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Full Length Research Paper

Isolation, chemical characterization and *in vitro* antioxidant activities of polysaccharides from *Aconitum coreanum*

Bin Li[1]*, Xian-Jun Meng[1] and Li-Wei Sun[2]

[1]College of Food Science, Shenyang Agricultural University, Shenyang 110866, China.
[2]College of Basic Medical, Beihua University, Jilin City 132013, China.

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The water-soluble crude polysaccharide *Aconitum coreanum* polysaccharides (ACPS) was extracted from *A. coreanum* and purified by Diethylaminoethyl cellulose and Sepharose CL-6B chromatography, giving three polysaccharide fractions coded as ACPSA-1, ACPSB-2 and ACPSB-3. Their chemical and physical characteristics of polysaccharide fractions, and antioxidant capacity, including scavenging activity against DPPH radicals, superoxide and hydroxyl radicals, and chelating ability, were valuated in this paper. Experiment results showed that ACPS-2 and ACPS-3 exhibited significantly antioxidant activity at a concentration-dependent manner. The polysaccharide fractions can be developed as new antioxidant agents.

Key words: *Aconitum coreanum*, polysaccharide, purification, characterization, antioxidant activity.

INTRODUCTION

The recent abundant evidences suggest that reactive oxygen species (ROS), such as superoxide anion, hydrogen peroxide and hydroxyl radical, involve in the pathogenesis of various disorders and diseases (Niki, 2010). According to the free-radical theory (Lin and Beal, 2003; Muller et al., 2007), the disruption of the delicate balance between generation of reactive oxygen species and antioxidant scavenging systems could lead to a shift to an oxidative cellular milieu, and eventually lead to serious health problems such as diabetes and Alzheimer's disease. So, much more attention has attracted to develop and utilize effective and natural antioxidants in the maintenance of human health and prevention and retardation the progress of many chronic diseases induced by free radical (Getoff, 2007).

Current researches on free radicals have confirmed that traditional Chinese medicine rich in antioxidants play an essential role in the prevention of cardiovascular diseases, cancers, neurodegenerative diseases, inflammation and other free radical induced problems (Liao et al., 2008; Wu et al., 2004), and search for novel type of antioxidants from traditional Chinese medicine (TCM) has achieved considerable attention (Kirby and Schmidt, 1997). Polysaccharides as important natural products from traditional Chinese medicine exhibit significant antioxidant activities, which protect cells against the damaging effects of reactive oxygen species, prevention of the chronic and degenerative diseases (Chen and Yan, 2005; Kardošová and Machová, 2006; Song et al., 2010).

The genus *Aconitum* is well known as poisonous and medicinal plants, which comprises ca. 400 species, and more than a half of them are growing in China. The most important variety of *Aconitum* genus, *Aconitum coreanum* (Lèvl.) Rapaics was distributed in Liaoning, Jilin, Neimenggu and Heilongjiang Province of north China. Its roots had been used as one of the most centuries-old traditional Chinese medicines documented in ancient Chinese medicinal literature. Supposedly originated from China, *A. coreanum* was discovered to be an antidote for poisonous herbs by a great herbalist, Shen Nong, about 4700 years ago.

To date, *A. coreanum* has been used to treat various kinds of disorders such as cardialgia, facial distortion, epilepsia, migraine headache, vertigo, tetanus, infantile

*Corresponding author. E-mail: libinsyau@163.com. Tel: + 86-24-88488277. Fax: + 86-24-88488277.

convulsion and rheumatic arthralgia (Alessandra et al., 2003; Liou et al., 2005).

However, there have been seldom reports on free radical scavenging activities of polysaccharide fractions from *A. coreanum*. In order to fully develop the wild resources and extend the potential use of *A. coreanum* in antioxidant biomedicine, the present study was carried out to investigate antioxidant activities of polysaccharide fractions from *Aconitum coreanum* with three *in vitro* antioxidant models, including 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity, superoxide radical scavenging activity, hydroxyl radical scavenging activity and ferrous ion-chelating activity, as well as their chemical and physical characteristics.

EXPERIMENTAL

Materials and chemicals

Aconitum coreanum was purchased from a local medicine market, and identified according to the identification standard of Pharmacopeia of the People's Republic of China. Phenazine methosulfate (PMS), nicotinamide adenine dinucleotide (NADH), nitroblue tetrazolium (NBT), safranine, ferroizine, T-series dextrans, Diethylaminoethyl cellulose, 1,1-diphenyl-2-picrylhydrazyl (DPPH) and standard sugars were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Sepharose CL-6B was purchased from Amersham Pharmacia Co. (Sweden). Trifluoroacetic acid (TFA), EDTA-Na_2, sodium hydroxide, hydroxylamine, inositol, acetic anhydride, pyridine, methanol and acetic acid were from Beijing Chemicals and Reagents Co. (Beijing, China). All other chemical reagents used were analytical grade.

Isolation and purification of polysaccharide fractions

After the roots of *A. coreanum* were cleaned, they were ground in an electric mill, and the powder was extracted with hot water at pH 7.0 for three times, 2 h for each time. The whole extract was filtered, centrifuged and concentrated, and then precipitated with 3 volumes of ethanol at 4°C overnight. The crude polysaccharide precipitate was collected by centrifugation, deproteinated by a combination of proteinase and Sevag method (Staub, 1965), and then obtained crude *A. coreanum* polysaccharides (cACPS).

The cACPS was dissolved in distilled water, and then loaded onto DEAE–cellulose column, eluted successively with distilled water and 0.5 M NaCl. Fractions were collected, and monitored with the phenol–sulfuric acid method. The two main fractions (ACPSA and ACPSB) was collected, dialyzed, lyophilized, and were further fractioned on a Sepharose CL-6B column, eluted with 0.15 M NaCl to yield three main fractions, codes as ACPSA-1, ACPSB-2 and ACPSB-3. All the fractions were collected, dialyzed and lyophilized.

Molecular weight determination

Molecular weights of the different polysaccharide fractions were determined by high performance liquid chromatography (HPLC). The samples of polysaccharide fractions were dissolved in distilled water, applied to Agilent HPLC system (Agilent Technologies, USA) equipped with a TSK-GEL G3000 PWXL column, eluted with 0.1 mol/L Na_2SO_4 solutions and detected by a RID-10A Refractive Index Detector. Dextran standards with different molecular weights (T-2000, T-70, T-40, T-20, and T-10) were to calibrated the column and establish a standard curve.

Monosaccharide composition analysis

Polysaccharide fractions were hydrolyzed and acetylated according to Lehrfeld (1985). Simply, the samples were hydrolyzed with TFA and then hydrolyzed product was reduced with KBH_4, followed by neutralization with acetic acid. After adding myo-inositol and Na_2CO_3, the residue was concentrated. The reduced products were added with pyridine–propylamine, and acetylated with pyridine–acetic anhydride. The acetylated products were analyzed by gas chromatography (GC), and identified and estimated with myo-inositol as the internal standard. GC was performed on a Agilent 6890 instrument (Agilent Technologies, USA) equipped with HP-5 capillary column (30 m × 0.32 mm × 0.2 μm) and flame-ionization detector (FID) and temperatures programmed from 120 to 250°C at a rate of 8°C /min.

Measurement of carbohydrate and protein contents

Total carbohydrate contents of the polysaccharide fractions were determined by phenol-sulfuric acid colorimetric method (Dubois et al., 1956). Total uronic acid contents were measured by *m*-hydroxydiphenyl method (Filisetti-Cozzi and Carpita, 1991). Protein contents were quantified according to the Bradford's method (Bradford, 1976).

DPPH free radical scavenging activity

Radical scavenging activity against the stable radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) was measured by the method of Fan et al. (2009) with a minor modification. Samples were dissolved in distilled water at 0 (control), 0.5, 1, 2, 4, and 8 mg/ml. One milliliter samples were mixed with 2 ml of freshly prepared DPPH (0.1 mM) in 50% ethanol. The mixture was incubated at 25°C for 30 min in the dark, and then the absorbance was measured at 517 nm. The experiment was carried out in triplicate and averaged. The scavenging activity of DPPH radicals was calculated by the following formula:

Scavenging effect (%) = (1 - *A*sample/*A*control) × 100%.

Superoxide radical scavenging assay

The scavenging effects of polysaccharide fractions on superoxide radicals were assayed by the method of photoreduction of NBT (Shu and Lung, 2008) with some modifications. Reaction mixtures in a final volume of 3 ml contained the following reagents at final concentration: 60 μM phenazine methosulfate (PMS), 468 μM nicotinamide adenine dinucleotide (NADH), 150 mM nitroblue tetrazolium (NBT), and various concentrations of samples. The mixture reacted at 20ºC for 10 min and then the absorbance was measured at 560 nm. Each value was expressed by the mean of triplicate measurements with standard deviation. The capability of scavenging the superoxide radical was calculated using the following equation:

Scavenging effect (%) = (1−Asample/Acontrol) ×100%

Hydroxyl radical scavenging activity

The hydroxyl radical assay was measured by the method of Fenton reaction (Xia et al., 2005) with a minor modification. Briefly, Samples were dissolved in distilled water at 0 (control), 0.5, 1, 2, 4, and 8 mg/ml. The reaction mixture contained 1 ml of safranine (0.36 mM), 0.5 ml of EDTA-Fe (2 mM), 1.5 ml of H_2O_2 (3.0%) and 1 ml samples of varying concentrations. After incubation at room temperature for 20 min, the absorbance of the mixture was measured at 520 nm. Hydroxyl radicals gave a crimson colour, so the absorbance change of the reaction mixture indicated the scavenging ability for hydroxyl radicals. The hydroxyl radical-scavenging activity was expressed as:

Scavenging effect (%) = (1 - Asample/Acontrol) × 100%.

Metal chelating assay

The ferrous ion chelating ability of all different fractions was investigated according to the method of Lin et al. (2009). Briefly, samples were dissolved in distilled water at 0 (control), 0.5, 1, 2, 4, and 8 mg/ml. The reaction mixture contained 0.1 ml $FeCl_2$ (2 mM), 0.4 ml ferroizine (5 mM) and 1 ml samples of varying concentrations. After shaken well and incubated for 10 min at room temperature, the absorbance of the mixture was measured at 562 nm. The ability of different fractions to chelate ferrous ion was calculated using the following equation:

Chelating ability (%) = (1−Asample/Acontrol) ×100%

RESULTS AND DISCUSSION

Isolation and purification of polysaccharides

The yield of the crude polysaccharide extracted from *A. coreanum* was 0.7%. After deproteinated by a combination of proteinase and Sevag method, the crude polysaccharide sample (cACPS) was loaded onto the DEAE-cellulose column (Figure 1), de-ionized water was used to elute the unbound component (ACPSA); and the retained components (ACPSB) was eluted with 0.5 M NaCl. Then ACPSA and ACPSB were loaded onto Sephacryl S-300 column, respectively, eluted with 0.15 M NaCl, and three main fractions (ACPSA-1, ACPSB-2 and ACPSB-3) were separated for further analysis of physicochemical properties and *in vitro* antioxidant activities.

Physicochemical properties and chemical compositions

The total sugar, protein, uronic acid contents, molecular weight and monosaccharides composition of the polysaccharide fractions are summarized in Table 1. The polysaccharide fractions ACPSB-2 and ACPSB-3 had higher total carbohydrate content (95.1% in ACPSB-2 and 96.5% in ACPSB-3) than ACPSA-1 (91.4%). According to Bradford method using bovine serum albumin (BSA) as standard, the protein contents of ACPSA-1, ACPSB-2 and ACPSB-3 were 7.5, 2.7 and 2.4%, respectively. Furthermore, the uronic acid content was measured by *m*-hydroxydiphenyl method, and the results showed that the uronic acid contents in ACPSB-2 and ACPSB-3 were 19.2 and 28.7%, respectively, not detected in ACPSA-1. The average molecular weights of ACPSA-1, ACPSB-2 and ACPSB-3 calculated by HPLC, according to the calibration curve with standard dextrans, were 74.5, 68,4 and 22,5 kDa, respectively.

Moreover, composition analysis of polysaccharide is an important procedure to control quality standard and give basic information of the polysaccharide fractions. According to GC analysis, ACPSA-1 was composed of arabinose, xylose, galactose and glucose with molar ratios of 23:12:10:41. ACPSB-2 and ACPSB-3 were both composed of five monosaccharides: arabinose, xylose, galactose, glucose and galacturonic acid with molar ratios of 15:8:10:43:16 and 11:14:10:32:21, respectively. Glucose was the predominant monosaccharide in all the fractions.

DPPH radical scavenging activity

The principle of scavenging the stable DPPH radical model, which is widely used to evaluate antioxidant activities, is based on the reduction of the stable DPPH solution (purple) in the presence of a hydrogen donating antioxidant, leading to the formation of non-radical form DPPH-H (yellow).

Figure 2 demonstrated DPPH scavenging activity caused by polysaccharide fractions at different concentrations. ACPSB-2 and ACPSB-3 both exhibited significant radical scavenging activities. When the concentration was more than 2 mg/ml, DPPH scavenging activity of ACPSB-2 and ACPSB-3 was significantly higher ($P < 0.01$) than that of ACPSA-1. The scavenging activity increased steadily at the concentration rang of 0.5 to 8 mg/ml. The DPPH radical scavenging rate of ACPSA-1, ACPSB-2 and ACPSB-3 reached 52.4, 77.5 and 70.2% at 8 mg/ml, respectively. There was no significant difference in scavenging activity between ACPSB-2 and ACPSB-3 at the concentration range of 0.5 to 8 mg/ml ($P >0.05$). The concentration required to inhibit 50% radical scavenging effect (IC_{50}) was determined from the results of a series of concentrations mentioned above. A lower IC_{50} value corresponds to a greater scavenging activity. The IC_{50} values of ACPSA-1, ACPSB-2 and ACPSB-3 were 6.6, 2.8, and 3.3 mg/ml, respectively, revealing that the fraction ACPSB-2 possessed the highest DPPH radical scavenging activity.

Superoxide radical scavenging activity

Superoxide anions are a precursor to active free radicals, which is normally formed first in cellular oxidation reactions. Although, it is not highly reactive, it can produce

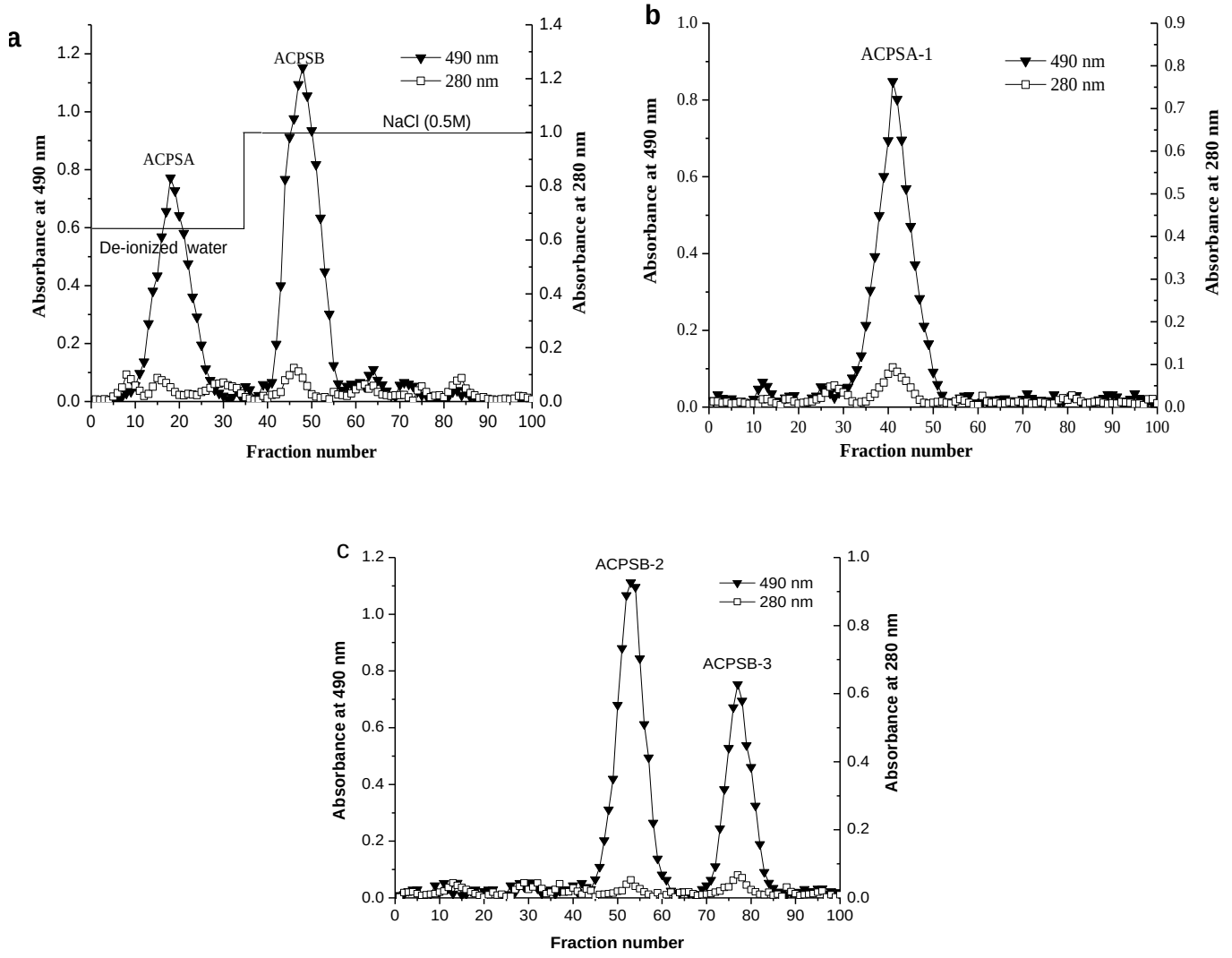

Figure 1. Polysaccharide fractionations isolated from *Aconitum coreanum* with DEAE-cellulose (a) and Sepharose CL-6B (b, c). Crude polysaccharide (cACPS) was loaded onto the DEAE-cellulose column, de-ionized water was used to elute the unbound component (ACPSA); and the retained components (ACPSB) was eluted with 0.5 M NaCl. Then ACPSA and ACPSB were loaded onto Sepharose CL-6B column, respectively, eluted with 0.15 M NaCl, giving three main fractions (ACPSA-1, ACPSB-2 and ACPSB-3).

other ROS such as hydrogen peroxide, hydroxyl radical, and singlet oxygen. Furthermore, superoxide anion radical and its derivatives can cause damage in lipids, proteins, and DNA. Therefore, it is of great important to scavenge superoxide anion radical (Xie et al., 2008).

As shown in Figure 3, superoxide scavenging activities of three fractions increased significantly ($P < 0.01$) with increasing concentrations from 0.5 to 8.0 mg/ml, and the superoxide radical scavenging rate of ACPSA-1, ACPSB-2 and ACPSB-3 at 8.0 mg/ml was 32.5, 47.1 and 78.5, respectively. The IC50 values of ACPSA-1, ACPSB-2 and ACPSB-3 were 11.5, 8.6, and 3.0 mg/ml, respectively. Scavenging activity of ACPSB-3 was significantly higher ($P < 0.01$) than that of ACPSA-1 and ACPSB-2.

Hydroxyl radical scavenging activity

Hydroxyl radicals are considered to be the most reactive oxygen radicals. The Fenton reaction, that is, $Fe^{2+} + H_2O_2 \rightarrow \cdot OH + OH^- + Fe^{3+}$, is a standard method used to determine the hydroxyl radicals scavenging capacity of antioxidant compound.

The hydroxyl radicals scavenging abilities of polysaccharide fractions were shown in Figure 4. ACPSA-1, ACPSB-2 and ACPSB-3 exhibited distinct scavenging ability, ACPSB-2 and ACPSB-3 against hydroxyl radical were better than that of ACPSA-1. Furthermore, the scavenging ability increased with the increasing concentrations from 0.5 to 8.0 mg/ml. At 8.0 mg/ml, the scavenging rate of ACPSA-1, ACPSB-2 and

Table 1. Molecular weight and composition of different polysaccharide fractions isolated from *Aconitum coreanum*.

	ACPSA-1	ACPSB-2	ACPSB-3
Molecular weight	74,500	68,400	22,500
Total sugar (%)	91.4	95.1	96.5
Protein (%)	7.5	2.7	2.4
Uronic acid (%)	nd[a]	19.2	28.7
Sugar components (mol%)			
Arabinose	23	15	11
Xylose	12	8	14
Galactose	10	10	10
Glucose	41	43	32
Galacturonic acid	nd	16	21

[a] nd: not detect.

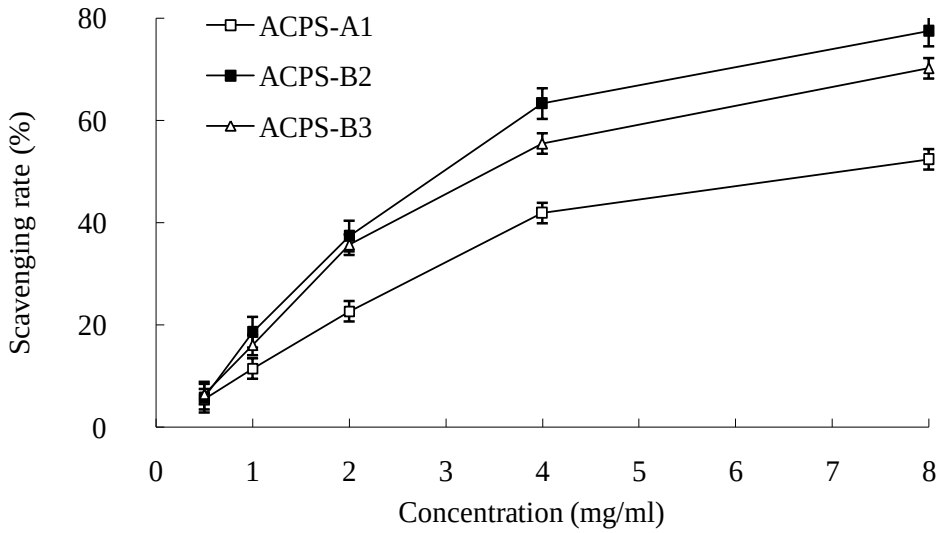

Figure 2. Scavenging rate of different polysaccharide fractions isolated from *Aconitum coreanum* against DPPH radical. Results were presented as means ± S.D. (n=3).

ACPSB-3 against hydroxyl radical was 29.4, 55.3 and 68.7%, respectively. The IC50 values of ACPSA-1, ACPSB-2 and ACPSB-3 were 16.2, 5.2 and 3.5 mg/ml, respectively. These results showed that ACPSB-2 and ACPSB-3 had a moderate hydroxyl radical scavenging activity.

Ferrous ion-chelating effect of polysaccharide fractions

Iron can stimulate lipid peroxidation and also accelerate peroxidation by degradation lipid hydroperoxides into peroxyl and alkoxyl radicals. Metal chelating capacity was significant since it reduced the concentration of the catalyzing transition metal in lipid peroxidation. Ferrous ions (Fe^{2+}) can catalyze and induce superoxide anion to form more harmful hydroxyl radicals. Ferrozine can form complexes with Fe^{2+} quantitatively, and the complex formation is disrupted with the result that the red color of the complex is decreased in the presence of chelating agents. Therefore, measurement of color reduction allows estimation of the chelating activity (Yuan et al., 2006).

The chelating ability of the samples are shown in Figure 5, all of the polysaccharide fractions ACPSB-2 and ACPSB-3 had an excellent chelating ability, while ACPSA-1 showed a moderate chelating ability. The chelating ability increased significantly ($P < 0.01$) with increasing concentrations from 0.5 mg/ml to 8.0 mg/ml, and the chelating ability of ACPSA-1, ACPSB-2 and ACPSB-3 at 8.0 mg/ml was 49.5, 75.3 and 68.7%, respectively, suggesting that they could capture ferrous

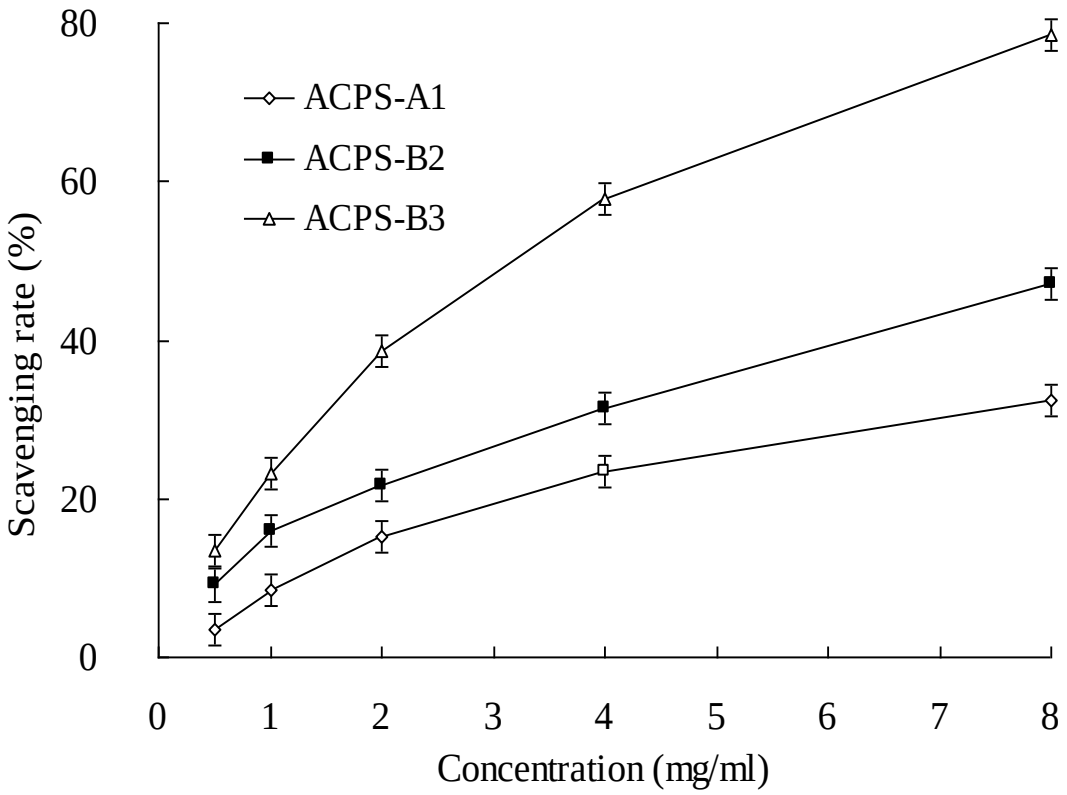

Figure 3. Scavenging rate of different polysaccharide fractions isolated from *Aconitum coreanum* against superoxide radical. Results were presented as means ±S.D. (n=3).

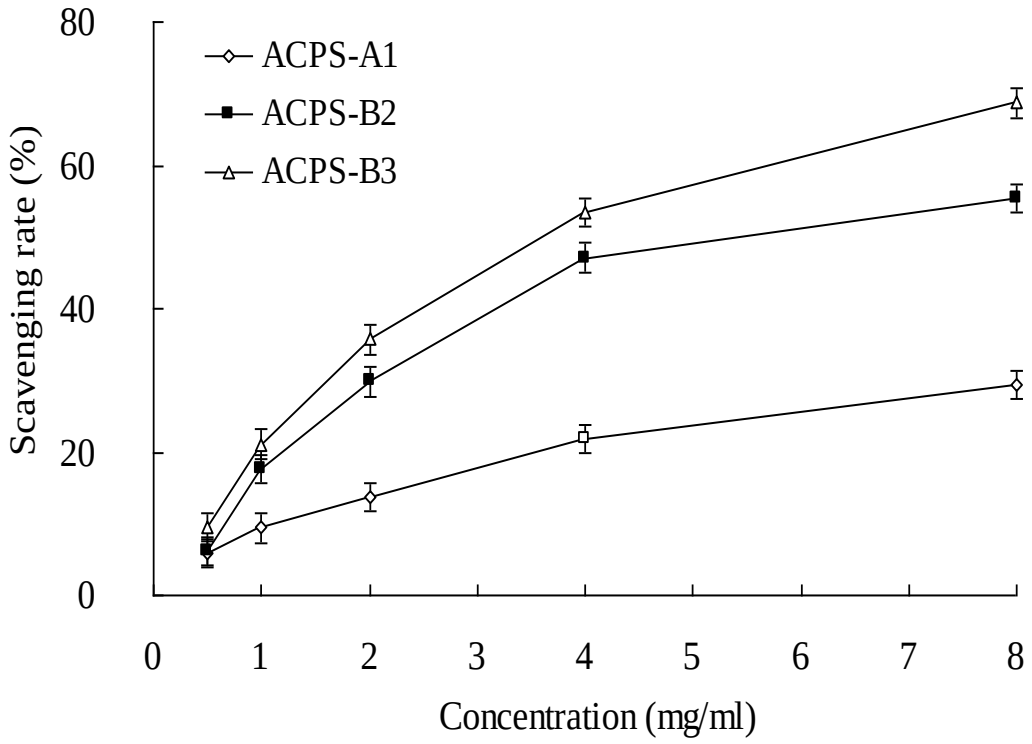

Figure 4. Scavenging rate of different polysaccharide fractions isolated from *Aconitum coreanum* against hydroxyl radical. Results were presented as means ± S.D. (n=3).

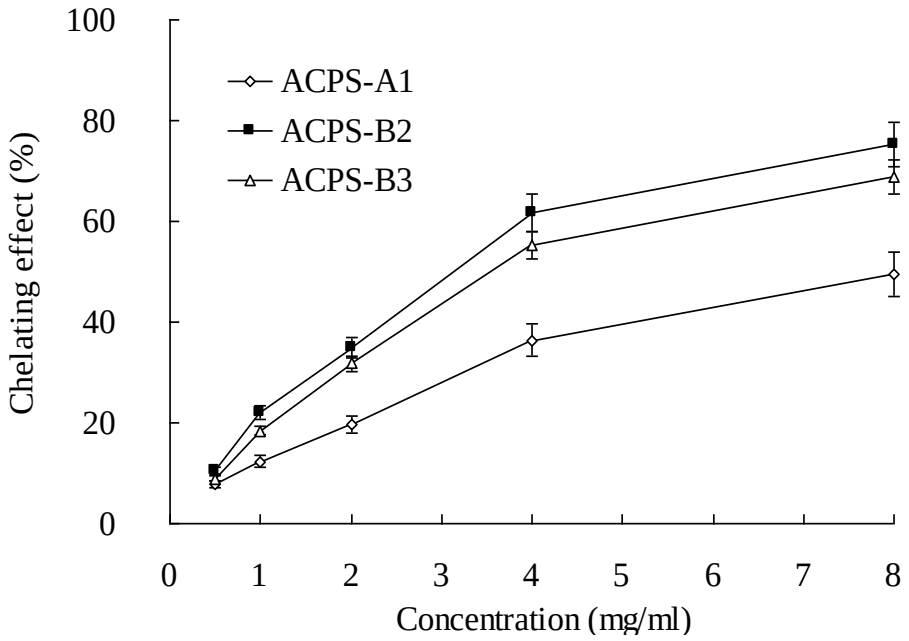

Figure 5. Ferrous ion-chelating ability of different polysaccharide fractions isolated from *Aconitum coreanum*. Results were presented as means ±S.D (n=3).

ion before ferrozine.

Conclusions

Highly reactive free radicals and reactive oxygen species (ROS) commonly exist in the biological systems of live organs, which can oxidize nucleic acids, proteins, lipids or DNA inducing many kinds of degenerative diseases. Natural antioxidant compounds, such as phenolic acids, polyphenols, flavonoids and polysaccharides can maintain human healthy, prevent and retard the progresses of many free radical-induced chronic diseases. In this paper, three polysaccharide fractions (ACPSA-1, ACPSB-2 and ACPSB-3) from the roots of *A. coreanum* were obtained by DEAE-cellulose and Sepharose CL-6B chromatography. Several *in vitro* models were applied to evaluate the antioxidant potential of *A. coreanum* polysaccharides. Experiment results showed that ACPSB-2 and ACPSB-3 exhibited significant antioxidant activity in a concentration-dependent manner. Further structural analysis of *A. coreanum* polysaccharides will be important for their application in medicinal fields.

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