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Chemical modification and antioxidant activities of polysaccharide from mushroom *Inonotus obliquus*

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ABSTRACT

Chemical modification polysaccharides exerted potent biological property which was related to the physicochemical properties. In the present study, polysaccharides from *Inonotus obliquus* were modified by suflation, acetylation and carboxymethylation. The physicochemical and antioxidant properties of *I. obliquus* polysaccharide (IOPS) and its derivatives were comparatively investigated by chemical methods, gas chromatography, size exclusion chromatography, scanning electron micrograph, infrared spectra and circular dichroism spectra, and ferric reducing power assay and lipid peroxidation inhibition assay, respectively. Results showed that physicochemical and antioxidant properties of IOPS were differed each other after the chemical modification of suflation, acetylation and carboxymethylation. Among the three derivatives, acetylationed polysaccharide (Ac-IOPS) resulted in lower molecular weight distribution, lower intrinsic viscosity, a hyperbranched conformation, higher antioxidant abilities on ferric-reducing power and lipid peroxidation inhibition activity compared with the native polysaccharide IOPS. Ac-IOPS might be explored as a novel potential antioxidant for human consumption.

1. Introduction

Mushrooms are well known for their medicinal and nutritional value owing to series of active compounds they contain. *Inonotus obliquus*, which is a white-rot fungus belonging to the family of *Hymenochaetaceae* Donk and distributed in Europe, Asia, and North America, has been used as folk medicine for cancer and many other diseases such as heart, liver, and stomach disease especially in Russia since 16th century. In recent years, wide concern on the health-promoting effects of *I. obliquus* has been aroused from scholars all over the world (Taji et al., 2007). *I. obliquus* has been documented to contain triterpenes, polysaccharides, steroid, polyphenolics and melanin, showing various biological activities including antioxidant activities (Zheng, Zhang, Zhao, Miao, & Jiang, 2009), hypoglycaemic effects (Lu, Chen, Fu, & Zhang, 2010), immune-stimulating properties (Kim et al., 2005), anticancer activities (Song et al., 2008), and antimutagenic effects (Ham et al., 2009).

Mushroom polysaccharides have been widely studied for their chemical and biological activities in food and medicinal industry owing to their health-promoting properties. Moreover, there are already many medicinal mushroom polysaccharides being commercially produced. Polysaccharides, one of the main active components of *I. obliquus*, were reported to exhibit many biological activities such as antioxidant (Fu, Chen, Dong, Zhang, & Zhang, 2010), anticancer (Kim et al., 2006), and immune-stimulating (Kim et al., 2005) effects. It is generally admitted that biological activities of polysaccharides depended on its molecular structure including monosaccharide composition, glycosidic bond of the main chain, degree of substitution, degree of branching, sugar component and conformation of the main chains. Therefore, increasing attention was attracted to molecular modification and structure-activity relationship of polysaccharides. In recent years, many reports concerning the chemical modifications of polysaccharides have been published demonstrating that the biological activities were relatively improved by molecular modification (Tao, Zhang, & Zhang, 2009; Wang et al., 2010). Chemical modification such as sulfation, carboxymethylation, acetylation and $NaIO_4$ oxidation for polysaccharide have been studied for preparing custom-made derivatives having desirable functional attributes (Peng & Zhang, 2003; Yang, Du, Huang, Wan, & Li, 2002). Introduction of ionic groups with appropriate degree of substitution can influence the bioactivities of polysaccharide to some extent. Furthermore, some studies have shown that the higher bioactivities of polysaccharides by sulfation, Carboxymethylation modifications were partially attributed to expanded chain conformation (Tao et al., 2009; Yuan et al., 2005). There are, however, still no reports on the chemical modifications for polysaccharides from *I. obliquus* in the literature.

Oxidation, induced by oxygen radicals, is pervasive in the organism and believed to attribute to various diseases including cancer, rheumatoid arthritis and atherosclerosis as well as in degenerative processes of aging. It is accepted that superoxide anion, hydroxyl radical and hydrogen peroxide generated by normal metabolic

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processes can easily initiate the peroxidation of membrane lipids, leading to various diseases (Finkel & Holbrook, 2000). In our previous study, we investigated the effects of different drying methods on the antioxidant activities of polysaccharides from *I. obliquus* (IOPS), which indicated that it exhibited strong radical scavenging ability and antioxidant properties (Ma et al., 2011). Studies on various chemical modification for polysaccharides have been carried out to elucidate the correlation of structure to antioxidant (Liu & Zhao, 2010; Zhang, Lu, Fu, Wang, & Zhang, 2011). However, there is still no study on the structure-antioxidant activity relationship for IOPS.

In the present study, the purpose is to modify IOPS by chemical methods (sulfation, carboxymethylation and acetylation modifications). The molecular weight of different samples was determined using size-exclusion chromatography and compared with the native samples. In addition, the viscosity and conformation were also investigated. The antioxidant activities of IOPS and various IOPS derivatives were evaluated and compared, and the relationship between the structure and conformation behavior of IOPS with antioxidant activities was discussed.

2. Materials and methods

2.1. Materials and chemicals

Commercially available wild *I. obliquus* sclerotium was obtained from Guandong Company in Harbin, China. Voucher specimens (No. TJC 200702) were deposited at the School of Pharmaceutical Science and Technology, Tianjin University. Rhamnose, arabinose, glucose, galactose, mannose and xylose were provided by Sigma Chemical Co. (St, Louis, MO, USA). Sephadex G-100 was purchased from Pharmacia (GE, USA). All other chemicals and reagents were purchased locally and were of analytical grade.

2.2. Extraction and purification of IOPS

The polysaccharides present in the sclerotium of *I. obliquus* were extracted according to our previous study (Fu et al., 2010). Briefly, the sclerotium of *I. obliquus* was refluxed with 80% (v/v) ethanol at 80 °C for 2 h for three times. The dried residues (500 g) of *I. obliquus* and 6000 mL of distilled water were used for each extraction. The extract was filtered through a Whatman No. 1 filter paper and the filtrate was then concentrated with a rotary evaporator at 50 °C under vacuum. The proteins in the extract were removed using the Sevag reagent (Zhang, Huang, Hou, & Wang, 2006). After removal of the protein, 95% ethanol was added and the mixture was maintained overnight at 4 °C to precipitate polysaccharides, which aimed at removing impurity again. The precipitate was collected by centrifugation at 3000 g for 10 min and then washed with acetone and petroleum ether in turns. Then the crude polysaccharide was obtained (named as IOPS). The crude polysaccharide IOPS (100 mg) was dissolved in 10 mL distilled water, centrifuged at 3000 × *g* for 15 min. The supernatant was then subjected to the DEAE-52 cellulose column (2.6 cm × 50 cm, pH = 7.0) which was eluted with 0.0 M, 0.1 M, 0.2 M, and 0.5 M NaCl in turns at a flow rate of 1.0 mL/min. The fractions were dialyzed, concentrated, freeze dried and named as IOPS-1, IOPS-2, IOPS-3 and IOPS-4, respectively, among which IOPS-2 was the target in the present study and named as Un-IOPS.

2.3. Chemical modification for Un-IOPS

2.3.1. Sulfation modification

The sulfation agent, SO_3·pyridine, was obtained by dropping 20 mL of $HClSO_3$ into 100 mL of pyridine under cooling in an ice-water bath. Un-IOPS (200 mg) was added to 84 mL of pyridine, and the mixture was stirred at 60 °C for 30 min in order to disperse it into the solvent. Then 16 mL of the SO_3·pyridine complex was added. After reaction for 4 h, the mixture was cooled to room temperature by an ice-water bath, neutralized with 30% NaOH solution, and concentrated under reduced pressure to evaporate the solvent. The residue was added to 20 mL of dimenthylformamide (DMF) and filtered. The filtrate was then precipitated with acetone. The precipitate was dissolved in distilled water and freeze dried. This sample was named as Su-IOPS.

The sulfur contents of Su-IOPS were determined by Wang's method (Wang, Zhang, Zhang, Zhang, & Li, 2009). A calibration curve was constructed with sodium sulfate as standard. The DS was calculated according to the equation:

$$\mathrm{DS} = \frac{1.62 \times S\%}{32 - 1.02 \times S\%} \quad (1)$$

2.3.2. Acetylation modification

Un-IOPS (200 mg) was dispersed in 20 mL of pyridine, and the mixture was stirred at 60 °C for 30 min, then 20 mL of a mixture of pyridine and AC_2O (1:1, v/v) were added. The reaction mixture was kept at 60 °C for 4 h. Distilled water (250 mL) was added to react with the excess AC_2O, and the mixture was concentrated under reduced pressure. Anhydrous EtOH was added to the concentrated solution, and the mixture was kept overnight at 0–5 °C. The precipitate was filtered off and washed three times with EtOH. The resulting precipitate was dissolved in distilled water and freeze dried. This sample was named as Ac-IOPS.

The acetyl group (AG) and degree of substitution (DS) of Ac-IOPS were determined as described by Das, Singh, Singh, and Riar (2010). Acetyl group and DS were calculated as follows:

$$\mathrm{AG}\ (\%) = \frac{[(b - s) \times N \times 0.043]}{W} \times 100 \quad (2)$$

where b = volume of 0.2 N HCl used to titrate blank (mL), s = volume of 0.2 N HCl used to titrate sample (mL), N = normality of 0.2 N HCl, W = mass of sample (g, db). DS was calculated as follows:

$$\mathrm{DS} = \frac{162 \times \mathrm{AG}}{4300 - 42\mathrm{AG}} \quad (3)$$

2.3.3. Carboxymethylation modification

The process of carboxymethylation was achieved by a suspension of 200 mg Un-IOPS in 20 mL of pyridine being vigorously stirred for 15 min at room temperature. Then 20% of aqueous NaOH solution was added by dropwise. After stirred at room temperature for 1 h, monochloroacetic acid (MCA, 4 M) was added. The flask was immersed in a thermostatic oil bath to keep at a specific temperature for the desired duration time. The reaction products were cooled to room temperature in an ice-water bath, neutralized with hydrochloric acid and dialyzed against distilled water for 72 h. The dialyzates were concentrated and precipitated with 95% ethanol. This sample was named as Ca-IOPS. The content of carboxymethyl group was determined using the method described in the literature (Zhang & Ding, 1995).

2.4. Chemical composition analysis

Natural polysaccharides are always conjugated with other components to show various bioactivities. It is, therefore, necessary to analyze the main compositions of IOPS-2 and its derivates. The neutral sugar content, uronic acid content and protein content were determined respectively. Neutral sugar content of IOPS was analyzed by the modified phenol–sulfuric method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). The modified carbazole assay was conducted to analyze the uronic acid content (Bitter & Muir, 1962). Protein content was analyzed by the coomassie brilliant blue method (Lowry, Rosebrough, Farr, & Randall, 1951). Neutral monosaccharide composition was analyzed according to our

previous procedure (Chen, Qu, Fu, Dong, & Zhang, 2009). Briefly, Un-IOPS and its derives samples (10 mg) were hydrolyzed with 1 mL of 2 M trifluoroacetic acid (TFA) at 100 °C for 6 h, followed by evaporation. The hydrolyzate was reduced with $NaBH_4$ for 1.5 h at room temperature. The excess $NaBH_4$ was decomposed with glacial acetic acid and removed by repeated evaporation to dryness with the addition HCl–MeOH (0.1%) (Mawhinney, Feather, Barbero, & Martinez, 1980). Alditol acetates of the reduced sugars and authentic standards (rhamnose, arabinose, xylose, mannose, glucose and galactose) were prepared with AC_2O at 100 °C for 1 h and subjected to gas chromatography (GC) on an Shimadzu GC-14B instrument with capillary column (HP-5, 30 m × 0.32 mm × 0.5 μm). The operation was performed in the following conditions: injection temperature: 240 °C; detector temperature: 240 °C; column temperature programmed: 150–210 °C increasing at 10 °C/min for 6 min; then increasing to 255 °Cat 15 °C/min for 3 min; and finally increasing to 260 °C at 1 °C/min for 5 min. Nitrogen was used as the carrier gas and maintained at 1.0 mL/min.

2.5. Molecular weight analysis

The molecular weight of Un-IOPS and its derivatives was determined by Gel Permeation Chromatography (GPC, Sephdex G-150) with series of dextran T10, T40, T70, T500 as molecular standardsusing the method of Yang's with a slight of modification (Yang et al., 2002). GPC was performed for different polysaccharides derivatives of *I. obliquus* and dextran standards by elution with phosphate buffered saline (PBS, 0.2 M) at a flow rate of 6 mL/h, which fractions were collected for every 3 mL. The total carbohydrate of each fraction was analyzed by phenol–sulfuric method (Dubois et al., 1956). The molecular weight of each fraction was obtained from the regression line of the standard molecular weight versus elution volume plot. The average molecular weight of polysaccharides was calculated as the following equation.

$$M_w = \frac{\sum M_i C_i}{\sum C_i} \quad (4)$$

where M_i represents the molecular weight of each fraction, while C_i the total carbohydrate concentration of each fraction (Lai & Yang, 2007).

2.6. Intrinsic viscosity analysis

A Ubbelohde glass capillary viscometer (Lunjie, ShangHai, China) equipped in viscometer bath was used to measure the passage time of every sample dissolved with 0.2 M phosphate buffer solution (PBS, pH 7.0) flowing through capillary while the temperature was controlled at 25 ± 0.5 °C. All samples were centrifuged (3000 × *g*, 10 min) and filtered through 0.45 μm filter membrane prior to being measured. The different passage time between two times was controlled under ± 0.1 s. Intrinsic viscosity, [η], is defined as

$$[\eta] = \lim_{c \to 0} \frac{\eta sp}{c} = \frac{\ln \eta r}{c} \quad (5)$$

where η_r, the relative viscosity, is the specific value of the viscosity of sample solution to pure solvent η_0; η_{sp} is the specific viscosity and calculated as $\eta_r - 1$; and c is the concentration of the samples solutions (Stivala & Bahary, 1978).

2.7. Morphological analysis

Scanning electron micrographs were obtained with an environmental scanning electron microscope (ESEM, Philips XL-30). The polysaccharide samples of Un-IOPS, Su-IOPS, Ac-IOPS, and Ca-IOPS were placed on a specimen holder with the help of double-sided adhesive tapes and coated with gold powder. Each sample was observed with 200, 500 and 1000 fold magnification at an accelerating potential of 20 kV during micrography.

2.8. FT-IR analysis

FT-IR spectra were recorded with KBr pellets on a Nicolet FT-IR 360 and Nicolet 170SX FT-IR spectrophotometers. 16 scans at a resolution of 4 cm^{-1} were averaged and referenced against air.

2.9. Circular dichroism spectroscopy

Circular dichroism (CD) spectra were collected on a J-180CD (JASCO, Japan) spectropolarimeter using solutions of samples concentration 0.5 mg/mL. Each CD spectrum was the accumulation of three scans at 50 nm/min with a 1-nm slit width and a time constant of 1 s. Data was collected from 190 nm to 450 nm at 1 nm interval.

2.10. Antioxidant activities in vitro

2.10.1. Assay for ferric reducing power (FRP)

FRP potential of Un-IOPS and its derivatives was determined according to the modified method by Yen and Chen (1995). Different content (0.5, 1, 2, 3, 5 mg) of Un-IOPS and its derivatives samples were mixed with 2.5 mL of 0.2 M PBS (pH 6.6) and 2.5 mL of 1% postassium ferricyanide $K_3[Fe(CN)_6]$. The mixture was incubated at 50 °C for 20 min. Aliquots (2.5 mL) of 10% trichloroacetic acid were added to the mixture, which was then centrifuged for 10 min at 1000 × *g*. The upper layer of solution (2.5 mL) was mixed well with 2.5 mL of distilled water and 0.5 mL of 0.1% $FeCl_3$. Finally, the absorbance was measured at 700 nm using UV-Vis spectrophotometer (UV-2450, Shmadzu, Japan).

2.10.2. Assay for inhibition of liver lipid peroxidation

The assay was performed by using the method described by Chen et al. (2009) but with slight of modification. Liver of rats were excised, rapidly washed and homogenized in 10 volumes (v/w) of normal saline at 4 °C. Reaction mixtures containing 2.5 mL of liver homogenate, 1.4 mL of 0.2 mM PBS (pH 7.4) containing 2.5 mL of 10 μM $FeSO_4 \cdot 7H_2O$, and 2.5 mL of 100 mM ascorbic acid. Various concentrations of Un-IOPS and its derivatives (0.5, 1, 2, 3, 5 mg/mL) were added to the reaction mixture and incubated at 37 °C for 30 min, followed by centrifugation (3000 × *g*, 10 min). After the addition of 1.0 mL of the TBA reagent to the supernatant, the tubes were placed in a boiling water bath for 15 min. Absorbance was then measured at 530 nm using UV–vis spectrophotometer (UV-2450, Shmadzu, Japan), and the percent inhibition of lipid peroxidation of samples was calculated.

2.11. Statistical analysis

Values were expressed as means ±standard deviation (SD) of three replicates, and Student's test was used for the statistical analysis.

3. Results and discussion

3.1. Chemical composition analysis

The neutral sugar content, uronic content, protein content and monosaccharide composition of Un-IOPS and its derivatives were summarized in Table 1. Compared with the native sample, for chemical modification derivatives, not only the neutral sugar content of Ca-IOPS decreased significantly ($p < 0.05$) but also the uronic acid content and protein content were decreased, which was in accordance with the results of Sara et al. (Saha et al., 2010).

Table 1
Chemical composition of *I. obliquus* polysaccharides and its derivatives.

Samples	Un-IOPS	Su-IOPS	Ac-IOPS	Ca-IOPS
Neutral sugar (W%)	30.01 ± 0.49	27.87 ± 0.35[a]	41.29 ± 0.40[a]	17.77 ± 0.56[a]
Uronic acid (W%)	14.47 ± 0.02	11.05 ± 0.85[a]	14.11 ± 0.76[b]	6.94 ± 0.45[a]
Protein (W%)	8.45 ± 0.01	2.72 ± 0.00[a]	6.80 ± 0.01[a]	0.49 ± 0.00[a]
Sugar composition (M%)				
Rha (M%)	2.67	1.57[b]	1.64[b]	0.39[a]
Ara (M%)	3.20	0.84[a]	1.68[a]	0.41[a]
Xyl (M%)	6.57	5.65	4.95	27.60[a]
Man (M%)	21.60	12.90[a]	16.60[a]	11.60[a]
Glc (M%)	48.00	77.90[a]	73.20[a]	59.20[a]
Gal (M%)	17.90	1.16[a]	1.95[a]	0.76[a]

Rha, rhamnose; Ara, arabinose; Man, mannose; Gal, galactose; Glc, glucose.
[a] Means there was very significant difference between Un-IOPS and its derivatives.
[b] Means there was significant difference between Un-IOPS and its derivatives.

The reason might be the β-elimination reaction during the carboxymethylation modifications under alkali condition. Under the sulfation condition, the sulfation degree of Su-IOPS was low. Compared with the native sample, there was a slight degradation occurred in the process of Su-IOPS preparation. At the same time, the neutral sugar and uronic acid content of Su-IOPS were 27.87% and 11.05% respectively and changed little compared with the content of Un-IOPS. These results were in accordance with the reports of Yang and Du (2003), in which the neutral sugar and uronic acid content of sulfated Chinese lacquer polysaccharides were changed little (Yang and Du, 2003).

Polysaccharide, which is one of the three main biopolymers, was reported to have exhibited many health-promoting effects. The results of GC analysis of the acetylated monosaccharides revealed that the types of monosaccharide were not changed by chemical modification. All the four polysaccharide samples were composed of rhamnose, arabinose, xylose, mannose, glucose and galactose. Chemical modification resulted in the changes of molar ratios on monosaccharide composition, which were 2.67:3.20:6.57:2.16:48.00:17.90, 1.57:0.84:5.65:12.90:77.90:1.16, 1.64:1.68:4.95:16.60:73.20:1.95 and 0.39:0.41:27.60:11.60:59.20:0.76 for Un-IOPS, Su-IOPS, Ac-IOPS and Ca-IOPS, respectively (Table 1). The results implied that IOPS consist mainly of glucose as the major neutral sugar together with small amount of mannose and galactose, which parallels with the findings that β-glucan was the prominent active composition of polysaccharide from *I. obliquus* by Rhee, Cho, Kim, Cha, & Park, 2008. Significantly increased glucose content in the modified polysaccharide could be found in the results, so it can be inferred that the antioxidant activities of the samples might be mount correspondingly. On the other hand, the change of molar ratio on monosaccharide composition might lead to the change of conformation. The degree of substitution of Su-IOPS, Ac-IOPS and Ca-IOPS were 0.44, 0.31 and 0.35, respectively.

3.2. Molecular weight distribution

The gel permeation chromatography was summarized in Table 2. The native polysaccharide, Un-IOPS, showed a relative wide group with the peak molecular weight of 12.2×10^4 Da (Fig. 1). When the polysaccharide was sulfated, the M_w value of its derivatives decreased to 5.22×10^4 Da, which might be owing to the degradation of the biopolymers during the sulfation process. There was the same phenomina in Ca-IOPS sample. Tao et al. (2009) also reported that the molecular weight of sulfated TM derivatives were lower than that of the native samples, which was according with the finding in the present study. Compared with Un-IOPS, there was slight of degradation in the process of Ac-IOPS's preparation. The M_w value of carboxymethylated derivatives Ca-IOPS were much lower than that of the native samples, which might be due to the O-glycosidic bonds in polysaccharide–protein complex are apt to break and the β-elimination in alkali condition, leading to the decrease of M_w value (Peng & Zhang, 2003). It is further indicated that the proteins links to the sugar unit mainly through O-glycosidic bonds in IOPS-2 samples.

3.3. Intrinsic viscosity properties

The intrinsic viscosity [η] is a characteristic property of polysaccharide. Infinitely dilute solutions of polysaccharide can be seemed as the optimum condition for the evaluation of contribution of individual molecules to rheological properties of the whole solutions owing to the reason that dilute solutions can be viewed as systems in which individual polysaccharide coil are independently and are free to move (Lai & Yang, 2007). The fitted linear equation between molecular weight ($\log M_w$) and elution volume (VE), calculated by the methods of least squares, was $\log M_w = 0.0169\,VE + 6.4391$ ($R^2 = 0.906$). Thus, the intrinsic viscosity can be obtained accordingly. As the polysaccharide IOPS and

Table 2
Intrinsic viscosity, molecular weight and degree of substitution parameters of Un-IOPS and its derivatives.

Samples	Un-IOPS	Su-IOPS	Ac-IOPS	Ca-IOPS
Molecular weight ($\times 10^4$ Da)	12.20	5.22	10.32	2.68
[η] (mL/g)	7.69	1.38	1.22	0.82
DS	–	0.44	0.31	0.35

Fraction was eluted using GPC with 0.2 M PBS and samples were dissolved in 0.2 M PBS (pH 7.0) to measure intrinsic viscosity. M_w, [η] represent molecular weight, intrinsic viscosity, respectively. DS means the degree of substitution of Su-IOPS, Ac-IOPS and Ca-IOPS.

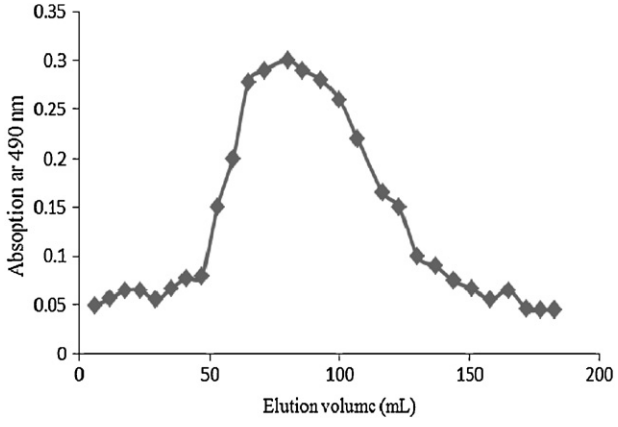

Fig. 1. Molecular size distribution of IOPS-2 (solvent: 0.2 M PBS). Error bars represent a standard deviation.

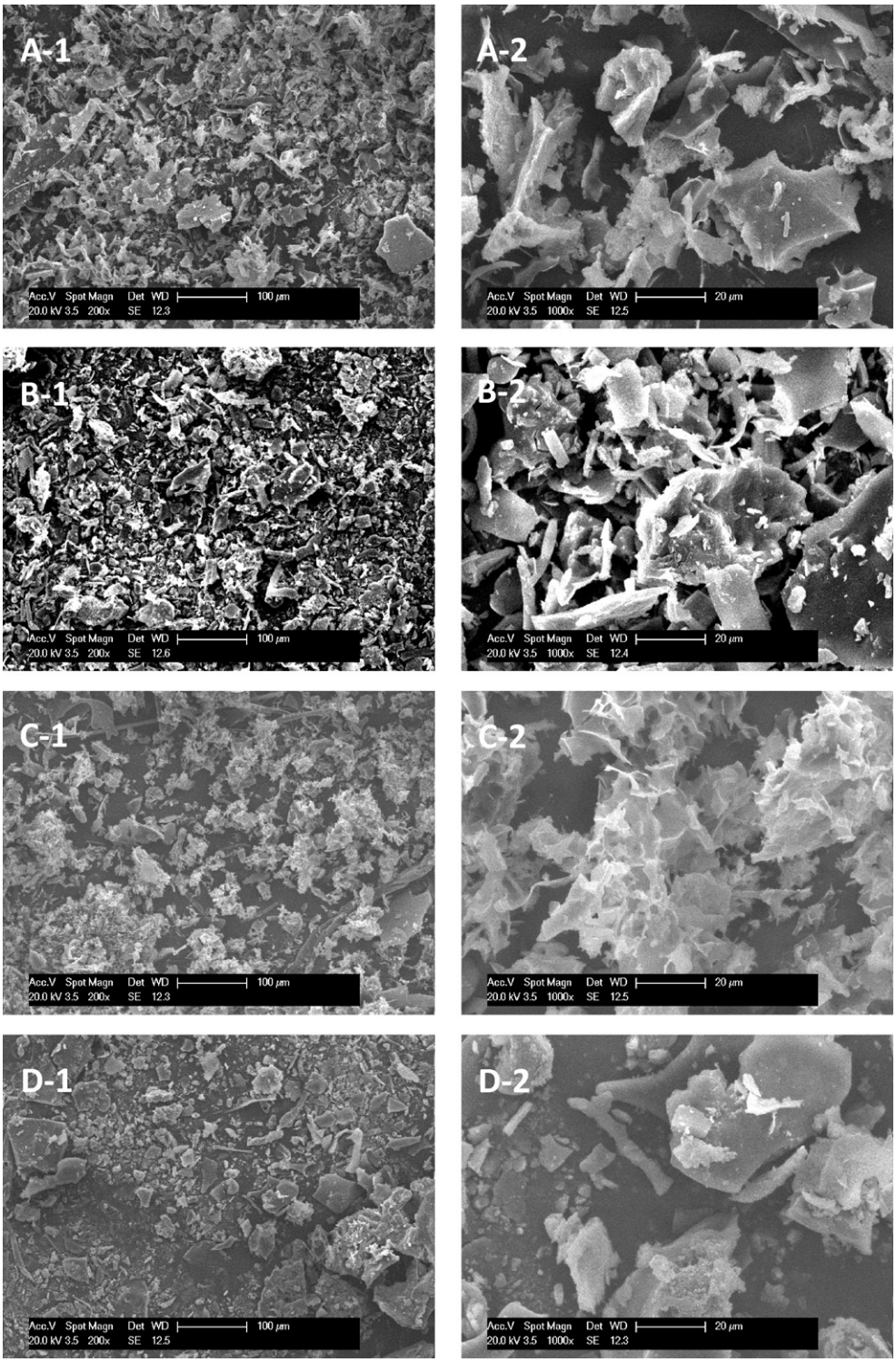

Fig. 2. Scanning electron micrographs of the four polysaccharides (A–D represent Un-IOPS, Su-IOPS, Ac-IOPS and Ca-IOPS, respectively. A-1, B-1, C-1 and D-1: ×200; A-2, B-2 C-2 and D-2: ×1000).

its derivates concentration increased, the effect of the interaction between the macromolecules over the reduced viscosity becomes prominent. Results were summarized in Table 2, showing that intrinsic viscosity of the samples were increased in the order of Ca-IOPS (0.82 mL/g), Ac-IOPS (1.22 mL/g), Su-IOPS (1.38 mL/g), and Un-IOPS (7.69 mL/g). The results implied that intrinsic viscosity of all Un-IOPS derivatives decreased, which might due to the decrease of the molecular weight.

3.4. Morphological analysis

Fig. 2 illustrates the scanning electron micrographs (SEM) of the native samples and its various derivatives at 200 and 1000 fold exaggeration condition. The surface of four polysaccharide samples shows significant variations in size and shape when viewed by SEM. The native sample exhibited a fragments appearance with not of uniform size. For chemical modifications samples,

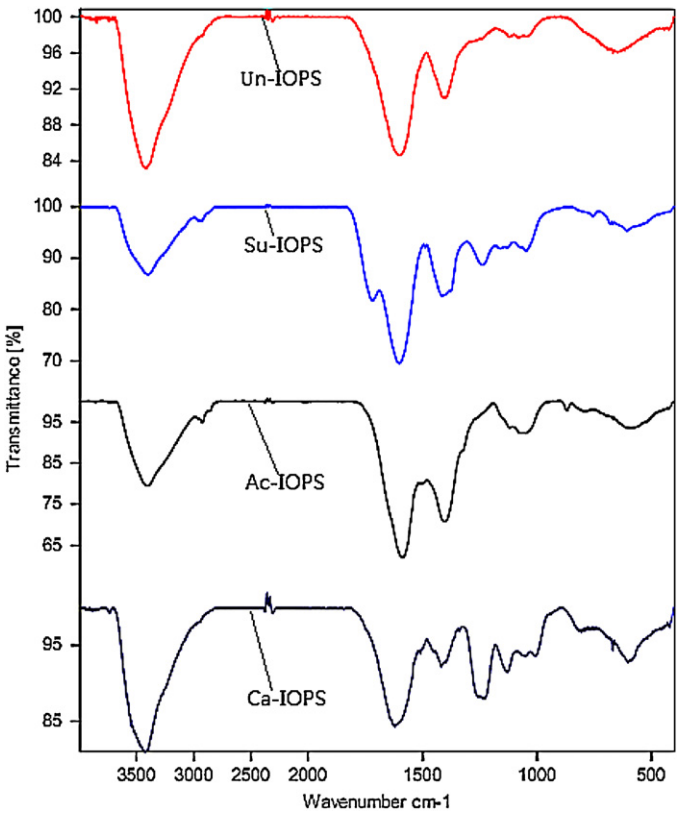

Fig. 3. FTIR spectra of the native polysaccharide Un-IOPS and its derivatives.

significant difference between them could be observed. Su-IOPS showed as irregular particles, whereas the surface of Ac-IOPS was rough, taking on island shape and plenty of bores, which indicated more branches. Ca-IOPS had particles similar to tile. The diameter of Un-IOPS, Su-IOPS, Ac-IOPS and Ca-IOPS were about 84.61 μm, 76.91 μm, 51.32 μm and 107.69 μm, respectively. These findings were in accordance with the molecular weight results. Zhang et al. (2010) reported that the SEM of sulfated polysaccharide exhibited a polyphasic convoluted structure, which was different with these results. The reason might be due to the different properties of different kind of polysaccharide from different resources.

3.5. Infrared spectra

The infrared spectra of Un-IOPS, Su-IOPS, Ac-IOPS and Ca-IOPS were illustrated in Fig. 3. For Su-IOPS, compared with native samples, the absorb band at 1229 cm^{-1} was due to asymmetrical S=O stretching vibration and the absorb band at 808 cm^{-1} was due to C—O—SO_3 stretching vibration, indicating that sulfation have actually occurred (Zhang, Zhang, Chen, & Zeng, 2000). Three new absorption peaks appeared at 1408, 1238 and 1717 cm^{-1} for the carboxymethylated derivatives, as a result of the existence of the H—C=O, C—O and C=O groups, respectively. In the spectrum of Ca-IOPS, the wake absorbance at 1550 and 2927 cm^{-1} was inferred to be COO^- and C—H stretching vibration, which suggested that the substitution degree of carboxymethyl was low (Zhang, Zhang, & Cheung, 2003).

3.6. Circular dichroism spectroscopy

The conformational changes of polysaccharides such as substitution by specific chromophores with optical activity including carboxyl groups, acetate groups and sulfenyl groups, may affect the polarizability, static field contributions, and orientation of the chromophores, leading to the change of the optical activity (Buffington, Pysh, Chakrabart, & Balazs, 1977). CD could provide a convenient method for investigating the conformational changes of polysaccharides. The CD spectrums were summarized in Fig. 4 in which every spectrum of the derivatives was compared with the native one. The native polysaccharide Un-IOPS conformation has a peak maximum at 211 nm and a minimum at about 190 nm. For Su-IOPS, on significant changes of polysaccharide main chain can be determined after sulfation (Duan & Fang, 2004). The native polysaccharide, does not give signal in the 190–340 nm whereas the N-acetyl polysaccharide, Ac-IOPS, gives an $n \rightarrow \pi$ transition with a c.d. band located near 205 nm. In Fig. 4C, there is a negative peak at 200 nm corresponding to the $n \rightarrow \pi$ transition of carboxymethyl group. These results were all in agreement with the findings in the infrared spectra analysis.

3.7. Antioxidant activities

3.7.1. Activity of ferric reducing power

The reducing capacity of a biopolymer would possibly serve as a significant indicator of its potential antioxidant effects. The mechanism for reducing property of reducer is that the presence of the antioxidant substances in the antioxidant samples causes the reduction of the Fe^{3+}/ferricyanide complex to the ferrous form. Briefly, Fe^{2+}can be monitored by measuring the absorption of the Prussian blue at 700 nm (Qi et al., 2006). As shown in Fig. 5A, the reducing power values of samples of Un-IOPS, Su-IOPS, Ac-IOPS and Ca-IOPS were 0.05, 0.06, 0.10, and 0.07, respectively, at the concentration of 0.5 mg/mL. The reducing capacity of all samples ascended with increasing concentration, which indicated that Un-IOPS and its derivatives were electron donors and could react with free radicals to convert them into more stable products. In the detecting concentration, Ac-IOPS showed pronounced advantage compared with the native sample Un-IOPS, while no obvious change could be detected between Su-IOPS, Ca-IOPS and Un-IOPS. Zou et al., however, reported that the reducing capacity of sulfated lacquer polysaccharides was enhanced (Zou et al., 2008), which could not be found in the present study. This might due to the different physicochemical properties of different kinds of polysaccharides. Tao et al. (2009) reported that bioactivity of two carboxymethylated polysaccharide–protein derivatives from Pleurotus tuber-regium was enhanced (Tao et al., 2009), however, no enhanced bioactivity could be detected for Ca-IOPS, which might be owing to the low substitution degree of carboxymethylation. Acetylation modification resulted in a significant increase of reducing power of the polysaccharides, which might be explained as that the increased solubility or the electron donor after modification (Lillo & Matsuhiro, 1997). On the basis of the assay of the in vitro reducing power, introduction of the ionic groups, decreased protein content, expanded chain, and relatively low M_w might be crucial to the enhancement of the reducing power activity of this polysaccharide.

3.7.2. Activity of inhibition on liver lipid peroxidation

Polysaccharide from *I. obliquus* has been widely defined as inhibitors of the oxidative deterioration (Ma et al., 2011). It is generally admitted that initiation of a peroxidation sequence in a cell or polyunsaturated fatty acid is due to the abstraction of a hydrogen atom from the double bond in the fatty acid. The free radical tends to stabilize by a molecular rearrangement to produce a conjugated diene, which then readily reacts with oxygen molecule to give a peroxy radical (Srivastava, Harish, & Shivanandappa, 2006). Malondialdehyde (MDA), the final product of the lipid peroxidation, was estimated as thiobarbituric acid (TBA) reactive substances. MDA is reacted with TBA under heat (100 °C) and acidic condition to produce red substance which has strong absorption (532 nm) in the

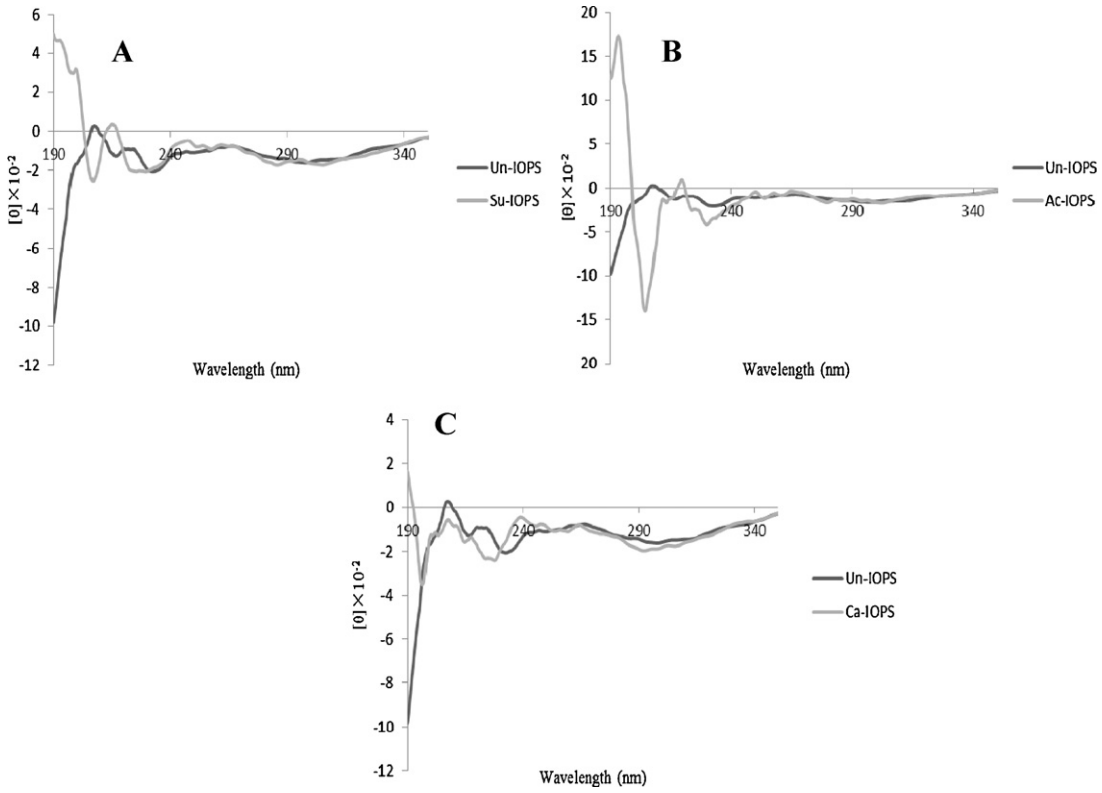

Fig. 4. The CD spectra of the native polysaccharide Un-IOPS and its derivates.

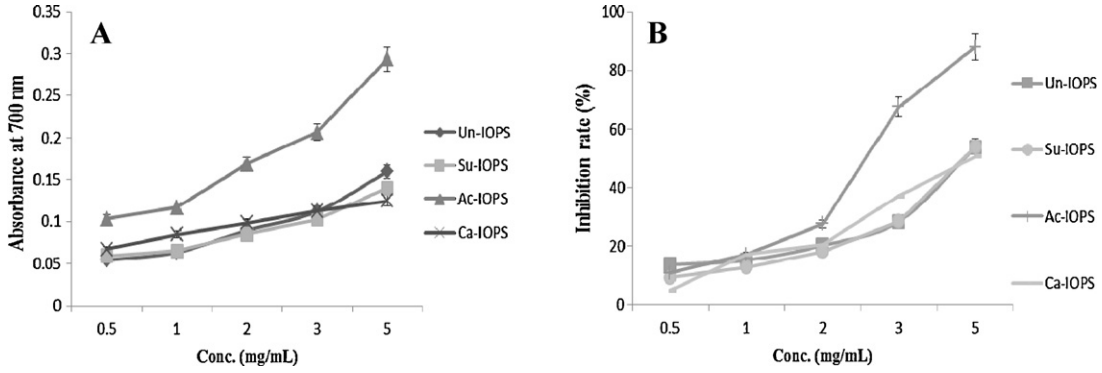

Fig. 5. Antioxidant activities of the native polysaccharide Un-IOPS. (A) Ferric reducing power activity. (B) Liver peroxidation inhibition activity; Results are means ± SD of three measurements.

aim of measuring of lipid peroxidation (Zhong, Jin, Lai, Lin, & Jiang, 2010).

The liver peroxidation inhibition activity of the four samples was summarized in Fig. 5B, indicating that the formation of MDA in Fe^{2+}-Vitamin C mediated lipid peroxidation in liver homogenate was inhibited in the presence of Un-IOPS and its derivatives and the inhibitory effects were in concentration dependent order. The inhibition rate of Un-IOPS, Su-IOPS, Ac-IOPS and Ca-IOPS were found as 53.56%, 53.85%, 87.81% and 50.51% respectively when concentration was at 5.0 mg/mL, suggesting that Ac-IOPS and Su-IOPS showed relatively strong liver peroxidation capacity. From the point of IC_{50}, the value of IC_{50} increased in the order of Ac-IOPS (IC_{50} = 2.73 mg/mL) < Ca-IOPS (IC_{50} = 4.75 mg/mL) < Su-IOPS (IC_{50} = 4.84 mg/mL) < Un-IOPS (IC_{50} = 5.56 mg/mL). The results suggested that chemical modification derivatives including Ac-IOPS, Ca-IOPS and Su-IOPS and showed stronger inhibition activity than the native polysaccharide Un-IOPS. Moreover, Ac-IOPS exhibited a relative strong liver peroxidation inhibition activity than Ca-IOPS and Su-IOPS, indicating that acetylation modification for IOPS could be seen as an efficient way to enhance physiochemical properties of polysaccharides with desired antioxidant activities. It was suggested that the *I. obliquus* polysaccharide and their derivatives had higher MDA inhibition activity, and the effect of introduction of ionic groups, the content of protein, and M_w on the bioactivity for the polysaccharide might not be neglected.

4. Conclusion

Polysaccharides from *I. obliquus* were firstly modified by chemical methods (suflation, acetylation and Carboxymethylation) in this study. Monosaccharide composition, molecular weight, viscosity, infrared spectra, CD spectra and antioxidant activities of Un-IOPS and its derivatives were comparatively investigated respectively. From the infrared spectra analysis, it was infered that sulfation, acetylation and carboxymethylation reaction all had actually

occurred, which was further verified by the CD spectra analysis. The molecular weight distribution and intrinsic viscosity of three polysaccharides derivatives were decreased compared with the native sample. Among the three modification product, Ac-IOPS exhibited pronounced advantage over the native polysaccharide according two antioxidant assays in vitro. It is noteworthy that Ac-IOPS might be explored as a novel potential antioxidant after its further study on the safety for human consumption and antioxidant activity in vivo.

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