSCR at 300°C was about 38.5% and 30.9%. The lower total weight loss of FSCR than that of USCR shows that FSCR has higher heat stability after fermentation.

Differential thermal analysis (TDA) is widely used in the pharmaceutical and food industries (Rey et al., 1988; Mano et al., 2003). Fig. 3-3 shows the DTA curves of USCR and FSCR. Two main peaks could be identified between 40-300°C. It is very interesting that FSCR has an exothermic peak at 44.5°C, while USCR has an endothermic peak at 55.0°C. The heat of vaporization of the free water is the dominant factor in determining the endothermic peak on the DTA curves of USCR. The exothermic peak of FSCR could attribute to hygroscopicity and antioxidant activity brought about by such ingredients as polysaccharides and polyphenol. They will absorb moisture or take an oxidation-reduction reaction with oxygen in the air, which are exothermic reactions. The second peaks of USCR and FSCR were observed at

270.5°C and 273.5°C, which were considered to be the result of the thermal degradation of polysaccharides, decarboxylation of acidic groups and dehydration of hydroxylate in aliphatic structures (Leinweber and Schulten, 1992; Dell'Abate et al., 2002). The higher decomposition temperature of FSCR again means its stability under high temperature conditions.

3.3.4 Color

Color is one of the most important attributes of food materials, since it influences consumer acceptability (Maskan, 2001). Besides consumer acceptability, color is also used for controlling the process and application. In this study, CIE L*, a* and b* parameters of USCR and FSCR were compared (Fig.3-4). It can be seen from this figure that the L*- values significantly decreased from the initial value of 87.06 to the final value of 57.91 after fermentation ($P < 0.05$). This was because of the pigments accumulation of *M. esculenta* thus resulting in reflection of light. The value of a* increased from the initial of -0.78 to the final of 6.69. This change was probably attributed to the formation of some new and stable pigment resistant not only to the polyphenol addition but also to pH changes and oxidation (Asenstorfer et al., 2001). The b*-value shows the degree of yellowness. A similar trend in a*-value was observed for the changes of b*- value during fermentation. The b*-value increased from the initial 19.65 to the final 29.61. This showed that the FSCR was dark-colored, with more red and more yellow; it contained more red and yellow pigments than USCR. Total color difference (ΔE) calculated by Eq. (3-1) is one of the best values for explaining the color change because it is a combination of L*, a* and b* values.

The total color difference value increased and the total color difference value recorded for FSCR was 25.8 lower than USCR. The results indicated that fermentation by *M. esculenta* caused a significant change in the color of SCR.

3.3.5 Proximate composition

The results of the proximate compositions of USCR and FSCR are shown in Table 3-1. The obtained data showed that protein and carbohydrate were significantly higher in the FSCR than USCR. On the other hand, fermentation significantly decreased the crude fat and fibre in SCR. The ash contents were not significantly different between the USCR and the FSCR. This finding in the present study is in agreement with those Sindhu, Khetarpaul (2005) and Arora et al. (2010) who observed no change in the ash contents of cereal-legume food blends occurred after single and sequential culture fermentation. The high level of crude protein (28.12 ±1.34%) of the FSCR sample makes the fermented SCR a good source of protein resulted from fermentation. The fermentation process has been used to create new proteins as reported by Obeta (1983), which probably explains the increase in protein during fermentation in this sutdy. By the end of fermentation, the contents of crude fat and fibre reduced by 6.47% and 5.81%, respectively. Studies have shown that they can be degraded and transformed into polysaccharides or short-chain fatty acids and small molecular substances (Bourquin et al., 1993; Guillon et al., 1995).

3.3.6 Free amino acids analysis

FAA is important in nutrition because of their biological significance. They are commonly used in nutritional supplements and food technology. In order to

investigate the effect of fermentation on FAA, the concentrations of FAA in the SCR before and after fermentation were detected. The contents of FAA in USCR and FSCR are shown in Table 3-2. After fermentation, 6 of the 8 essential amino acids increased while lysine decreased. Phenylalanine wasn't detected in USCR and FSCR. The content of methionine and isoleucine increased by 35.70 times and 4.66 times respectively. Moreover, most of the no essential FAA increased except for histidine and glutamic acid. Among them, the taurine content increased 19.54 times, compared with that in USCR. Taurine has many fundamental biological roles such as conjugation of bile acids, antioxidation, osmoregulation, membrane stabilization and modulation of calcium signaling (Huxtable, 1992; Sturman, 1993). The content of 20 free amino acids in FSCR was 5060.25±3.80 nmol/g representing an increase of more than 2-folds. The increase of FAA in the fermented product could be attributed to protein hydrolysis and the increased metabolic activities of *M. esculenta*. The results obtained in this study agree with the observation of Yong and Wood (1977) who found an increase in amino acids level during the fermentation of soybeans to produce "koji".

3.3.7 Polysaccharides and total polyphenol

Polysaccharides from fruiting bodies, cultured mycelium or culture broths have potential antitumor and immunomodulating properties (Ooi and Liu, 2000; Wasser, 2002; Masuda, et al., 2009). The polysaccharides in SCR increased from 25.22±2.23 to 95.82±1.37 mg/g after fermentation. In addition, its health-protection capacity has developed a great interest in the study on polyphenols. A number of traditional plant-

derived folk medicines are rich in polyphenol (Pandey and Rizvi, 2009). It has antioxidant properties that can help prevent cancer and age-related diseases (Gursoy et al., 2009). The polyphenols in USCR and FSCR are 5.99±0.27 and 7.70±0.18 mg/g respectively.

3.3.8 Fourier-transform infrared spectrometric analysis

Fourier transform infrared spectra of USCR and FSCR are shown in Fig. 3-5. The results of functional group analysis revealed the existence of various characteristic functional groups. As shown in Fig. 3-5, the FTIR spectra of FSCR and USCR were similar and varied only in the relative intensity of absorption bands. The FTIR spectra of USCR displayed a number of absorption peaks, reflecting the complex properties of raw SCR, which is consistent with Gao et al. (2011). There were three new bands of FSCR, the band at 1456 cm^{-1} attributed to aliphatic C-H deformation, 1364 cm^{-1} was due to CH_3– group symmetric bending vibrations, and the band at 616 cm^{-1} could be attributed to the asymmetric and symmetric O＝S＝O deformation of sulfates (Sekkal and Legrand, 1993). Aliphatic C-H deformation peak at 1456 cm^{-1} showed an aliphatic structure, the most probably aromatic compounds. The fermenting process is reported to be able to enhance nutritional value and digestibility while releasing delicious new flavors and aromas (Nout and Kiers, 2005). Meanwhile, an identifiable peak near 1364 cm^{-1} in the FSCR spectrum might indicate the production of organic acids (Guo et al., 2012). According to Pielesz and Biniaś (2010), the peaks at the band of 616 cm^{-1} in the FTIR spectra were characterized by small changes in the molecular structure of oligosaccharide. The fibre in the SCR might have been degraded to

oligosaccharides by *M. esculenta* during the fermentation. Production of low molecular polysaccharides by fungi is a universal phenomenon and there might be synergic effects of the polysaccharides during native cellulose degradation.

3.3.9 pH and TA

The change in pH is a reflection of fermentation (Hu et al., 2013). The initial pH value was about 6.99±0.02 in USCR and it decreased sharply to 4.28±0.01 after fermentation. In parallel with this decrease in pH was an increase in TA. From an initial value of about 0.35±0.06% the TA rose to greater than 2.73±0.09% after fermentation. This can be explained by the rapid growth of *M. esculenta*, resulting in a rapid production of organic acids, such as lactic acid and amino acid mentioned above, which also contributed to the nutrient supply for fungus and the inhibition of undesirable microorganisms (Xu et al., 2009; Cizeikiene et al., 2013). Based on the reports, rapid growth of CIELAB can cause pH to decrease below 4.5 within 2 days, which is essential to prevent spoilage and to ensure safety of the product (Adams et al., 1987). The low pH may offer high protection against oxidative stress, relatively good shelf life.

3.4 Summary

The influence of fermentation by *M. esculenta* on the physicochemical properties of SCR was investigated in this study. Fermentation led to morphological change and higher powder hygroscopicity and decomposition temperature. Results also indicated that FSCR showed some advantages over USCR owing to its higher nutrient composition such as free amino acids, polysaccharides and polyphenols, which maybe

of nutritional importance during the application of FSCR. SCR fermented by *M. esculenta* is a good choice for reuse of SCR and could be utilized to produce antioxidants for food, pharmaceutical and cosmetics.

Table 3- 1 Proximate analysis of USCR and FSCR (% by mass, dry basis)

Chemical parameters	Protein	Crude fat	Carbohydrate	Fibre	Ash
USCR	24.93±2.07	12.77±1.35	43.53±1.16	14.79±1.23	3.98±0.72
FSCR	28.12±1.34	6.30±1.26	52.31±0.98	8.98±1.06	4.29±1.08

Table 3- 2 Free amino acid in USCR and FSCR

Free amino acid (nmol/g)	USCR	FSCR
Methionine*	26.28±0.44	938.32±32.56
Isoleucine*	59.16±1.00	275.60±3.60
Threonine*	56.00±2.16	160.68±4.20
Leucine*	56.84±0.36	113.76±0.00
Valine*	342.80±6.24	403.6±5.28
Tryptophane*	185.12±6.32	244.36±1.32
Lysine*	23.48±0.68	11.73±1.17
Phenylalanine*	0	0
Taurine	31.16±1.08	608.96±0.80
Serine	47.76±0.08	239.32±2.36
Cysteine	2.48±0.00	10.32±0.08
Glycine	83.32±0.60	145.32±1.96
Alanine	313.16±3.80	826.52±11.40
Proline	111.08±0.60	187.40±0.12
Asparagine	70.84±1.00	110.161+0.32
Glutamine	54.76±1.56	89.40±2.04
Arginine	224.28±3.80	248.8±1.36
Tyrosine	126.84±4.28	340.76±0.20
Histidine	34.64±1.76	8.28±0.12
Glutamic acid	493.32±10.44	96.96±3.28
Total amino acid	2343.32±2.43	5060.25±3.80

*Essential amino acid (EAA).

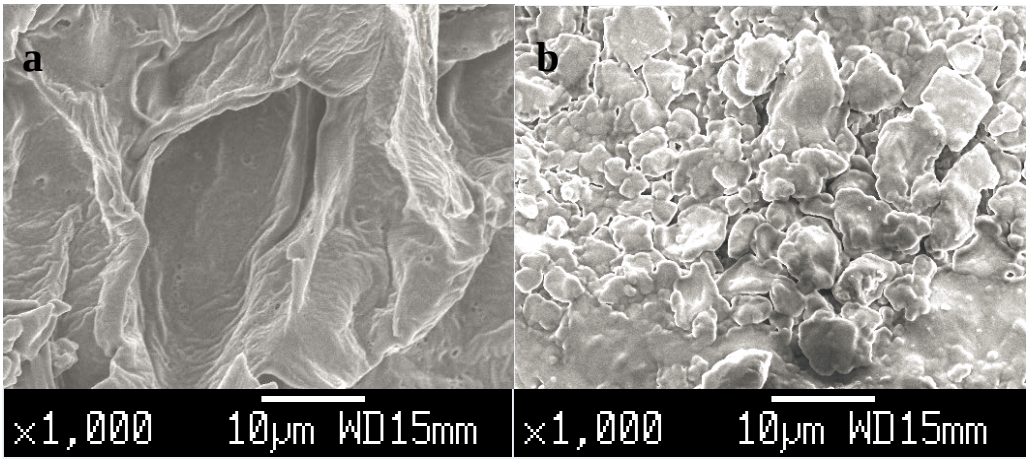

Fig. 3- 1 Scanning electron micrograph (SEM) images of USCR (a), FSCR (b)

at magnification of 1000× and 5 kV. Bar = 10 μm.

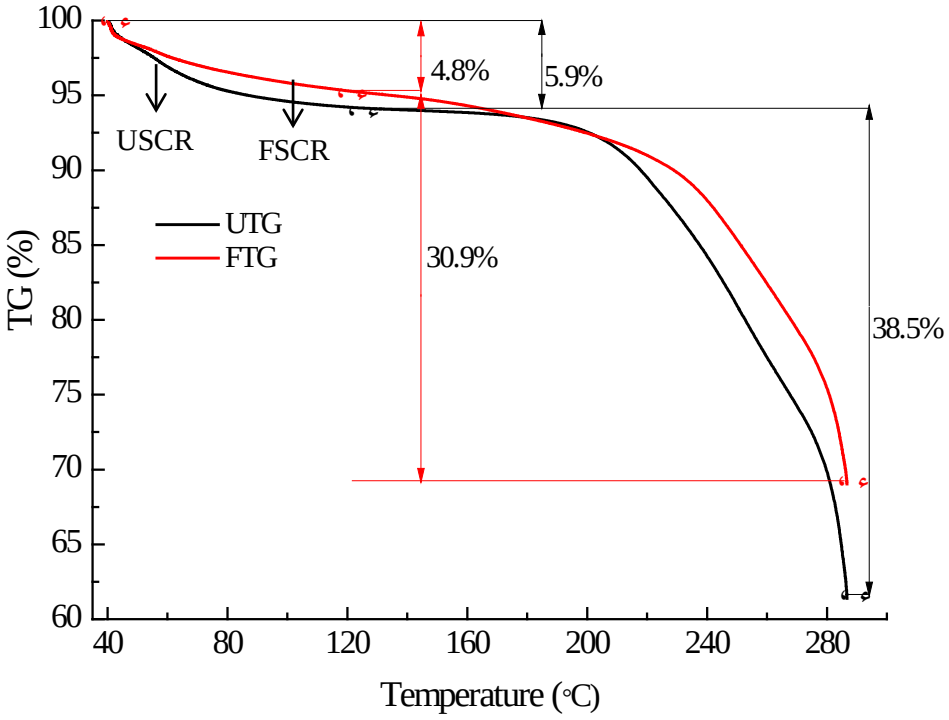

Fig. 3- 2 TG curves of USCR (UTG) and FSCR (FTG).

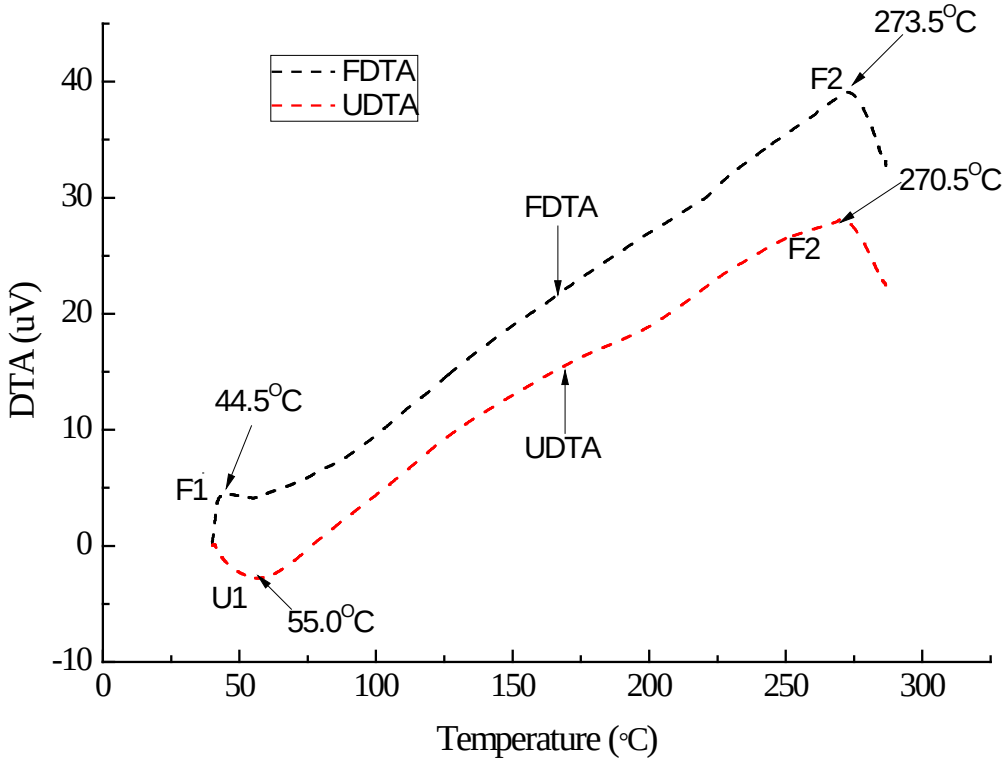

Fig. 3- 3 DTA curves of USCR (UDTA) and FSCR (FDTA).

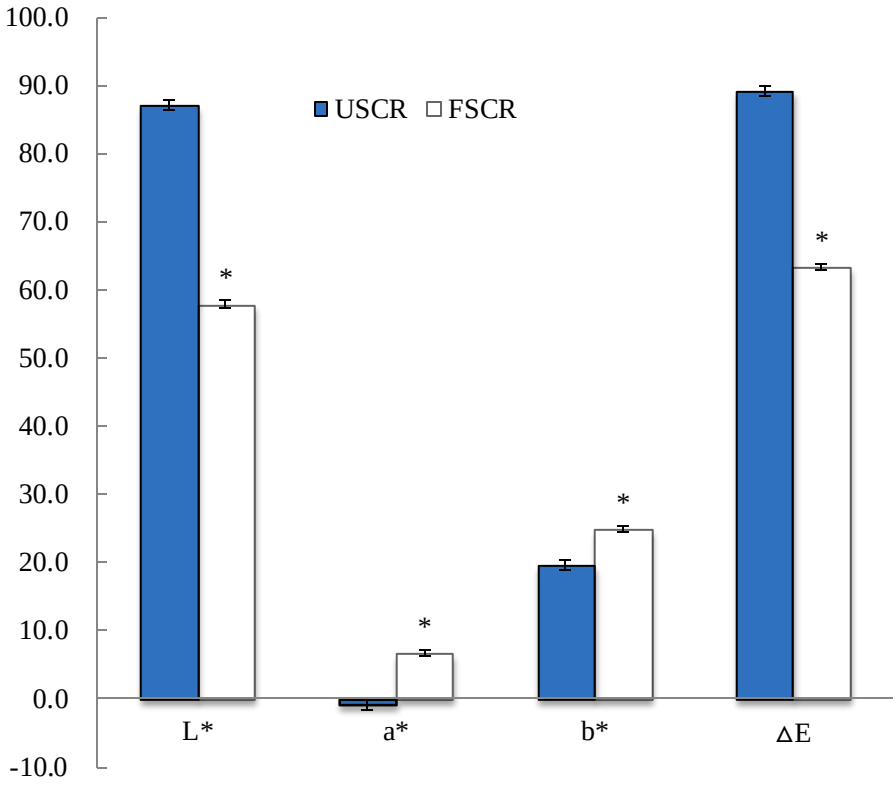

Fig. 3- 4 CIELab coordinates (L*; a*; b*, ΔE) of USCR and FSCR.

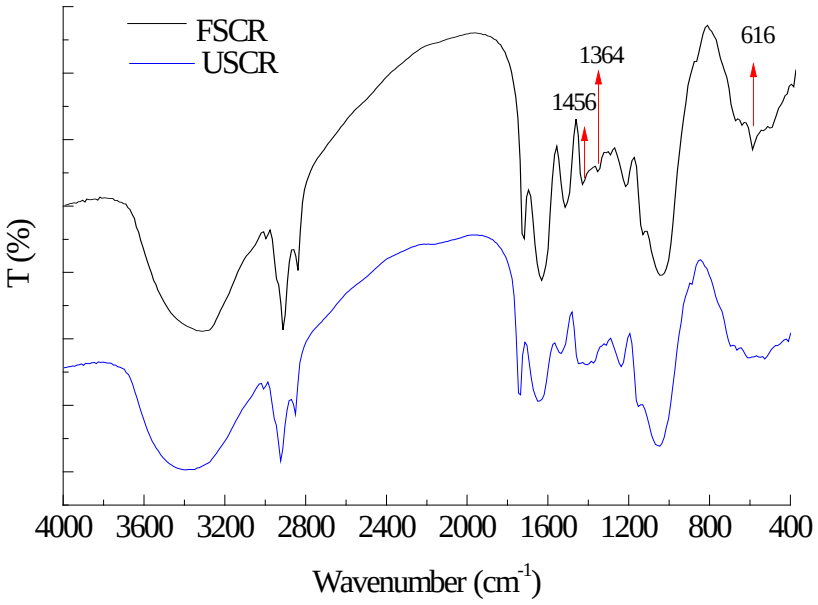

Fig. 3- 5 FTIR spectra of USCR and FSCR.

Chapter 4 Characteristics, antioxidant activity of polysaccharides

4.1 Introduction

Much of attention has been paid to polysaccharides due to their unique bioactivities and chemical structures in recent years (Schepetkin and Quinn, 2006; Chen et al., 2008). Moreover, polysaccharides are biological macromolecules and they have immunomodulatory, antitumor, anti-inflammatory, and anti-fatigue effects, which are related to the antioxidant properties of polysaccharides (Chen et al., 2012). Reactive oxygen species are generated endogenously through normal metabolic activity and they can react with critical cellular biomolecules such as lipids, proteins and DNA and initiate events that lead to increased risk of chronic disease (Zhou and Chen, 2011). In order to improve human health condition, synthetic antioxidants are used for industrial processing at present (Xu et al., 2009). However, the presence of unwanted side effects is believed to be unavoidable, and those most generally observed have been suspected of being responsible for liver damage and other diseases (Qi et al., 2005). Therefore, a basic understanding of both the characteristics for the polysaccharides is essential for successful interpretation of their bioactivities.

There is also very little information on the physicochemical parameters, and bioactivities of the polysaccharides. To address the relationship between the characteristics and the antioxidant activity, a comparison was made between two polysaccharides named PS and MPS which were extracted from unfermented soybean curd residue (USCR) and fermented soybean curd residue (FSCR). Morphological

change, hygroscopicity, fourier transform infrared spectroscopy (FTIR), UV spectra analysis and monosaccharide composition were evaluated. Moreover, in vitro antioxidant activities for two polysaccharides were compared.

4.2 Materials and methods

4.2.1 Chemicals and reagents

Ethanol, Na_2CO_3, phenol, sulphuric acid, ascorbic acid, hydrogen peroxide, chloride ferric, potassium ferricyanide, ferrous sulfate, potassium persulphate, trichloracetic acid, sodium salicylate and ethylene diamine tetra acetic acid (EDTA) were obtained from Wako Pure Chemical, Osaka, Japan. SOD Assay Kit-WST was purchased from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan). Gallic acid, 2, 2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 2,2-diphenyl-1-picry-hydrazyl radical (DPPH) were purchased from Sigma Aldrich, Inc. (Saint Louis, MO, USA). All the other chemical reagents were of analytical grade.

4.2.2 Pre-treatment of SCR

The pre-treatment of SCR was the same with 3.2.2.

4.2.3 Strain and culture media

The strain and culture media were the same with 3.2.3.

4.2.4 Extraction and purification of polysaccharides

In order to compare the antioxidant activity of polysaccharides before and after fermentation, the polysaccharides from unfermented SCR (PS) and fermented SCR (MPS) were extracted according to Meng et al. (2010) with some modification. Briefly, unfermented and fermented SCR were ground in a sample mill to pass

through NO. 60 mesh after oven drying for 4 days at 60°C. The powdered material was refluxed in 80% ethanol for 6 h to remove some colored substance, monosaccharide, oligosaccharides, and small molecule materials (Peng et al., 2012). Then the cooled extract was discarded and the residue was washed with 95% ethanol, anhydrous ethyl alcohol, acetone and diethyl ether respectively. The residue was dried at room temperature for 24 h prior to extraction. Subsequently, the extraction was carried out using boiling water for 2 h. After that, the syrup was centrifuged at 7500×g for 15 min and the residue was re-extracted under the same conditions. The combined supernatant fluids were concentrated to minimum volume using a rotary evaporator at 60°C under low pressure. The protein in the concentrated solution was removed by Sevag reagent (chloroform and n-butanol in 4:1 ratio) (Staub, 1965). The extract was dialyzed by deionized water for 72 h. To obtain the polysaccharides, the extract was precipitated with 4 volumes of anhydrous ethanol at 4°C for overnight and the precipitation was centrifuged at 7500×g for 15 min. The precipitate was dissolved in distilled water, collected, frozen and freeze-dried, then the obtained MPS and PS were used to study the antioxidant activities.

4.2.5 Physical properties

Physical properties including morphological analysis, powder hygroscopicity and color determination of crude polysaccharides were studied. The methods were the same as 3.2.4.

4.2.6 Chemical characteristics

(1) Spectra analysis of polysaccharides

The FTIR spectra (4000–400 cm^{-1}) of the polysaccharides fractions were determined by a Jasco FTIR 3000 spectrophotometer (Jasco, Wakayama, Japan). Polysaccharides solution (0.2 mg/mL, dissolved in ultrapure water) was scanned on a spectrophotometer UV-1800 (Shimadzu, Tokyo, Japan) from 200 nm to 400 nm. Ultrapure water was used as solvent and control.

(2) Monosaccharide composition analysis

The crude polysaccharides (10 mg) and 50 nmol of inositol (as an internal standard), dissolved in 2 M trifluoroacetic acid (TFA, 2 mL), was hydrolyzed at 120°C for 3 h in a sealed glass tube on the mechanism of acid-catalyzed hydrolysis (Yu et al., 2009). The hydrolyzate was repeatedly co-concentrated with methanol to remove the excess acid at 50°C, and then the hydrolyzed products were prepared for acetylation. The acetylation was carried out with 10 mg hydroxylamine hydrochloride and 0.5 mL pyridine by heating in a water bath (90°C) for 30 min. After incubation, the tubes were removed from the heat block, allowed to cool to room temperature, and then 0.5 mL of acetic anhydride was added and mixed thoroughly by vortexing. The tubes were sealed and incubated in a water bath shaker set at 90°C for 30 min again. After cooling, approximately 0.1 mL of clear supernatant was added to the auto sampler vials with inserts for injection into the gas chromatograph on a GCMS-QP2010 Plus (Shimadzu, Japan) instrument equipped with a hydrogen flame ionization detector, using a DB-5 column (30 m × 0.25 mm × 0.25 mm). The following chromatographic conditions were used: high-purity helium was used as the carrier gas at a flow rate of 1 mL/min. The temperature of the injector and detector was 250°C. An initial column

temperature held at 60°C for 2 min followed by 15°C /min to 180°C and then 4°C /min to 250°C for 3 min (Chen et al., 2008). Injections were made in the splitless mode. The temperature of mass spectrometer ion source was 250°C. 1 μL sample was injected into the column with the split ratio of 10:1. Peaks of neutral monosaccharide in the MPS were identified by comparison of retention time and mass spectral fragmentation patterns with monosaccharide standards. The amount of individual monosaccharide was calculated by comparison of the peak areas to the peak area of inositol.

4.2.7 Assay for antioxidant activities

(1) Radical scavenging activity on DPPH

Radical scavenging activities on DPPH were evaluated using the method described by Blois (2002) with a slight modification. Aliquots (0.5 mL) of various concentrations (0.156-10.00 mg/mL) of MPS and PS were mixed with 2 mL (25 μg/mL) of a MeOH solution of DPPH. Then the mixture was shaken vigorously and allowed to stand in the dark for 30 min. The absorbance was measured at 517 nm against a blank. Decrease in the DPPH solution absorbance indicated an increase of the DPPH radical-scavenging activity. Ascorbic acid was used as the positive control. The radical scavenging activity on DPPH was calculated according to the following equation:

$$\text{Scavenging activity } (\%) = (1 - \frac{A_1 - A_2}{A_0}) \times 100 \qquad (4\text{-}1)$$

where A_1 was the absorbance with the presence of DPPH and sample; A_0, with the presence of DPPH but without sample; and A_2, with the presence of sample but

without DPPH.

(2) Hydroxyl free radical scavenging activity

Hydroxyl free radical scavenging activity was measured according to Nicholas et al. (1989) with a few modifications. Hydroxyl free radicals were generated from $FeSO_4$ and H_2O_2, and detected by their ability to hydroxylate salicylate. The reaction mixture (2.5 mL) contained 0.5 mL $FeSO_4$ (1.5 mM), 0.35 mL of H_2O_2 (6 mM), 0.15 mL of sodium salicylate (20 mM), and 1 mL of different concentrations of MPS. Ascorbic acid was used as the positive control. After incubation for 1 h at 37°C, the absorbance of the hydroxylated salicylate complex was measured at 562 nm. The percentage scavenging effect was calculated as:

$$\text{Scavenging activity } (\%) = (1 - \frac{A_1 - A_2}{A_0}) \times 100 \qquad (4\text{-}2)$$

where A_0 is the absorbance of the solvent control, A_1 is the absorbance of the sample or ascorbic acid, whereas A_2 is the absorbance of the reagent blank without sodium salicylate.

(3) Ferrous metal ions chelating activity

Ferrous metal ions chelating activity of MPS was measured according to Yuan et al. (2008) with a few modifications. A sample of ethylene diamine tetraacetie acid (EDTA) solution (1 mL) was mixed with 50 μL of ferrous chloride (2 mM) and 0.2 mL of ferrozine (5 mM), shaken well, settled for 10 min at room temperature, and the absorbance of the mixture was determined at 562 nm. EDTA was included as the positive control. The ion chelating activity was calculated as:

$$\text{Chelating activity } (\%) = (1 - \frac{A_1 - A_2}{A_0}) \times 100 \qquad (4\text{-}3)$$

where A_0 is the absorbance of the control (without sample), A_1 is the absorbance in the presence of the sample and A_2 is the absorbance without ferrozine.

(4) ABTS radical scavenging activity

ABTS assay was based on the method of Re et al. (1999). ABTS radical cation ($ABTS^{\bullet+}$) was produced by reacting 7 mM ABTS solution with 2.45 mM potassium persulphate and allowing the mixture to stand in the dark at room temperature for 12-16 h before use. The $ABTS^{\bullet+}$ solution was diluted with ethanol to an absorbance of 0.70 ± 0.02 at 734 nm.

Then 0.15 mL of various concentration of the sample (0.156-10.00 mg/mL) was mixed with 2.85 mL of $ABTS^{\bullet+}$ solution. Finally, the absorbance was measured at 734 nm after incubation at room temperature for 10 min. The scavenging activity of ABTS free radical was calculated using the following equation:

$$\text{Scavenging activity } (\%) = (1 - \frac{A_1}{A_0}) \times 100 \qquad (4\text{-}4)$$

where A_0 is the absorbance of control without sample and A_1 is the test sample without $ABTS^{\bullet+}$.

4.2.8 Statistical analysis

All experiments were carried out in triplicate. Data were processed and analyzed using Design Expert Software (version 8.0.6, Stat-Ease. Inc., Minneapolis, USA) and Data Processing System (version 7.05 Fujitsu Ltd). P-values below 0.05 were regarded as statistically significant.

4.3 Results and discussion

4.3.1 Morphological properties

The SEM offered a clear view of the preservative of the microstructure of polysaccharides before and after fermentation. The microstructures of the PS and MPS were illustrated in Fig. 4-1. The surfaces of the two polysaccharides showed significant variations in size and shape when viewed by SEM. SEM micrograph (Fig.4-1 a) of PS shows that it is composed of rough lines and wrinkles. The surfaces of MPS are illustrated in Fig. 4-1 b. For the image at 1,000 fold magnification, the surface of MPS was rough, an irregular spray shape. This was quite different from the results of Ma et al. (2013) in which it was reported that polysaccharides extracted from mushroom *Inonotus obliquus* were black particles similar to anomalistic stones. Because of these different characteristics, the micrographs of fermented polysaccharides might be used as standards to qualitatively identify different polysaccharides. The mechanism is possibly due to the changes in molecular weight, intermolecular distance and interconnection caused by the growth metabolism of *M. esculenta*.

4.3.2 Hygroscopicity

The hygroscopicity of PS and MPS are 10.82±0.83 and 15.46±0.11 g water/100 g solids respectively. According to Cai and Corke (2000), as the molecular weight decreased, the hygroscopicity increased. The MPS were more hygroscopic than the PS. This difference can be attributed to the SCR being degraded to low molecule weight polysaccharides during fermentation, making it more hygroscopic.

4.3.3 Color

People perceive an object as food via their visual perception system before tasting it and predict its taste before making a decision about whether or not to purchase or eat it. Therefore, color is sometimes the most decisive element as an indicator for food selection and evaluation (Abdullah et al., 2004, Afshari-Jouybari and Farahnaky, 2011, Fernández et al., 2005 and Mendoza et al., 2006). In this study, CIE L*, a* and b* parameters of PS and MPS were compared in Fig. 4-2. No statistically significant differences were found in the color parameters between the polysaccharides after fermentation, although all the PS showed higher values of color intensity and yellow tones and lower green tones than MPS. The results indicated that the extract process remove the color effectively.

4.3.4 Spectra analysis of MPS and PS

Protein absorbance is dominated by tryptophans, tyrosines, and disulfide bonds. Protein absorbance has a peak near 280 nm and a characteristic shoulder at 290 nm. The 260 nm/280 nm ratio for protein is 0.6 (Glasel, 1995; Goldfarb et al., 1951). The UV spectra of PS and MPS are shown in Fig. 4-3. A week peak around 280 or 260 nm were detected in the UV spectrum, indicating they probably have protein and nucleic acid (Glasel, 1995; Porterfield and Zlotnick, 2010).

FTIR spectroscopy is a powerful technique for the identification of characteristic organic groups in the polysaccharides. The PS and MPS were characterized by FTIR spectroscopy as shown in Fig. 4-4. The peaks of them are close to each other. The strong bands at 3000-3500 cm^{-1} are characteristic of glycosidic structures and are

related to OH stretching. The samples exhibited a specific absorption peak at 1720 cm^{-1}, suggesting the presence of uronic acid (Wang et al., 2004). They exhibited the characteristic absorption of polysaccharides at 1650 and 1400 cm^{-1} (Huang et al., 2007). The C–O–C stretching has characteristic absorption at 1124 cm^{-1}. The band at 890 cm^{-1} is characteristic for the β-glycosidic linkage (Kozarski et al., 2012). It has been reported that the β-glycosidic linkage is the essential structural feature for immunostimulatory and antitumoral effects (Demleimer et al., 1992; Hung et al., 2008; Song and Du, 2012). The absorption peak at 800 cm^{-1} for the sample might be the characteristic absorption of mannose (Widjanarko et al., 2011).

4.3.5 Monosaccharide component analysis

To further investigate effect of fermentation on monosaccharide composition of polysaccharides, GC-MS analysis was used. The Monosaccharide composition of PS and MPS are shown in Table 4-1. It was indicated that PS was composed mainly of glucose, galactose, arobinose and xylose in a molar ratio of 27.88:10.09:8.34:2.93. Similar monosaccharide compositions have been reported by Mateos-Aparicio et al. (2010). MPS showed some difference in its monosaccharide composition, with the presence of glucose, mannose, galactose, arobinose, xylose, and rhamnose with a molar ratio of 36.13:10.05:9.44:7.35:4.69:3.18. Glucose and mannose were the main sugar units, their content nearly exceeded 46 mol % of the total sugar. Some reports explain that rhamnose and arabinose are associated with the antioxidant activities of polysaccharides isolated from mushrooms and *Hovenia dulcis* (He et al., 2012; Wang et al., 2012). Also, previous studies reported that aloe vera gel was mainly composed

of mannose (60.9%), glucose (13.1%) and galactose (1.5%) (Ni et al., 2004). The results demonstrate that the increase of rhamnose and arabinose in the monosaccharide composition might lead to strong antioxidant activity. These results also demonstrated that MPS isolated from fermented SCR might have a different chemical composition. The diversity in monosaccharide compositions between MPs and PS could be the reason that MPS possessed higher activity than PS according to GC-MS analysis (Shi et al., 2013).

4.3.6 Evaluation of antioxidant activity

The material with antioxidant activity may fight inflammation, neutralize the free radicals that damage cells and can prematurely age, which plays an important role in body's health (Tehranifar et al., 2011). The extraction rate of MPS and PS were 9.03% and 2.44%, respectively. To compare the antioxidant activity of the PS and MPS, as the main index of antioxidant activities in vivo, several methods have been used for the determination of the antioxidant activities such as ABTS assay, DPPH test, hydroxyl radical scavenging activities and ferrous metal ions chelating activity method.

DPPH is a free radical that accepts an electron or hydrogen radical to become a stable diamagnetic molecule (Soares et al., 1997). The effect of polysaccharides on DPPH free radical scavenging activity was believed to be due to their hydrogen donating ability (Chen et al., 2008). The results of DPPH free radical scavenging activity of the PS and MPS are shown in Fig. 4-5 with ascorbic acid (Vc) as control standard. As can be seen from Fig. 4-5, the DPPH radical scavenging activity

increased from 11.96% to 93.94%, when the concentration of MPS increased from 0.156 to 10 mg/mL. While when PS concentration increased from 0.156 mg/mL to 10 mg/mL, the DPPH radical scavenging ration increased from 12.69% to 27.51%. Compared with PS, MPS had significantly higher DPPH radical scavenging activity.

Hydroxyl radical removal is important for the protection of living systems. It can damage virtually all types of macromolecules in our body such as carbohydrates, nucleic acids, lipids and amino acids, which is a very dangerous to an organism (Gulcin, 2006; Ke et al., 2009). Therefore, it is important to discover chemicals with good scavenging capacity for these reactive oxygen species. The hydroxyl radical scavenging capacity of an extract is directly related to its antioxidant activity (Babu et al., 2001). Fig. 4-6 depicts the scavenging activity of MPS and PS on hydroxyl radical. The scavenging ratio of MPS and PS correlated well with increasing concentrations, increasing from 16.57% to 100%, and from 6.58% to 42.13% respectively, when the concentrations increased from 0.156 mg/mL to 10 mg/mL. The scavenging activity of MPS was lower than Vc, but still much higher than that of PS. The results indicated that MPS exhibits stronger antioxidant effects than PS on hydroxyl radical scavenging.

ABTS assay is often used to evaluate total antioxidant power of single compound and complex mixtures of various plants (Katalinic et al., 2006; Huang et al., 2008). In this experiment, the scavenging ability of MPS and PS on ABTS free radical is shown in Fig. 4-7. MPS and PS were found to have the ability to scavenge hydroxyl radicals at concentrations between 0.156 mg/mL and 10 mg/mL compared to the same

concentration of Vc. MPS had a higher scavenging effect for hydroxyl radicals than PS. Their scavenging powers correlated well with increasing concentrations, but were significantly lower than ascorbic acid when the concentration was below 5.0 mg/mL.

Chelation of metal ions has an antioxidant effect because the transition metals iron and copper promote oxidative damage at different levels (Saiga et al., 2003). As shown in Fig. 4-8, the metal chelating activity of MPS and PS increased with increasing concentrations used in the test. Compared with EDTA, the chelating activity of MPS and PS on ferrous ion was weaker when the concentration was below 5.0 mg/mL. The result showed that PS had negligible Fe^{2+} chelating activity, and the maximal chelating activities of MPS and PS were 95.94% and 27.51% at 10 mg/mL, respectively.

4.4 Summary

In this study, the characteristics and antioxidant activity of PS and MPS are investigated. Compared with PS, MPS had different micrographs and higher hygroscopicity as well as dark color. Furthermore, MPS contained diverse monosaccharide than PS. The obtained MPS demonstrated higher positive antioxidant activities than PS. The results will provide references for the large-scale production of polysaccharides by *M.esculenta* and point to a new direction for the utilization of SCR.

Table 4- 1 Monosaccharide compositions of PS and MPS (mol %)

Polysaccharides	Glucose	Mannose	Galactose	Araribose	Xylose	Rhamnose
PS	27.88	-	10.09	8.34	2.93	-
MPS	36.13	10.05	9.44	7.35	4.69	3.18

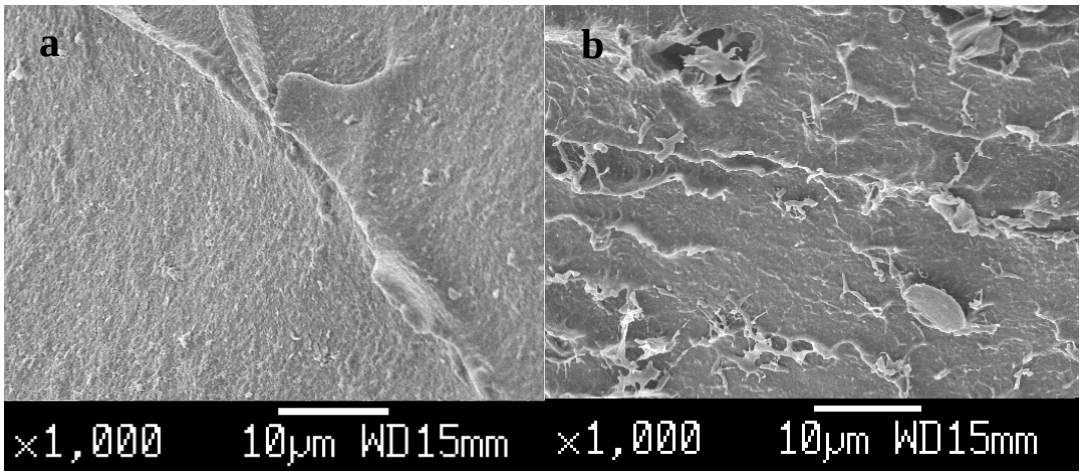

Fig. 4- 1 Scanning electron micrograph (SEM) images of PS (a), MPS (b)

at magnification of 1000× and 5 kV. Bar = 10 μm.

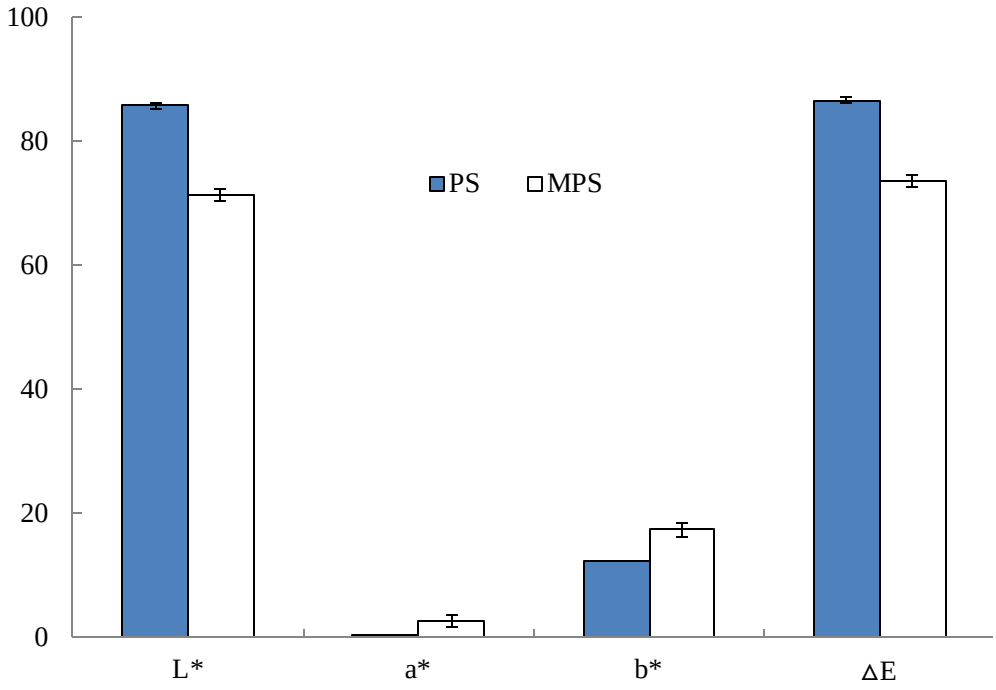

Fig. 4- 2 CIELab coordinates (L*; a*; b*, ΔE) of PS and MPS.

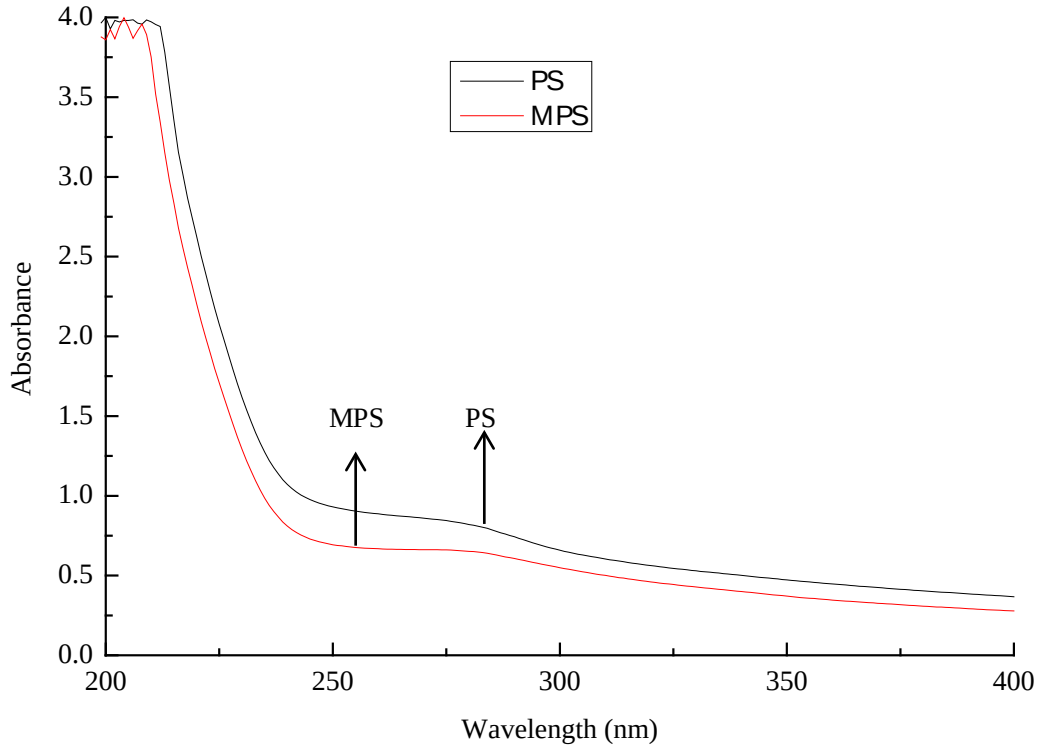

Fig. 4- 3 UV spectra of PS and MPS

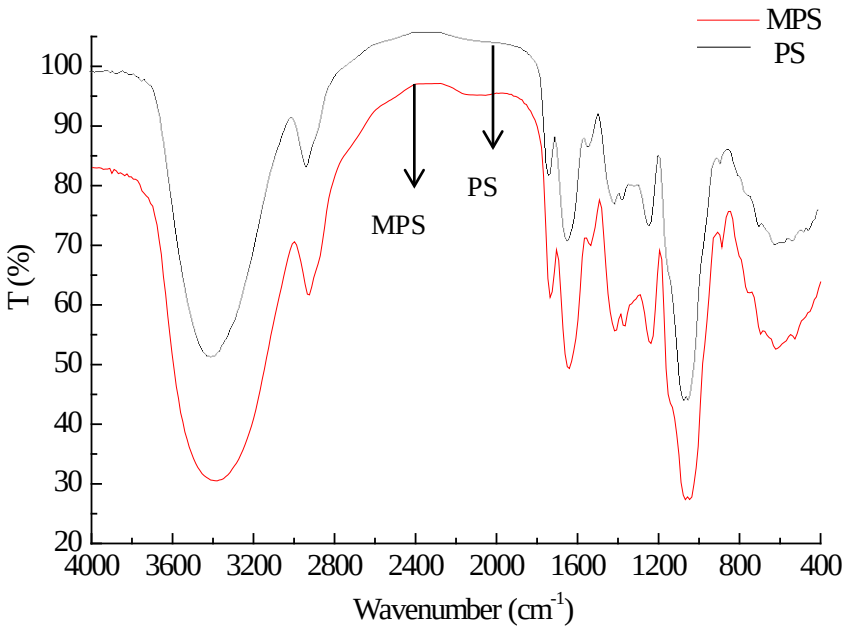

Fig. 4- 4 FTIR spectra of PS and MPS

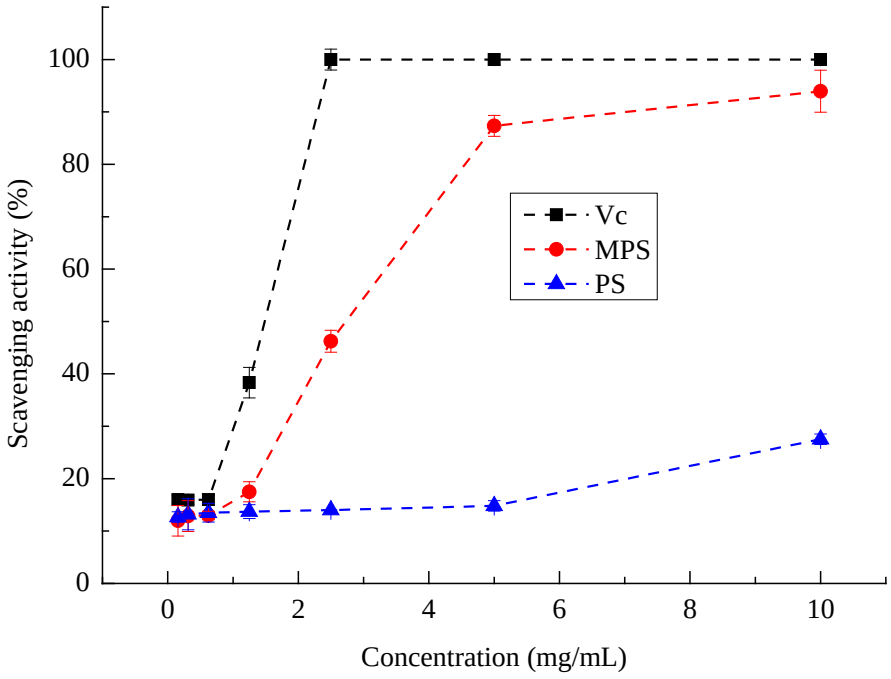

Fig. 4- 5 Scavenging activity of the MPS and PS on DPPH radical.

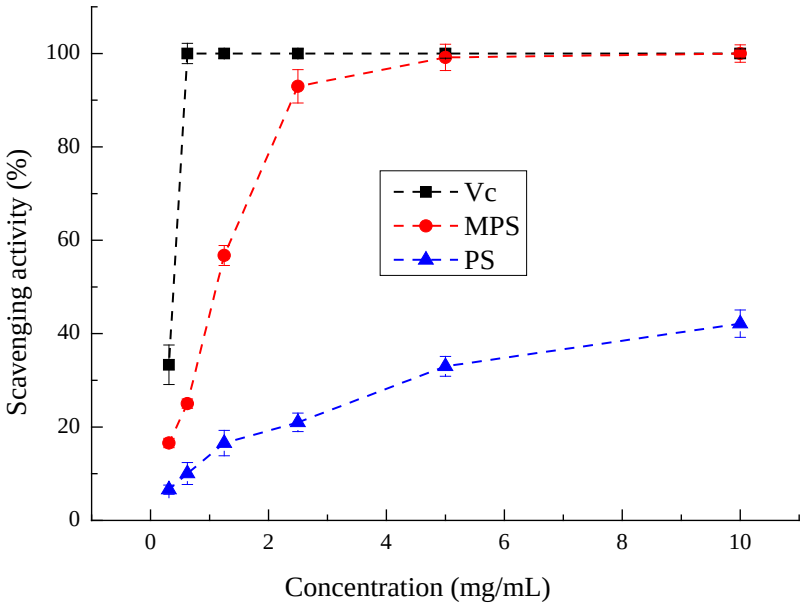

Fig. 4- 6 Scavenging activity of MPS and PS on hydroxyl radical.

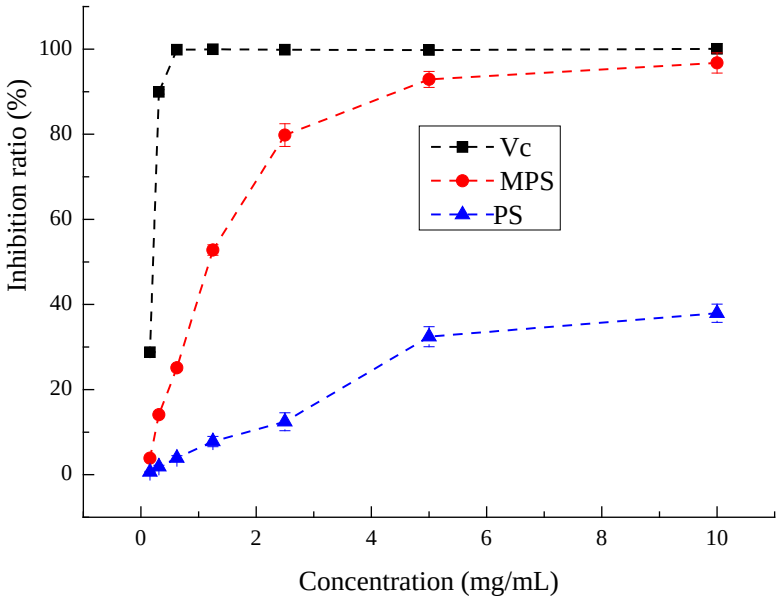

Fig. 4- 7 Scavenging activity of the MPS and PS on ABTS radical.

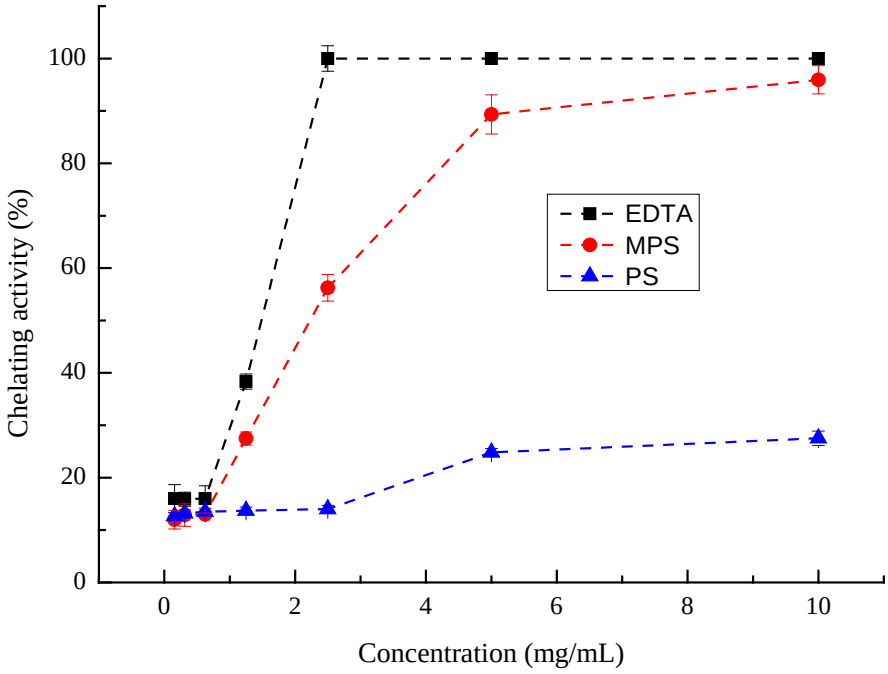

Fig. 4- 8 Chelating activity of the MPS and PS.

Chapter 5 Antitumor and immunomodulatory activity of polysaccharides

5.1 Introduction

Cancer, one of the most serious diseases affects human health. According to GLOBOCAN 2008 statistical reports, about 12.7 million cancer cases and 7.6 million cancer deaths were recorded worldwide in 2008. Furthermore, it is predicted that almost 21.4 million new cancer cases and over 13.2 million deaths are likely to occur by 2030 (Ahmedin et al., 2011). The efficiency of antitumor therapies would improve if it benefits immune response against tumor development. Therefore it is necessary to work on antitumor agents with low toxic effects and effects that improve host defense mechanisms against tumor growth. In recent years, there is increasing attention to polysaccharides from natural sources as an efficient medicine to prevent and treat cancer because of their antitumor and immunomodulatory activity (Song and Du, 2012).

The growing global prevalence of cancer has made cancer therapy one of the most investigated aspects in recent years. The most considerable challenges facing effective cancer therapy are systemic toxicity of antitumor drugs, their lack of tumor localizing and an even distribution throughout the whole body. Besides, anticancer drugs short half-lives in blood circulation and their undesirable pharmacokinetic behavior are among other drawbacks which are present in the way of cancer chemotherapy. Various macromolecules including proteins, antibodies and polysaccharides have been conjugated to cytotoxic drugs. Among them polysaccharide-based systems have

gained increasing attention due to their cost effectiveness, abundance in nature, remarkable physicochemical, biological characteristics and simplicity of chemical reactions required for specific modifications (Goodarzi et al., 2013). They exhibit a wide range of biological functions, such as anti-inflammatory (Kang et al., 2011), antioxidant, antitumor (Song and Du, 2012), immunostimulatory (Dai et al., 2009), anticancer activity (Xie et al., 2013) and so on, which are related to the antioxidant properties of polysaccharides. The anti-cancer efficacy of polysaccharides was first recognized by Nauts et al. (1946) who found that certain polysaccharides could induce complete remission in patients with cancer. It has been well documented that the polysaccharides of ginseng possess various anti-tumor activities including preventive and inhibitory effects against tumors as well as enhanced immunological functions (Choi, 2008). Numerous studies have suggested that polysaccharides can inhibit tumor growth through the following common mechanisms: (1) the prevention of tumorigenesis by oral consumption of active preparations; (2) direct anti-cancer activity, such as the induction of tumor cell apoptosis; (3) immunopotentiation activity in combination with chemotherapy; and (4) the inhibition of tumor metastasis (Zong et al., 2012).

However, little information could be found with respect to polysaccharides isolated from SCR fermented by *M. esculenta*, let alone the immunomodulatory and antitumor activity. In this chapter, immunomodulatory and antitumor activity of MPS and PS were compared.

5.2 Materials and methods

5.2.1 Chemicals and reagents

Propidium iodide (PI), Minimum Essential Medium Eagle (MEM) medium, fetal bovine serum (FBS), Griess reagent, Rhamnose, fucose, arabinose, d-ribose, xylose, allose, mannose, galactose, glucose, inositol and penicillin-streptomycin solution were purchased from Sigma Aldrich, Inc. (Saint Louis, MO, USA). Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan). All the other reagents were of analytical grade.

5.2.2 Extraction and purification of polysaccharides

Fresh SCR (80% of moisture content) was obtained from Inamoto Co., Ltd., Tsukuba, Japan. Then the SCR was fermented according to the conditions Chapter 2. After fermentation, the polysaccharides were extracted from the unfermented SCR and fermented SCR with the method in Chapter 4.2.2. The polysaccharides obtained from unfermented SCR and fermented SCR by *M. esculeta* were named as PS and MPS, respectively.

5.2.3 Cell line and cell culture

The murine macrophage cell line RAW 264.7, human colon cancer cell line (DLD-1), human cervical cancer *Hela* and human hepatocarcinoma cell line *HepG 2* were obtained from RIKEN Bioresource Center (Tsukuba, Japan). RAW 264.7 and Hela were grown in MEM medium containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. HepG2 was grown in DMEM medium containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Culture was maintained at 37°C

in a humidified 5% CO_2 atmosphere (ASTEC APC-30D CO_2 Incubator, Fukuoka, Japan). Cells were cultured for 2-3 days to reach the logarithmic phase and used for experiment.

5.2.4 Immunomodulatory activity

(1) Measurement of cell proliferation ability

The effect of polysaccharides on RAW 264.7 proliferation was estimated by Cell Counting Kit-8 (CCK-8). RAW 264.7 cells were cultured in 96-well plate at a density of 5×10^4 cells/mL at 37°C in a 5% CO_2 atmosphere for 24 h. Then cells were incubated with various concentrations of extracts (0, 6.25, 12.5, 25, 50 and 100 μg/mL) at 37°C for 24 h. After incubation, 10 μL of CCK-8 reagent solution was added and incubated at 37°C for 4 h. The cell viability was determined by the optical density (O.D.) at a wavelength of 450 nm with a microplate reader (BIO-RAD Model 550). Data was expressed as percentages of control. Cell proliferation rate was calculated by the following equation:

Cell proliferation rate (%) = $(A_{sample}-A_{blank1})/(A_{control}-A_{blank2})\times 100$

A_{sample}, optical density value of tested samples with cells, A_{blank1}, optical density value of samples with medium, $A_{control}$, optical density value of control with cells, A_{blank2}, optical density value of control with medium.

(2) Nitric oxide assay

The macrophage cells (1×10^5 cells/mL) were dispensed into a 96-well plate for 24 h. Next the cells were stimulated with Lipopolysaccharide (LPS) 1 μg/mL and various concentrations of polysaccharides (0, 6.25, 12.5, 25, 50 and 100 μg/mL) for 24 h.

After the incubation, the nitrite concentration in the culture medium was measured as an indicator of NO production based on the Griess reaction. 50 μL of each supernatant was mixed with the same volume of Griess reagent and the resultant mixture was then incubated at room temperature for 10 min. The absorbance at 570 nm was then measured using a microplate reader. Freshly cultured medium was used as the blank in all experiments. $NaNO_2$ was used as a standard to calculate the nitrite concentrations.

(3) Phagocytosis assay

The phagocytic ability of the macrophages was measured by neutral red uptake. The macrophages RAW 264.7 cells (5×10^4 cells/mL) were cultured in the presence of various concentrations of polysaccharides and LPS (1 μg/mL) in a 96-well plate in a total volume of 100 μL per well for up to 48 h at 37°C. 100 μL/well of 0.075% neutral red was added and incubated for another 1 h. The media were discarded and the macrophages were washed twice with PBS (pH: 7.2-7.4). Then, 100 μL/well of the cell lysing solution (50% ethanol: 50% acetic acid (v/v) = 1:1) was added and incubated for 2 h. The absorbance at 570 nm was measured using a microplate reader.

(4) Protective activity

The RAW 264.7 cells were cultured in a 96-well plate at a density of 5×10^4 cells/mL for 24 h at 37°C in a 5% CO_2 atmosphere. Then the cells were incubated with DOX (5 μM) in the presence or absence of various concentrations of MPS and PS for 24 h. After drug exposure, 10 μL of the CCK-8 solution was added and

incubated at 37°C for 4 h. The cell numbers were quantitated by reading the absorbance at 450 nm. The data were expressed as percentages of the control.

5.2.5 Antitumor activity

An aliquot (100 μL) of DLD-1, Hela and HepG2 cells suspension (5×10^4 cells/mL) were dispensed into a 96-well plate and pre-incubated at 37°C in a 5% CO_2 atmosphere for 24 h. Then the cells were exposed to various concentrations of extracts (0, 50, 100, 150, 200 and 250 μg/mL) for 12 h, 24 h, and 48 h. After drug exposure, 10 μL of CCK-8 solution was added and incubated at 37°C for 4 h. Cell numbers were quantitated by reading the O.D. at 450 nm with a microplate reader (BIO-RAD Model 550). Cell viability was calculated by the following equation:

Cell survival rate (%) = $(A_{sample}-A_{blank1})/(A_{control}-A_{blank2})\times 100$ (5-1)

A_{sample}, optical density value of tested samples with cells, A_{blank1}, optical density value of samples with medium, $A_{control}$, optical density value of control with cells, A_{blank2}, optical density value of control with medium.

5.2.6 Statistical analysis

The data were presented as means ± SD of three determinations. Statistical analyses were performed using student's t-test and one way analysis of variance. Multiple comparisons of means were done by the least significance difference test. All computations were done by employing the statistical software (SPSS, version 11.0). P-values below 0.05 were regarded as statistically significant.

5.3 Results and discussion

5.3.1 Immunomodulatory activity

(1) Proliferation assay

Proliferation of macrophage is one important indicator of immunoactivation. So it is necessary to evaluate the cytotoxic effect or proliferation effect of MPS and PS on RAW 264.7 cells before further tests being carried out. The proliferation effects of MPS and PS on the macrophages are shown in Fig. 5-1. CCK-8 assay indicated that both polysaccharides did not show cytotoxicity on the proliferation of RAW 264.7 cells in 24 h but a stimulation activity. At a concentration of 50 μg/mL, MPS showed the strongest stimulatory effect on cells proliferation, about 317.8%. The result shows that MPS is more active than PS. It is noteworthy to mention that a similar kind of activation has been observed with mushroom polysaccharides (Rout et al., 2005; Roy et al., 2009). However, PS has a stability proliferation at different concentrations. These results suggest that MPS has stronger biological activity than PS.

(2) Effect of polysaccharides on NO production

In the immune system, macrophages play a stabilizing role in regulating the body. Nitric oxide (NO) plays an important role in the cytotoxic activity of macrophages towards tumor cells and microbial pathogens (Zamora et al., 1997). Lipopolysaccharides (LPS), are large molecules consisting of a lipid and a polysaccharide joined by a covalent bond; they are found in the outer membrane of Gram-negative bacteria, act as endotoxins and elicit strong immune responses in cells. When the macrophages are exposed with LPS, they will release NO to fight off an

assault of LPS. The immunomodulatory activity is decreased in concordance with the increase of NO released from macrophages. The effect of PS and MPS on the NO production in murine macrophage RAW 264.7 cells is shown in Fig. 5-2. A small amount of NO was produced when macrophages were exposed to medium while a large of when they exposed to LPS. Incubation of these cells with MPS at the concentration 6.25 μg/mL induced a significant decrease in NO release ($P < 0.05$) compared with negative control. On the other hand, PS had significantly influence on NO production at the concentration between 25 and 100 μg/mL. Moreover the level of NO production of MPS at concentration of 6.25 μg/mL was comparable to that elicited by medium.

(3) Phagocytosis assay

Macrophage is the most important professional phagocyte and it plays an essential and pivotal role in host defense against any type of invading cells including tumor cells (Katsiari et al., 2010). The assessment of phagocytosis function can be a critical parameter of immunotoxicology. PS and MPS were studied for its influence on peritoneal macrophage phagocytosis, and the effect on phagocytosis of macrophages for 48 h is shown in Fig. 5-3. Compared with the control group, MPS at the concentration of 50 and 100 μg/mL significantly enhanced the proliferation of macrophages, but no significant improvement was observed between the dosages of 6.25 and 50 μg/mL. PS had no significant effect on RAW 264.7 cell ($P <0.05$). Thus the results suggested that administration of MPS may result in the initiation of immune reaction against foreign materials such as pathogen and tumors.

(4) Protective activity

Doxorubicin (DOX) is a potent, broad-spectrum chemotherapeutic drug used all over the world. Despite its effectiveness, it has a wide range of toxic side effects, many of which most likely result from its inherent pro-oxidant activity (Tangpong et al., 2011). Treatment with DOX could result in a decrease of cancer cell as well as macrophage survival rate. To confirm the effects of polysaccharides on the protective activity on macrophages, DOX was added to the cells for 24 h. Macrophages without any pretreatment were used as control group. Fig. 5-4 shows the effect of MPS and PS on DOX-induced macrophage RAW 264.7 cells survival. PS showed potential protective activity on RAW 264.7 about 65.0%, whereas MPS has significant protective activity in a dose-dependent manner. In the antioxidant experiment, MPS showed high DPPH radical-scavenging activity. So, MPS might reduce the reactive oxygen species level in cells thereby mitigate the apoptosis induced by DOX.

5.3.2 Antitumor activity

As recently demonstrated, some extracts from mushroom markedly inhibit the growth of cancer cells directly, whereas other extracts show some different functions. To determine which function the extracted polysaccharides have, the antitumor activity of the two kinds of polysaccharides was determined.

Fig. 5-5 shows the inhibition activity of MPS and PS on Hela cells for 48 h. The inhibition ratio was slightly enhanced with the increase in concentration of MPS. At a high concentration of 200 μg/mL, the inhibition ratio in suppressing the growth of Hela tumor cells was 32.6%. The results showed MPS exhibited low antitumor

activity. At the same time, PS also showed very poor inhibition effect. The explanation of this may indicated that MPS had selective antitumor activity.

HepG2 cells, a human hepatoma cell line, are considered to be a good system to study in vitro xenobiotic metabolisms and liver toxicity, as they retain many of the specialized functions that characterize normal human hepatocytes (Knasmuller et al., 1998). In particular, HepG2 cells retain the activity of many phase I, phase II, and antioxidant enzymes, ensuring that they constitute a good tool to study the cytoprotective, genotoxic, and antigenotoxic effects of compounds (Knasmuller et al., 2004 and Mersch-Sundermann et al., 2004). In vitro antitumor activities of MPS and PS against HepG 2 cells were investigated, and the results are shown in Fig. 5-6. As shown in Fig. 5-6, PS inhibited the growth of the transplanted tumor, with the inhibitory rates of 25.49%, 36.25%, and 17.01% at the dose of 150, 200, 250 μg /mL respectively. When the cells were exposed to MPS, the tumor cell growth inhibition rates largely increased first but later decreased. The inhibition ratio got to highest value of 77.35% at concentration of 100 μg/mL. The result indicated that MPS had a potent anti-proliferation effect on HepG2 cells. These differences in antitumor activities may be attributed to their different molecular weights, charge characteristics and monosaccharide distributions (Dias et al., 2005).

In vitro inhibition ratio of DLD-1 cell growth by PS and MPS at different concentration (50, 100, 200 and 250 μg/mL) are shown in Fig. 5-7. With the concentration increase from 50 to 250 μg/mL, the inhibition ratio of MPS on DLD-1 cells was significantly enhanced from 5.90% to 37.7%. On the other hand, PS

exhibited a relatively lower cytotoxicity to DLD-1 cells. The inhibition ratio of PS on DLD-1 cells at the concentrations from 50 to 250 μg/mL was no more than 20%, much lower than that of MPS.

5.4 Summary

Macrophage cells (RAW 264.7) were used as experimental objects to evaluate immunomodulatory. The proliferation effect of MPS on cells was 317.8% at 50 μg/mL and MPS induced 14.0 μM NO at the concentration 6.25 μg/mL. At the same time, MPS showed phagocytosis activity (O.D. 540 nm) which was 0.58 at concentration of 100μg/mL and proliferation effect was 96.0% at 50μg/mL on the macrophages when exposed to Doxorubicin (DOX). In addition, antitumor experiment indicated that MPS had inhibition effect on human cervical carcinoma cells (Hela), human colon cancer cell line (DLD- 1) and human hepatocarcinoma cell line (HepG2) than PS, the inhibition ratio were 32.60%, 37.70% and 77.39% respectively. Therefore, the current data show that these polysaccharides have potential for being utilized as medicinal, pharmacological and functional food ingredients. Further studies regarding the detailed structures of the polysaccharides should be extended.

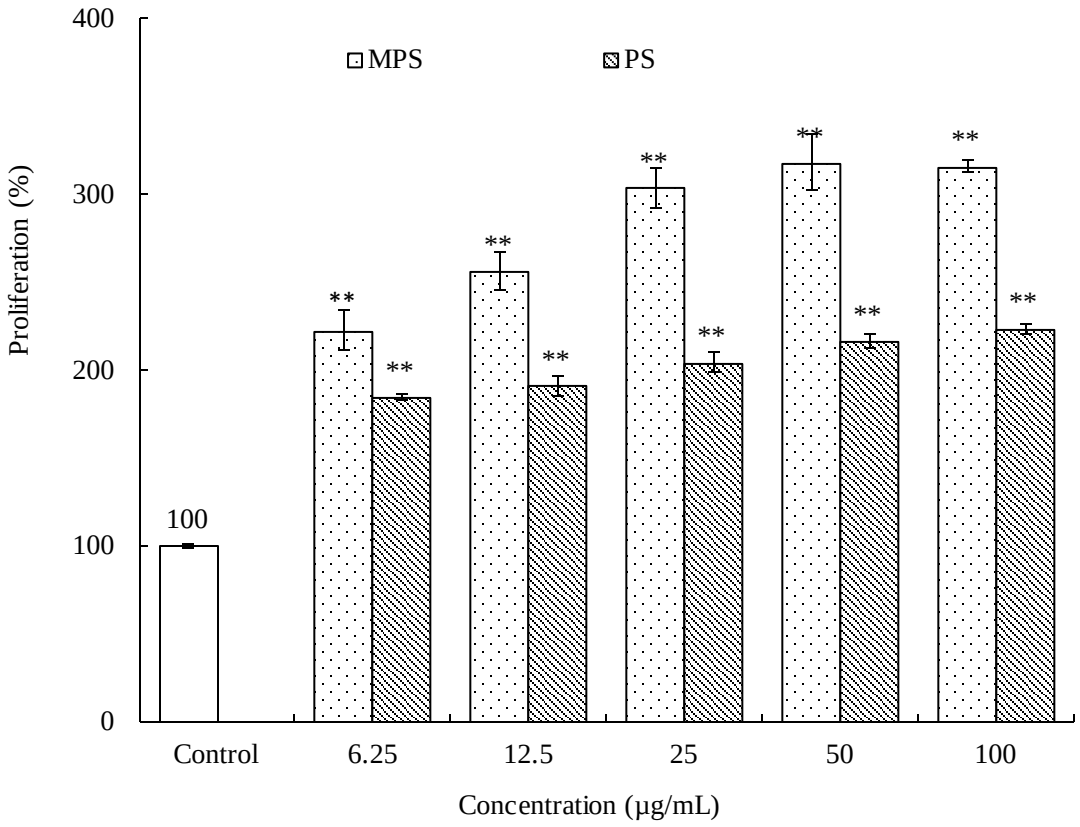

Fig. 5- 1 Proliferation effects of MPS and PS on the macrophage cells.

** indicates statistically significant difference ($P<0.01$).

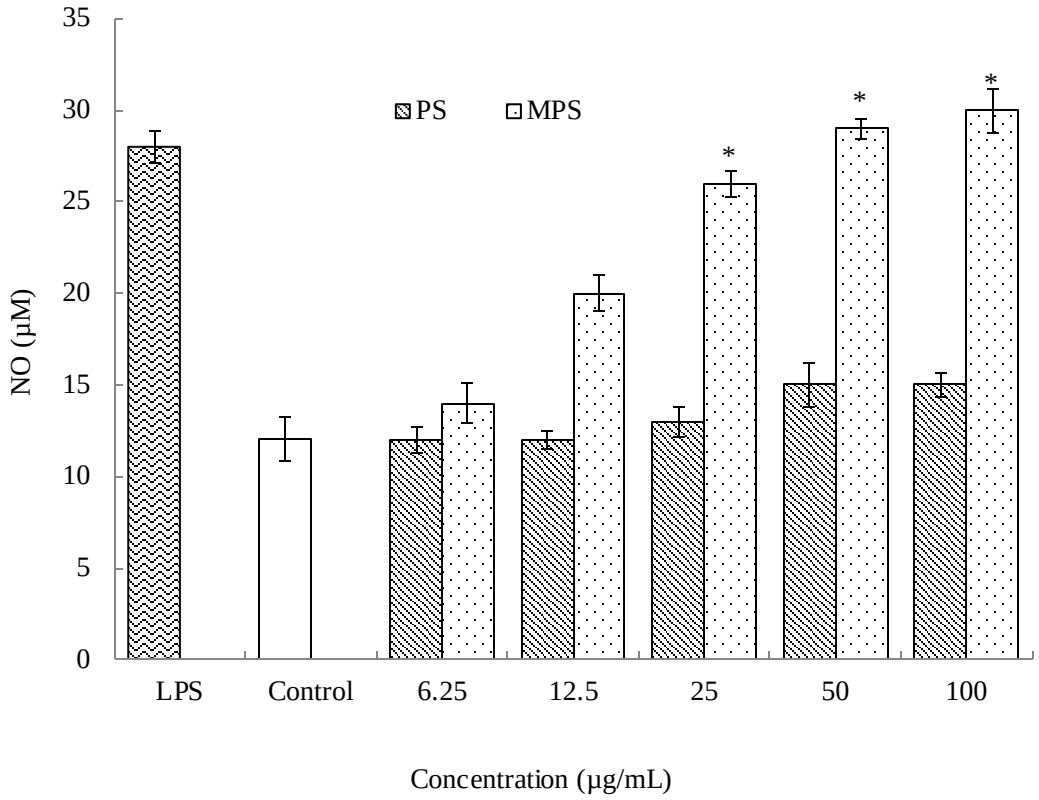

Fig. 5- 2 Effect of PS and MPS on NO production of macrophage cells.

* indicates statistically significant difference ($P<0.05$).

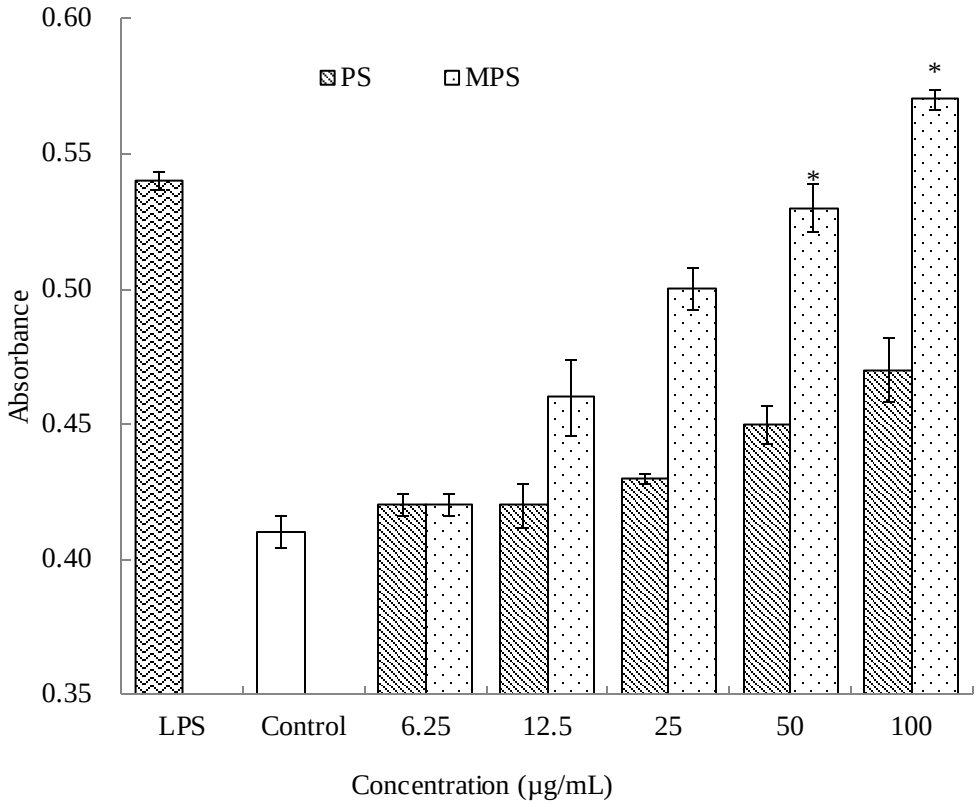

Fig. 5- 3 Effect of treatment with PS and MPS on phagocytosis of macrophage cells.

* indicates statistically significant difference ($P<0.05$).

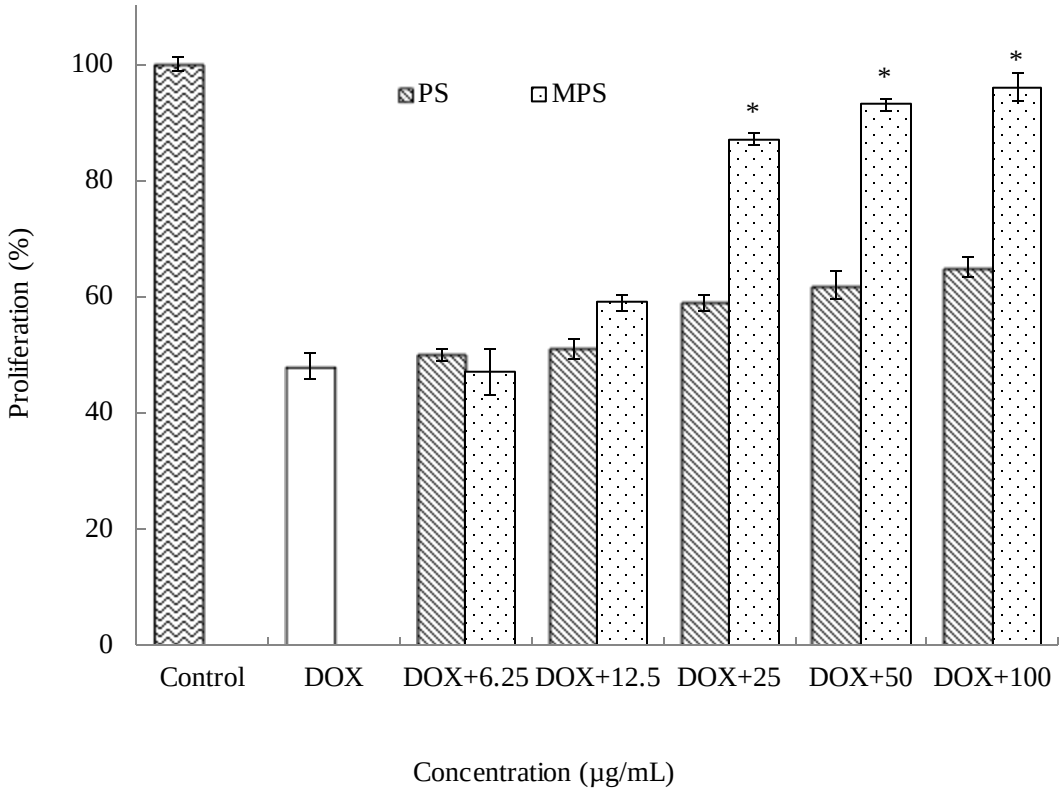

Fig. 5- 4 Protective activity of MPS and PS on DOX-induced macrophage cells.

* indicates statistically significant difference ($P<0.05$).

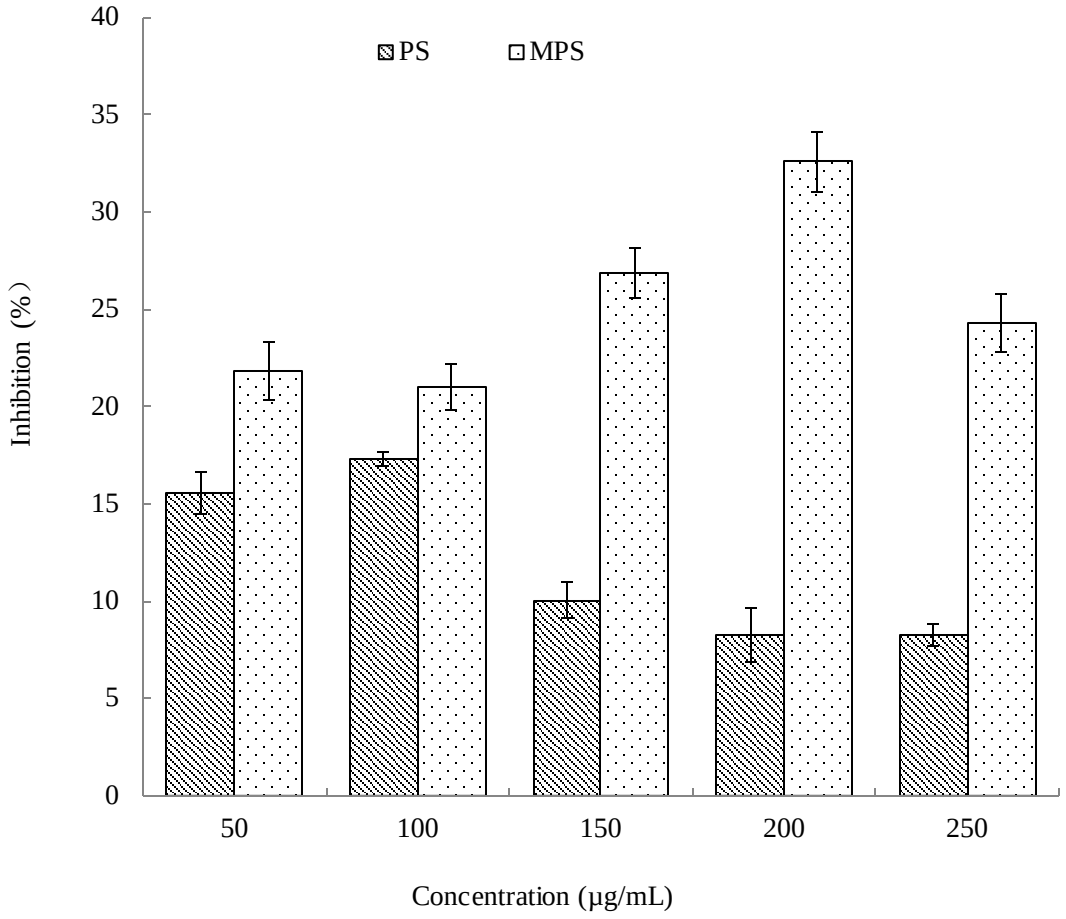

Fig. 5- 5 Inhibition effect of MPS and PS on Hela cells (48 h).

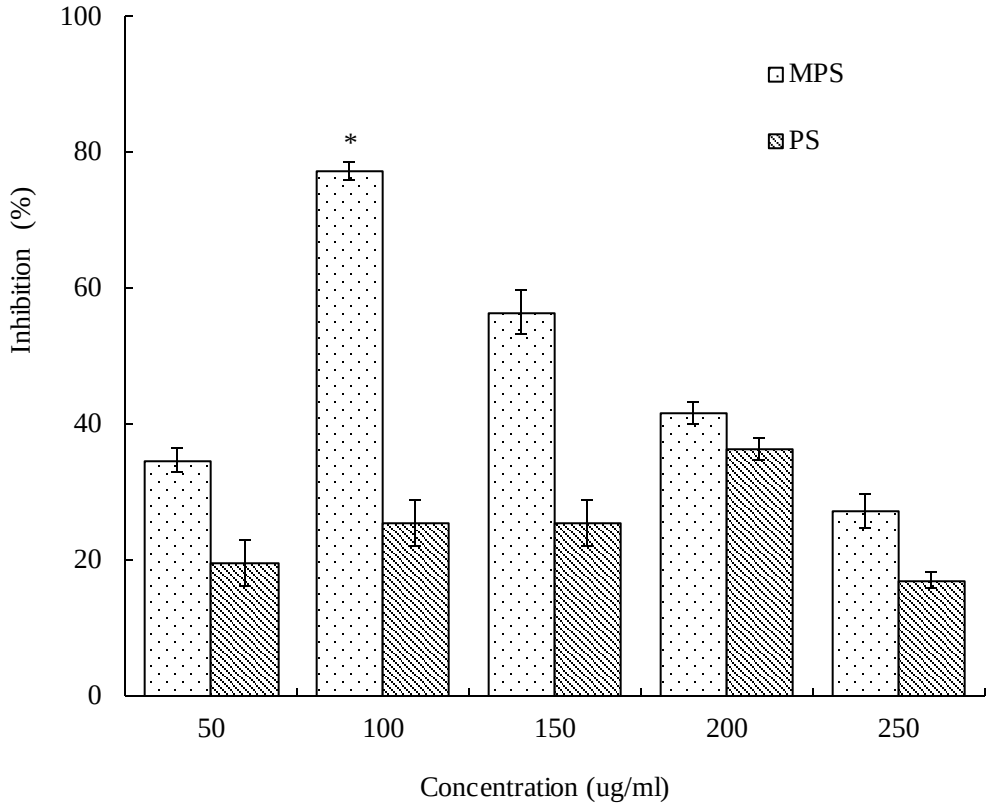

Fig. 5- 6 Inhibition effect of MPS and PS on HepG 2 cells (24 h).

* indicates statistically significant difference ($P<0.05$).

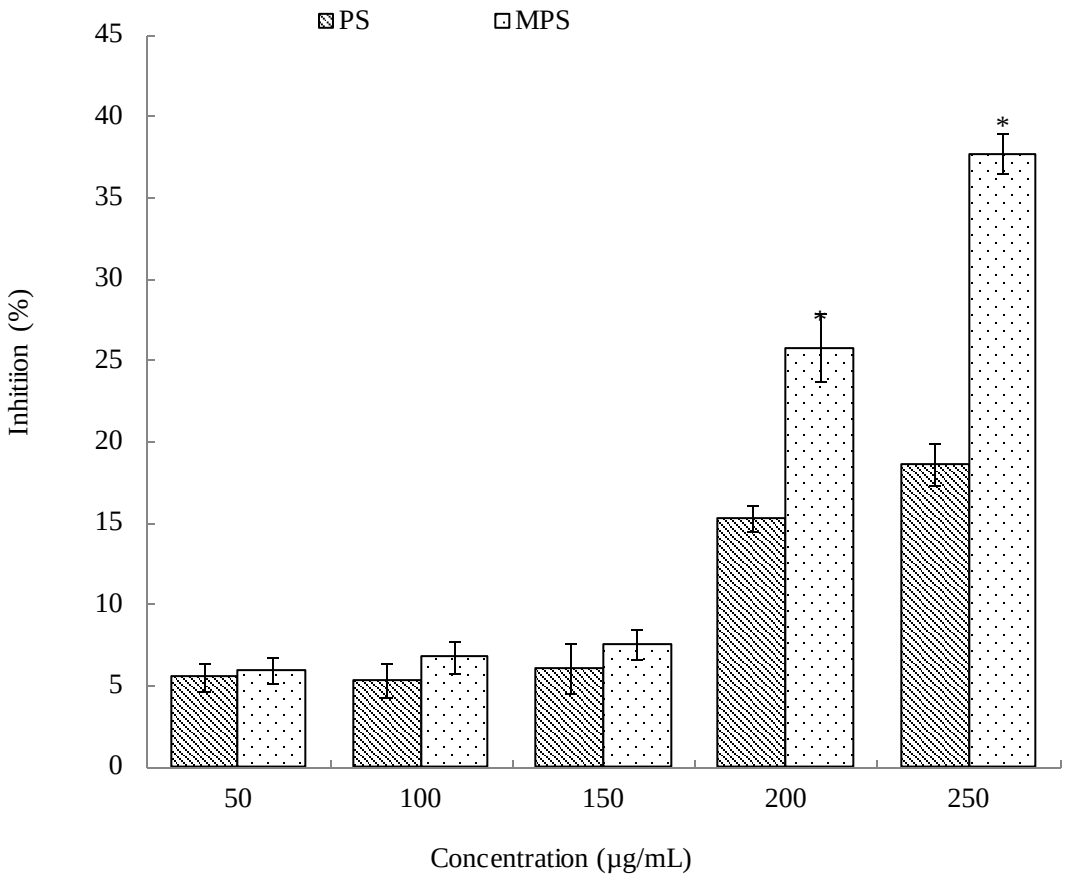

Fig. 5- 7 Inhibition effect of MPS and PS on DLD1 cells (48 h).

* indicates statistically significant difference ($P<0.05$).

Chapter 6 Immunomodulatory and antitumor activity of purified polysaccharides

6.1 Introduction

Currently chemotherapy is the main tool for cancer treatment, but it induces severe side effects in many cases. Extensive studies have been carried out to search for new active extracts or components from various plants which can be used in the treatment of cancer with high efficacy and safety. In the last decades, many polysaccharides and polysaccharide-protein complexes extracted from mushrooms, fungi, yeast, algae, lichens, plants and animals have been reported to exhibit a variety of biological activities, especially antitumor and immunomodulatory activities. They cause no harm and have no additional stress on the body, but help the body to adapt to environmental and biological stress. Therefore it is essential to explore the pharmacological effect of the polysaccharides from natural source for the access to safe and potent antitumor agents that protect cancer patients from the harmful side effects of chemotherapy.

Polysaccharides can be purified using a combination of techniques, such as ethanol precipitation, factional precipitation, ion-exchange chromatography, gel filtration, and affinity chromatography (Wasser, 2002). Basically, gel filtration could separate polysaccharides by molecular weight, and ion-exchange chromatography could separate neutral polysaccharides from acidic ones by eluent. It was found that neutral polysaccharides fraction eluted with water was rich in glucose, galactose and arabinose and acidic polysaccharides fraction eluted with NaCl solution ranging from 0.2 to 0.6 M consisted mainly of galacturonic acid along with rhamnose, arabinose,

and galactose (Jin et al, 2012).

Immunomodulation through natural substances may be considered an alternative for the prevention and cure of neoplastic diseases (Mitchell, 2003). There is growing evidence suggesting that polysaccharides from natural plants can significantly enhance the immune system (Lim et al., 2004; Yang et al., 2006 ; Zhu et al., 2007). They are regarded as promising immunomodulatory agents which are relatively nontoxic and have no significant side effects. Therefore, the discovery and identification of novel plant polysaccharides as ideal immunomodulators has become an important research goal.

In this study, the results from Chapter 4 indicated that the polysaccharides from fermented SCR have antioxidant activity. The activity of polysaccharides complexes are reported to be most closely related to their physicochemical properties, such as chemical composition polysaccharides content, protein content, , molecular weight, viscosity, conformation, types of sugar residues, infrared spectra and degree of branching (Zhang et al., 2007).

This study was performed to investigate and compare the anti-inflammatory and antitumor effects of the purified polysaccharides. In this chapter, polysaccharides from fermented SCR were isolated by water extraction, filtration, DEAE-SephadexA-50 chromatography and Sephadex LH-20 gel permeation column in sequence, analyzed the elementary characterization of the polysaccharides fractions, and evaluated the influence of the polysaccharides fractions upon the activity of macrophage cell (RAW 264.7) and antitumor activity of HepG2 cell in vitro.

6.2 Materials and methods

6.2.1 Chemicals and reagents

Propidium iodide (PI), Minimum Essential Medium Eagle (MEM) medium, fetal bovine serum (FBS), Griess reagent, Rhamnose, fucose, arabinose, d-ribose, xylose, allose, mannose, galactose, glucose, inositol and penicillin-streptomycin solution were purchased from Sigma Aldrich, Inc. (Saint Louis, MO, USA). Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan). All the other reagents were of analytical grade.

6.2.2 Preparation of polysaccharides

Fresh SCR (80% of moisture content) was obtained from Inamoto Co., Ltd., Tsukuba, Japan. Then the SCR was fermented according to conditions in Chapter 2. After fermentation, the polysaccharides were extracted from the unfermented SCR and fermented SCR according to Chapter 5. The polysaccharides obtained from fermented SCR by *M. esculeta* were named as MPS.

The crude polysaccharides was re-dissolved in 5 mL distilled water, filtered through Whatman filters and applied to a DEAE-SephadexA-50 column (2.5×40 cm) equilibrated with distilled water. The polysaccharides was fractionated and eluted with distilled water and different concentrations of stepwise NaCl solution (0, 0.05, 0.1 and 0.5 M NaCl) at a flow rate of 2.0 mL/min. The elutes were concentrated to obtain the main fractions, which were then fractionated by size-exclusion chromatography Sephadex LH-20 gel permeation column (2.5×40 cm) eluted with 0.05 M NaCl at a flow rate of 0.5 mL/min. The fractions obtained were combined

according to the total carbohydrate content quantified by the phenol-sulfuric acid method (Dubois et al., 1956). The relevant fractions were collected, concentrated, dialyzed and lyophilized. For analysis of biological activity, the fractions were diluted in distill ultrapure water and filtered through sterile 0.45 μm filters.

6.2.3 Characterization of polysaccharides fractions

(1)Ultraviolet analysis

Two fractions were dissolved and diluted to 0.2 mg/mL respectively, and the solutions of the polysaccharides fractions were scanned from 200 to 400 nm with UV-1800 spectrophotometer (Shimadzu, Tokyo, Japan). Ultrapure water was used as solvent and control.

(2) Fourier transform infrared analysis

FTIR spectra were recorded using the KBr-disk method by a Jasco FTIR 3000 spectrophotometer (Jasco, Wakayama, Japan) in the range 400-4000 cm^{-1}.

(3) Monosaccharide composition analysis

The analytical methods are same with in 4.2.6 (2) in Chapter 4.

6.2.4 Cell culture

The human hepatocarcinoma cell line (HepG 2) was obtained from RIKEN Bioresource Center (Tsukuba, Japan). It was grown in DMEM medium containing 10% of fetal bovine serum (FBS) and 1% of penicillin/streptomycin. Culture was maintained at 37°C in a humidified 5% CO_2 atmosphere (ASTEC APC-30D CO_2 Incubator, Fukuoka, Japan). Cells were cultured for 2-3 days to reach the logarithmic phase and used for experiment.

6.2.5 Immunomodulatory activity

Immunomodulatory activities including cell proliferation ability, nitric oxide assay, phagocytosis assay and protective activity were tested with the methods in Chapter 5.2.4.

6.2.6 Antitumor activity

Antitumor activity was determined by the method mentioned in Chapter 5.2.5.

6.2.7 Statistical analysis

The data were presented as means ± SD of three determinations. Statistical analyses were performed using student's t-test and one way analysis of variance. Multiple comparisons of means were done by the least significance difference test. All computations were done by employing the statistical software (SPSS, version 11.0). P-values below 0.05 were regarded as statistically significant.

6.3 Results and discussion

6.3.1 Extraction and purification of polysaccharides

In the present study, MPS was isolated from SCR and the yield was about 9.58%. Furthermore, the solution was firstly separated through an anion-exchange chromatography of DEAE-SephadexA-50, with three independent elution peaks (P-1, P-2 and P-3) being detected by the phenol-sulfuric acid assay (Fig. 6-1). The three fractions were collected, concentrated and purified by gel filtration chromatography of Sephadex LH-20, respectively. As a result, the two peaks generated from P-1, were named as MP-1 and MP-2, respectively (Fig. 6-1b). P-2 and P-3 generated only one single elution peak, named as MP-3 and MP-4 respectively (Fig. 6-1c and Fig. 6-1d).

The recovery rates of MP-1, MP-2, MP-3 and MP-4 based on the amount of MPS were 26.2%, 3.0%, 29.1% and 18.7%, respectively. Because of the low extraction ratio of MP-2, it was not studied in the follow-up experiment.

6.3.2 Spectra analysis

The UV spectra are shown in Fig. 6-2. The three polysaccharides had no absorption at 280 or 260 nm in the UV spectrum, indicating the absence of protein and nucleic acid. FTIR spectroscopy is a powerful technique for the identification of characteristic organic groups in the polysaccharides. The MP-1, MP-3 and MP-4 were characterized by FTIR spectroscopy as shown in Fig. 6-3. The peaks of them are close to each other. Three characteristic absorptions of polysaccharides, a strong and wide absorption band of about 3000-3500 cm^{-1} for O–H stretching vibrations and a strong absorption peak of about 2800-3000 cm^{-1} for C–H stretching vibrations were observed. The relatively strong absorption peak at around 1640 cm^{-1} indicated the characteristic absorption of C=O. (Ge et al, 2009). They all exhibited a specific absorption peak at 1050 cm^{-1}, suggesting the presence of –C–O group (Mecozzia et al., 2011). The band at 890 cm^{-1} is a characteristic absorption for the β-glycosidic linkage (Kozarski et al., 2012). It has been reported that the β-glycosidic linkage is the essential structural feature for immunostimulatory and antitumoral effects (Demleimer et al., 1992; Hung et al., 2008; Song and Du, 2012). The absorption peak at 800 cm^{-1} for the sample was the characteristic absorption of mannose (Widjanarko et al., 2011). The bands in the range of 350-600 cm^{-1} are assigned to skeletal modes of pyranose rings (Yang and Zhang, 2009). In particular, the band towards 1735 cm^{-1} of MP-3 and MP-4 was

assigned to C=O stretching, which indicates the existence of ester carbonyl groups. Moreover, the band at 1235 cm^{-1} of MP-3 and MP-4 is associated to C-O stretching according to Kolts et al. (1994).

6.3.3 Monosaccharide component analysis

To further investigate the effect of fermentation on monosaccharide compositions of polysaccharides, GC-MS analysis was used. The molar ratios of the three parts are shown in Table 6-1. Analysis of monosaccharide composition of the polysaccharides fractions indicated that the three polysaccharides fractions primarily consisted of glucose mannose and galactose, which accounted for the majority of monosaccharide present. Small amounts of araribose, rhamnose and xylose were detected in them. The molar ratio results of the three polysaccharides fraction were in agreement with the findings of Jin et al. (2012) to some extent.

6.3.4 Immunomodulatory activity

(1) Proliferation assay

As the first step towards understanding the immunomodulatory activity of polysaccharides, the effect of MP-1 and MP-3 and MP-4 on macrophage cells proliferation was investigated. As seen from the Fig.6-4, the three fractions greatly promoted the proliferation of macrophages. Especially, at the concentration of 25 μg/mL, proliferation activity of MP-3 and MP-4 on macrophages was up to their maximum. Interestingly, MP-1 has a proliferation effect in a dose dependent manner while MP-3 and MP-4 have highest proliferation ratio at concentration 25 μg/mL. While with the increase of doses, the proliferation ratio decreased. Although the exact

reason is unclear, it may be about the osmotic pressure. When at the low concentration, polysaccharides will provide nutrient for macrophages. However, with the increase of concentration, the osmotic pressure also increase, which is not benefit for the proliferation. Also, the results suggest that MP-3 had stronger biological activity than the other two polysaccharides fractions on macrophages RAW 264.7.

(2) Effect of polysaccharides on NO production

Macrophages play important roles in the host defense system against microbial infections, and they produce various inflammatory mediators, cytokines, and phagocytic activities (Gopinath et al., 2006). Stimulation of macrophages by lipopolysaccharide (LPS) enhances the production of mediators, such as nitric oxide (NO), reactive oxygen species (ROS), tumor necrosis factor-α (TNF-α), and interleukin-1β (IL-1β) (Kim et al., 2012). NO mediates a number of the host-defense functions of activated macrophages. NO is a highly reactive signaling molecule and inflammatory mediator that acts as a cytotoxic agent and modulates immune responses (Korhonen et al., 2005). The effect of purified polysaccharides on the NO production in murine macrophage RAW 264.7 cells is shown in Fig. 6-5. All polysaccharides increased NO production in macrophages in a dose-dependent manner in a descending order of MP-3, MP-4 and MP-1, compared to that of LPS-treated group.

(3) Phagocytosis assay

Phagocytosis is a major mechanism of cellular protection and manifestation of inflammatory and immunological responsiveness (Suganuma et al., 2011). Based on

the above results, the effect of MP-1, MP-3 and MP-3 on phagocytic activity of macrophages was investigated. Fig 6-6 shows the phagocytosis effect of MP-1, MP-3 and MP-3. All polysaccharides have phagocytosis effect on macrophages in a dose-dependent manner. MP-3 shows the best phagocytosis effect on macrophages among the three polysaccharides.

(4) Protective activity

DOX is a cytotoxic anthracycline antibiotic isolated from the cultures of *Streptomyces peucetius* (Lee et al., 2008). Animal and human cancer cells treated with DOX have been shown to manifest the characteristic morphologic changes associated with apoptosis or programmed cell death (Weiss, 1992). Despite its effectiveness, it has a wide range of toxic side effects, many of which most likely result from its inherent pro-oxidant activity (Tangpong et al., 2011). Treatment with DOX could result in a decrease of cancer cell as well as macrophage survival rate. To confirm the effects of purified polysaccharides on the protective activity on macrophages, DOX was added to the cells for 24 h. Fig. 6-7 shows the effect of purified polysaccharides on DOX-induced macrophage RAW 264.7 cells survival. It indicates that the three kind of purified polysaccharides have potential protective activity on RAW 264.7 in a dose-dependent and they all got to maximum proliferation at concentration of 50 μg/mL. It indicate that they could reduce the reactive oxygen species level in cells thereby mitigate the apoptosis induced by DOX.

6.3.5 Antitumor activity

The tumor growth inhibitory effect of MP-1, MP-3 and MP-4 against HepG2 at the doses of 6.25, 12.5, 25, 50 and 100 μg/mL is summarized in Fig.6-8. The three fractions exhibited relatively strong inhibition effect at all concentration 50 and 100 μg/mL. Especially, MP-1 sample had the highest inhibition ratio of 68.01 ± 2.61% at 50 μg/mL, but the in vivo inhibition ratios of the three fractions decreased slightly at 100 μg/mL, and no obvious dose–dependency relationship was found between concentration of the different fractions and growth inhibition of HepG2 cells. However, it's worth mentioning that MP-3 and MP-4 have similar inhibition ratio on HepG2 cells, which may be contribute to by their similar monosaccharide composition.

6.3.6 Biological activity comparation of crude and purified polysaccharides

Table 6-2 and Table 6-3 show the biological activity of crude and purified polysaccharides. From the data of Table 6-2, it can be seen that crude polysaccharides had better immunomodulatory activity than purified polysaccharides except for the index about nitric oxide production. On the other hand, the inhibition ratio of crude polysaccharides on HepG2 cells was 77.39% at concentration of 100μg/mL while the best inhibition ratio came from MP-1, about 68.01% at concentration of 50μg/mL. Overall, the crude polysaccharides had better biological activity than the purified polysaccharides. As it is known that biological activity are related to the monosaccharide composition, molecular weight, and structure of polysaccharides. Crude polysaccharides have abundant monosaccharide composition and their different

molecular masses will have synergistic enhancement effect on the observed bioactivity, which is probably the most important factor.

6.4 Summary

The crude polysaccharides were purified by filtration, DEAE-SephadexA-50 chromatography and Sephadex LH-20 size-exclusion chromatography in sequence. Three main fractions, MP-1, MP-3 and MP-4 were obtained through the extraction and purification steps. The three purified polysaccharides were found to contain similar monosaccharide composition but at different mol ratio. Furthermore, the influence of polysaccharides fractions upon activation of macrophage cell RAW 264.7 and antitumor activities to HepG2 in vitro were evaluated. The results indicated that the proliferation of MP-3 on RAW 264.7 was 313.57% at 100μg/mL while MP-1 had higher growth inhibition effect on HepG2 cells, about 68.01% at concentration of 50 μg/mL.

Table 6- 1 Monosaccharide composition of polysaccharides fractions (mol %)

Polysaccharides	Glucose	Mannose	Galactose	Arabinose	Xylose	Rhamnose
MP-1	38.06	14.43	17.06	9.25	2.08	0.94
MP-3	27.04	28.66	11.12	9.07	6.71	3.22
MP-4	24.69	20.46	10.22	7.91	4.05	2.83

Table 6- 2 Immunomodulatory activity of crude polysaccharides and purified polysaccharides

Polysaccharides	Immunomodulatory activity (24h)			
	Proliferation of the macrophages (%)	Production of nitric oxide (μM)	Phagocytosis (O.D.540 nm)	Protection (Proliferation %)
Crude polysaccharides	317.8% (50μg/mL)	14.0μM (6.25μg/mL)	0.58 (100μg/mL)	96.0% (50μg/mL)
Purified MP-1	199.9% (100μg/mL)	10.0μM (6.25μg/mL)	0.41 (100μg/mL)	90.6% (50μg/mL)
Purified MP-3	313.57% (100μg/mL)	10.3μM (6.25μg/mL)	0.46 (100μg/mL)	92.3% (50μg/mL)
Purified MP-4	184.99% (25μg/mL)	11.1μM (6.25μg/mL)	0.41 (100μg/mL)	84.1% (50μg/mL)

Table 6- 3 Antitumor activity of crude polysaccharides and purified polysaccharides

Polysaccharides		Antitumor activity (Inhibition %- concentration-time)		
		Hela cells	HepG2 cells	DLD-1 cells
Crude polysaccharides		32.6 0% (200μg/mL 48h)	77.39%(100μg/mL 24h)	37.70%(250μg/mL48h)
Purified	MP-1	____	68.01%(50μg/mL 24h)	____
	MP-3	____	56.37%(100μg/mL24h)	____
	MP-4	____	57.29%(100μg/mL24h)	____

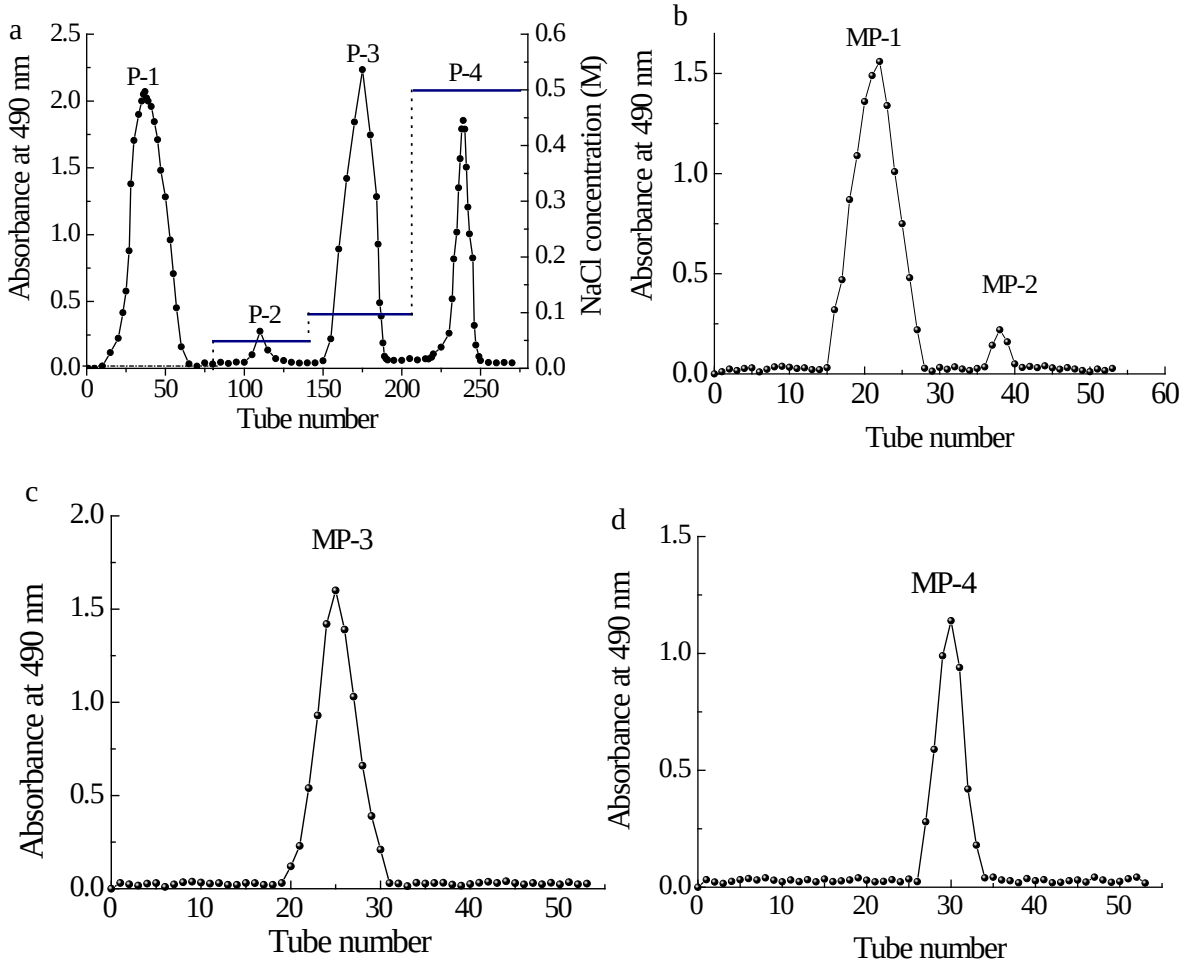

Fig. 6- 1 Stepwise elution curve of crude MPS.

Stepwise elution curve of crude MPS on anion-exchange chromatography column DEAE-SephadexA-50 (a) and elution curve of polysaccharides fractions (MP-1, MP-3 and MP-4) from DEAE-SephadexA-50 on size-exclusion chromatography column of Sephadex LH-20 (b–d).

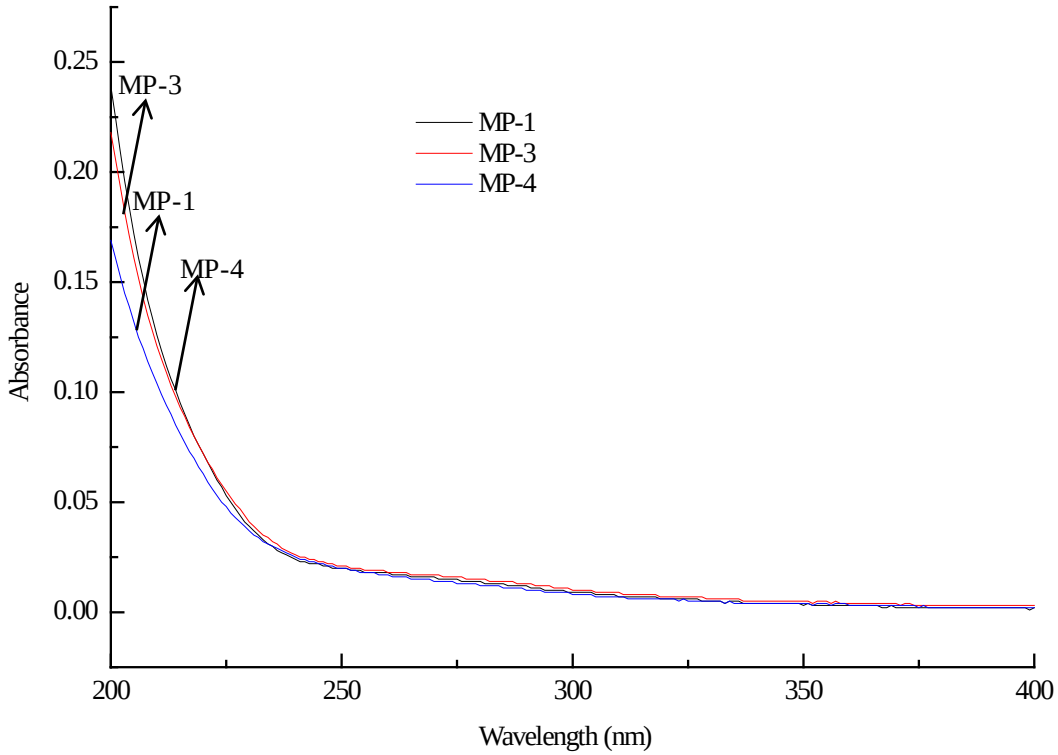

Fig. 6- 2 UV spectra of MP-1, MP-3 and MP-4.

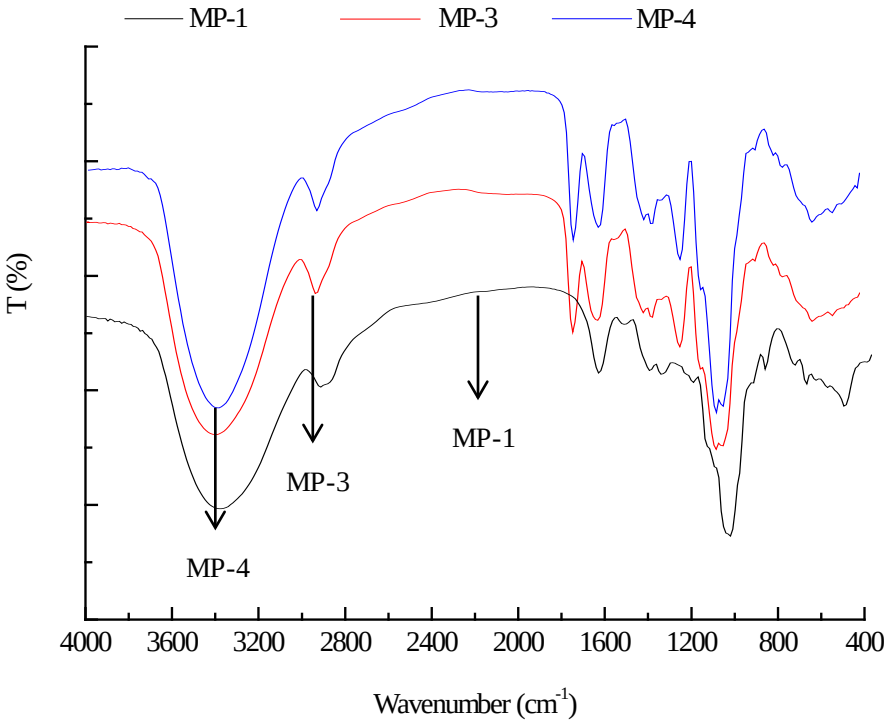

Fig. 6- 3 FTIR spectra of MP-1, MP-3 and MP-4.

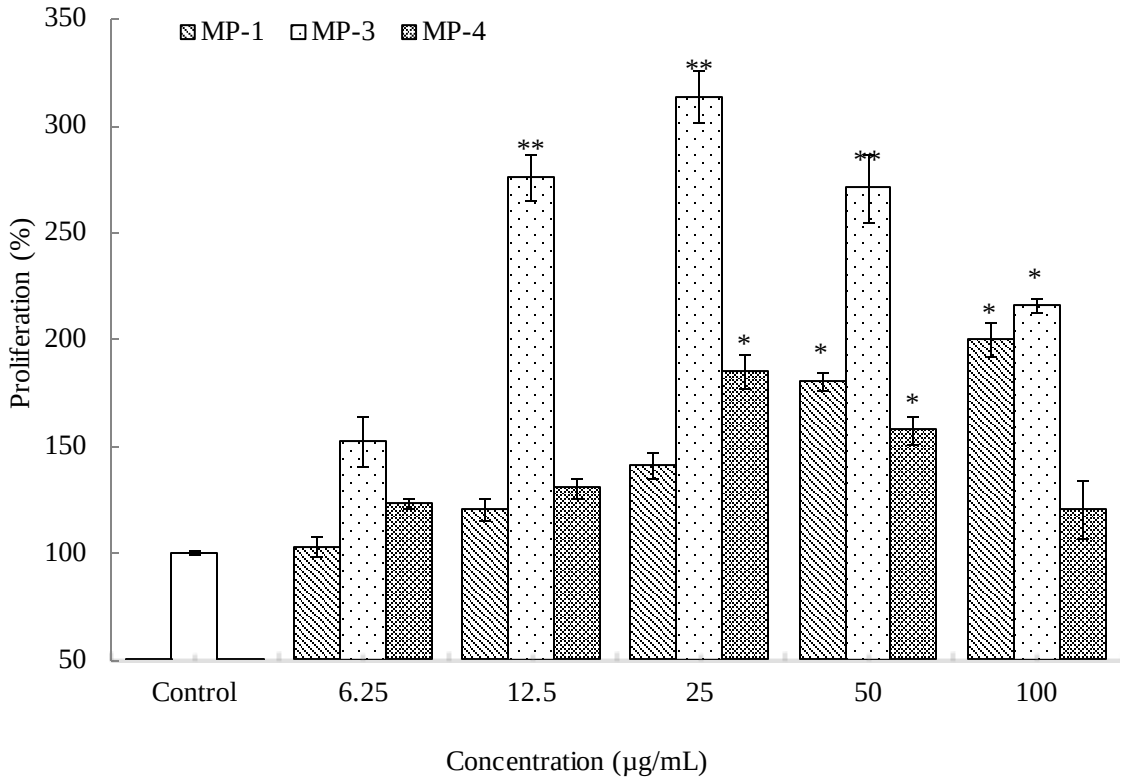

Fig. 6- 4 Proliferation effect of purified polysaccharides on macrophage viability.

* indicates statistically significant difference ($P<0.05$).

** indicates statistically significant difference ($P<0.01$).

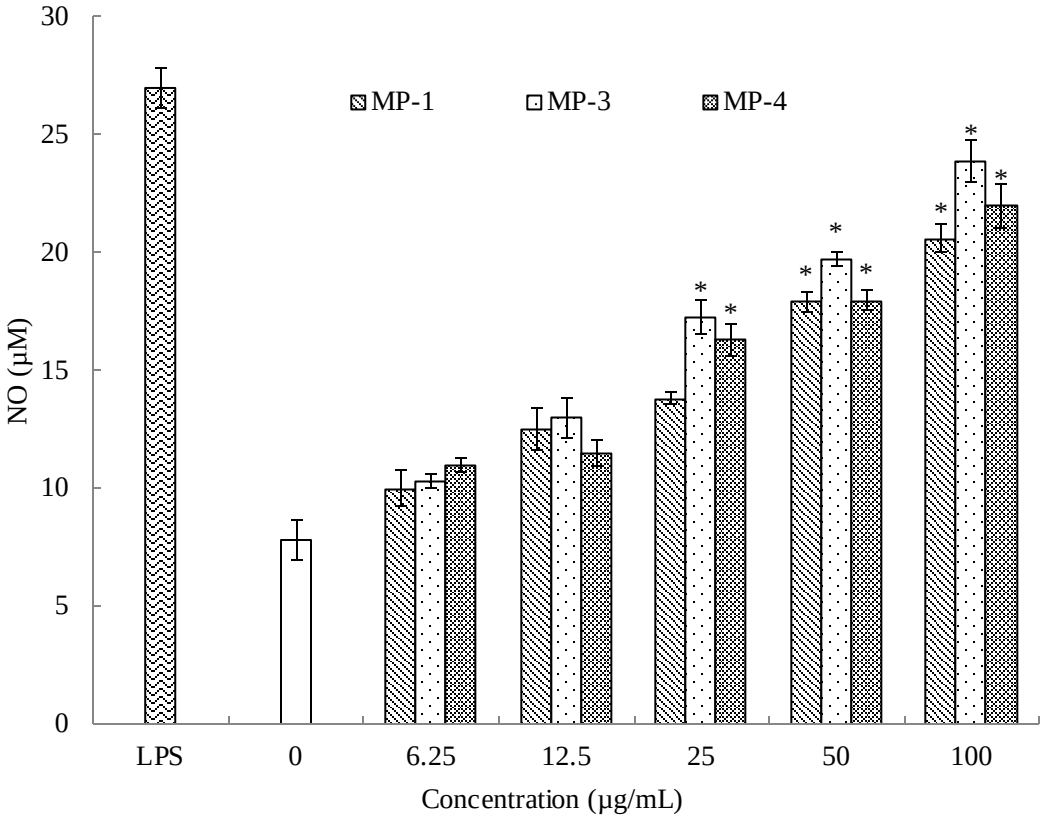

Fig. 6- 5 Effect of MP-1, MP-3 and MP-4 on NO production of macrophage cells.

* indicates statistically significant difference ($P<0.05$).

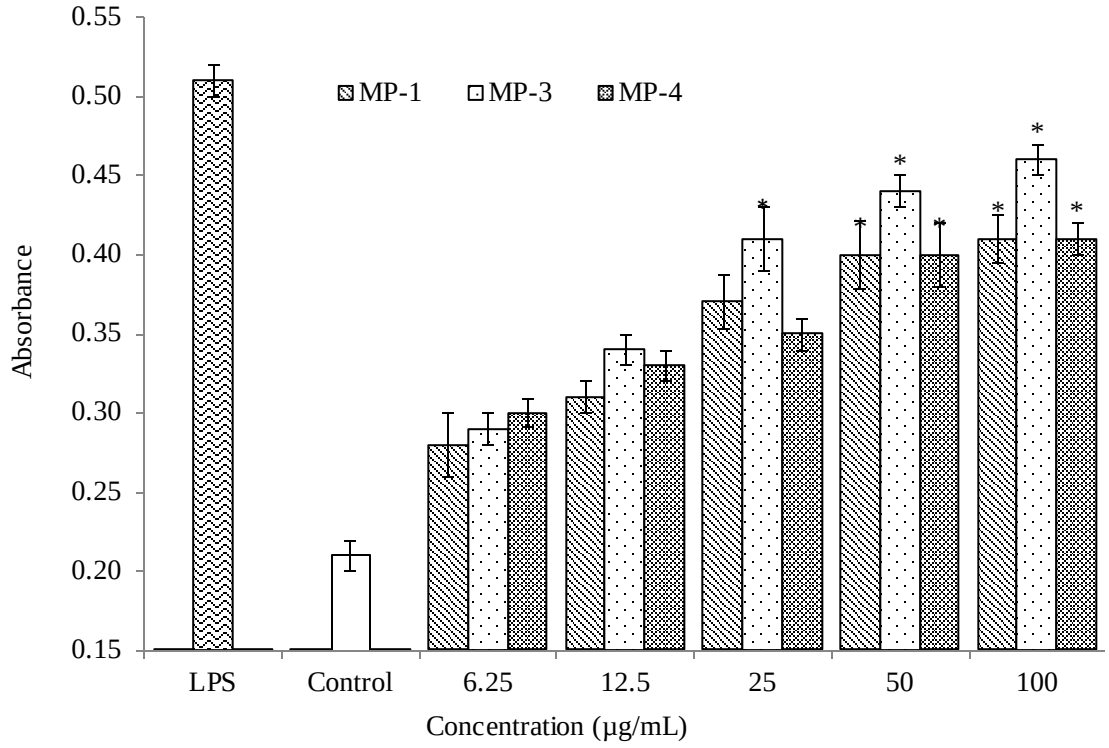

Fig. 6- 6 Effect of MP-1, MP-3 and MP-4 on phagocytosis of macrophage cells (24 h).

* indicates statistically significant difference ($P<0.05$).

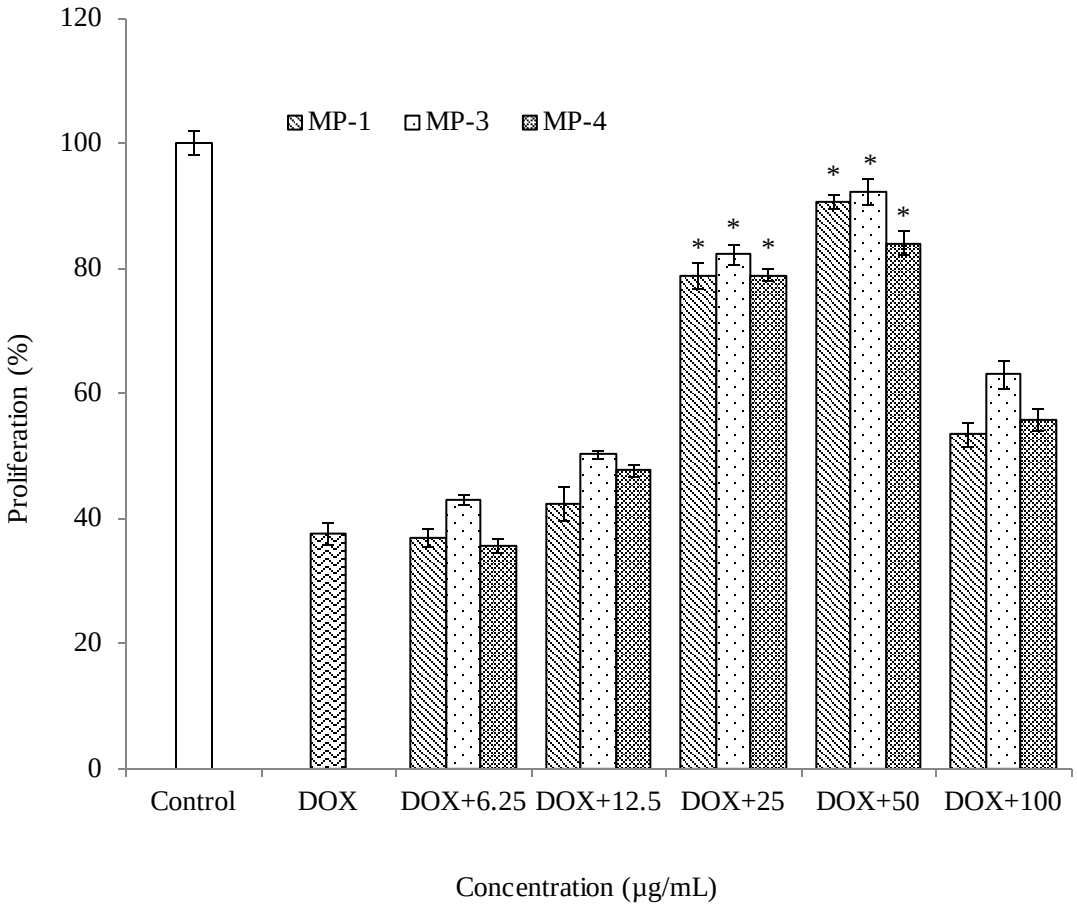

Fig. 6- 7 Effect of MP-1, MP-3 and MP-4 on DOX-induced macrophage cells.

* indicates statistically significant difference ($P<0.05$).

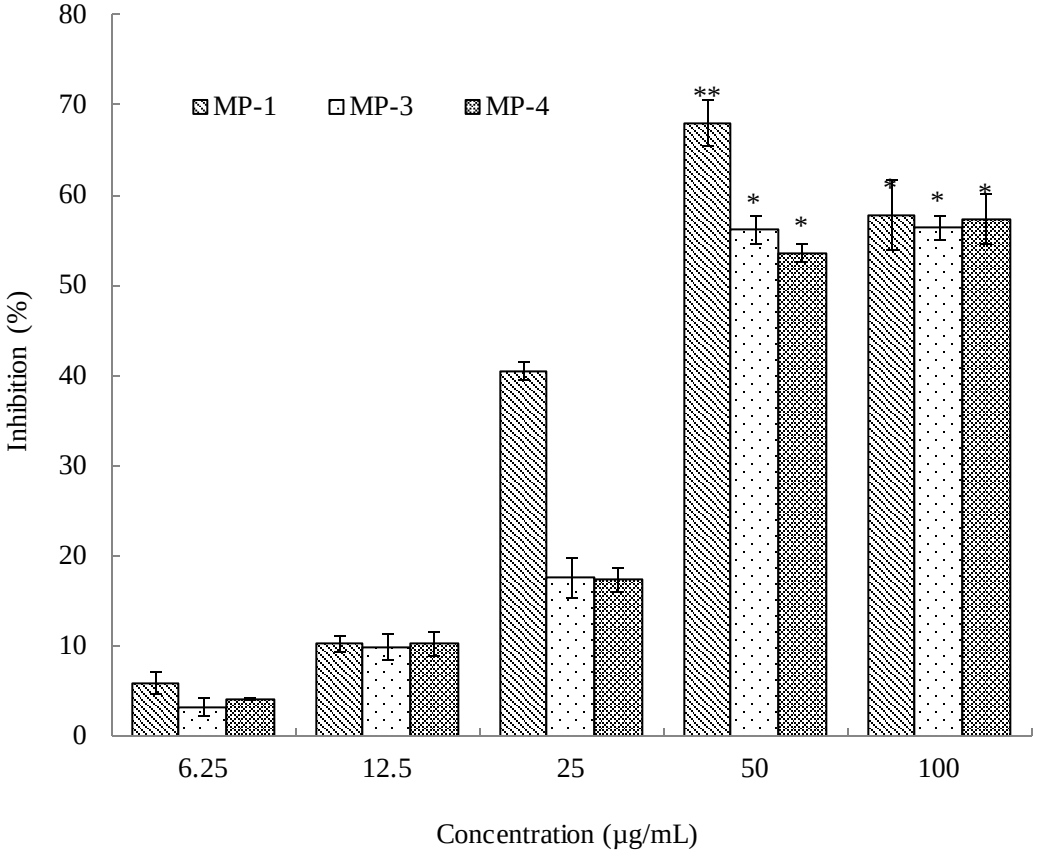

Fig. 6- 8 Inhibition effect of MP-1, MP-2 and MP-3 on HepG 2 cells (24 h).

* indicates statistically significant difference ($P<0.05$).

** indicates statistically significant difference ($P<0.01$).

Chapter 7 Conclusion and future research

In this study, an industrial byproduct, SCR was used as main nutrient media to ferment polysaccharides *by M. esculenta*. The culture media component and fermentation conditions were optimized to improve the polysaccharides production by orthogonal experimental design and response surface methodology, respectively. After fermentation, the physicochemical properties of fermented SCR and unfermented SCR were compared. Furthermore, the biological activity of crude polysaccharides from SCR and purified products were evaluated based on their antioxidant activity, immunomodulatoy and antitumor activity.

7.1 Conclusions

The main conclusions of present work can be summarized as follows:

(1) The culture media component and fermentation condition were optimized to improve the polysaccharides production by orthogonal experimental design and response surface methodology, respectively. The optimal fermentation conditions for the highest MPS production of 95.82±1.37 mg/g were determined as glucose 4.0%, $(NH_4)_2SO_4$ 1.5%, water 75.0% and $MgSO_4 \cdot 7H_2O$ 0.2%, fermentation temperature 22.6°C, fermentation time 21.0 days, and inoculum size 2.67%.

(2) Fermentation led to morphologic change and higher powder hygroscopicity and decomposition temperature. Results also indicated that FSCR showed some advantages over USCR owing to its higher nutrient composition such as free amino acids, polysaccharides and polyphenols, which will be of nutritional importance during the application of the FSCR.

(3) Compared with PS, MPS has different micrographs and higher hygroscopicity as well as dark color. Furthermore, MPS have diverse monosaccharide than PS. Results showed that MPS exhibited stronger scavenging activities against DPPH radical, hydroxyl radical, hydrogen oxide, ABTS radical cation decolorization as well as ferrous chelating effect.

(4) MPS can stimulate macrophage cells and induce considerable NO, phagocytosis and protection effect on the macrophages. In addition, antitumor experiment indicated that MPS exhibited higher inhibition effect on Hela, DLD-1 and HepG2 cells than PS. In particular, the inhibition ratio of MPS reached to 77.39% at the concentration of 100 μg/mL on HepG2 cells.

(5) Three purified polysaccharides were found to have a similar monosaccharide composition. The results indicated that MP-1, MP-3 and MP-4 can increase the proliferation activity of macrophage significantly and play an inhibition effect on cancer cells. Moreover, MP-3 had a higher immunity function on macrophages RAW 264.7 while MP-1 exhibited a higher growth inhibition on HepG2 cells.

The results obtained in this study will provide references for the large-scale production of polysaccharides by *M. esculenta* and point to a new direction for the utilization of SCR. The present study also affords a theoretical foundation for low-cost production of polysaccharides in industrial scale. Furthermore, animal models in vivo of the polysaccharides should be validated to confirm and extend these findings.

7.2 Further research

(1) The roles of purified polysaccharides remain unclear owing to its structure and function although there are some indications that they have high biological activity. Further works are in progress on characterization and structure of the purified polysaccharides, which is of potential research and development value in the field of functional foods.

(2) Results indicate that fermented polysaccharides have functions of anti-immunity, anticancer effect. The mechanisms of anti-immunity, anticancer effect of polysaccharides should be elucidated in detail.

(3) All previous results derived from in vitro while the results may lack sufficiency to convince in vivo applications. To confirm and extend these findings, animal models in vivo is necessary.

(4) Antioxidant material is highly unstable and may be lost during processing and storage. The stability, in uses and in the storage process as well as the storage condition on the biological activity of polysaccharides should be included in further study.

References

Abdullah, M.Z., Guan, L.C., Lim, K.C., Karim, A.A., 2004. The applications of computer vision system and tomographic radar imaging for assessing physical properties of food. Journal of Food Engineering, 61, 125-135.

Adams, M.R., Cooke, R.D., Twiddy, D.R., 1987. Fermentation parameters involved in the production of lactic acid preserved fish-glucose substrates. International Journal of Food Science and Technology, 22, 105-114.

Afshari-Jouybari, H., Farahnaky, A., 2011. Evaluation of Photoshop software potential for food colorimetry. Journal of Food Engineering, 106, 170-175.

Ahmedin, J., Freddie, B., Center, M.M., Jacques, F., Elizabeth, W., David, F., 2011. Global cancer statistics. CA - Cancer Journal for Clinics, 61, 69-90.

Almaraz, J.J., Zhou, X.M., Mabood, F., Madramootoo, C., Rochette, P., Ma, B.L., Smith, D.L., 2009. Greenhouse gas fluxes associated with soybean production under two tillage systems in southwestern Quebec. Soil and Tillage Research, 104, 134-139.

Alves, M.J., Ferreira, I.C., Dias, J., Teixeira, V., Martins, A., Pintado, M.A., 2012. Review on antimicrobial activity of mushroom (Basidiomycetes) extracts and isolated compounds. Planta Medica, 78, 1707-1718.

Ao, T., Cantor, A.H., Pescatore, A.J., Pierce, J.L., Dawson, K.A., 2010. Effects of citric acid, alpha-galactosidase and protease inclusion on in vitro nutrient release

from soybean meal and trypsin inhibitor content in raw whole soybeans. Animal Feed Science and Technology, 162, 58-65.

Arora, S., Jood, S., Khetarpaul, N., 2010. Effect of germination and probiotic fermentation on nutrient composition of barley based food mixtures. Food Chemistry, 119, 779-784.

Asenstorfer, R.E., Hayasaka, Y., Jones, G.P., 2001. Isolation and structures of oligomeric wine pigments by bisulphite mediated ion-exchange chromatography. Journal of Agricultural and Food Chemistry, 49, 5957-5963.

Association of Official Analytical Chemists (AOAC), 1998. Official methods of analysis, (16th ed.). Virginia: Arlington.

Babu, B.H., Shylesh, B.S., Padikkala, J., 2001. Antioxidant and hepatoprotective effect of *Alanthus icicifocus*. Fitoterapia, 72, 272-277.

Bisaria, V.S., 1998. Bioprocessing of agro-residues to value added products, In Martin, A.M. (ed.), Bioconversion of waste materials to industrial products. Chapman & Hall, U.K. 197-246.

Blois, M.S., 2002. Antioxidant determinations by the use of a stable free radical. Nature, 26, 1199-1200.

Bosaeus, I., 2004. Fibre effects on intestinal functions (diarrhoea, constipation and irritable bowel syndrome). Clinical Nutrition, Supplement, 1, 33-38.

Bourne, M.C., Clemente, M.G., Banzon, J., 1976. Survey of the suitability of thirty cultivars of soybeans for soymilk manufacture. Journal of Food Science, 41, 1204-1208.

Bourquin, L.D., Titgemeyer, E.C., Fahey, G.C.Jr., 1993. Vegetable fiber fermentation by human fecal bacteria: cell wall polysaccharide disappearance and short-chain fatty acid production during in vitro fermentation and water-holding capacity of unfermented residues. Journal of Nutrition, 123, 860-869.

Cai, Y.Z., Corke, H., 2000. Production and properties of spray-dried Amaranthus betacyanin pigments. Journal of Food Science, 65, 1248-1252.

Champagne, P., 2007. Feasibility of producing bio-ethanol from waste residues: A Canadian perspective Feasibility of producing bio-ethanol from waste residues in Canada. Resources, Conservation and Recycling, 50, 211-230.

Chen, G., Ma, X., Liu, S., Liao, Y., Zhao, G., 2012. Isolation, purification and antioxidant activities of polysaccharides from *Grifola frondosa*. Carbohydrate Polymers, 89, 61-66.

Chen, H.X., Zhang, M., Qu, Z.S., Xie, B.J., 2008. Antioxidant activities of different fractions of polysaccharide conjugates from green tea (*Camellia Sinensis*). Food Chemistry, 106, 559-563.

Chen, X., Zhong, H.Y., Zeng, J.H., Ge, J., 2008. The pharmacological effect of polysaccharides from *Lentinus edodes* on the oxidative status and expression of

VCAM-1m RNA of thoracic aorta endothelial cell in high-fat-diet rats. Carbohydrate Polymers, 74, 445-450.

Chen, Y., Xie, M.Y., Nie, S.P., Li, C., Wang, Y.X., 2008. Purification, composition analysis and antioxidant activity of a polysaccharide from the fruiting bodies of *Ganoderma atrum*. Food Chemistry, 107, 231-241.

Cheng, Y., Shimizu, N., Klmura, T., 2005. The viscoelastic properties of soybean curd (tofu) as affected by soymilk concentration and type of coagulant. International Journal of Food Science and Technology, 40, 385-390.

Choi, K.T., 2008. Botanical characteristics, pharmacological effects and medicinal components of Korean Panax ginseng C A Meyer. Acta Pharmacologica Sinica, 29, 1109-1118.

Chung, I.M., Seo, S.H., Ahn, J.K., Kim, S.H., 2011. Effect of processing, fermentation, and aging treatment to content and profile of phenolic compounds in soybean seed, soy curd and soy paste. Food Chemistry, 127, 960-967.

Cizeikiene, D., Juodeikiene, G., Paskevicius, A., Bartkiene, E., 2013. Antimicrobial activity of lactic acid bacteria against pathogenic and spoilage microorganism isolated from food and their control in wheat bread. Food Control, 31, 539-545.

Dai, Z., Zhang, H., Zhang, Y., Wang, H., 2009. Chemical properties and immunostimulatory activity of a water-soluble polysaccharide from the clam of *Hyriopsiscumingii Lea*. Carbohydrate Polymers, 77, 365-369.

Dajanta, K., Apichartsrangkoon, A., Chukeatirote, E., Frazier, R.A., 2011. Free-amino acid proiles of thua nao, a thai fermented soybean, Food Chemistry, 125, 342-347.

Dell'Abate, M.T., Benedetti, A., Trinchera, A., Dazzi, C., 2002. Humic substances along the profile of two Typic Haploxerert. Geoderma, 107, 281-296.

Demleimer, S., Krous, J., Franz, G., 1992. Synthesis and antitumour activity of derivatives of curdlan and lichenan branched at C-6. Carbohydrate Research, 226, 239-246.

Dias, P.F., Siqueira, J.M., Vendruscolo, L.F., Neiva, T.J., Gagliardi, A.R., Maraschin, M., Ribeiro-do-Valle, R.M., 2005. Antiangiogenic and antitumoral properties of a polysaccharide isolated from the seaweed Sargassum stenophyllum. Cancer Chemotherapy and Pharmacology, 56, 436-446.

Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., Smith, F., 1956. Colorimetric method for determination of sugars and related substances. Analytical Chemistry, 28, 350-356.

Dursun, N., Musa, M.Ö., G.ıyasettin, K., Celaleddin, Ö., 2006. Mineral contents of 34 species of edible mushrooms growing wild in Turkey. Journal of the Science of Food and Agriculture, 86, 1087-1094.

Elmastas, M., Turkekul, I., Ozturk, L., Gulcin, I., Isildak, O., Aboul-Enein, H.Y., 2006. Antioxidant activity of two wild edible mushrooms (*Morchella vulgaris*

and *Morchellaesculanta*) from North Turkey. Combinatorial Chemistry and High Throughput Screening, 9, 443-448.

Fafaungwithayakul, N., Hongsprabhas, P., Hongsprabhas, P., 2011. Effect of soy soluble polysaccharide on the stability of soy-stabilised emulsions during in vitro protein digestion. Food Biophysics, 6, 407-415.

Falade, K.O., Okafor, C.A., 2013. Physicochemical properties of five cocoyam (*Colocasia esculenta* and *Xanthosoma sagittifolium*) starches. Food Hydrocolloids, 30, 173 -181.

Fernández, L., Castillero, C., Aguilera, J.M., 2005. An application of image analysis to dehydration of apple discs. Journal of Food Engineering, 67, 185-193.

Food and Agriculture Organization of the United Nations, 2009; Available from: http://faostat.fao.org/site/339/default.aspx. [accessed on Nov. 9, 2012].

Gao, J.F., Wang, J.H., Yang, C., Wang, S.Y., Peng, Y.Z., 2011. Binary biosorption of Acid Red 14 and Reactive Red 15 onto acid treated okara: Simultaneous spectrophotometric determination of two dyes using partial least squares regression. Chemical Engineering Journal, 171, 967-975.

Ge, Y., Duan, Y., Fang, G., Wang, S., 2009. Polysaccharides from fruit calyx of *Physalis alkekengivar*. Francheti: Isolation, purification, structural features and antioxidant activities. Carbohydrate Polymers, 77, 188-193.

Glasel, J.A., 1995. Validity of nucleic acid purities monitored by 260 nm/280 nm

absorbance ratios. Biotechniques, 18, 62-63.

Goldfarb, A.R., Saidel, L.J., Mosovich, E., 1951. The ultraviolet absorption spectra of proteins. Journal of Biological Chemistry, 193, 397-404.

Goodarzi, N., Varshochian, R., Kamalinia, G., Atyabi, F., Dinarvand, R., 2013. A review of polysaccharide cytotoxic drug conjugates for cancer therapy. Carbohydrate Polymers, 92, 1280-1293.

Gopinath, V.K., Musa, M., Samsudin, A.R., Sosroseno, W., 2006. Role of interleukin-1 beta and tumour necrosis factor-alpha on hydroxyapatite-induced phagocytosis by murine macrophages (RAW 264.7 cells). British Journal of Biomedical Science, 63, 176-178.

Guermani, L., Villaume, C., Bau, H.W., Chandrasiri, V., Nicolas, J.P., Mejean, L., 1992. Composition and nutritional value of okara fermented by Rhizopus oligosporus. Science Aliment, 12, 441-451.

Guillen, M.D., Cabo, N., 2000. Some of the most significant changes in the Fourier transform infrared spectra of edible oils under oxidative conditions, Journal of Science, Food and Agriculture, 80, 2028-2036.

Guillon, F., Renard, C., Hospers, J., Thibault, J.F., Barry, J. L., 1995. Characterisation of residual fibres from fermentation of pea and apple fibres by human colonic bacteria. Journal of the Science of Food and Agriculture, 68, 521-529.

Gulcin, I., 2006. Antioxidant and antiradical activities of L-carnitine. Life Science, 78,

803-811.

Guo, X., He, X., Zhang, H., Deng, Y., Chen, L., Jiang, J., 2012. Characterization of dissolved organic matter extracted from fermentation effluent of swine manure slurry using spectroscopic techniques and parallel factor analysis (PARAFAC). Microchemical Journal, 102, 115-122.

Gursoy, N., Sarikurkcu, C., Cengiz, M., Solak, M.H., 2009. Antioxidant activities, metal contents, total phenolics and flavonoids of seven *Morchella* species, Food and Chemical Toxicology, 47, 2381-2388.

Hattori, T., Ohishi, H., Yokota, T., Ohoami, H., Watanabe, K., 1995. Antioxidative effect of crude antioxidant preparation from soybean fermented by Bacillus natto. LWT - Food Science and Technology, 28, 135-138.

Hayashi, S., Matsuzaki, K., Kawahara, T., Takasaki, Y., Imada, K., 1992. Utilisation of soybean residue for the production of fl-fructofuranosidase. Bioresource technology, 41, 231-233.

He, J.Z., Ru, Q.M., Dong, D.D., Sun, P.L., 2012. Chemical characteristics and antioxidant properties of crude water soluble polysaccharides from four common edible mushrooms. Molecules, 17, 4373-4387.

Heleno, S.A., Stojković, D., Barros, L., Glamočlija, J., Soković, M., Martins, A., Queiroz, M.J.R.P., Ferreira, I.C.F.R., 2013. A comparative study of chemical

composition, antioxidant and antimicrobial properties of *Morchella esculenta* (L.) Pers. from Portugal and Serbia, Food Research International, 51, 236-243.

Hinks, C.F., Hupka, D., 1995. The effects of feeding leaf sap from oats and wheat, with and without soybean trypsin inhibitor, on feeding behaviour and digestive physiology of adult males of *Melanoplus sanguinipes*. Insect Physiological, 41, 1007-1015.

Hölker, U., Juörgen, L., 2005. Solid-state fermentation-are there any biotechnological advantages. Current Opinion in Microbiology, 8, 301-306.

Hsieh, C., Yang, F.C., 2004. Reusing soy residue for the solid-state fermentation of *Ganoderma lucidum*. Bioresource Technology, 91,105-109.

Hu, J.L., Nie, S.P., Li, C., Xie, M.Y., 2013. In vitro fermentation of polysaccharide from the seeds of *Plantago asiatica L*. by human fecal microbiota. Food Hydrocolloids, 33, 384-392.

Huang, S.S., Huang, G.J., Ho, Y.L., Lin, Y.H., Hung, H.J., Chang, T.N., 2008. Antioxidant and antiproliferative activities of the four Hydrocotyle species from Taiwan. Botanical Studies, 49, 311-322.

Hung, W.T., Wang, S.H., Chen, C.H., Yang, W.B., 2008. Structure determination of β -Glucans from *Ganoderma lucidum* with Matrix-assisted Laser Desorption/ionization (MALDI) Mass Spectrometry. Molecules, 13, 1538-1550.

Huxtable, R.J., 1992. The physiological actions of taurine. Physiological Reviews, 72, 101-163.

Jenkins, D.J.A., Kendall, C.W.C., Augustin, L.S.A., Franceschi, S., Hamidi, M., Marchie, A., Jenkins, A.L., Axelsen, M., 2003. Glycemic index: overview of implications in health and disease. The American Journal of Clinical Nutrition, 76, 266-273.

Jin, M., Lu, Z., Huang, M., Wang, Y., Wang, Y., 2011. Sulfated modification and antioxidant activity of exopolysaccahrides produced by *Enterobacter cloacae* Z0206. International Journal of Biological Macromolecules, 48, 607-612.

Jin, M., Zhao, K., Huang, Q., Xu, C., Shang, P., 2012. Isolation, structure and bioactivities of the polysaccharides from *Angelica sinensis (Oliv.)* Diels: A review. Carbohydrate Polymers, 89, 713-722.

Jin, M.L., Lu, Z.Q., Huang, M., Wang, Y.M., Wang, Y.Z., 2011. Sulfated modification and antioxidant activity of exopolysaccahrides produced by *Enterobacter cloacae* Z0206. International Journal of Biological Macromolecules, 48, 607-612.

Kang, S.M., Kim, K.N., Lee, S.H., Ahn, G., Cha, S.H., Kim, A.D., Yang, X.D., Kang, M.C., Jeon, Y.J., 2011. Anti-inflammatory activity of polysaccharide purified from AMG-assistant extract of *Ecklonia cava* in LPS-stimulated RAW 264.7 macrophages. Carbohydrate Polymers, 85, 80-85.

Katalinic, V., Milos, M., Kulisic, T., Jukic, M., 2006. Screening of 70 medical plant extracts for antioxidant capacity and total phenols. Food Chemistry, 94, 550-557.

Katsiari, C.G., Liossis, S.C., Sfikakis, P.P., 2010. The pathophysiologic role of monocytes and macrophages in systemic lupus erythematosus: A reappraisal. Seminars in Arthritis and Rheumatism, 39, 491-503.

Katsuhiko, M., Ryohei, A., Atsushi, A., 2006. Effect of pretreatment on lactic acid fermentation of bean curd refuse with simultaneous saccharification. Studies in Surface Science and Catalysis, 159, 133-136.

Katsuhiko, M., Takeshi, M., Tadasi, W., 2001. Methane fermentation of bean curd refuse. Journal of Bioscience and Bioengineering, 91, 208-212.

Ke, C.L., Qiao, D.L., Gan, D., Sun, Y., Ye, H., Zeng, X.X., 2009. Antioxidant activity in vitro and in vivo of the capsule polysaccharides from Streptococcus equi subsp. zooepidemicus. Carbohydrate Polymer, 275, 677-682.

Khare, S.K., Jha, K., Gandhi, A.P., 1995. Citric acid production from Okara (soy-residue) by solid-state fermentation. Bioresource Technology, 54, 323-325.

Kim, H.S., Kim, Y.J., Lee, H.K., Ryu, H.S., Kim, J.S., Yoon, M.J., Kang, J.S., Hong, J.T., Kim, Y., Han, S.B., 2012. Activation of macrophages by polysaccharide isolated from *Paecilomyces cicadae* through toll-like receptor 4. Food and Chemical Toxicology, 50, 3190-3197.

Klots, T.D., Chirico, R.D., Steele, W.V., 1994. Complete vapor phase assignment for the fundamental vibrations of furan, pyrrole and thiophene. Spectrochimica Acta, 50, 765-795.

Knasmuller, S., Mersch-Sundermann, V., Kevekordes, S., Darroudi, F., Huber, W.W., Hoelzl, C., et al., 2004. Use of human-derived liver cell lines for the detection of environmental and dietary genotoxicants; current state of knowledge. Toxicology, 198, 315-328.

Knasmuller, S., Parzefall, W., Sanyal, R., Ecker, S., Schwab, C., Uhl, M., et al., 1998. Use of metabolically competent human hepatoma cells for the detection of mutagens and antimutagens. Mutation Research, 402, 185-202.

Korhonen, R., Lahti, A., Kankaanranta, H., Moilanen, E., 2005. Nitric oxide production and signaling in inflammation. Current Drug Targets Inflammation Allergy, 41, 471-479.

Kozarski, M., Klaus, A., Niksˇic, M., Vrvic′, M.M., Todorovic′, N., Jakovljevic′, D., Van Griensven, L.J.L.D., 2012. Antioxidative activities and chemical characterization of polysaccharide extracts from the widely used mushrooms *Ganoderma applanatum*, *Ganoderma lucidum*, *Lentinus edodes* and *Trametes versicolor*. Journal of Food Composition and Analysis, 26, 144-153.

Lee, J.W., Sung, N.Y., Kim, J.K., Kim, J.H., Raghavendran, H.R.B., Yoo, Y.C., Shin, M.H., Byun, M.W., 2008. Effect of gamma irradiation on spleen cell

function and cytotoxicity of doxorubicin. Chemico-Biological Interactions, 173, 205-214.

Leinweber, P., Schulten, H.R., 1992. Differential thermal analysis, thermogravimetry and in-source pyrolysis-mass spectrometry studies on the formation of soil organic matter. Thermochimica Acta, 200, 151-167.

Lim, T.S., Na, K., Choi, E.M., Chung, J.Y., Hwang, J.K., 2004. Immunomodulating activities of polysaccharides isolated from Panax ginseng. Journal of Medicinal Food, 7, 1-6.

Litchfteld, J.H., Vely, V.G., Overbeck, R.C., 2006. Nutrient content of morel mushroom mycelium: amino acid composition of the protein. Journal of Food Science, 28, 741-743.

Ma, L., Chen, H., Zhu, W., Wang, Z., 2013. Effect of different drying methods on physicochemical properties and antioxidant activities of polysaccharides extracted from mushroom *Inonotus obliquus*. Food Research International, 50, 633-640.

Mano, J.F., Koniarova, D., Reis, R.L., 2003. Thermal properties of thermoplastic starch/synthetic polymer blends with potential biomedical applicability. Journal of Materials Science: Materials in Medicine, 2003, 14, 127-135.

Maskan, M., 2001. Kinetics of color change of kiwifruits during hot air and microwave drying. Journal of Food Engineering, 48, 169-175.

Masuda, Y., Matsumoto, A., Toida, T., Oikawa, T., Ito, K., Nanba, H., 2009. Characterization and antitumor effect of a novel polysaccharide from Grifola frondosa. Journal of Agricultural and Food Chemistry, 57, 10143-10149.

Mateos-Aparicio, I., Mateos-Peinado, C., Jimenez-Escrig, A., Rupérez, P., 2010. Multifunctional antioxidant activity of polysaccharide fractions from the soybean byproduct okara. Carbohydrate Polymers, 82, 245-250.

Mateos-Aparicio, I., Mateos-Peinado, C., Rupérez, P., 2010b. High hydrostatic pressure improves the functionality of dietary fibre in okara by-product from soybean. Innovative Food Science and Emerging Technologies, 11, 445-450.

Mateos-Aparicio, I., Redondo-Cuenca, A., Villanueva-Suárez, M.J., 2010c. Isolation and characterisation of cell wall polysaccharides from legume by-products: okara (soymilk residue), pea pod and broad bean pod. Food Chemistry, 122, 339-345.

Mau, J.L., Chang, C.N., Huang, S.J., Chen, C.C., 2004. Antioxidant properties of methanolic extracts from *Grifola frondosa, Morchella esculenta* and *Termitomyces albuminosus* mycelia. Food Chemistry, 87, 111-118.

Mecozzia, M., Pietroletti, M., Tornambè, A., 2011. Molecular and structural characteristics in toxic algae cultures of Ostreopsis ovata and Ostreopsis spp. evidenced by FTIR and FTNIR spectroscopy. Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy, 78, 1572-1580.

Mendoza, F., Dejmek, P., Aguilera. J.M., 2006. Calibrated color measurements of

agricultural foods using image analysis. Postharvest Biology and Technology, 41, 285-295.

Meng, F., Liu, X., Jia, L., Song, Z., Deng, P., Fan, K., 2010. Optimization for the production of exopolysaccharides from *Morchella esculenta* SO-02 in submerged culture and its antioxidant activities in vitro. Carbohydrate Polymer, 79, 700-704.

Meng, F., Zhou, B., Lin, R., Jia, L., Liu, X., Deng, P., Fan, K., Wang, G., Wang, L., Zhang, J., 2010. Extraction optimization and in vivo antioxidant activities of exopolysaccharide by *Morchella esculenta SO-01*. Bioresource Technology, 101, 4564-4569.

Mersch-Sundermann, V., Knasmuller, S., Wu, X.J., Darroudi, F., Kassie, F., 2004. Use of a human-derived liver cell line for the detection of cytoprotective, antigenotoxic and cogenotoxic agents. Toxicology, 198, 329-340.

Mitchell, M.S., 2003. Immunotherapy as part of combinations for the treatment of cancer. International Immunopharmacology, 3, 1051-1059.

Muroyama, K., Atsumi, R., Andoh, A., 2006. Effect of pretreatment on lactic acid fermentation of bean curd refuse with simultaneous saccharification. Studies in Surface Science and Catalysis, 159, 133-136.

Muroyama, K., Mochizuki, T., Wakamura, T., 2001. Methane fermentation of bean curd refuse. Journal of Bioscience and Bioengineering, 91, 208-212.

Nauts, H.C., Swift, W.E., Coley, B.L., 1946. The treatment of malignant tumors by bacterial toxins as developed by the late William B. Coley, reviewed in the light of modern research. Cancer Research, 6, 205-214.

Ni, Y., Turner, D., Yates, K.M., Tizard, I., 2004. Isolation and characterization of structural components of Aloe vera L. leaf pulp. International Immunopharmacology, 1745-1755.

Nicholas, S., Cumbes, Q.J., 1989. Hydroxyl radical scavenging activity of compatible solutes. Phytochemistry, 28, 1057-1060.

Nie, S.P., Xie, M.Y., 2011. A review on the isolation and structure of tea polysaccharides and their bioactivities. Food Hydrocolloids, 25, 144-149.

Nitha, B., Fijesh, P.V., Janardhanan, K.K., 2013. Hepatoprotective activity of cultured mycelium of Morel mushroom, *Morchella esculenta*. Experimental and Toxicologic Pathology, 65, 105-112.

Nitha, B., Janardhanan, K.K., 2008. Aqueous-ethanolic extract of morel mushroom mycelium *Morchella esculenta*, protects cisplatin and gentamicin induced nephrotoxicity in mice. Food Chemistry Toxicology, 46, 3193-3199,

Nout, M.J.R., Kiers, J.L., 2005. Tempe fermentation, innovation and functionality: update into the third millennium. Journal of Applied Microbiology, 98, 789-805.

O'Toole, D.K., 1999. Characteristics and use of okara, the soybean residue from soy milk production-A review. Journal of Agricultural and Food Chemistry, 47, 363-371.

Obeta, J.A.N., 1983. A note on microorganisms associated with the fermentation of African locust bean (*Parkia filicoidea*) during iru preparation. Plant Food for Human Nutrition, 3, 245-250.

Ooi, V.E., Liu, F., 2000. Immunomodulation and anticancer activity of polysaccharide-protein complexes. Current Medicinal Chemistry, 7, 715-729.

Pandey, K.B., Rizvi, S.I., 2009. Current understanding of dietary polyphenols and their role in health and disease. Current Nutrition and Food Science, 5, 249-263.

Peng, K., Lv, S.L., Wang, X.Y., Sun, L.J., Yu, J.W., 2012. Polyporus umbellatus polysaccharides ameliorates carbon tetrachloride-induced hepatic injury in mice. African Journal of Pharmacy and Pharmacology, 37, 2686-2691.

Periago, M.J., Ros, G., Rinc ´on, F., Mart´ınez, C., 1997. Nutritional meaning of dietary fibre and phytic acid in meat-based homogenised weaning foods, Food Research International, 30, 223-230.

Pielesz, A., Biniaś, W., 2010. Cellulose acetate membrane electrophoresis and FTIR spectroscopy as methods of identifying a fucoidan in Fucusvesiculosus Linnaeus. Carbohydrate Research, 345, 2676-2682.

Porterfield, J.Z., Zlotnick, A., 2010. A simple and general method for determining the

protein and nucleic acid content of viruses by UV absorbance. Virology, 407, 281-288.

Qi, H.M., Zhang, Q.B., Zhao, T.T., Chen, R., Zhang, H., Niu, X.Z., Li, Z.E., 2005. Antioxidant activity of different sulfate content derivatives of polysaccharide extracted from Ulva pertusa (Chlorophyta) in vitro. International Journal of Biological Macromolecules, 37, 195-199.

Quettier-Deleu, C., Gressier, B., Vasseur, J., Dine, T., Brunet, C., Luyckx, M., Cazinc, M., Cazinc, J.C., Bailleulb, F., Trotin, F., 2000. Phenolic compounds and antioxidant activities of buckwheat (*Fagopyrum esculentum Moench*) hulls and flour. Journal of Ethnopharmacology, 72, 35-42.

Rashad, M.M., Mahmoud, E.A., Abdou, H.M., Nooman, M.U., 2011. Improvement of nutritional quality and antioxidant activities of yeast fermented soybean curd residue. African Journal of Biotechnology, 10, 5504-5513.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., Rice-Evans, C., 1999. Antioxidant activity applying an improved ABTS radical cation decolorisation assay. Free Radical Biological and Medicine, 26, 1231-1237.

Redondo-Cuenca, A., Villanueva-Suárez, M. J., Mateos-Aparicio, I., 2008. Soybean seeds and its by-product okara as sources of dietary fibre. Measurement by AOAC and Englyst methods. Food Chemistry, 108, 1099-1105.

Rey, F.J., Ramos-Sánchez, M.C., Rodríguez, M.L., Martín-Gil, J., Martín-Gil, F.J., 1988. DTG and DTA studies on sugar derivatives. Thermochimica Acta, 134, 67-72.

Rinaldi, V.E.A., Ng, P.K.W., Bennink, M.R., 2000. Effects of extrusion on dietary fiber and isolavone contents of wheat extrucdates enriched with wet okara. Cereal Chemistry, 77, 237-240.

Rotaoll, N., Dunkel, A., Hofmann, T., 2005. Activity-guided identification of (S)-malic acid 1-O-D-glucopyranoside (morelid) and gamma-aminobutyric acid as contributors to umami taste and mouth-drying oral sensation of morel mushrooms (*Morchella deliciosa Fr.*). Journal of Agricultural and Food Chemistry, 53, 4149-4156.

Rout, D., Mondal, S., Chakraborty, I., Pramanik, M., Islam, S.S., 2005. Chemical analysis of a new (1-3)-, (1-6)-branched glucan from an edible mushroom, Pleurotus florida. Carbohydrate Research, 340, 2533-2539.

Roy, S.K., Das, D., Mondal, S., Maiti, D., Bhunia, B., Maiti, T.K., Islam, S.S., 2009. Structural studies of an immunoenhancing water-soluble glucan isolated from hot water extract of an edible mushroom, Pleurotus florida, cultivar. Assam Florida Carbohydrate Research, 344, 2596-2601.

Saiga, A., Tanabe, S., Nishimura, T., 2003. Antioxidant activity of peptides obtained from porcine myofibrillar proteins by protease treatment. Journal of Agricultural and Food Chemistry, 51, 3661-3667.

Schepetkin, I.A., Quinn, M.T., 2006. Botanical polysaccharides: Macrophage immunemodulation and therapeutic potential. International Immunopharmacology, 6, 317-333.

Schieber, A., Stintzing, F.C., Carle, R., 2001. By-products of plant food processing as a source of functional compounds-recent developments. Trends in Food Science and Technology, 12, 401-413.

Sekkal, M., Legrand, P., 1993. A spectroscopic investigation of the carrageenans and agar in the 1500-100 cm^{-1} spectral range. Spectrochimica Acta Part A: Molecular Spectroscopy, 49, 209-221.

Sheu, S.C., Lyu, Y., Lee, M.S., Cheng, J.H., 2013. Immunomodulatory effects of polysaccharides isolated from *Hericiumerinaceus* on dendritic cells. Process Biochemistry, 48, 1402-1408.

Shi, M., Yang, Y., Guan, D., Wang, Y., Zhang, Z., 2012. Bioactivity of the crude polysaccharides from fermented soybean curd residue by *Flammulina velutipes*. Carbohydrate Polymers, 89, 1268-1276.

Shi, M., Zhang, Z., Yang, Y., 2013. Antioxidant and immunoregulatory activity of *Ganoderma lucidum* polysaccharide (GLP). Carbohydrate Polymers, 95, 200-206.

Sindhu, S.C., Khetarpaul, N., 2005. Development, acceptability and nutritional evaluation of an indigenous food blend fermented with probiotic organisms. Nutrition and Food Science, 35, 20-27.

Soares, J.R., Dins, T.C.P., Cunha, A.P., Almeida, L.M., 1997. Antioxidant activity of some extracts of Thymus zygis. Free Radical Research, 26, 469-478.

Song, G., Du, Q., 2012. Structure characterization and antitumor activity of an α β-glucan polysaccharide from *Auricularia polytricha*. Food Research International, 45, 381-387

Song, G., Du, Q., 2012. Structure characterization and antitumor activity of an α, β-glucan polysaccharide from Auricularia polytricha. Food Research International, 45, 381-387.

Staub, A.M., 1965. Removeal of protein-Sevag method. Methods in Carbohydrate Chemistry, 5, 5-6.

Sturman, J.A., 1993. Taurine in development. Physiological Reviews, 73, 119-148.

Suganuma, H., Fahey, J.W., Bryan, K.E., Healy, Z.R., Talalay, P., 2011. Stimulation of phagocytosis by sulforaphane. Biochemical and Biophysical Research Communications, 405, 146-151.

Surel. O., Couplet, B., 2005. Influence of the dehydration process on active compounds of okara during its fractionation, Journal of the Science of Food and Agriculture, 85, 1343-1349.

Suruga, K., Kato, A., Kadokura1, K., Hiruma, W., Sekino, Y., Buffington, C.A.T., Komatsu, Y., 2007. "Okara" a new preparation of food material with antioxidant activity and dietary fiber from soybean. Soybean and Nutrition, 16, 311-326.

Tangpong, J., Miriyala, S., Noel, T., Sinthupibulyakit, C., Jungsuwadee, P., Clair, D.K.St., 2011. Doxorubicin-induced central nervous system toxicity and protection by xanthone derivative of Garcinia Mangostana. Neuroscience, 175, 292-299.

Tatsuya, N., Takabatake, H., Mizuno, O., Oham, M., 2002. Inhibition of hydrogen fermentation of organic wastes by lactic acid bacteria. International Journal of Hydrogen Energy, 27, 1367-1371.

Tehranifar, A., Selahvarzia, Y., Kharrazia, M., Bakhsh, V.J., 2011. High potential of agro-industrial by-products of pomegranate (Punica granatum L.) as the powerful antifungal and antioxidant substances. Industrial Crops and Products, 34, 1523-1527.

Tian, Y., Xu, X., Xie, Z., Zhao, J., Jin, Z., 2011. Starch retrogradation determined by differential thermal analysis (DTA). Food Hydrocolloids, 25, 1637-1639.

Turhan, S., Temiz, H., Sagir, I., 2007. Utilization of wet okara in low-fat beef patties. Journal of Muscle Foods, 18, 226-235.

Van der Riet, W.B., Wight, A.W., Cilliers, J.J.L., Datel, J.M., 1989. Food chemical investigation of tofu and its byproduct okara. Food Chemistry, 34, 193-202.

Villanueva, M.J., Yokoyama, W.H., Hong, Y.J., Barttley, G.E., P. Rupérez, P., 2011. Effect of high-fat diets supplemented with okara soybean by-product on lipid

proiles of plasma, liver and faeces in Syrian hamsters. Food Chemistry, 124, 72-79.

Wachiraphansakul, S., Devahastin, S., 2007. Drying kinetics and quality of okara dried in a jet spouted bed of sorbent particles. LWT-Food Science and Technology, 40, 207-219.

Wahid, M., Sattar, A., Khan, S., 1988. Composition of wild and cultivated mushrooms of Pakistan. Mushroom Journal for the Tropics, 8, 47-51.

Wang, J.C., Hu, S.H., Su, C.H., Lee, T.M., 2001. Antitumor and immune enhancing activities of polysaccharide from culture broth of *Hericiumspp*. Kaohsiung Journal of Medical Science, 17, 461-467.

Wang, M., Zhu, P., Jiang, C., Ma, L., Zhang, Z., Zeng, X., 2012. Preliminary characterization, antioxidant activity in vitro and hepatoprotective effect on acute alcohol-induced liver injury in mice of polysaccharides from the peduncles of *Hovenia dulcis*. Food and Chemical Toxicology, 50, 2964-2970.

Wang, S.Y., Hsu, M.L., Hsu, H.C., Tzeng, C.H., Lee, S.S., Shiao, M.S., Ho, C.K., 1997. Theanti-tumor effect of *Ganoderma lucidum* is mediated by cytokines released from activated macrophages and T lymphocytes. International Journal of Cancer, 70, 699-705.

Wang, Y., Zhang, M., Ruan, D., Shashkov, A.S., Kilcoyne, M., Savageb, A.V., Zhang, L., 2004. Chemical components and molecular mass of six polysaccharides

isolated from the sclerotium of *Poria cocos*. Carbohydrate Research, 339, 327-334.

Wasser, S.P., 2002. Medicinal mushrooms as a source of antitumor and immunomodulating polysaccharides. Applied Microbiology and Biotechnology, 60, 258-274.

Weeks, B.A., Keisler, A.S., Myrvik, Q.N., Warinner, J.E., 1987. Differential uptake of neutral red by macrophages from three species of estuarine fish. Developmental & Comparative Immunology, 11, 117-124.

Weiss, R.B., 1992. The anthracyclines: will we ever find a better doxorubicin. Seminar in Oncology, 19, 670-686.

Widjanarko, S.B., Nugroho, A., Estiasih, T., 2011. Functional interaction components of protein isolates and glucomannan in food bars by FTIR and SEM studies. African Journal of Food Science, 5, 12-21.

Wong, J.W.C., Mak, K.F., Chan N.W., Lam, A., Fang, M., Zhou, L.X., Wu, Q.T., Liao, X.D., 2001. Co-composting of soybean residues and leaves in Hong Kong. Bioresource Technology, 76, 99-106.

Xie, J., Liu, X., Shen, M., Nie, S., Zhang, H., Li, C., Gong, D., Xie, M., 2013. Purification, physicochemical characterizationandanticancer activity of a polysaccharide from *Cyclocaryapaliurus* leaves. Food Chemistry, 136, 1453-1460.

Xu, H., Sun, L.P., Shi, Y.Z., Wu, Y.H., Zhang, B., Zhao, D.Q., 2008. Optimization of cultivation conditions for extracellular polysaccharide and mycelium biomass by *Morchella esculenta* As51620. Biochemical Engineering Journal, 39, 66-73.

Xu, W.T., Zhang, F.F., Luo, Y.B., Ma, L.Y., Kou, X.H., Huang, K.L., 2009. Antioxidant activity of a water-soluble polysaccharide purified from Pteridium aquilinum. Carbohydrate Research, 344, 217-222.

Xu, Y., Xia, W., Yang, F., Kim, J., Nie, X., 2009. Effect of fermentation temperature on the microbial and physicochemical properties of silver carp sausages inoculated with *Pediococcus pentosaceus*. Food Chemistry, 118, 512-518.

Xu, Y., Xia, W., Yang, F., Nie, X., 2010. Physical and chemical changes of silver carp sausages during fermentation with *Pediococcus pentosaceus*. Food Chemistry, 122, 633-637.

Yang, L., Zhang, L.M., 2009. Chemical structural and chain conformational characterization of some bioactive polysaccharides isolated from natural sources. Carbohydrate Polymers, 76, 349-361.

Yang, T., Jia, M., Meng, J., Wu, H., Mei, Q., 2006. Immunomodulatory activity of polysaccharide isolated from Angelica sinensis. International Journal of Biological Macromolecules, 39, 179-184.

Yang, X., Guo, D., Zhang, J., Wu, M., 2007. Characterization and anti-tumor activity of pollen polysaccharide. International Immunopharmacology, 7, 427-434.

Yong, F.M., Wood, B.J.B., 1977. Biochemical changes in experimental soy sauce koji. International Journal of Food Science and Technology, 12, 163-175.

Yu, R.M., Yin, Y., Yang, W., Ma, W.L., Yang, L., Chen, X.J., Zhang, Z., Yea, B., Song, L., 2009. Structural elucidation and biological activity of a novel polysaccharide by alkaline extraction from cultured *Cordyceps militaris*. Carbohydrate Polymers, 75, 166-171.

Yuan, J.F., Zhang, Z.Q., Fan, Z.C., Yang, J.X., 2008. Antioxidant effects and cytotoxicity of three purified polysaccharides from Ligusticum chuanxiong Hort. Carbohydrate Polymer, 74, 822-827.

Zaidman, B.Z., Yassin, M., Mahajna, J., Wasser, S.P., 2005. Medicinal mushroom modulators of molecular targets as cancer therapeutics. Applied Microbiology and Biotechnology, 67, 453-468.

Zamora, R., Matthys, K.E., Herman, A.G., 1997. The protective role of thiols against nitric oxide-mediated cytotoxicity in murine macrophage J774 cells. European Journal of Pharmacology, 321, 87-96.

Zhang, M., Cui, S.W., Cheung, P.C.K., Wang, Q., 2007. Antitumor polysaccharides from mushrooms: a review on their isolation process, structural characteristics and antitumor activity. Trends in Food Science and Technology, 18, 4-19.

Zhou, L., Chen, B., 2011. Bioactivities of water-soluble polysaccharides from Jisongrong mushroom: Anti-breast carcinoma cell and antioxidant potential. International Journal of Biological Macromolecules, 48, 1-4.

Zhou, Y., Zhang, Z., Nakamoto, T., Li, Y., Yang, Y., Utsumi, M., Sugiura, N., 2011. Influence of substrate-to-inoculum ratio on the batch anaerobic digestion of bean curd refuse-okara under mesophilic conditions. Biomass and Bioenergy, 35, 3251-3256.

Zhu, X.L., Chen, A.F., Lin, Z.B., 2007. *Ganoderma lucidum* polysaccharides enhance the function of immunological effector cells in immunosuppressed mice. Journal of Ethnopharmacology, 111, 219-226.

Zhu, Y.P., Fan, J.F., Cheng, Y.Q., Li, L.T., 2008. Improvement of the antioxidant activity of chinese traditional fermented okara (Meitauza) using *Bacillus subtilis B2*. Food Control, 19, 654-661.

Zong, A., Cao, H., Wang, F., 2012. Anticancer polysaccharides from natural resources: A review of recent research. Carbohydrate Polymers, 90, 1395-1410.

Acknowledgements

This dissertation is a milestone in my academic career. I have been fortunate to learn theories and concepts which would have been impossible if I had not extensively carried out the research. I am grateful to a number of people who have guided and supported me throughout the research process.

Foremost, I would like to express my sincere gratitude to my advisor Prof. Zhang for the continuous support of my Ph.D study and research, for his patience, motivation, enthusiasm and immense knowledge. His guidance helped me in all the time of research and writing of this thesis. I also want to thank Prof. Yang, Prof. Lei and Prof. Utsumi, their encouragement, insightful comments and instructions have enabled me to assemble and finish the dissertation effectively.

I also would like to thank the China Scholarship Council (CSC). The scholarship let me past a comfortable study life in the last three years.

I thank my fellow lab mates, for the stimulating discussions, for the sleepless nights we were working together before deadlines, and for all the fun we have had in the last three years.

Finally, my deep gratitude is to my beloved husband and parents for their love and mental support.

Publications

[1] **Shuhong Li**, Yaxin Sang, Dan Zhu, Yingnan Yang, Zhongfang Lei, Zhenya Zhang (2013). Optimization of fermentation conditions for crude polysaccharides by *Morchella esculenta* using soybean curd residue. Industrial Crops and Products, 50, 666-672.

[2] **Shushing Li**, Linbo Wang, Chunfeng Song, Xuansheng Hu, Hongyi Sun, Yingnan Yang, Zhongfang Lei, Zhenya Zhang (2013). Utilization of soybean curd residue for polysaccharides by *Wolfiporia extensa (Peck) Ginns* and the antioxidant activities in vitro. Journal of the Taiwan Institute of Chemical Engineers, In press, DOI: http://dx.doi.org/10.1016/j.jtice. 2.

[3] **Shuhong Li**, Dan Zhu, Kejuan Li, Yingnan Yang, Zhongfang Lei, and Zhenya Zhang (2013). Soybean Curd Residue: Composition, utilization, and related limiting factors. ISRN Industrial Engineering, DOI: http://dx.doi.org/10.1155/2013/423590.

[4] **Shuhong Li**, Xuansheng Hu, Kejuan Li, Xi Yuan, Zhenya Zhang (2013). Effect of fermentation by *Morchella esculenta* on the physicochemical properties of soybean curd residue. Food Hydrocolloids, under review.

[5] Hongyi Sun, Shaofei Wang, **Shuhong Li**, Xi Yuan, Jian Ma, Zhenya Zhang (2013). Antioxidant activity and immunomodulatory of extracts from roots of Actinidia kolomikta. International Journal of Biology, 5 (3), 1-12.

[6] Xuansheng Hu, **Shuhong Li**, Linbo Wang, Dan Zhu, Yuepeng Wang, Yiting Li, Yingnan Yang, Zhenya Zhang, Delin Cheng (2013). Anti-diabetic activities of aqueous extract from Actinidia kolomikta root against α-glucosidase. Journal of Pharmacognosy and Phytochemistry, 2 (4), 53-57.