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Purification, composition analysis and antioxidant activity of different polysaccharide conjugates (APPs) from the fruiting bodies of *Auricularia polytricha*

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ABSTRACT

In this study, four purified polysaccharides (APPsA-1, APPsB-1, APPsB-2 and APPsC-1) were derived from the fruiting bodies of *Auricularia polytricha* by size-exclusion chromatography and ion-exchange chromatography on an ÄKTA explore 100 purification system. Chemical and physical characteristics of four purified polysaccharides were investigated by a combination of chemical and instrumental analysis methods. On the basis of hydroxyl radical assay, superoxide radical assay and Fe^{2+}-chelating ability assay, the antioxidant activities of APPsA-1, APPsB-1, APPsB-2 and APPsC-1 were investigated. The results showed that all fractions exhibited antioxidant activities in a concentration-dependent manner, and the higher the content of uronic acid, the stronger the antioxidant activities of APPs. Therefore they should be developed as new antioxidant agents.

1. Introduction

Oxygen is one of the most important elements on the earth, which occupies 53.8% of the earth crust and forms about 21% of the atmosphere. Oxygen is involved in life metabolism and become the material base of vital movement in addition to anaerobe. The generation of reactive oxygen species (ROS) is an unavoidable consequence of life in an aerobic environment. On the one hand, production of ROS is essential to many organisms for the production of energy to fuel biological processes; on the other hand, excessive free radicals are implicated in the pathogenesis of many human diseases, including cancer, aging, atherosclerosis, reperfusion injury and hepatic injury (Barry & Gutteridge, 1989; Ke et al., 2009; Luo & Fang, 2008; Zha et al., 2009; Zou et al., 2008) through lipid peroxidation (Barry & Susanna, 1993), DNA damage (Barry & Aruoma, 1991) and inhibition of protein synthesis (Martin & Dean, 1991) and so on. In order to reduce the oxidative damage of ROS, many synthetic antioxidants, such as butylated hydroxyanisole, butylated hydroxytoluene, *tert*-butyl-hydroquinone and propyl gallate, are used widely at present. However, recent research suggested that synthetic antioxidants were restricted due to potential hazards related to health (Grice, 1988). Therefore, enhancement of body's antioxidant defences through natural and safe antioxidants would seem to provide a reasonable and practical approach to reduce the oxidative stress to human body. Published data indicated that natural polysaccharides and their conjugates, which are widely distributed in animals, plants, and microorganisms, in general possessed potential and potent antioxidant activities and could be explored as novel potential antioxidants (Cui et al., 2008; Ge, Duan, Fang, Zhang, & Wang, 2009; Matkowski, Tasarz, & Szypula, 2008; Yuan, Zhang, Fan, & Yang, 2008; Zhu et al., 2009).

Auricularia polytricha, which belongs to Auriculariaceae family, is widespread in many districts of China, such as Heilongjiang, Hebei, Jiangxi, Anhui, and Guangdong province. The modern pharmacology research indicated that it has the functions of lowering blood-fat, antioxidant, antitumor, antinociceptive, and immunomodulatory activities (Luo et al., 2009; Mau, Chao, & Wu, 2001; Yang et al., 2002). However, up to now, no detailed investigations have been conducted on composition characterization and antioxidative capacities of different polysaccharides isolated from *A. polytricha*.

In view of the above, the present work was for the first time reported on the extraction and purification of different fractions of polysaccharide conjugates from the fruiting bodies of *A. polytricha* on an ÄKTA explore 100 purification system. In addition, the properties and antioxidant activities of these polysaccharides were also identified.

2. Materials and methods

2.1. Materials and chemicals

DEAE Sepharose Fast Flow and Sepharose 6 Fast Flow were purchased from Amersham (Sweden). T-series dextran, dimethyl

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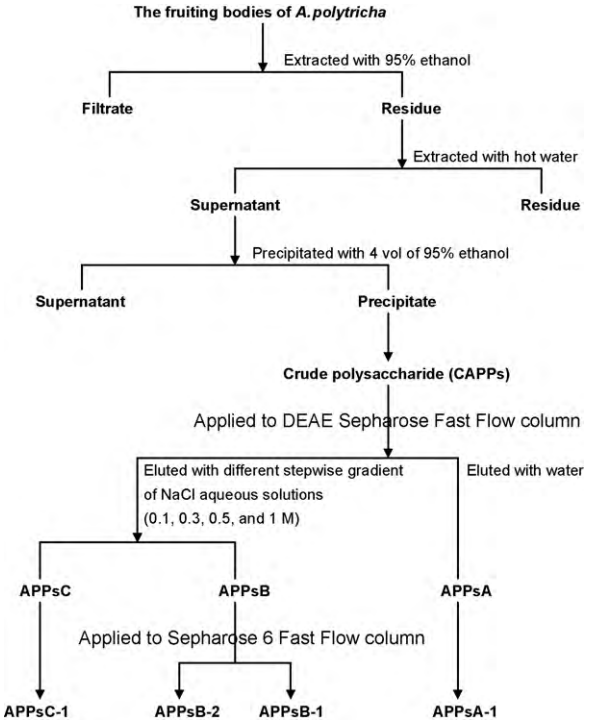

Fig. 1. Summarized extraction scheme of APPs from the fruiting bodies of *A. polytricha*.

sulfoxide (DMSO), standard sugars, D-glucuronic acid, deoxyribose, trichloride ferric ($FeCl_2$), ferrous sulfate ($FeSO_4$), ethylene diamine tetra-acetic acid (EDTA), ferrozine, nitroblue tetrazolium (NBT), phenazine methosulfate (PMS), dihydromicotineamidadenine dinucleotide (NADH), Tris–HCl buffer, H_2O_2, ascorbate acid, and thiobarbituric acid (TBA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All of other reagents were analytical grade from Peking Chemical Co. (Peking, China).

2.2. Extraction and purification of polysaccharide conjugates fractions

As shown in Fig. 1, the fruiting bodies of *A. polytricha* were extracted with 95% ethanol (5000 ml) at 75 °C for three times and 3 h for each time under reflux to remove lipid. The residue was then extracted with distilled water (8000 ml) at 75 °C for three times and 3 h for each time. After centrifugation (1700 × *g* for 10 min, at 20 °C), the supernatant was concentrated to one tenth of the volume, and precipitated with 4 vol of 95% ethanol at 4 °C for 24 h. The precipitate was washed with absolute ethanol, acetone and ether, respectively. The washed precipitate was the crude polysaccharide (CAPPs).

The CAPPs were purified on an ÄKTA explore 100 purification system equipped with a pump P-900, a UV-900 monitor, a pH/C-900 monitor, a fraction collector 950 (Frac-950) and an auto-sampler A-900. The CAPPs were dissolved in distilled water, centrifuged, and then the supernatant was applied to a DEAE Sepharose Fast Flow column (2.6 cm × 40 cm) equilibrated with ultrapure water. 1 column volume (CV) is about 160 ml. After loading with sample, the column was eluted with 1 CV of ultrapure water and then with 1 CV of different stepwise gradient of NaCl aqueous solutions (0.1, 0.3, 0.5, and 1 M) at a flow rate of 4 ml/min, respectively. Different fractions (8 ml in each tube) were collected using the Frac-950, and then purified further on a Sepharose 6 Fast Flow column (2.6 cm × 100 cm) with 0.15 M/l NaCl at a flow rate of 1 ml/min to yield four main fractions (APPs), coded as APPsA-1, APPsB-1, APPsB-2 and APPsC-1. All the fractions were collected, dialyzed and lyophilized to give white purified polysaccharide fractions. Total carbohydrate content of each tube was measured at 490 nm by Dubois's method, and protein absorption at 280 nm was measured for each fraction.

2.3. General methods

The total carbohydrate content was determined by the phenol-H_2SO_4 method, with glucose as the standard (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). Protein was measured by the Bradford's method (Bradford, 1976) using bovine serum albumin (BSA) as the standard. Uronic acid contents were determined by measuring the absorbance at 525 nm using the m-hydroxybiphenyl colourimetric procedure and with D-glucuronic acid as the standard (Blumenkrantz & Asboe-Hansen, 1973). UV–vis absorption spectra were recorded with a UV–vis spectrophotometer (Model SP-752, China). The IR spectrum of the polysaccharide was determined using a Fourier transform infrared spectrophotometer (FTIR, Bruker, Germany). The purified polysaccharides were dried at temperature in vacuum over P_2O_5 for 48 h prior to making pellet with KBr powder for FTIR measurement in a range of 400–4000 cm^{-1}. Gas chromatography (GC) was performed on a Shimadzu GC-14C instrument (Shimadzu, Japan) equipped with a DB-1 capillary column (30 m × 0.25 mm × 0.25 μm) and flame-ionization detector (FID). The column temperature was kept at 120 °C for 2 min, and increased to 250 °C (maintained for 3 min) at a rate of 8 °C/min. The injector and detector heater temperature were 250 and 300 °C, respectively. The rate of N_2 carrier gas was 10 ml/min.

2.4. Monosaccharide composition

GC was used for identification and quantification of the monosaccharides. Samples were hydrolyzed and acetylated according to Lehrfeld (1985). Firstly, the samples (10 mg) were hydrolyzed with 2 M TFA (2 ml) at 120 °C for 2 h, and the excess acid was completely removed by co-distillation with ethanol. Then the hydrolyzed product was reduced with KBH_4 (30 mg), followed by neutralization with dilute acetic acid and evaporated at 45 °C after adding 1 mg myo-inositol and 0.1 M Na_2CO_3 (1 ml) at 30 °C with stirring for 45 min. The residue was concentrated by adding methanol. Finally the reduced products (alditols) were added with 1:1 pyridine–propylamine at 55 °C with stirring for 30 min, and acetylated with 1:1 pyridine–acetic anhydride in a boiling water bath for 1 h. The acetylated products were analyzed by GC, and identified and estimated with myo-inositol as the internal standard.

2.5. Molecular weight determination

The average molecular weight of APPs was determined by high-performance size-exclusion chromatography (HPSEC) (Sun et al., 2008), which was performed on a SHIMADZU HPLC system fitted with one TSK-G3000PW_{XL} column (7.8 mm ID × 30.0 cm) and a SHIMADZU RID-10A detector. The data were processed by GPC processing software (Millennium^{32} version). The mobile phase was 0.7% Na_2SO_4, and the flow rate was 0.5 ml/min at 40 °C, with 1.6 mPa. A sample (3 mg) was dissolved in the mobile phase (0.5 ml) and centrifuged (10,000 rpm; 3 min), and 20 μl of supernatant were injected in each run. The molecular mass was estimated by reference to a calibration curve made from a set of Dextran T-series standards of known molecular masses (T-130 80, 50, 25, 10).

2.6. Assay for antioxidant activity

2.6.1. Hydroxyl radical assay

Assessment of the scavenging ability of APPs on hydroxyl radicals was performed by the method previously described by Halliwell, Gutteridge, and Aruoma (1987), with a minor modification. Reaction mixtures in a final volume of 1.0 ml contained deoxyribose (60 mM), phosphate buffer (pH 7.4, 20 mM), ferric trichloride (100 μM), EDTA (100 μM), H_2O_2 (1 mM), ascorbic acid (100 μM) and different concentrations of APPs (0, 0.25, 0.5, 1, 2, or 4 mg/ml). Solutions of ferric trichloride and ascorbic acid were made immediately before use. The reaction solution was incubated for 1 h at 37 °C, and then 1 ml of 1% TBA and 1 ml of 20% (v/v) HCl were added to the mixture. The mixture was boiled for 15 min and cooled on ice. Deionized water and ascorbic acid served as blank and positive control, respectively. The absorbance of the resulting mixture was measured at 532 nm. The scavenging activity of hydroxyl radical (%) was calculated according to the following equation:

$$\text{Scavenging effect (\%)} = \frac{A_{532(\text{blank})} - A_{532(\text{sample})}}{A_{532(\text{blank})}} \times 100$$

where $A_{532(\text{blank})}$ was the absorbance of the control (deionized water, instead of sample); $A_{532(\text{sample})}$ was the absorbance of the test sample mixed with reaction solution.

2.6.2. Superoxide radical assay

The superoxide radical scavenging activity of APPs was evaluated according to the method detailed by Sun, Wang, Fang, Gao, and Tan (2004). Briefly, superoxide radicals were generated in 3.0 ml of 16 mM Tris–HCl buffer (pH 8.0), containing 78 mM reduced nicotinamide adenine dinucleotide (NADH), 50 μM nitroblue tetrazolium (NBT), 10 μM phenazin methosulfate (PMS), and APPs at given concentrations of 0, 25, 50, 100, 200 and 400.0 μg/ml. The coloration reaction of superoxide radicals with NBT was determined at 560 nm. The deionized water was used as the blank control and ascorbic acid was used as positive control. The scavenging activity of superoxide radicals (%) was calculated according to the following equation:

$$\text{Scavenging effect (\%)} = \frac{A_{560(\text{blank})} - A_{560(\text{sample})}}{A_{560(\text{blank})}} \times 100$$

where $A_{562(\text{blank})}$ was the absorbance of the control (deionized water, instead of sample); $A_{562(\text{sample})}$ was the absorbance of the test sample mixed with reaction solution.

2.6.3. Determination of Fe^{2+}-chelating ability

The chelating activity of sample on Fe^{2+} was measured as reported (Dinis, Madeira, and Almeida, 1994) by measuring the formation of ferrous iron–ferrozine complex. Different concentration of sample (0, 0.25, 0.5, 1, 2, or 4 mg/ml) was mixed with 3.7 ml of deionized water, and then reacted with $FeSO_4$ (2 mM, 0.1 ml). The reaction was allowed to proceed for 30 s. After 0.2 ml of 5 mM ferrozine was added, the solution was mixed, left to stand for 10 min at room temperature, and then the absorbance of the mixture was determined at 562 nm. Ethylenediaminetetraacetic acid (EDTA, 10 Ag/ml) was co-assayed as a positive control. A lower level of absorbance indicated stronger chelating activity. The chelating activity of APPs on Fe^{2+} (%) was calculated according to the following equation:

$$\text{Chelating ability (\%)} = \frac{A_{562(\text{blank})} - A_{562(\text{sample})}}{A_{562(\text{blank})}} \times 100$$

where $A_{562(\text{blank})}$ was the absorbance of the control (deionized water, instead of sample); $A_{562(\text{sample})}$ was the absorbance of the test sample mixed with reaction solution.

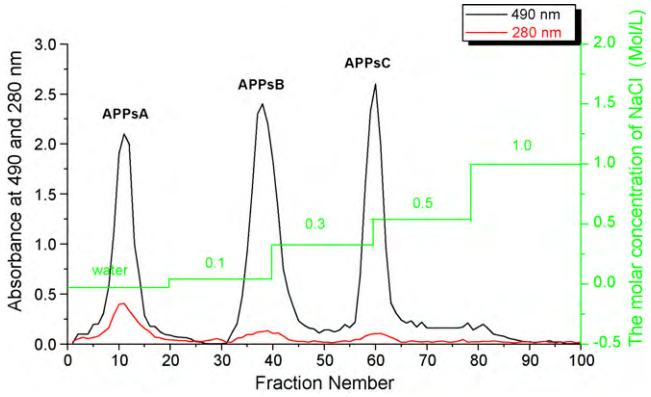

Fig. 2. The profile of APPs isolated from the fruiting bodies of *A. polytricha* on a DEAE Sepharose Fast Flow column eluted with distilled water and stepwise gradient of NaCl aqueous solutions (0.1, 0.3, 0.5, and 1 M) at a flow rate of 4 ml/min.

3. Results and discussion

3.1. Isolation, purification and physicochemical properties of different polysaccharide conjugates fractions

ÄKTA explore 100 purification system was successfully employed to purify the polysaccharide from the fruiting bodies of *A. polytricha*. According to the charge difference, three fractions of APPsA, APPsB and APPsC were isolated from distilled water elute and NaCl elute by the means of ion-exchange chromatography method, respectively (Fig. 2). On account of molecular weight difference, and then different charged polysaccharide was further purified by size-exclusion chromatography on a Sepharose 6 Fast Flow column, giving four homogeneous fractions of APPsA-1, APPsB-1, APPsB-2 and APPsC-1 (Fig. 3).

The polysaccharide content, protein content, uronic acid content, molecular weight, and monosaccharides composition of APPsA-1, APPsB-1, APPsB-2 and APPsC-1 were given in Table 1. The infrared spectra of APPsA-1, APPsB-1, APPsB-2 and APPsC-1 all displayed a broad stretching intense characteristic peak at around 3407 cm^{-1} due to hydroxyl stretching vibration of the polysaccharides, and a weak C–H stretching vibration band at 2931 cm^{-1}. Two stretching peaks at 1077 and 1154 cm^{-1} suggested the presence of C–O bonds. In addition, signals at 1700 cm^{-1} standed for stretch vibration of C=O of uronic acids and 1410 cm^{-1} for stretch vibration of C–O within COOH (data not shown).

Table 1
Components of monosaccharide and properties of APPs from the fruiting bodies of *A. polytricha*.

Samples	APPsA-1	APPsB-1	APPsB-2	APPsC-1
Total sugar (%)	85.3	89.4	92.7	93.1
Protein (%)	14.2	9.1	8.8	6.3
Uronic acid (%)	nd[a]	23.2	24.1	32.8
Average molecular weights	4.3×10^4	4.6×10^4	1.4×10^4	2.7×10^4
Sugar components (mol%)				
Mannose	4.2	3.3	3.5	2.5
Galactose	2.3	1.7	2.1	2.1
Glucose	1.1	0.4	0.6	0.2
Glucuronic acid	nd	1.7	2.1	2.4

[a] nd: not detected.

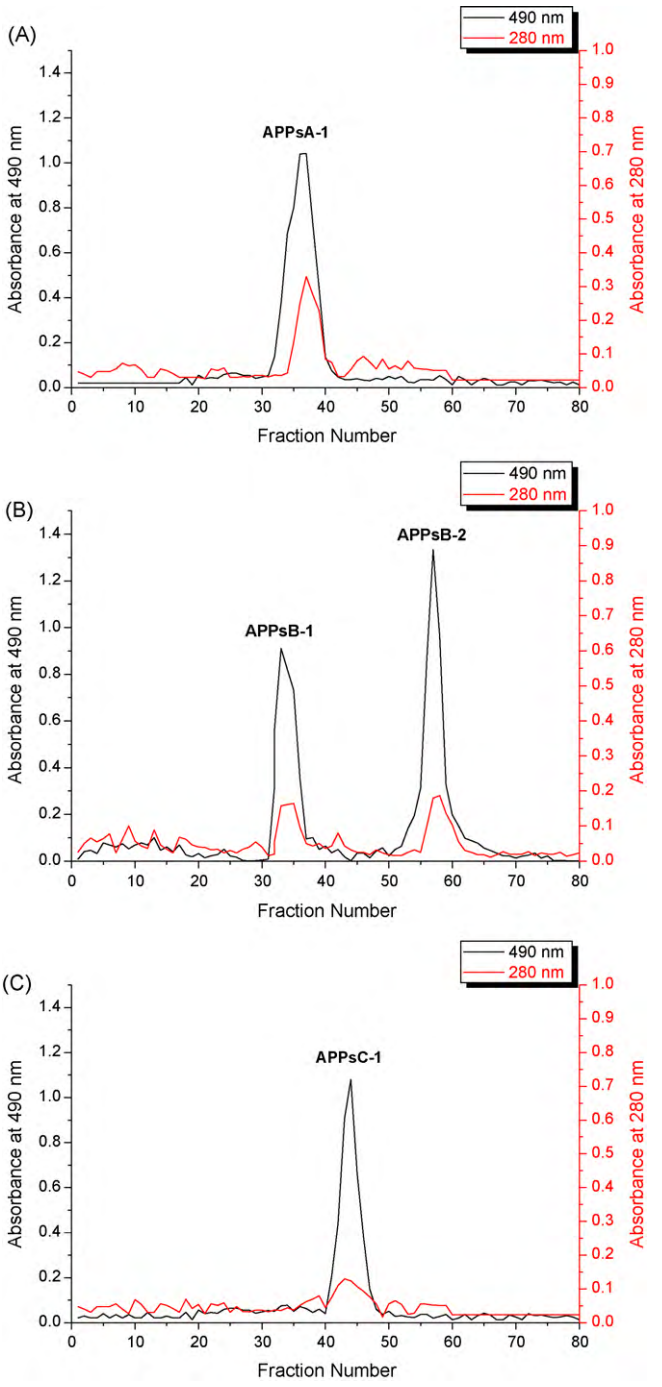

Fig. 3. The profile of APPs isolated from the fruiting bodies of *A. polytricha* on a Sepharose 6 Fast Flow column eluted with 0.15 M/l NaCl aqueous solutions at a flow rate of 1 ml/min.

3.2. Antioxidant activity

3.2.1. Scavenging effects of polysaccharide conjugates on hydroxyl radicals

Among the reactive oxygen species, the hydroxyl radical is the most reactive one and induces severe damage to adjacent bio-molecules. Hydrogen peroxide and superoxide molecules can lead to oxidative injury in the bio-molecules indirectly by producing hydroxyl radical via Fenton reaction and/or iron-catalyzed Haber–Weiss reaction, which can be prevented and/or inhibited by antioxidants (Erel, 2004). The scavenging effects of various samples (0–4 mg/ml) on hydroxyl radicals are shown in Fig. 4. APPsC-1

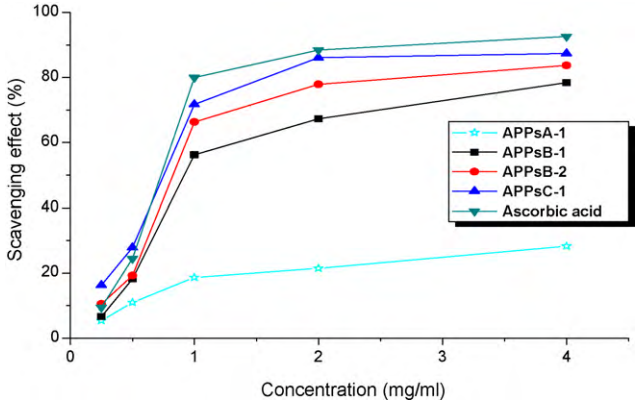

Fig. 4. Scavenging effects of APPs on hydroxyl radicals with ascorbic acid as a positive control. Results were presented as mean value ($n = 3$).

possessed higher hydroxyl radical scavenging activity than other fractions, namely APPsA-1, APPsB-1, and APPsB-2, which was close to that of ascorbic acid. There was on significant difference on scavenging activity between APPsB-1 and APPsB-2 at the concentration rang of 0–4 mg/ml. The hydroxyl radical scavenging ability and content of uronic acid decreased in the same order of ascorbic acid > APPsC-1 > APPsB-2 > APPsB-1 > APPsA-1.

3.2.2. Scavenging effects of polysaccharide conjugates on superoxide radicals

Although superoxide is a relatively weak oxidant, and is likely looked upon as alkali in water solution. But it decomposes to form stronger reactive oxidative species, such as singlet oxygen and hydroxyl radicals, which possesses greater oxidative and oleophilic ability than the precursor to initiate peroxidation of lipids in a longer time. In addition, superoxides are also known to indirectly induce lipid peroxidation as a result of H_2O_2 formation, creating precursors of hydroxyl radicals (Dahl & Richardson, 1978). From Fig. 5, we can see that their scavenging ability on superoxide radicals correlated positively well with increasing concentrations (0–400 μg/ml) and uronic acid content (0–32.8%). APPsC-1 exhibited the highest scavenging effect on superoxide radicals among all fractions, followed by APPsB-2 and APPsB-1, which were similar to that of ascorbic acid. The APPsA-1 containing no uronic acid did not present any scavenging activity on superoxide radicals, as compared with others containing uronic acid and ascorbic acid. Their scavenging ability on superoxide radicals decreased in the order of APPsC-1 > ascorbic acid > APPsB-2 > APPsB-1 > APPsA-1. Accordingly this order conformed to their content of uronic acid contained.

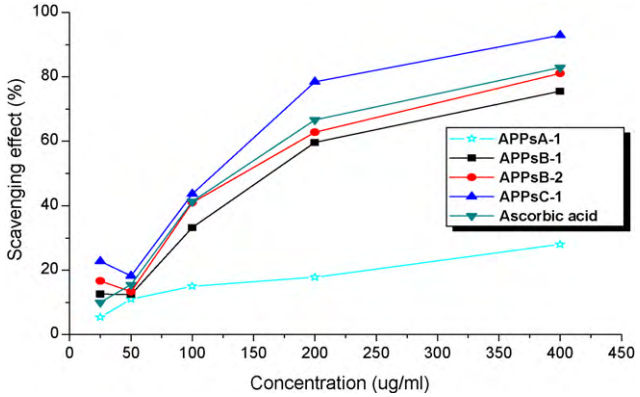

Fig. 5. Scavenging effects of APPs on superoxide radicals with ascorbic acid as a positive control. Results were presented as means ± S.D. ($n = 3$).

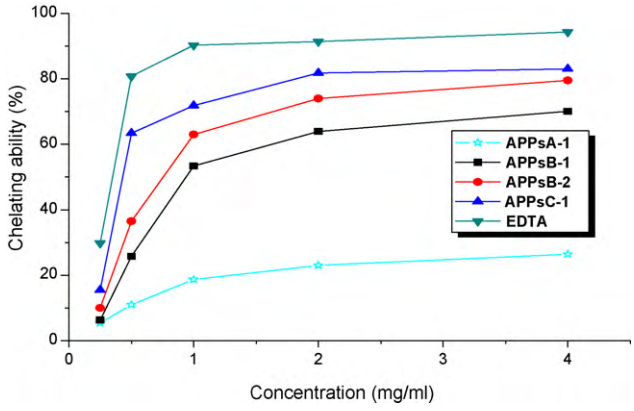

Fig. 6. Chelating ability of APPs on Fe^{2+} with EDTA as a positive control. Results were presented as mean value ($n = 3$).

3.2.3. *Chelating ability of polysaccharide conjugates on Fe^{2+}*

Some research reported some transition metals, such as Fe^{2+}, Cu^{+}, Pb^{2+}, Co^{2+} and so on, could trigger process of free radical reaction to magnify the cellular damage. Fe^{2+} is known as the most powerful pro-oxidant among various species of metal ions due to its high reactivity, which accelerates lipid oxidation by breaking down hydrogen and lipid peroxidase to reactive free radicals via the Fenton type reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\bullet} + OH^{-}$). Fe^{3+} ion also produces radicals from peroxides, although the rate is 10-fold less than that of Fe^{2+} ion ($Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + OOH^{\bullet} + H^{+}$). Metal chelating activity is claimed as one of antioxidant mechanisms, since it reduces the concentration of the catalyzing transition metal in lipid peroxidation (Qiao et al., 2009). Under methanol or ethanol solution, ferrozine can react with Fe^{2+} to form red complexes of ferrozine–Fe^{2+}. When there is other chelating agent, the ferrozine–Fe^{2+} formation is disrupted with the result that the red color of the complexes decreases. Therefore measurement of absorption value of reaction solution in 562 nm could be used to estimate the metal chelating activity of antioxidant (Wang et al., 2009). The chelating ability of APPsA-1, APPsB-1, APPsB-2, APPsC-1, and EDTA on Fe^{2+} were shown in Fig. 6. Except for APPsA-1, the other three fractions and EDTA were found to have more potent chelating ability on Fe^{2+} in a concentration-dependent manner from 0 to 4.0 mg/ml. Especially the concentration below 1 mg/ml, their chelating ability on Fe^{2+} changed in a steep slop, and beyond this concentration there was a slow augment. Their chelating ability on Fe^{2+} decreased in the order of EDTA > APPsC-1 > APPsB-2 > APPsB-1 > APPsA-1, as well as in which the content of uronic acid among them.

4. Conclusions

ROS are a family of molecules derived from oxygen, and characterized by their high chemical reactivity, paramagnetism and short life. ROS encompass free radicals (species containing highly reactive unpaired electrons) such as superoxide (O^{2-}) and hydroxyl radicals (OH^{-}), as well as other molecules such as hydrogen peroxide (H_2O_2) and peroxynitrite (ONOO), which are not free radicals, but can also act as oxidizing agents in biological systems. Under physiological conditions, there is a balance between ROS generation and the activity of enzymatic (superoxide dismutase, catalase and glutathione peroxidase) and non-enzymatic (glutathione, alpha-tocopherol, ascorbate and thioredoxin) antioxidant defences that decrease ROS concentrations. The crucial balance between ROS generation and antioxidant defence is a determinant in disease prevention (Cotgreave, Moldéus, & Orrenius, 1988; Machlin & Bendich, 1987). Normally ROS exert important physiological functions in low concentrations. However, over-production of ROS or decreased antioxidant defences could lead to oxidative stress. Oxidative stress can result in free radical-induced oxidation and damage to biomolecules such as lipids, DNA, and proteins (Wu, Xu, Shan, & Tan, 2006). To the best of our knowledge, superoxide and hydroxyl radicals are the most important active oxygen radicals *in vivo*. Superoxide radical is one important cause of oxidative stress in that it is the first oxygen radical produced *in vivo* and lasts for longer time than other radicals. Hydroxyl radical is the most active free radical that attacks all the biological molecules by setting off free radical chain reactions (Barry & Susanna, 1993). Furthermore, it is well known that Fe^{2+} is the most powerful pro-oxidant to indirectly initiate lipid peroxidation.

In this paper, we successfully obtained four purified homogeneous polysaccharide (APPsA-1, APPsB-1, APPsB-2, and APPsC-1) and their in vitro antioxidant activities were evaluated by the means of hydroxyl radical scavenging assay, superoxide radical scavenging assay, and chelating effects on Fe^{2+}. We found that they exhibited different antioxidant abilities in a concentration-dependent manner. The higher the content of uronic acid, the stronger the antioxidant activity of APPs. However, the underlying mechanism on their different effect on the antioxidant activity is not very clear. One of the mechanisms involved in antioxidant activity may originated from hydrogen atom-donating ability of a molecule to a radical, which result in terminating radical chain reactions and converting free radicals to unharmful products (Hu, Zhang, & Kitts, 2000). The electron-withdrawing carboxyl groups substituted in C-5 of sugar residue could activate the hydrogen atom of sugar residues through field and inductive effects. The higher activated capacity of the group, the stronger hydrogen atom-donating capacity of the APPs. In addition, it was reported that the compounds with structures containing two or more of the following functional groups: –OH, –SH, –COOH, $–PO_3H_2$, C=O, $–NR_2$, –S– and –O– in a favorable structure–function configuration can show metal chelating activity (Yuan, Bone, & Carrington, 2005). Based on the above studies, further detailed structural characterization of polysaccharides should be carried out to provide a good opportunity for scientists to elucidate the structure–function relationship and to explore high potential antioxidant agent.

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