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Antioxidant activities of different polysaccharide conjugates (CRPs) isolated from the fruiting bodies of *Chroogomphis rutilus* (Schaeff.: Fr.) O. K. Miller

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ABSTRACT

Four water-soluble purified polysaccharides (CRPsA-1, CRPsA-2, CRPsB-1 and CRPsB-2) were obtained from the fruiting bodies of *Chroogomphis rutilus* by DEAE Sepharose Fast Flow column chromatography and Sepharose 6 Fast Flow column chromatography. Their chemical and physical characteristics were determined by chemical methods, gas chromatography (GC) and high-performance size-exclusion chromatography (HPSEC). On the basis of hydroxyl radical assay, superoxide radical assay and Fe^{2+}-chelating ability assay, the antioxidant activities of CRPsA-1, CRPsA-2, CRPsB-1 and CRPsB-2 were investigated. Among these fractions, CRPsB-1 and CRPsB-2 containing glucuronic acid had the higher scavenging effects on hydroxyl radicals and superoxide radicals, and more potent Fe^{2+}-chelating activity than CRPsA-1and CRPsA-2 containing no glucuronic acid in a concentration-dependent manner.

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1. Introduction

It is well known that active free radicals, including oxygen free radicals and non-oxygen free radicals, are by-products of normal metabolism. The generation of reactive oxygen species (ROS) is an unavoidable consequence of life in an aerobic environment. However, the uncontrolled production of ROS is involved in onset of many human diseases such as cancer, atherosclerosis, reperfusion injury and hepatic injury, as well as in degenerative processes associated with aging (Barry & Gutteridge, 1989; Ke et al., 2009; Luo & Fang, 2008; Zha et al., 2009; Zou et al., 2008) through lipid peroxidation (Barry and 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, such as liver damage and carcinogenesis (Grice, 1988; Yuan, Zhang, Fan, & Yang, 2008). Therefore, enhancement of body's antioxidant defenses 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 were 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 et al., 2008; Zhu et al., 2009).

Chroogomphis rutilus is a traditional Chinese medicinal and edible fungus distributed in the Northeast Provinces of China, which is a Gomphidius fungus belonging to the Basidiomycotina. In our previous research, we successfully isolated one water-soluble polysaccharide from the fruiting bodies of *C. rutilus* and explored its scavenging effect on hydroxyl radicals (Sun, Li, Yang, Liu, & Kennedy, 2010). However, up to now, no detailed investigation has been conducted on composition characterization and antioxidative capacity of different polysaccharides isolated from *C. rutilus*. Therefore, this paper was concerned with the purification and properties of different fractions of polysaccharide conjugates from the fruiting bodies of *C. rutilus* and explored their antioxidant activities for seeking new biological functional principle used in food and pharmaceutical industry.

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 sulfoxide (DMSO), standard sugars, D-glucuronic acid, deoxyribose, trichloride ferric ($FeCl_2$), ferrous sulfate ($FeSO_4$), ethylene diamine tetra-acetic acid (EDTA), ferrozine, nitro blue tetrazolium (NBT), phenazine methosulfate (PMS), dihydromicotineamidade-

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nine 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

The fruiting bodies of *C. rutilus* (0.2 kg) were extracted with 95% ethanol (5000 ml × 3 times) at 75 °C for 6 h under reflux to remove lipid. The residue was then extracted with distilled water (8000 ml × 3 times) at 75 °C 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 (CCRPs).

The CCRPs 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 CCRPs 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 (CRPs), coded as CRPsA-1, CRPsA-2, CRPsB-1 and CRPsB-2. 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. Monosaccharide composition and properties

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. 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. 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.

The IR spectrum of the polysaccharide was determined using a Fourier transform infrared spectrophotometer (FTIR, Bruker, Germany). The purified polysaccharides were dried at room 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}.

2.4. Molecular weight determination

The average molecular weight of CRPs was determined by HPSEC (Sun et al., 2008), which was performed on a SHIMADZU HPLC system fitted with one TSK-G3000PW_{XL} column (7.8 mm i.d. × 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).

2.5. Assay for antioxidant activity

2.5.1. Hydroxyl radical assay

Assessment of the scavenging ability of CRPs 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 CRPs (0, 0.5, 1, 2, 4 and 8 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 $A_{532(\text{blank})}$ is the absorbance of the control (deionized water, instead of sample), and $A_{532(\text{sample})}$ is the absorbance of the test sample mixed with reaction solution.

2.5.2. Superoxide radical assay

The superoxide radical-scavenging activity of CRPs 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 nicotinamide adenine dinucleotide (NADH), 50 μM nitroblue tetrazolium (NBT), 10 μM phenazin methosulfate (PMS), and CRPs at given concentrations of 0, 0.5, 1, 2, 4 and 8 mg/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 $A_{560(\text{blank})}$ is the absorbance of the control (deionized water, instead of sample), and $A_{560(\text{sample})}$ was the absorbance of the test sample mixed with reaction solution.

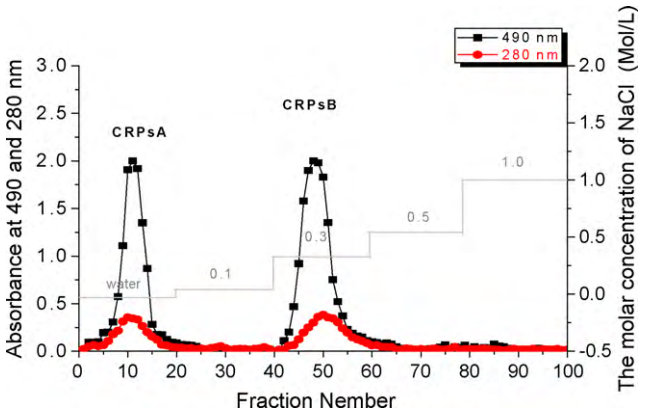

Fig. 1. The profile of CRPs isolated from the fruiting bodies of *C. rutilus* on a DEAE Sepharose Fast Flow column eluted with distilled water and stepwise gradient of NaCl aqueous solutions (0.1, 0.3, 0.5, and 1 M) at a flow rate of 4 ml/min.

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

The chelating activity of sample on Fe^{2+} was measured as reported (Dinis, Madeira, & Almeida, 1994) by measuring the formation of ferrous iron–ferrozine complex. Different concentration of sample (0, 0.5, 1, 2, 4 and 8 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) was co-assayed as a positive control. A lower level of absorbance indicated stronger chelating activity. The chelating activity of CRPs 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), and $A_{562(\text{sample})}$ was the absorbance of the test sample mixed with reaction solution.

3. Results and discussion

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

We successfully purified the polysaccharides from the fruiting bodies of *C. rutilus* on the ÄKTA explore 100 purification system. According to the charge difference, two fractions of CRPsA and CRPsB were isolated from distilled water elute and NaCl elute by the means of ion exchange chromatography method, respectively (Fig. 1). On account of molecular weight difference, CRPsA and CRPsB were further purified by size-exclusion chromatography on a Sepharose 6 Fast Flow column to yield four homogeneous fractions of CRPsA-1, CRPsA-2, CRPsB-1 and CRPsB-2, respectively (Fig. 2).

The polysaccharide content, protein content, uronic acid content, molecular weight and monosaccharides composition of CRPsA-1, CRPsA-2, CRPsB-1 and CRPsB-2 are given in Table 1. The infrared spectra of CRPsA-1, CRPsA-2, CRPsB-1 and CRPsB-2 all displayed a broad stretching intense characteristic peak at around 3403 cm^{-1} due to hydroxyl stretching vibration of the polysaccharides, and a weak C–H stretching vibration band at 2934 cm^{-1}. Two stretching peaks at 1076 and 1149 cm^{-1} suggested the presence of C–O bonds. In addition, signals at 1703 cm^{-1} standed for stretch vibration of C=O of uronic acids and1403 cm^{-1} for stretch vibration of C–O within COOH (data not shown).

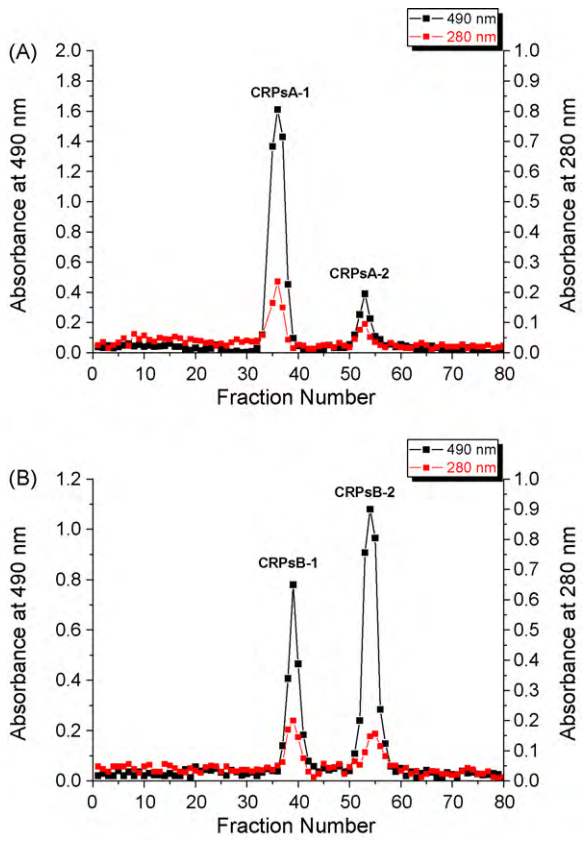

Fig. 2. The profile of CRPs isolated from the fruiting bodies of *C. rutilus* on a Sepharose 6 Fast Flow column eluted with 0.15 M/L NaCl aqueous solutions at a flow rate of 1 ml/min.

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 and induces severe damage to adjacent biomolecules. Hydrogen peroxide and superoxide molecules can lead to oxidative injury in the biomolecules 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 (0–8 mg/ml) on hydroxyl radicals are shown in Fig. 3. CRPsB-1 and CRPsB-2 possessed higher hydroxyl radical-scavenging activity than CRPsA-1 and CRPsA-2 in a concentration-dependent manner. Especially above the concentration of 4 mg/ml, scavenging effect of CRPsB-1 and CRPsB-2 on hydroxyl radical was close to that of ascorbic acid.

Table 1
Components of monosaccharide and properties of CRPs from the fruiting bodies of *C. rutilus*.

Samples	CRPsA-1	CRPsA-2	CRPsB-1	CRPsB-2
Total sugar (%)	90.3	89.4	86.1	89.4
Protein (%)	9.6	10.1	13.8	9.6
Average molecular weights	4.1×10^4	2.3×10^4	3.2×10^4	1.9×10^4
Sugar components (mol%)				
Mannose	nd[a]	1	0.5	1.1
Arabinose	nd[a]	0.1	nd[a]	0.1
Galactose	2.0	3.9	2.1	1.1
Glucose	1	1	1	1
Glucuronic acid	nd[a]	nd[a]	1.1	1.2

[a] nd: not detected.

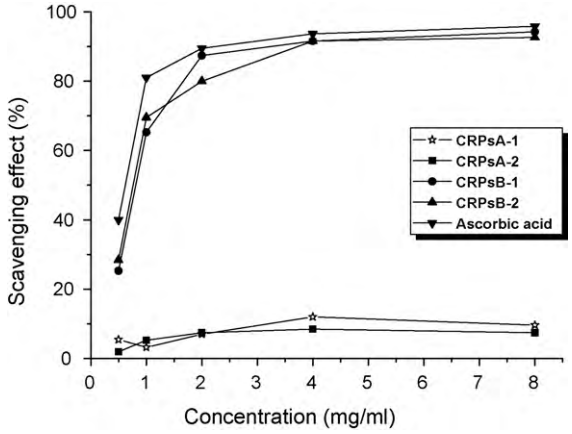

Fig. 3. Scavenging effects of CRPs on hydroxyl radicals with ascorbic acid as a positive control. Results were presented as mean value ($n = 3$).

There was no significant difference on scavenging activity between CRPsA-1 and CRPs A-2 at the concentration rang of 0–8 mg/ml. Their scavenging abilities on hydroxyl radicals decreased in the order of ascorbic acid > CRPsB-1 (CRPsB-2) > CRPsA-1 (CRPsA-2).

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 lipids peroxidation 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. 4, it was obviously that the scavenging ability of CRPsB-1, CRPsB-2 and ascorbic acid on superoxide radicals correlated positively well with increasing concentrations (0–8 mg/ml). The superoxide radicals scavenging ability of CRPsB-1 and CRPsB-2 were close to that of ascorbic acid when the concentration was beyond 2 mg/ml. The CRPsA-1 and CRPsA-2 containing no glucuronic acid did not present any scavenging activity on superoxide radicals, as compared with CRPsB-1, CRPsB-2, and ascorbic acid. Their scavenging abilities on superoxide radicals decreased in the order of ascorbic acid > CRPsB-1 (CRPsB-2) > CRPsA-1 (CRPsA-2).

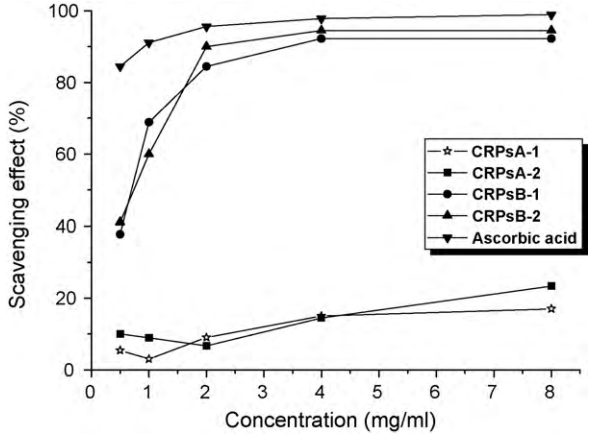

Fig. 4. Scavenging effects of CRPs on superoxide radicals with ascorbic acid as a positive control. Results were presented as mean value ($n = 3$).

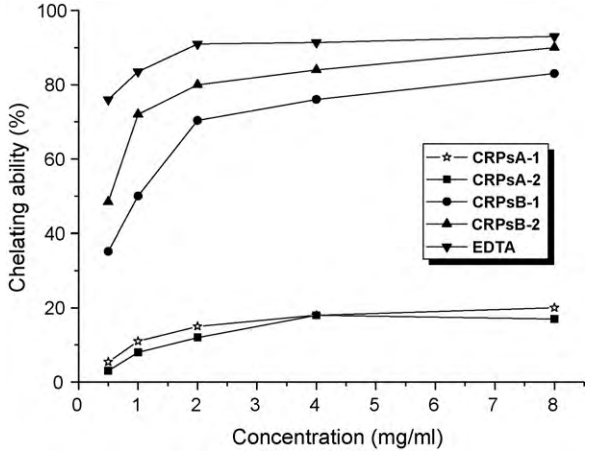

Fig. 5. Chelating abilities of CRPs on Fe^{2+} with EDTA as a positive control. Results were presented as mean value ($n = 3$).

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

Some research report 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 tenfold 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 abilities of CRPsA-1, CRPsA-2, CRPsB-1, CRPsB-2 and EDTA on Fe^{2+} are shown in Fig. 5. Except for CRPsA-1 and CRPsA-2, CRPsB-1, CRPsB-2 and EDTA were found to have more potent chelating ability on Fe^{2+} in a concentration-dependent manner from 0 to 8.0 mg/ml. Their chelating abilities on Fe^{2+} decreased in the order of EDTA > CRPsB-2 > CRPsB-1 > CRPsA-1 (CRPsA-2).

4. Conclusions

In the present study, we successfully obtained four purified homogeneous polysaccharide, termed as CRPPsA-1, CRPsA-2, CRPsB-1 and CRPsB-2. According to the results stated above, CRPsB-1 and CRPsB-2 were found to have more potent antioxidant potential by means of the *in vitro* evaluation of hydroxyl radical-scavenging, superoxide radical-scavenging and chelating effects on Fe^{2+} in a concentration-dependent manner, which may be comparable to ascorbic acid. Furthermore, whether the polysaccharides contain the glucuronic acid play a key role in their antioxidant activities. However, the underlying mechanism on its 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 actively release the hydrogen atom of the sugar residue through field and inductive effects. The higher activated capacity of the group, the stronger hydrogen atom-donating capacity of the CRPs has. 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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