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The isolation and antioxidant activity of polysaccharides from the marine microalgae *Isochrysis galbana*

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ABSTRACT

Three polysaccharides, IPSI-A, IPSI-B and IPSII, were successfully isolated from the marine microalgae *Isochrysis galbana* through a combination of anion-exchange column chromatography and repeated gel chromatography. These three polysaccharides were demonstrated to have moderate scavenging activities against superoxide and hydroxyl radicals and moderate reductive power in a concentration-dependent manner. The IPSII demonstrated more effective antioxidant activities than IPSI-A and IPSI-B. IPSII had a molecular weight of 15.934 kDa belonging to a β-type heteropolysaccharide with a pyran group and primarily contained mannose with variable amounts of glucose, galactose and rhamnose based on an analysis of infrared spectroscopy (IR), electrospray ionization-mass spectrometry (ESI-MS) and ^{1}H nuclear magnetic resonance (^{1}H NMR).

1. Introduction

Active oxygen products (AOS), such as singlet oxygen ($^1O_2$), superoxide radicals ($O_2^{\bullet-}$), hydroxyl radicals (•OH), and hydroxyl peroxide (H_2O_2), are generated by normal metabolism or from exogenous factors and agents. These oxygen species can react with macromolecules and lead to cellular damages (Malanga & Puntarulo, 1995; Martin & Burch, 1990). To reduce the damage to the human body and prolong the storage stability of food, synthetic antioxidants are used in industrial processing. Antioxidants can minimize the oxidative damage by increasing cells' natural defenses and by scavenging the free radical (Liu & Ng, 2000; Schinella et al., 2002). Butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG) and tertiary butylhydroxyquinone (TBHQ) are authorized as synthetic antioxidants for use in food (Abdalla et al., 1999). There are some serious problems concerning the toxicity of these compounds. The main concern about the safety of these synthetic compounds is related to their metabolism and possible absorption and accumulation in body organs and tissues (Hayashi, Morimoto, Miyata, & Sato, 1993). Thus, it is essential to develop and utilize effective natural antioxidants to protect against free radicals and retard the progress of many chronic diseases (Luo, 2008; Nandita & Rajini, 2004).

Natural antioxidants isolated from plants, fungi and marine algae represent the most useful so-called "nutraceuticals" and functional foods for health protection and disease prevention (Gutteridge & Halliwell, 1994, chap. 4). In the search for new natural antioxidants, a number of polysaccharides obtained from plants and microorganisms have been demonstrated to possess potent antioxidant activity (Duan et al., 2006; Herrero et al., 2005; Hu, Xu & Hu, 2003; Jiang et al., 2005; Kuda et al., 2005; Chidambara Murthy et al., 2005; Qi et al., 2006; Ramarahnam et al., 1995; Tannin-Spitz et al., 2005; Ye et al., 2012). In recent years, the use of photosynthetic microorganisms such as microalgae has received increasing attention due to their diverse phytometabolic activity with various chemical structures and biological activities. The use of microalgae for human consumption has also increased. Studies have shown them to be a high value health food with many valuable biochemical products including polyunsaturated fatty acids, phytosterols, heat-induced protein, phenolic compounds, pigments and sulfated polysaccharides, *etc.* (Spolaore et al., 2006). Polysaccharides isolated from microalgae have significant antioxidant properties in *vitro* and had effective scavenging abilities on superoxide radicals, hydroxyl radicals and hydroxyl peroxide including: *Spirulina platensis* (Herrero et al., 2005; Li, 2010; Li et al., 2007), *Porphyridium cruentum* (Shi, 2007; Sun, Wang, et al., 2010; Sun, Lei, et al. 2010; Tannin-Spitz et al., 2005; Wang, Sun & Zhao, 2012; Zhu et al., 2008), *Dunaliella salina* (Chidambara Murthy et al., 2005), *Rhodella reticulata* (Chen et al., 2010) and *Schizochytrium* sp. (Wang et al., 2011).

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Isochrysis galbana is a species of marine microalgae of substantial interest in aquaculture with significant attention worldwide. It feeds mollusk larvae, fish and crustaceans in their early stages of growth and has good nutritive characteristics (Sukenik & Wahnon, 1991). It is rich in lipids with a high content of polyunsaturated fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) known to play an important role the prevention of heart and circulatory diseases and encourages brain development (Napolitano, Ackman & Ratnayake, 1990). Our previous work found that *Isochrysis galbana* was rich in polysaccharides and its contents were up to 25% (dry cell weight) (Dai & Wu, 2000; Napolitano et al., 1990; Wang & Sun, 2005). There are a number of reports on the extraction of *Isochrysis galbana* polysaccharides (Guo et al., 2011; Pu, Wang & Sun, 2012; Sun, Wang, et al., 2010; Sun, Lei, et al., 2010; Wu et al., 2004), and infrared spectroscopy (IR) showed that *Isochrysis galbana* polysaccharides (IPS) contained sulfuric group and amidogen (Sun, Wang, et al., 2010; Sun, Lei, et al., 2010). However, isolation, purification and antioxidant activity were not reported. The aim of this study is to isolate and purify polysaccharides from the marine microalgae *Isochrysis galbana* and investigate their physical properties and antioxidant properties *in vitro*.

2. Materials and methods

2.1. Microalgae strain

Isochrysis galbana was offered by Marine Microalgae Research Center, Ocean University of China and cultured in Erlenmeyer flasks with f/2 medium (Guillard & Ryther, 1962). Cultures were incubated at 23 °C and illuminated with fluorescent lamps in a 16/8 dark/light cycle, at an irradiance level of 70 μmol photons m^{-2} s^{-1}.

2.2. Isolation and purification

Cell suspensions were centrifuged at 5000 × *g* for 15 min, and the pellets were rinsed thoroughly with distilled water, collected and then lyophilized to attain a dry powder using vacuum freeze drier (FD-1), and then kept in plastic bags at room temperature before use. The dry powder (20 g) was suspended in 400 mL distilled water, sonicated for 5 min, adjusted to pH 9.0, extracted for 180 min at 70 °C, and then centrifuged at 5000 × *g* for 10 min. The supernatant was collected, concentrated under reduced pressure at 40 °C and mixed with 3 volumes of 75% ethanol (v/v) and left overnight at 4 °C to isolate the polysaccharides. The precipitate was collected by centrifugation, and washed sequentially with the lowest possible volume of ethanol, acetone and ether, respectively.

The above mentioned all precipitate was dissolved in 50 mL hot water. Proteins were excluded with the Sevag method (Staob, 1965) and dialyzed in cellulose membrane tubing (molecular weight cutoff 3500) against distilled water at room temperature—first with reverse flowing distilled water for 48 h followed by still distilled water for 48 h that was changed every 4 h—to remove low-molecular weight material (chromones and anthranoids). The product was then concentrated and precipitated with 4-fold volumes of 95% ethanol to obtain the polysaccharide-enriched fraction (IPS, 4.0 g).

The crude polysaccharide (0.5 g) was dissolved in 5 mL distilled water, centrifuged, and then the supernatant was applied to an anion exchange column with DEAE-52 (2.4 cm × 50 cm) eluting with distilled water, 0.1 mol L^{-1} NaCl, 0.2 mol L^{-1} NaCl, 0.3 mol L^{-1} NaCl, 0.5 mol L^{-1} NaCl, 0.1 mol L^{-1} NaOH, 0.5 mol L^{-1} NaOH and 1.0 mol L^{-1} NaOH at a flow rate of 0.5 mL/min. The volume fraction was 3 mL per tube. Total sugar content of the eluate was determined by the phenol-sulfuric acid method. The main fractions eluted with distilled water and 1.0 mol L^{-1} NaOH. These were pooled, desalted and further purified by a Sephadex G-50 (2.2 cm × 50 cm) and Sephadex G-100 column (2.2 cm × 50 cm) eluting with distilled water at a flow rate of 0.3 mL/min, respectively. The main fraction was collected, dialyzed, concentrated and lyophilized, resulting in purified IPS used for subsequent studies. The three purified polysaccharides were obtained and named IPSI-A, IPSI-B and IPSII through repeated an anion exchange column and gel chromatography above, respectively.

2.3. Physical and chemical properties of the purified polysaccharides

2.3.1. Appearance and solubility of the purified polysaccharides

The color, smell and texture of the purified polysaccharides were noted. 0.01 g of the purified polysaccharide powder was added into 10 mL ethanol, 10 mL ether and 10 mL acetone, respectively, mixed well and observed if it was soluble.

2.3.2. Purity of the purified polysaccharides

The purified polysaccharide solution was placed at −35 °C overnight, thawed rapidly and centrifuged (12,000 rpm, 20 min) to examine for precipitation. If no precipitation occurred, the absorption spectrum was recorded using a Lambda 35 spectrophotometer (Perkin Elmer, U.S.) between 190 and 800 nm to assay for proteins and nucleic acids (Wang, Liu, et al., 2007; Wang, Cui, & Xu, 2007).

Purity of IPSI-A, IPSI-B and IPSII was calculated using a size-exclusion HPLC chromatography system. The following system was used: Waters Linear Ultrahydrogel Column, Waters guard column, and a differential refractometric detector (Shimadzu Corp., Japan). The eluent was 0.2 M Na_2Ac in HPLC-grade water at 0.3 mL/min. The samples were dissolved in 0.2 M Na_2Ac and filtered through a 0.2-mm filter prior to injection.

2.3.3. Chemical reactions

The structural properties and functional groups of polysaccharides could be tested by chemical reactions. These tests help enhance the understanding of structure identification and relationship. All the assays following were performed according to the reports (Kang, Yun, & Lee, 2003; Li et al., 1999).

The carbazole and sulphuric acid reaction can be used to determine the existence of uronic acid in the purity of polysaccharides (Bitter & Muir, 1962).

Fehling reagent reaction was usually used to determine the existence of reducing sugar in the molecular structure (Li et al., 1999).

The reaction between iodine and starch was used to determine the purity of polysaccharides in terms of the existence of starch residual in those polysaccharides (Kang et al., 2003).

Molish reaction was used to identify whether the analyte belonged to carbohydrate (Kang et al., 2003).

Sulfate content was determined after hydrolysis with trifluoroacetic acid according to the methods of Therho and Hartiala (1971).

2.3.4. Infrared (IR) analysis

For IR spectroscopy, the polysaccharide was mixed with KBr powder, ground and then pressed into a 1 mm pellets for Fourier transform infrared (FT-IR) measurement in the frequency range of 4000–500 cm^{-1}. The FT-IR spectra of the polysaccharides were measured on a Nicolet Nexus 470 spectrometer.

2.3.5. Electrospray ionization-mass spectrometry (ESI-MS) spectroscopy

MS analysis used 2 μL of sample by quantitative ring sampling. The main parameters of ESI-MS analysis were: spray voltage 4 kV;

sheath gas 30 ARB; 5 ARB auxiliary gas; ion transport capillary temperature 275 °C; and tube voltage of 150 V lens.

2.3.6. ^1H nuclear magnetic resonance (^1H NMR)

Samples were dissolved in D_2O (1.0 mL). One and two-dimensional ^{1}NMR spectra were recorded in D_2O at 313 K on a Bruker DRX-400 spectrometer operating at 400 MHz for ^{1}H. The spectra were processed with Bruker software (Bruker, Wissembourg, France).

2.4. Antioxidant activities

2.4.1. Scavenging ability on superoxide radicals

The superoxide radical scavenging activity was determined based on the method reported by Marklund and Marklund (1974) with minor modifications. Briefly, 3 mL of 0.05 mol L^{-1} Tris–HCl buffer (pH 8.2) and 1 mL of samples at different concentrations (0, 0.1, 0.2, 0.4, 0.8, 1.6, and 3.2 mg/mL) were incubated at 25 °C for 10 min, then 200 μL of pyrogallol at the same temperature were added to the mixture and the reaction was proceed at 25 °C for 4 min. Finally, the reaction was terminated by the addition of 0.5 ml of HCl. The absorbance was measured at 320 nm against the blank. Ascorbic acid (Vc) was used as a positive control. The superoxide radical scavenging activity was calculated as:

$$\text{The scavenging ability } (\%) = \left(\frac{1 - A_{\text{sample}}}{A_{\text{control}}}\right) \times 100 \quad (1)$$

where A_{control} is the absorbance of control without samples, and A_{sample} is the absorbance of the samples.

2.4.2. Scavenging ability on hydroxyl radical

The hydroxyl radical scavenging activity was measured based on the report of Smirnoff and Cumbes (1989) with some modifications. First, 0.3 mL of 5 mmol L^{-1} orthophenanthroline, 0.8 mL of 0.75 mol L^{-1} phosphate buffer (pH 7.4) and 0.3 mL of 7.5 mmol L^{-1} $FeSO_4$ were added to 0.5 mL of sample solution at different concentrations (0, 0.1, 0.2, 0.4, 0.8, 1.6, and 3.2 mg/mL). Finally, 0.2 mL of 1% H_2O_2 was added, and the reaction mixture was incubated at 37 °C for 60 min. The absorbance of the product was measured at 510 nm. Ascorbic acid (Vc) was used for comparison. Hydroxyl radical scavenging activity was calculated as described above.

2.4.3. Scavenging ability on hydrogen peroxide

The ability of samples to quench hydrogen peroxide (H_2O_2) was determined spectrophotometrically (Ruch, Cheng & Klauning, 1989). Samples were dissolved in 3.4 mL of 0.1 M pH 7.4 phosphate buffered saline (PBS) and mixed with 0.6 mL of 40 mM solution of H_2O_2. The absorbance of H_2O_2 at 230 nm was determined 10 min later in a spectrophotometer. For each concentration, a blank sample was used for background subtraction. Ascorbic acid (Vc) was used as a positive control. The inhibition of H_2O_2 production was calculated as follows:

$$\text{The inhibition rate } (\%) = \left[1 - \frac{A_{\text{sample}} - A_{\text{sample blank}}}{A_{\text{control}}}\right] \times 100 \quad (2)$$

where A_{control} is the absorbance of the control group in H_2O_2 generation system, A_{sample} is the absorbance of the sample group and $A_{\text{sample blank}}$ is the absorbance of the blank only.

2.4.4. Reducing power

The method used here is based on Oyaizu (1986) and Yenhum (1997) with some modifications. The reaction mixtures contained 2.5 mL phosphate buffer saline (pH 6.6, 0.2 mol L^{-1}), 2.5 mL potassium ferricyanide [$K_3Fe(CN)_6$] (1%, w/v) and 0.5 mL sample solution. After incubating at 50 °C for 20 min, 2.5 mL of trichloroacetic acid (10%, w/v) was added to the mixture, and then centrifuged at 1400 rpm for 10 min. Then 0.5 mL of the upper layer of the solution was collected and mixed with 2.0 mL deionized water and 0.4 mL $FeCl_3$ (0.1%, w/v). After incubating at room temperature for 10 min, the absorbance of the mixture was measured at 700 nm. Increased absorbance of the reaction mixture indicated greater reducing power. Ascorbic acid (Vc) was used as a positive control.

2.5. Statistical analysis

All bioassay results were expressed as means ± standard deviation (SD). The experimental data were subjected to an analysis of variance (ANOVA) for a completely random design. For each polysaccharide, three samples were prepared for assays of every antioxidant attribute.

3. Results and discussion

3.1. Isolation and purification of the polysaccharides

By ion-exchange chromatography, unabsorbed and acidic fractions were separated from IPS. These fractions eluted in deionized water (IPSI) and 1.0 M NaOH (IPSII) and were 17.37% and 70.10% of the total, respectively, according to the collected polysaccharide mass and these two peaks regions: 30–42 mL and 732–786 mL, respectively. The polysaccharide (IPSI) was further fractionated into IPSI-A and IPSI-B as shown in Fig. 1A. The gel chromatography of the three polysaccharides (IPSI-A, IPSI-B and IPSII) gave only a single peak and symmetrically sharp peak regions at 35–65 mL, 35–80 mL and 35–60 mL, respectively (Fig. 1B).

Since DEAE is an anion exchanger, positive charged groups and neutral groups in the samples were removed with eluate, whereas negative ions in the samples could exchange with counter ions and combine with the chromatography column. Thus, the purified IPSI-A and IPSI-B belonged to a kind of weak alkaline polysaccharides, and IPSII to a kind of acidic polysaccharides.

Other authors have reported methods for the purification of polysaccharides from microalgae. Chen, Zhang and Su (2012) reported that a polysaccharide fraction was isolated and purified by ion exchange chromatography on DEAE-cellulose 52 from *Chlorella pacifica*. Wang et al. (2011) used a 732 cationic exchange resin to separate extracted exopolysaccharide from marine microalga *Schizochytrium* sp. TIO1101 to obtain three major polysaccharide components: acidic polysaccharide, weak alkaline polysaccharides and alkaline polysaccharides. Chen et al. (2010) also demonstrated a method by using anion exchange chromatography on DEAE Sepharose Fast Flow and Sepharose 6B gel filtration chromatography to obtain polysaccharides PEPS1 and PEPS2 from *Rhodella reticulata*. Sun, Wang, and Shi (2009) also used a chromatography column equipped with Sephacryl S-400 for the separation and purification of the degraded compounds of *Porphyridium cruentum* extracellular polysaccharides. Yim, Kim, and Ahn (2004) used a Sepharose 4B column chromatography to purify sulfated extracellular polysaccharide p-KG03 from the marine microalga *Gyrodinium impudicum* strain KG03. Structurally diverse polysaccharides from microalgae are a renewable resource of biotechnologically important materials, some of which may be useful for niche applications.

3.2. Physical and chemical properties of IPSI-A, IPSI-B and IPSII

The IPSI-A, IPSI-B and IPSII were light yellow, odorless powder. It was soluble in water but insoluble in ethanol and other organic

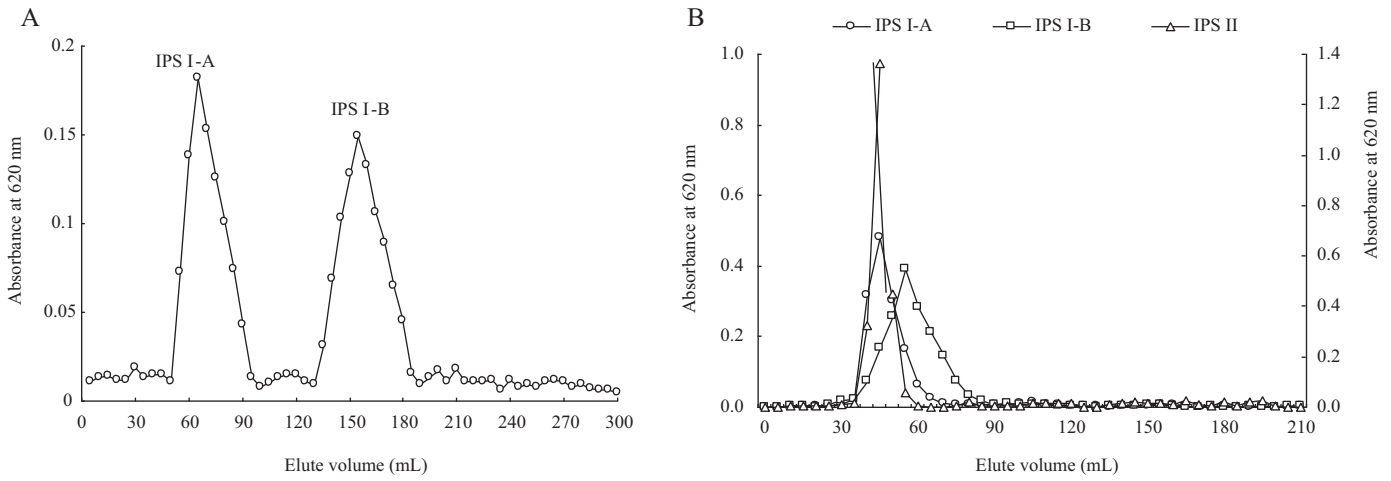

Fig. 1. Purification of the polysaccharides obtained from *Isochrysis galbana*. (A) IPSI obtained on a DEAD-52 column was applied to a Sephadex G-50 and eluted as described in Section 2. The fractions containing the polysaccharides were pooled and named as IPSI-A and IPSI-B, respectively. (B) IPSI-A, IPSI-B and IPSII were loaded on Sephadex G-100 column and eluted as described in Section 2.

solvents, such as ether and acetone, which accorded with general properties of polysaccharides (Ye et al., 2012).

The purified polysaccharides solution was a uniform liquid with a light yellow in color, and no precipitation occurred after centrifugation. An absorbance spectrum scan of IPSI-A, IPSI-B and IPSII indicated an absence of proteins and nucleic acids due to the negative response – no UV absorption at 260 or 280 nm.

Responses of the chemical reactions between the three purified polysaccharides and several reagents were illustrated in Table 1, indicating those purified polysaccharides belonged to carbohydrate material, which could be oxidized into uronic acid, while there were no reducing sugar and starch in the purified material.

"+" means positive; "−" means negative. Carbazole and sulphuric acid: 6 mL of concentrated sulfuric acid were added to 1 mL sample solution in an ice water bath, and fully mixed. The reaction mixture was incubated at 85 °C for 20 min, removed to room temperature after cooling. Finally, 0.2 mL of carbazole liquid (0.1%), kept at room temperature for 2 h. The color changes were observed in the test tube. The reaction between uronic acid and carbazole in forming a chromogen, where no color developed in the mixture suggests the absence of uronic acid; Fehling reagent: 2 mL of sample solution and 1 mL of Fehling reagent were added to test tube, and fully mixed. The reaction mixture was incubated at 50–60 °C for 2 min. The color changes were observed in the test tube. The aldehyde group in the reducing sugar could reduce Cu^{2+} in Fehling reagent into Cu^{+} in forming a red-colored compound which precipitates; Molish reaction: First, two drops of Morse reagent were add to 1 ml of sample solution and then fully mixed. Finally, 1 mL of concentrated sulfuric acid was slowly added along the tube wall, and then slowly up the tube and the reaction mixture was not shake. The color changes were observed in the test tube. Polysaccharides dehydrated into furfural and other derivatives under the treatment of concentrated sulphuric acid or hydrochloric acid. Furfural and its derivatives react with α-naphthol and form purple compounds containing rings in the structure.

Table 1
Responses of chemical reactions between the three purified polysaccharides and several reagents.

Reactions	Results	Analysis
Carbazole and sulphuric acid	+	An uronic acid group presented in the three purified polysaccharides
Fehling reagent	−	No reducing sugar in the three purified polysaccharides structure
I-KI	−	No starch residual in the three purified polysaccharides
Molish reaction	+	Carbohydrate materials present

The uronic acids and sulfate contents of the three purified polysaccharides were given in Table 2. From the table, IPSII had the most uronic acids and IPSI-B had the least. Mei et al. (2005) reported that the uronic acids content of intra- and extra-cellular polysaccharides from *Microcystic aeruginosa* was 12.8% and 10.7%, respectively. Sun and Wang (2013) reported that the uronic acids content of extracellular polysaccharide from *Isochrysis galbana* was 17.1%. But in our study as shown in Table 2, the uronic acids content of IPSII reached 25.6%. Coincidentally, IPSII had the most sulfate content and IPSI-B had the least. Mei et al. (2005) reported that the sulfate content of extracellular polysaccharide from *Microcystic aeruginosa* was 44.4 μg/mg. The sulfate content of extracellular polysaccharides from *Nitzschia closterium* and *Porphyridium cruentum* was not more than 4 μg/mg (Xia & Yao, 2006; Zheng et al., 2005). Thus, the IPSII obtained from *Isochrysis galbana* presented higher content of uronic acid and sulfate than those from other microalgae. For polysaccharides from natural products, the existence of uronic acid and sulfate in the molecular structure has vital significance-those with a high content of uronic acid and sulfate may have radical scavenging activity (Xue et al., 1998; Duh, Tu & Yen, 1999; Zhang et al., 2003), this requires further investigations.

The three purified polysaccharides appeared as a single and symmetrical sharp peak in high-performance liquid chromatography (data not show). These results indicated that IPSI-A, IPSI-B and IPSII were homogeneous polysaccharides and did not contain any contaminants such as chromones and anthranoids. This was also confirmed by thin-layer chromatography (TLC) analysis (data not show).

Table 2
The uronic acids and sulfate contents of the three purified polysaccharides.

Samples	Uronic acids (%)	Sulfate (μg/mg)
IPSI-A	15.6	21.9
IPSI-B	6.59	11.2
IPSII	25.6	54.9

3.3. Antioxidant properties

3.3.1. Scavenging ability on superoxide radicals, hydroxyl radicals and hydrogen peroxide

Although, the superoxide radical was a weak oxidant in most organisms, it could produce hydrogen peroxide and hydroxyl radicals through dismutation and other reactions and is the source of free radicals formed *in vivo*. Moreover, superoxide radical and its derivatives are cell-damaging through causing damage to the DNA and membrane of the cell (MacDonald, Galley & Webster, 2003). Except for the superoxide radical, the hydroxyl radical is considered to be a highly potent oxidant, which can react with all biomacromolecules in living cells (Gülçin, 2006). Hydrogen peroxide can be formed *in vivo* by many oxidizing enzymes such as superoxide dismutase (SOD). It can cross membranes and may slowly oxidize a number of compounds (Yildírím, Mavi, & Kara, 2001). Thus, removing superoxide radicals, hydroxyl radicals and hydrogen peroxide are important for antioxidant defense in cell or food systems.

A significant increase of the scavenging activity was observed at the concentration range (0–3.2 mg/mL) of the polysaccharides (Fig. 2A–C). The scavenging ability of polysaccharides on superoxide radicals, hydroxyl radicals and hydrogen peroxide was in a concentration-dependent way. At 3.2 mg/mL, the abilities of IPSI-A, IPSI-B and IPSII to scavenge superoxide radical were 33.8%, 32.3% and 53.5%, respectively; the hydroxyl radicals scavenging rate of IPSI-A, IPSI-B and IPSII was 34.3%, 37.5% and 63.6%, respectively; the scavenging capability of IPSI-A, IPSI-B and IPSII to hydrogen peroxide were 11.4%, 10.2% and 48.7%, respectively. Scavenging activity of IPSII was significantly higher ($P < 0.05$) than that of IPSI-A and IPSI-B. There was no significant difference ($P > 0.05$) in scavenging activity against superoxide radicals, hydroxyl radicals and hydrogen peroxide between IPSI-A and IPSI-B. In the system of pyrogallol's autoxidation, IPSI-A, IPSI-B and IPSII could inhibit the autoxidation of pyrogallol, which indicated that those three polysaccharides played important roles in antioxidation.

At 1.6 mg/mL, scavenging ability of ascorbic acid (Vc) on superoxide radicals, hydroxyl radicals and hydrogen peroxide was in the range of 57.3%, 68.8% and 100%, respectively. The scavenging abilities of IPSII on superoxide radicals, hydroxyl radicals and hydrogen peroxide were all relatively lower than that of ascorbic acid at the same concentrations. These results showed that IPSII had a moderate superoxide radicals, hydroxyl radicals and hydrogen peroxide scavenging activity.

Although the polysaccharide concentrations required for antioxidant activity were relatively high, *i.e.*, 3.2 mg/mL, others have shown that even crude polysaccharide from *Spirulina platensis* could scavenge superoxide radical and hydroxyl radical efficiently (Li, 2010; Li et al., 2007). At 0.6 mg/mL, the capacity of this crude polysaccharide to scavenge superoxide and hydroxyl radicals was higher than 70.0% (Li et al., 2007). The scavenging abilities of sulfated polysaccharide isolated from *Spirulina platensis* on hydroxyl radicals were all relatively higher than that of ascorbic acid (Li, 2010). Also, the *Nostoc* polysaccharides are capable of scavenging both superoxide anion and hydroxyl radical *in vitro*, and the highest scavenging rates to superoxide anion and hydroxyl radical were 65% and 51% at 0.5 mg/mL, respectively (Li et al., 2011).

3.3.2. Reducing power

Earlier authors have revealed that there was a direct correlation between antioxidant activity and reducing power (Duh, Du & Yen, 1999). The reducing capacity of a compound may be an indicator of its potential antioxidant activity. Fig. 2D showed the reductive activity of IPSI-A, IPSI-B and IPSII using the potassium ferricyanide reduction method. The reducing power of the three polysaccharides increased significantly ($P < 0.05$) with increasing concentration of samples. Among the three fractions of polysaccharides, IPSII showed obviously ($P < 0.05$) higher reducing strength than IPSI-A and IPSI-B, but not ascorbic acid. In all cases except ascorbic acid, the highest value of reducing power was observed in the treatment of IPSII at 3.2 mg/mL, which was 1.61-fold that of IPSI-A and 1.80-fold that of IPSI-B at the dose of 3.2 mg/mL. There was no significant difference ($P > 0.05$) in reductive power between IPSI-A and IPSI-B. Also, ascorbic acid showed little reductive power and its reducing power had no significant changes ($P > 0.05$) in the test ranges. The present results revealed that the three polysaccharides isolated from *Isochrysis galbana* had effective reducing power.

As indicated in the literature, acidic polysaccharides are likely to contain more uronic acids with negative charges (Duh, Tu, et al., 1999). The atoms of polysaccharides with a greater proportion of uronic acid were negatively charged, resulting in less steric hindrance when a superoxide anion radical ($O_2^{-\bullet}$) attacks. This explains the reason for the high content of uronic acid in acidic polysaccharides having higher radical scavenging activities, especially for superoxide anion radicals (Duh, Tu, et al., 1999). Ye et al.'s (2012) study also suggest that the high content of uronic acid in the EPS (an acidic exopolysaccharide) might be the main reason among the factors which may attribute to the strong antioxidant activities. In our research, acidic polysaccharide IPSII with the higher content of uronic acids showed higher radical scavenging activity than weak alkaline polysaccharide IPSI-A or IPSI-B.

Furthermore, the sulfate content of IPSII (54.9 μg/mg) was significantly higher than that of IPSI-A (21.9 μg/mg) and IPSI-B (11.2 μg/mg). Tehila-Spitz et al. tested the possible involvement of SO_4^{2-} in its antioxidant activity. Their results indicated that there is indeed a correlation between the sulfate content and antioxidant activity in that the antioxidant activity exhibited by the polysaccharide with the high sulfur content (4.5%) that exceeded the polysaccharide containing 3% sulfur by about 20%. This finding is in keeping with the suggestion of Xue et al. (1998) and Zhang et al. (2003) that the difference in antioxidant abilities of different polysaccharides may be due to differences in their sulfate content.

The antioxidant activity of the polysaccharides may be related to monosaccharide design, molecular size, structure and conformation. These monosaccharides in the polysaccharides are potent reductive agents as they can supply hydrogen that combines with a radical to form a stable radical to terminate the radical reaction. The differences in the antioxidant abilities of the polysaccharides were directly due to their chemical features. To elucidate the mechanism for this antioxidant behavior, further investigation into the structure of the polysaccharides isolated from the marine microalgae *Isochrysis galbana* are in progress. Future work will focus on the understanding of its structure–activity relationship and its *in vivo* antioxidant activities/mechanisms.

The overall results suggest that IPSII obtained from *Isochrysis galbana* has potential as a natural antioxidant in certain applications, where synthetic chemicals would fail in the requirements of safety and efficiency. Therefore, in the following research, structural information of IPSII was analyzed by IR, ESI-MS and ^{1}H NMR.

3.4. IR analysis of the IPSII

The IR analysis of the IPSII is shown in Fig. 3. There were many peaks from 3403.16 to 602.71 cm^{-1}. The band at 3403.16 cm^{-1} region was attributed to the stretching vibration of O-H in the constituent sugar residues (Kanmani et al., 2011). The band at 2924.85 cm^{-1} was associated with the stretching vibration of C—H in the sugar ring. The band at 1662.88 cm^{-1} was due to the stretching vibration of C=O and carboxyl group. The absorptions around 1423.37 cm^{-1} represented CH_2 and OH bonding. The strong absorption at 1145.64 cm^{-1} was dominated by glycosidic linkage ν(C—O—C)-stretching vibration (Sun et al., 1998). Typical

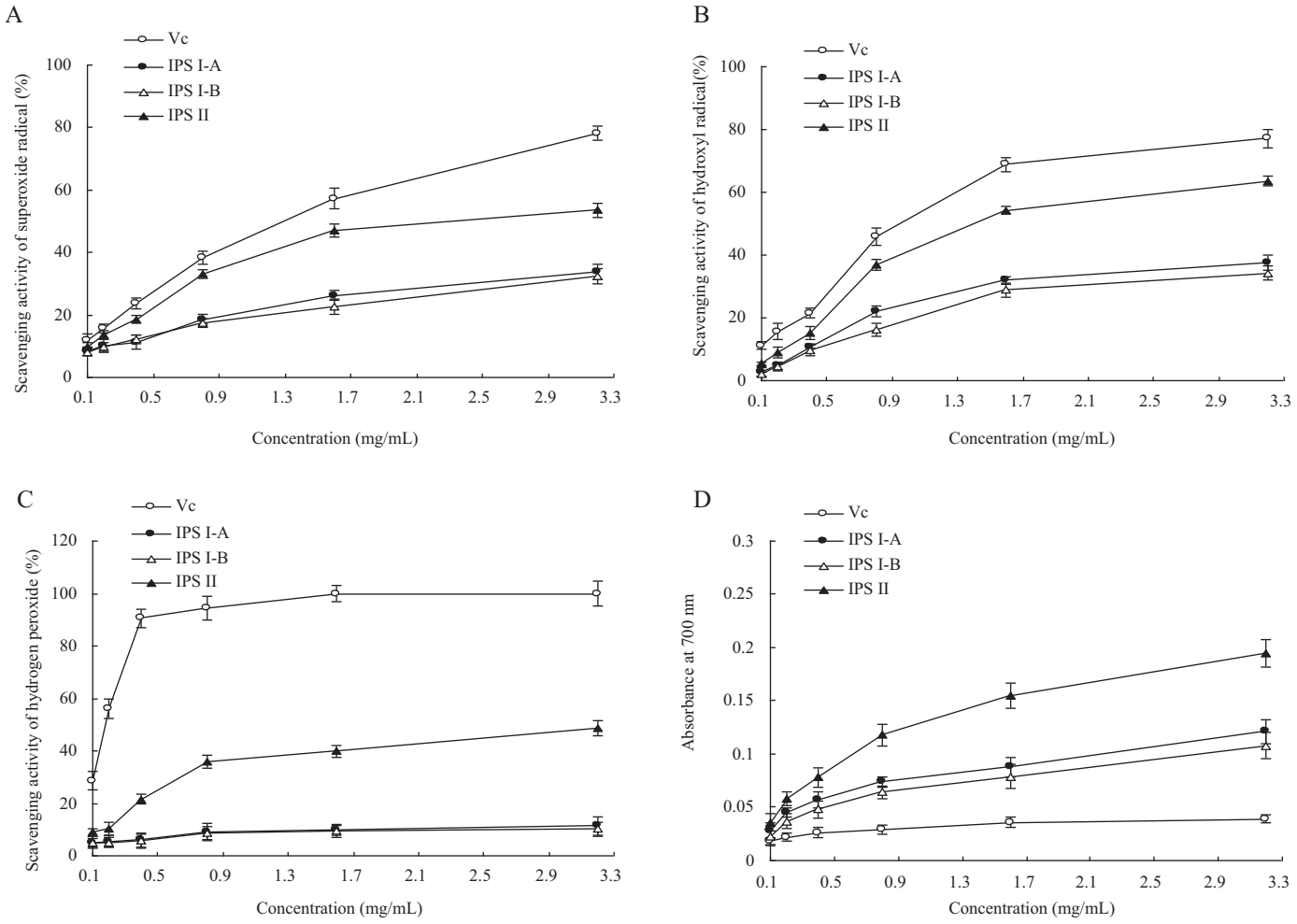

Fig. 2. Scavenging activity of IPSI-A, IPSI-B and IPSII from *Isochrysis galbana* against superoxide radical (A), hydroxyl radical (B), hydrogen peroxide (C) and total reductive potential with ascorbic acid (Vc) applied as a positive control.

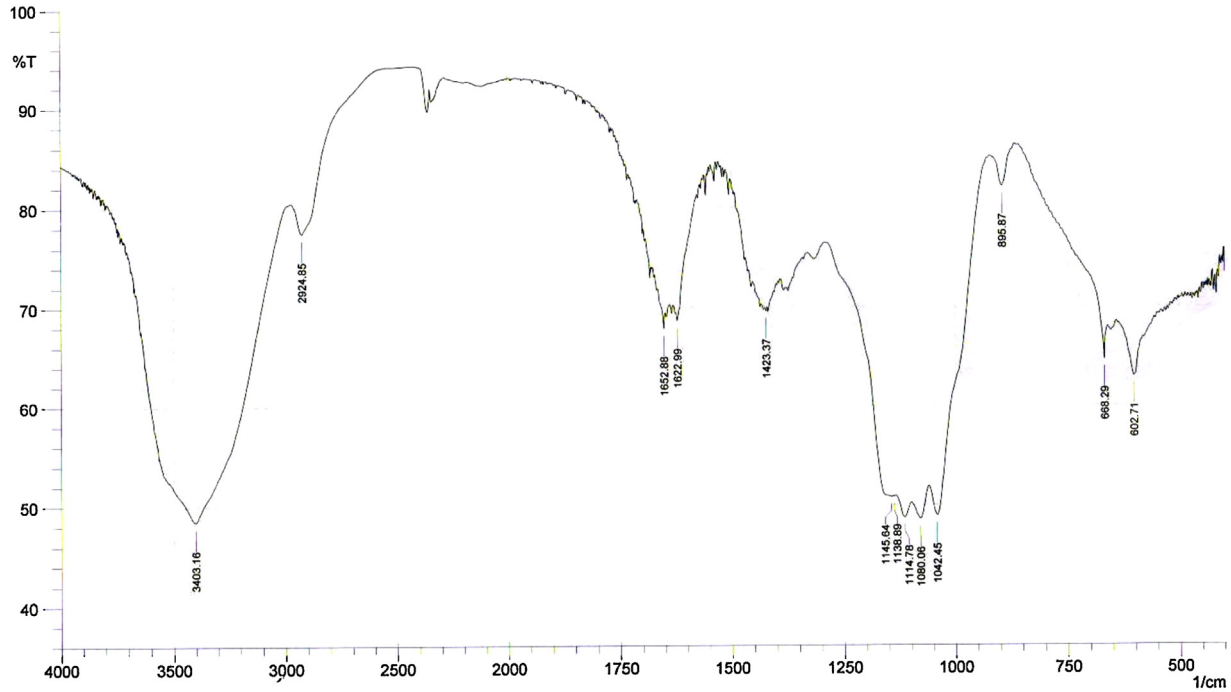

Fig. 3. Infrared spectroscopy of IPSII.

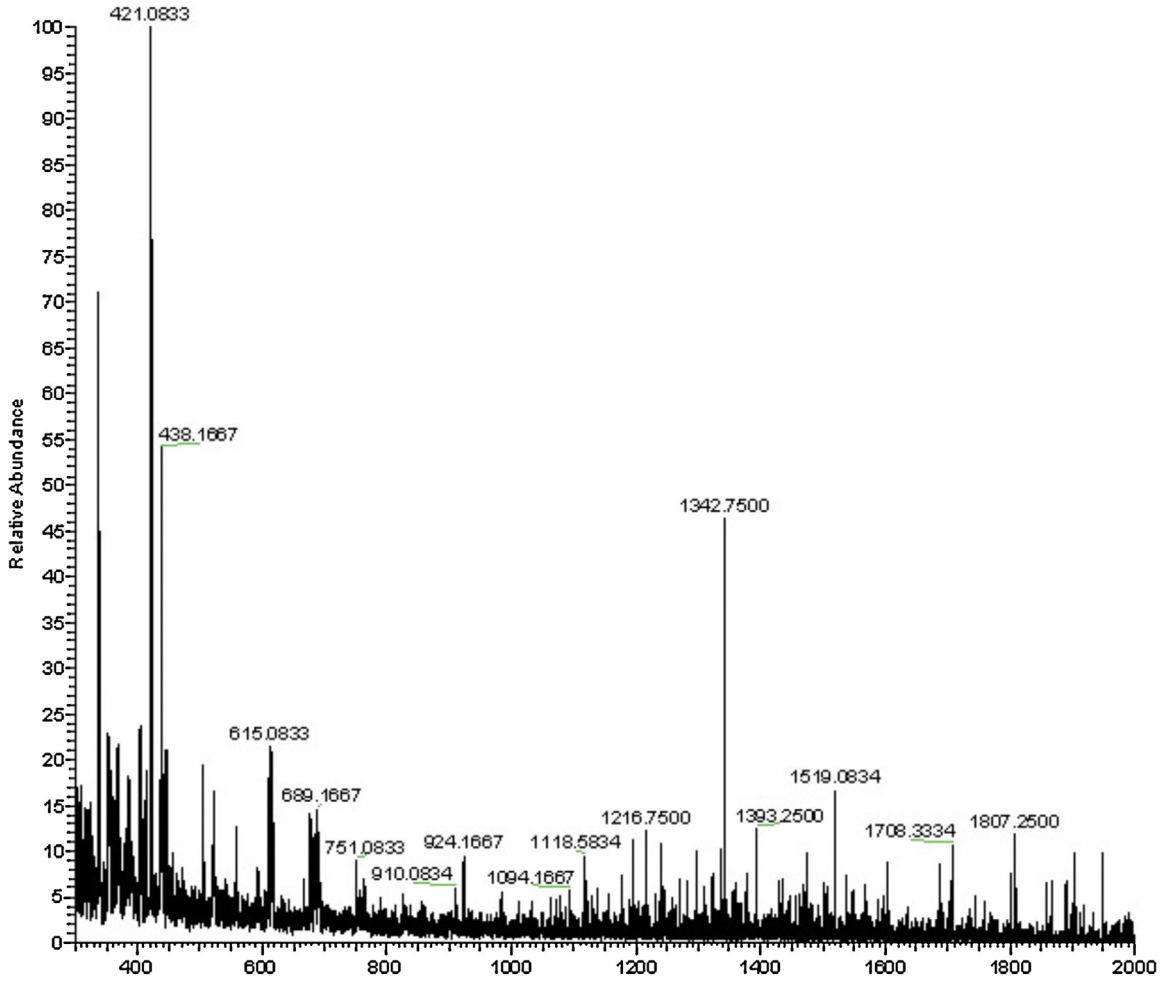

Fig. 4. Electrospray ionization-mass spectrometry spectroscopy of IPSII.

absorption at 1042.45 cm^{-1} revealed galactose frame with pyranglycoside linkage. Moreover, the characteristic band at 895.87 cm^{-1} belonged to the β-anomeric configuration, and the characteristic bands at 956 and 873 cm^{-1} were due to the pyranoid ring (Synytsya et al., 2003; Zhbankov, Andrianov & Marchewka, 1997).

3.5. ESI-MS analysis of the IPSII

ESI-MS can produce a multi-charged ion peak across a large mass range with high sensitivity (Fenn et al., 1989). ESI-MS can analyze even monosaccharide residues containing carboxyl or sulfate groups of polysaccharide, while fast atom bombardment ionization mass spectrometry (FAB-MS) enables analysis. Because ESI-MS can detect non-derived sugars in pmol quantity, it has become one of the best current analysis methods of macromolecular polysaccharide and its complexes (Chen & Wang, 1997, chap. 3; Hu, Wang & Luo, 2000). Wang, Liu, et al. (2007) and Wang, Cui, et al. (2007) introduced the use of ESI-MS to polysaccharides and proteins. In this paper, the average molecular weight of IPSII was 15.934 kDa (Fig. 4). A polysaccharide with a molecular weight between 100 and 200 Ku (91.66–181.33 kDa) had the highest biological activity. Alternatively, species with a molecular weight from 5 to 10 Ku (4.58–9.17 kDa) was inactive (Zhu, Zhao & Fang, 2002). However, Haslin, Lahaye, and Pellegrini (2001) found that the polysaccharide with a molecular weight of 10 Ku (9.17 kDa) from *Asparagopsis armata* had the strongest activity against HIV virus. In our research, IPSII with a molecular weight of 15.934 kDa had moderate scavenging activities against superoxide radical, moderate scavenging activities of hydroxyl radical and moderate reductive power. Tsiapali et al.'s (2001) study showed that the spatial structure and molecular weight of polysaccharides could affect the bioactivity.

3.6. ^{1}H NMR analysis of the IPSII

NMR spectroscopy was used to verify the structural characterization of IPSII obtained from the above data (Fig. 5). There are the three anomeric hydrogen signals in ^{1}H NMR: δ 4.30 ppm, δ 4.45 ppm and δ 5.25 ppm. These correspond to three monosaccharides and combined with chemical analysis and literature were identified as D-galactose (β-configuration), D-glucose (β-configuration) and L-rhamnose (α-configuration), respectively. The H atom signals between 4.4 and 4.8 ppm were assigned to β-anomeric configuration with a pyran group, whereas the signal beyond 5.0 ppm pointed to α-anomeric configuration. Through a comparison with the literature data, it was identified as a β-anomeric configuration. The preponderant signals between 3.3 and 3.5 ppm were assigned to MeO, those between 2.5 and 3.0 ppm pointed to N-AC, and the other signals between 1.1 and 1.3 ppm indicated a 6-deoxy sugar methyl doublet.

The results indicated that IPSII was composed of D-galactose, D-glucose and L-rhamnose with a molecular weight of 15.934 kDa and belonging to a β-type heteropolysaccharide with a pyran group and glycosidic linkage. Due to the absence of ^{13}C NMR data, linkage data on the sugar chains is unclear.

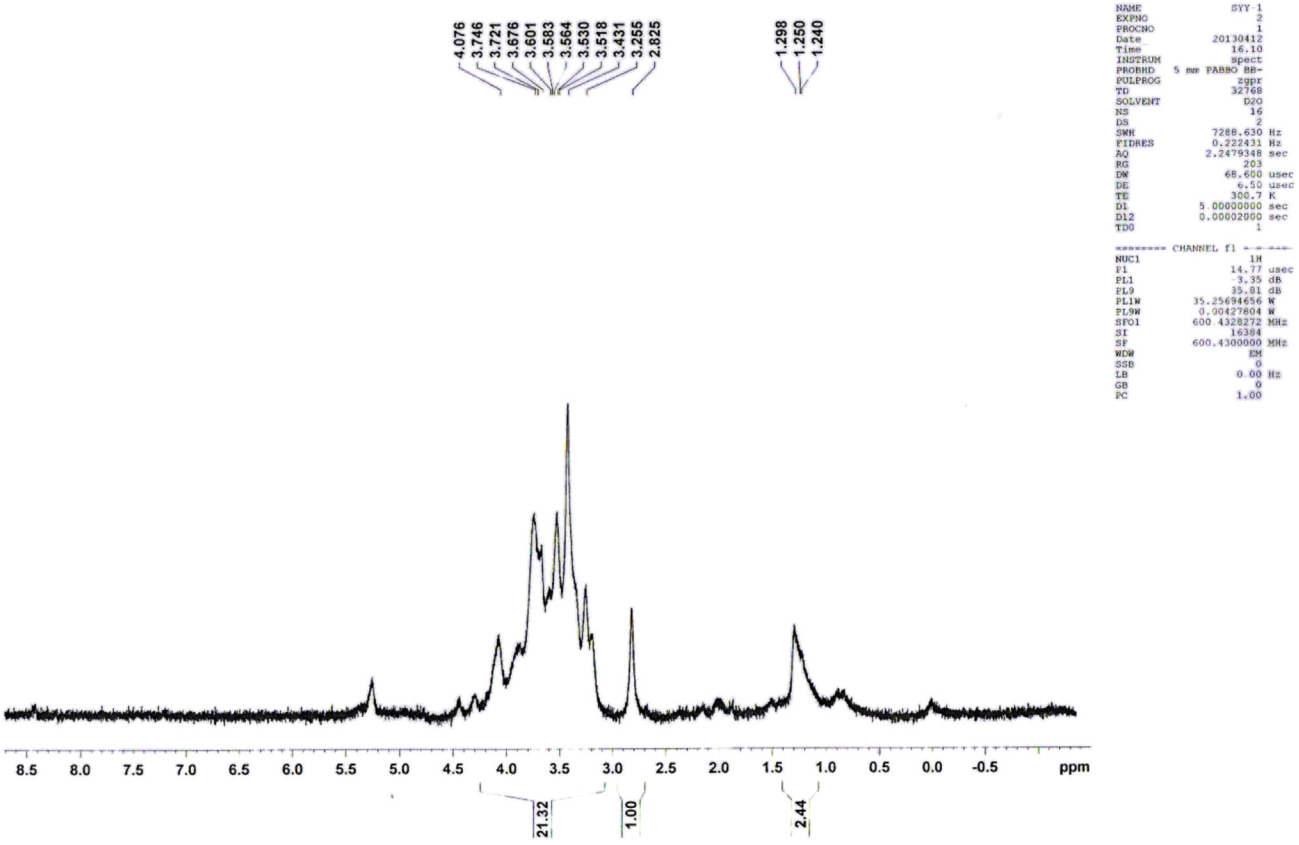

Fig. 5. ^{1}H NMR spectroscopy of IPSII.

Many studies suggest that the bioactivities of polysaccharides are closely associated with their structures such as the type of glycosyl units, the configuration of glycosidic bonds, and the substituents of the polysaccharides. In addition, the spatial structure and molecular weight of polysaccharides could also affect the bioactivity (Tsiapali et al., 2001). Therefore, the antioxidative activity of the IPSII was not the result of any single factor. It is the result of many factors when combined in the variation of monosaccharide composition, structure configuration, mode of attending glycosidic bonds, molecular weight and other structural characteristics of IPSII. Considering the complexity of antioxidation mechanisms, a comprehensive understanding of these effects is required (Moure, Dominguez, & Parajo, 2006). However, due to the lack of results from ^{13}C NMR data in understanding the connection mode of sugar chain of IPSII in the present study, it is difficult to thoroughly interpret the structure–activity relationship of the IPSII during the antioxidation mechanisms. However, this points out a direction for our future studies.

4. Conclusions

The marine microalgae *Isochrysis galbana* contains rich bioactive materials and fatty acids and promises to produce both value-added compounds and biofuel. In this study, the three polysaccharides, IPSI-A, IPSI-B and IPSII, were extracted and purified from *Isochrysis galbana via* hot water extraction, ethanol precipitation, sevage purification, ion exchange fractionation, size exclusion, dialysis and freeze drying. The products were odorless, soluble in water and insoluble in commonly used organic solvents. These three polysaccharides were carbohydrate materials and were polysaccharide rich in uronic acid and sulfate with significant free radical scavenging and antioxidant activities *in vitro*. These findings are important in the development of *Isochrysis galbana* toward new and safe therapeutic agents to be utilized for the management of oxidative damage-derived disorders such as coronary heart ailment, arthritis, Parkinson's and Alzheimer's diseases.

IPSII had a molecular weight of 15.934 kDa, and belonged to a β-type heteropolysaccharide with a pyran group and was primarily consisted of mannose with variable amounts of glucose, galactose and rhamnose based on an analysis of IR, ESI-MS and ^{1}H NMR. Clearly, IPSII is a novel polysaccharide that is distinctly different both in molecular weight and chemical characterization from any of other microalgae polysaccharides that have been isolated or prepared (Chen et al., 2010; Li, 2010; Shi, 2007; Sun et al., 2009; Wang et al., 2012; Yim et al., 2004).

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References

Abdalla, A. E., Tirzite, D., Tirzitis, G., & Roozen, J. P. (1999). Antioxidant activity of 1,4-dihydropyridine derivatives in-carotenemethyl linoleate, sunflower oil and emulsions. *Food Chemistry*, *66*, 189–195.

Bitter, T., & Muir, H. M. (1962). A modified uronic acid carbazole reaction. *Analytical Biochemistry*, *4*, 330–334.

Chen, H. L., & Wang, K. Y. (1997). *Structure and function of glycoconjugates*. Shanghai: Shanghai Medical University Press.

Chen, B. L., You, W. L., Huang, J., Yu, Y., & Chen, W. P. (2010). Isolation and antioxidant property of the extracellular polysaccharide from *Rhodella reticulate*. *World Journal of Microbiology and Biotechnology*, *26*, 833–840.

Chen, X. Q., Zhang, Y., & Su, Y. C. (2012). Isolation and purification of antimicrobial polysaccharides from *Chlorella pacifica*. *Food Science and Technology*, *37*(4), 168–170.

Chidambara Murthy, K. N., Vanitha, A., Rajesha, J., Mahadeva Swamy, M., Sowmya, P. R., & Ravishankar, G. A. (2005). In vivo antioxidant activity of carotenoids from *Dunaliella salina* – A green microalga. *Life Sciences*, *76*, 1381–1390.

Dai, J. B., & Wu, Q. Y. (2000). The growth and biochemical quality of marine unicellular microalga *Isochrysis galbana* in outdoor and indoor mass culture. *Marine Sciences*, *24*, 29–32.

Duan, X. J., Zhang, W. W., Li, X. M., & Wang, B. (2006). Evaluation of antioxidant property of extract and fractions obtained from a red alga, *Polysiphonia urceolata*. *Food Chemistry*, *95*, 37–43.

Duh, P. D., Du, P. C., & Yen, G. C. (1999). Action of methanolic extract of mung bean hulls as inhibitors of lipid peroxidation and non-lipid oxidative damage. *Food and Chemical Toxicology*, *37*, 1055–1061.

Duh, P. D., Tu, Y. Y., & Yen, G. C. (1999). Antioxidant activity of water extract of Harng Jyur (*Chrysanthemum morifolium* Ramat). *Lebensmittel-Wissenschaft & Technologie*, *32*, 269–277.

Fenn, J. B., Mann, M., Meng, C. K., Wong, S. F., & Whitehouse, C. M. (1989). Electrospray ionisation for mass spectrometry of large biomolecules. *Science*, *246*, 64–71.

Guillard, R. R. L., & Ryther, J. H. (1962). Studies of marine planktonic diatom. I. *Cyclotella* nana Hustedt and *Detonula confervacea* (Cleve) Gran. *Canadian Journal of Microbiology*, *17*, 309–314.

Gülçin, İ. (2006). Antioxidant and antiradical activities of L-carnitine. *Life Sciences*, *78*, 803–811.

Guo, G. L., Xu, S. Z., Li, W. H., Sun, Y. Y., & Wang, C. H. (2011). Optimization of microwave-assisted extraction of polysaccharides from *Isochrysis galbana*. *Food Science*, *32*(14), 113–117.

Gutteridge, J. M. C., & Halliwell, B. (1994). *Antioxidants in nutrition, health, and disease*. Oxford: Oxford University Press.

Haslin, C., Lahaye, M., & Pellegrini, M. (2001). In vitro anti-HIV activity of sulfated cell-wall polysaccharides from gametic, carposporic and tetrasporic stages of the Mediterranean red alga *Asparagopsis armata*. *Planta Medica*, *67*, 301–305.

Hayashi, Y., Morimoto, K., Miyata, N., & Sato, H. (1993). Quantitative cancer risk analysis of BHA based on integration of pathological and biological/biochemical information. *Toxicology and Industrial Health*, *9*, 243–249.

Herrero, M., Martin-Alvarez, P. J., Señoráns, F. J., Cifuentes, A., & Ibáñez, E. (2005). Optimization of accelerated solvent extraction of antioxidants from *Spirulina platensis* microalga. *Food Chemistry*, *93*, 417–423.

Hu, Y. N., Wang, M. Y., & Luo, G. A. (2000). Applications of mass spectrometry in carbohydrate studies. *Acta Pharmaceutica Sinica B*, *35*(5), 397–400.

Hu, Y., Xu, J., & Hu, Q. H. (2003). Evaluation of antioxidant potential of Aloe vera (*Aloe barbadensis* Miller) extracts. *Journal of Agricultural and Food Chemistry*, *51*, 7788–7791.

Jiang, Y. H., Jiang, X. L., Wang, P., & Hu, X. K. (2005). In vitro antioxidant activities of water-soluble polysaccharides extracted from *Isaria farinosa* B05. *Journal of Food Biochemistry*, *29*, 323–335.

Kang, D. G., Yun, C. K., & Lee, H. S. (2003). Screening and comparison of antioxidant activity of solvent extracts of herbal medicines used in Korea. *Journal of Ethnopharmacology*, *87*, 231–236.

Kanmani, P., Kumar, R. S., Yuvaraj, N., Paari, K. A., Pattukumar, V., & Arul, V. (2011). Production and purification of a novel exopolysaccharide from lactic acid bacterium *Streptococcus phocae* P180 and it's functional characteristics activity *in vitro*. *Bioresource Technology*, *102*, 4827–4833.

Kuda, T., Tsunekawa, M., Hishi, T., & Araki, Y. (2005). Antioxidant properties of dried 'kayamo-nori', a brown alga *Scytosiphon lomentaria* (Scytosiphonales, Phaeophyceae). *Food Chemistry*, *89*, 617–622.

Li, X. B. (2010). *The optimization of purification process of sulfate polysaccharide (SPS) from Spirulina and its antioxidative and resistance activity to Pyricularia oryzae*. Hangzhou: Zhejiang University.

Li, H., Zhou, Q., Cai, Y., Zhang, S., & Zhong, Y. C. (1999). Studies on polysaccharides from *Holothuria leucospilota*. *Chinese Medicinal Materials*, *9*, 328–329.

Li, L., Gao, Y. T., Dai, Y., Yang, Y. L., & Wang, X. M. (2007). Scavenging effects of *Spirulina* and polysaccharides *Spirulina platensis* on active oxygens and its antioxidation *in vitro*. *Chemistry & Bioengineering*, *24*, 55–57.

Li, H. F., Xu, J., Liu, Y. M., Ai, S. B., Qin, F., Li, Z. W., et al. (2011). Antioxidant and moisture-retention activities of the polysaccharide from *Nostoc commune*. *Carbohydrate Polymers*, *83*, 1821–1827.

Liu, F., & Ng, T. B. (2000). Antioxidative and free radical scavenging activities of selected medicinal herbs. *Life Sciences*, *66*, 725–735.

Luo, D. H. (2008). Identification of structure and antioxidant activity of a fraction of polysaccharide purified from *Dioscorea nipponica* Makino. *Carbohydrate Polymers*, *71*, 544–549.

MacDonald, J., Galley, H. F., & Webster, N. R. (2003). Oxidative stress and gene expression in sepsis. *British Journal of Anaesthesia*, *90*, 221–232.

Malanga, G., & Puntarulo, S. (1995). Oxidative stress and antioxidant content in *Chlorella vulgaris* after exposure to ultraviolet β-radiation. *Physiologia Plantarum*, *94*, 672–679.

Marklund, S., & Marklund, G. I. (1974). Involvement of superoxide anion radicals in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. *European Journal of Biochemistry*, *47*, 469–471.

Martin, J. P., & Burch, P. (1990). Production of oxygen radicals by photosensitzation. *Methods in Enzymology*, *186*, 635–645.

Mei, Q. H., Miao, Y. Q., Zhang, C. W., Lu, C. M., & Wu, G. R. (2005). Preliminary studies on the isolation, purification and physicochemical properties of extracellular acidic polysaccharides from Microcystis aeruginosa var. major. *Journal of Lake Sciences*, *17*(4), 322–326.

Moure, A., Dominguez, H., & Parajo, J. C. (2006). Antioxidant properties of ultrafiltration-recovered soy protein fractions from industrial effluents and their hydrolysates. *Process Biochemistry*, *41*, 447–456.

Nandita, S., & Rajini, P. S. (2004). Free radical scavenging activity of an aqueous extract of potato peel. *Food Chemistry*, *85*, 611–616.

Napolitano, G. E., Ackman, R. G., & Ratnayake, W. M. N. (1990). Fatty acid composition of three cultured algal species (*Isochrysis galbana*, *Chaetoceros gracilis* and *Chaetoceros calcitrans*) used as food for bivalve larvae. *Journal of the World Aquaculture Society*, *21*, 122–130.

Oyaizu, M. (1986). Studies on products of browning reaction: antioxidative activities of products of browning reaction prepared from glucosamine. *Japanese Journal of Nutrition*, *44*, 307–315.

Pu, Y. F., Wang, C. H., & Sun, Y. Y. (2012). Microwave extraction process of extracellular polysaccharide in *Isochrysis galbana*. *Journal of Food Science and Technology*, *37*(7), 186–190.

Qi, H. M., Zhang, Q., Zhao, T., Hu, R. G., Zhang, K., & Li, Z. E. (2006). In vitro antioxidant activity of acetylated and benzoylated derivatives of polysaccharide extracted from *Ulva pertusa* (Chlorophyta). *Bioorganic & Medicinal Chemistry Letters*, *16*, 2441–2445.

Ramarahnam, N., Osawa, T., Ochi, H., & Kawakishi, S. (1995). The contribution of plant food antioxidants to human health. *Trends in Food Science & Technology*, *6*, 75–82.

Ruch, K. J., Cheng, S. J., & Klauning, J. E. (1989). Prevention of cytotoxicity and inhibition of intercellular communication by antioxidant catechin isolated from Chinese green tea. *Carcinogenesis*, *10*, 1003–1008.

Schinella, G. R., Tournier, H. A., Priet, J. M., Mordujovich de Buschiazzo, P., & Ríos, J. L. (2002). Antioxidant activity of anti-inflammatory plant extracts. *Life Sciences*, *70*, 1023–1033.

Shi, Q. J. (2007). *The molecular modification of polysaccharides from Porphyridium cruentum and its antioxidation in vitro*. Yantai: Yantai University.

Smirnoff, N., & Cumbes, Q. J. (1989). Hydroxyl radical scavenging activity of compatible solutes. *Phytochemistry*, *28*, 1057–1060.

Spolaore, P., Joannis-Cassan, C., Duran, E., & Isambert, A. (2006). Commercial applications of microalgae. *Journal of Bioscience and Bioengineering*, *101*, 87–96.

Staob, A. M. (1965). Removal of proteins from polysaccharides methods. *Carbohydrate Chemistry*, *5*, 1–5.

Sukenik, A., & Wahnon, R. (1991). Biochemical quality of marine unicellular algae with special emphasis on lipid composition (I): *Isochrysis galbana*. *Aquaculture*, *97*, 61–72.

Sun, Y. Y., & Wang, H. (2013). Study of *in vitro* antioxidation and physical and chemical characteristics analysis of extracellular polysaccharides isolated from *Isochrysis galbana*. *Marine Sciences*, *5*, 45–49.

Sun, R. C., Fang, J. M., Goodwin, A., Lawther, J. M., & Bolton, A. J. (1998). Fractionation and characterization of polysaccharides from abaca fibre. *Carbohydrate Polymers*, *37*, 351–359.

Sun, L. Q., Wang, C. H., Shi, Q. J., & Ma, C. H. (2009). Preparation of different molecular weight polysaccharides from *Porphyridium cruentum* and their antioxidant activities. *International Journal of Biological Macromolecules*, *45*, 42–47.

Sun, L. Q., Wang, C. H., Shi, Q. J., Chen, C. L., & Wang, L. (2010). Degradation of polysaccharide from *Porphyridium cruentum* and its antioxidant activities *in vitro*. *East China University of Science and Technology (Natural Science Edition)*, *36*, 211–215.

Sun, Y. Y., Lei, Q. Y., Liu, X. X., Zhu, M., & Wang, C. H. (2010). Extraction process of intracellular and extracellular polysaccharide from *Isochrysis galbana*. *Food Science*, *31*, 55–59.

Synytsya, A., Čopíková, J., Matějka, P., & Machovic, V. (2003). Fourier transform Raman and infrared spectroscopy of pectins. *Carbohydrate Polymers*, *54*, 97–106.

Tannin-Spitz, T., Bergman, M., van-Moppes, D., Grossman, S., & Arad, S. (2005). Antioxidant activity of the polysaccharide of the red microalga *Porphyridium* sp. *Journal of Applied Phycology*, *17*, 215–222.

Therho, T. T., & Hartiala, K. (1971). Method for determination of the sulfate content of glycosamino glycans. *Analytical Biochemistry*, *41*, 471–476.

Tsiapali, E., Whaley, S., Kalbfleisch, J., Ensley, H. E., Browder, I. W., & Williams, D. L. (2001). Glucans exhibit weak antioxidant activity, but stimulate macrophage free radical activity. *Free Radical Biology and Medicine*, *30*, 393–402.

Wang, C. H., & Sun, Y. Y. (2005). The effect of nutrients on the growth and biochemical components of *Isochrysis galbana*. *Food and Fermentation Industries*, *31*, 13–17.

Wang, F., Liu, A., Zhang, H. Y., & Li, D. F. (2007). Electrospray ionization-mass spectrometry and its application in the polysaccharides and proteins. *Journal of Changchun University of Technology (Natural Science Edition)*, *28*(4), 444–447.

Wang, H. W., Cui, C. S., & Xu, Y. Q. (2007). Study on extraction of pumpkin polysaccharide with complex enzymes and its purification. *Food Science*, *8*, 247–249.

Wang, S. Y., Jiang, Y., Meng, C., OuYang, Y. H., & Lin, X. Z. (2011). Preparation of extracellular polysaccharide from fermentation liquor of marine microalgae *Schizochytrium* and study on the bioactivities. *Journal of Fuzhou University (Natural Science Edition)*, *39*, 786–791.

Wang, L., Sun, L. Q., & Zhao, X. H. (2012). Physicochemical property analysis of polysaccharides from marine microalgae and their antioxidation, hygroscopicity and moisture retention activities. *Fine Chemicals*, *1*, 20–25.

Wu, M., Li, W. Q., Zhang, S. J., & Chen, Q. H. (2004). Study on the conditions of extracting and purifying of polysaccharide from marine microalgae. *Journal of Ocean Technology*, *23*(1), 9–12.

Xia, H. F., & Yao, S. J. (2006). Cultivation of *Porphyridium* spp. and separation its sulfated polysaccharide. *Food Chemistry*, *27*(3), 75–78.

Xue, C., Yu, G., Hirata, T., Terao, J., & Lin, H. (1998). Antioxidative activities of several marine polysaccharides evaluated in a phosphatidylcholine–liposomal suspension and organic solvents. *Bioscience, Biotechnology, and Biochemistry*, *62*, 206–209.

Ye, S. H., Liu, F., Wang, J. H., Wang, H., & Zhang, M. P. (2012). Antioxidant activities of an exopolysaccharide isolated and purified from marine *Pseudomonas* PF-6. *Carbohydrate Polymers*, *87*, 764–770.

Yenhum, S. (1997). Antioxidant activity of green tea and its catechins in fish meat model system. *Journal of Agricultural and Food Chemistry*, *45*, 4262–4265.

Yildírím, A., Mavi, A., & Kara, A. A. (2001). Determination of antioxidant and antimicrobial activities of *Rumex crispus* L. extracts. *Journal of Agricultural and Food Chemistry*, *49*, 4083–4089.

Yim, J. H., Kim, S. J., Ahn, S. H., Lee, C. K., Rhie, K. T., & Lee, H. K. (2004). Antiviral effects of sulfated exopolysaccharide from the marine microalga *Gyrodinium impudicum* strain KG03. *Marine Biotechnology*, *6*, 17–25.

Zhang, Q. B., Yu, P. Z., Li, Z. E., Zhang, H., Xu, Z. H., & Li, P. C. (2003). Antioxidant activities of sulfated polysaccharide fractions from *Porphyra haitanensis*. *Journal of Applied Phycology*, *15*, 305–310.

Zhbankov, R. G., Andrianov, V. M., & Marchewka, M. K. (1997). Fourier transform IR and Raman spectroscopy and structure of carbohydrates. *Journal of Molecular Structure*, *436–437*, 637–654.

Zheng, W. F., Chen, C. F., Bao, K. D., Wang, Y. Q., & Chu, C. C. (2005). Components of exopolysaccharides of *Nitzschia closterium* and preparation of their sulfated products. *Chinese Traditional and Herbal Drugs*, *36*(12), 1790–1793.

Zhu, G. J., Zhao, Z. F., & Fang, H. Y. (2002). The relationship between structure and function of polysaccharides. *Journal of Wuxi University of Light Industry*, *21*(2), 209–212.

Zhu, J. H., Wang, M., Zhang, W., Li, C. J., & Zhang, C. W. (2008). Study on the toxicities and the antioxidative activities of selenium exopolysaccharide from *Porphyridium* sp. (Se-PSP). *Chinese Journal of Marine Drugs*, *27*, 48–51.