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Antioxidant activity of a water-soluble polysaccharide purified from *Pteridium aquilinum*

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ABSTRACT

A water-soluble crude polysaccharide, obtained from fern *Pteridium aquilinum*, was fractionated by DEAE–Sepharose Fast-Flow column chromatography, and purified by Sephacryl S-400 HR column chromatography. The average molecular weight (M_w) of the purified polysaccharide (PLP) is 458,000 Da. The monosaccharide components of PLP were characterized by gas chromatography (GC), and the majority of the monosaccharide components was glucose (relative mass 58.1%) with low levels of galactose, mannose, rhamnose, and arabinose (relative mass 18.7%, 6.8%, 10.2%, and 6.1%, respectively). The Fourier-transform infrared spectra (FTIR) of PLP revealed typical characteristics of polysaccharides. On the basis of the ferric-reducing antioxidant power assay (FRAP), DPPH radical-scavenging, the superoxide radical assay, and self-oxidation of 1,2,3-phentriol assay, the antioxidant activities of PLP were investigated. The purified polysaccharide was demonstrated to have strong reductive power (FRAP value: 827.6 μmol/L), moderate scavenging activities against DPPH radicals (83.1%) and superoxide radicals (60.5%), and moderate inhibiting power for self-oxidation of 1,2,3-phentriol (52.4%).

1. Introduction

Oxidation is essential to many organisms for the production of energy to fuel biological processes. However, reactive oxygen species are often over-produced under pathological conditions, resulting in oxidative stress.[1,2] And over-production of various forms of activated oxygen species, such as free-radical and non-free-radical species, is involved in the onset of many diseases such as cancer, cardiovascular diseases, rheumatoid arthritis, and atherosclerosis, as well as in degenerative processes associated with aging.[3,4] In order to reduce damage to the human body, synthetic antioxidants are used for industrial processing at the present time. However, the presence of unwanted side effects is almost unavoidable, and those most commonly observed have been suspected of being responsible for liver damage and carcinogenesis.[5] Thus, it is essential to develop natural antioxidants so that they can protect the human body from free radicals and retard the progress of many chronic diseases.[6] Several constituents of plant extracts have been shown to have antioxidant activity, such as ascorbic acid, tocopherol, β-carotene, flavonoids, tannins, and anthocyanins.[7,8]

Polysaccharides, which are widely distributed in animals, plants, and microorganisms, exhibit varied biological activities, such as antitumor, anticancer, antiviral, anticoagulant, and immunological activities.[9–11] Recently, polysaccharides from edible and medicinal plants have provoked interest as sources of novel potential antioxidants, since more and more published data indicate that plant polysaccharides in general have strong antioxidant activities.[12]

The fern is a sporous plant with fascicular vascular cambia, and now approximately 12,000 species are documented around the globe. Ferns are common in warm, dark areas of both torrid and subtorrid zones.[13] In China, there are a multitude of fern types that make up about 61 families, 223 genuses, and 2600 species. Wild brake (*Pteridium aquilinum* (L) Kuhn. var. *latiusculum*) has been described as one of the most common ferns in China. The use of its rhizomes and fronds as food appears to be widespread. Young croziers are consumed fresh, canned, dried, or pickled, and they are regarded as a delicacy in many countries. However, consumption of the bracken fern *P. aquilinum* has been shown to induce bladder and intestinal carcinomas in cattle and to cause a number of diseases in other farm animals. Some human populations also eat young bracken shoots, and epidemiological studies in Japan and Brazil have shown a close association between bracken consumption and the occurrence of certain cancers. An unstable glucoside named ptaquiloside, containing a reactive cyclopropane ring, has been isolated from the fern and has been proven to have potent carcinogenicity.[14,15] Various biologically active compounds such as flavonoids, terpenoids, and steroids, pterosins A and B, isolated

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from *P. aquilinum*, have also been reported in many studies.[16,17] However, to date, no investigation has been carried out on polysaccharides that may account for the textural properties and antioxidant activities of *P. aquilinum*.

In this work, crude polysaccharides from the fern *P. aquilinum* were extracted, isolated, and further purified. The toxicity and carcinogenicity of the bracken fern to domestic and experimental animals have been extensively described.[18] The extraction and purification steps in our study are necessary to remove most of the toxic compounds in *P. aquilinum*. In addition, the properties and antioxidant activities of the water-soluble polysaccharides were also identified.

2. Materials and methods

2.1. Chemicals

2,4,6-Tripyridyl-*s*-triazine (TPTZ), 1,1-diphenyl-2-picrylhydrazyl (DPPH), and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Nicotinamide adenine dinucleotide (NADH), nitroblue tetrazolium (NBT), and phenazine methosulfate (PMS) were purchased from E. Merck (Darmstadt, Germany). The standard monosaccharides (D-glucose, D-xylose, D-galactose, L-rhamnose, D-mannose, and D-arabinose) were purchased from E. Merck (Darmstadt, Germany) and Sigma Chemical Co. (St. Louis, MO, USA). DEAE-Sepharose Fast-flow column, Sephacryl S-400 HR prep grade and dextrans of different molecular weights were from Pharmacia Co. (Uppsala, Sweden). All other chemicals and reagents were analytical grade.

2.2. Preparation of crude polysaccharides from *Pteridium aquilinum*

The young croziers of *P. aquilinum* (L) Kuhn. var. *latiusculum* were obtained from Beijing Shengdeyuan Co. (Beijing, China). The samples were thoroughly washed with tap water, dried at 50 °C, and finely powdered with a mixer. The powder was subjected to successive extraction with 90% EtOH to remove lipids and pigments. The defatted powder was then subjected to water extraction. The supernatant was treated with SEVAG reagent[19] to remove free proteins, dialyzed (72 h), and then concentrated by rotary evaporation at reduced pressure below 50 °C. EtOH (3–4 times by vol) was added to precipitate the crude polysaccharides. After centrifugation, the precipitate was washed with anhydr EtOH and then dissolved in water and lyophilized.

2.3. Purification of polysaccharide

2.3.1. Anion-exchange chromatographic method

The crude polysaccharides (100 mg) were dissolved in distilled water and membrane filtered (0.45 μm, Nucleopore). Then the solution was applied to a DEAE-Sepharose Fast-flow column (1.6 × 20 cm) equilibrated previously with 20 mM Tris–HCl (pH 7.8) with the AKTA Purifier system (Amersham Pharmacia Biotech, Sweden). Then the column was eluted with water (500 mL), at a flow rate of 2 mL/min, with collection of 4-mL fractions for each tube. The polysaccharide content in each fraction was monitored by the phenol–sulfuric acid method.[20] Appropriate fractions were concentrated, dialyzed against water, and finally lyophilized.

2.3.2. Gel-filtration chromatography

Size-exclusion chromatography of the purified polysaccharide from *P. aquilinum* was performed on Sephacryl S-400 HR (50 × 1.0 cm) with the AKTA Purifier system (Amersham Pharmacia Biotech, Sweden), and 0.2 M NaCl was used as eluant. Preliminary calibration of the column was conducted using dextrans of different molecular weight (Dextran Blue, Dextran, T500, T70, T40, and T10). The molecular weight was calculated by the calibration curve obtained by using various standard dextrans.[21]

2.4. Determination of the physical characteristics of the polysaccharide

Total sugar content in the purified polysaccharide was determined by the phenol–sulfuric acid method, using D-glucose as standard. Protein content was also determined by the method of Bradford (1976),[22] with bovine serum albumin as standard.

2.5. Monosaccharide composition and properties

GC was performed on a HP-6890 instrument (Hewlett–Packard Component, Chelmsford, Massachusetts, USA) with an Elite-17ms column (30 m × 0.32 mm × 0.25 μm). First, the polysaccharide (10 mg) was hydrolyzed with 4 M CF_3CO_2H (5 mL) for 6 h at 110 °C in a sealed glass tube. The excess acid was completely removed at 70 °C by a steady stream of nitrogen, and then the hydrolyzed products were prepared for acetylation. The acetylation was carried out with 10 mg of hydroxylamine hydrochloride and with 0.5 mL of pyridine by heating in a water bath for 30 min at 90 °C. After incubation, the tube was allowed to cool down to room temperature, and then 0.5 mL of acetic anhydride was added and mixed thoroughly by vortexing. The tube was sealed and incubated in a water bath shaker set at 90 °C for 30 min. The derivatives were loaded onto an Elite-17ms column equipped with a flame-ionization detector (FID), using *myo*-inositol as the internal standard. Alditol acetates of authentic standards (D-glucose, D-xylose, D-galactose, L-rhamnose, D-mannose, and D-arabinose) with *myo*-inositol as the internal standard were prepared and subjected to GC analysis separately in the same way. The operation was performed using the following conditions: H_2: 30 mL/min; air: 400 mL/min; N_2: 40 mL/min; injection temperature: 210 °C; detector temperature: 240 °C; column temperature programmed from 160 to 190 °C at 5 °C/min, then increasing to 210 °C at 3 °C/min and holding for 10 min at 210 °C.

2.6. Infrared spectra of water-soluble polysaccharides

The IR spectra of the polysaccharides were determined using a Fourier-transform infrared spectrophotometer (Nexus 5DXC FTIR, Thermo Nicolet, America). The purified polysaccharide was ground with spectroscopic grade potassium bromide (KBr) powder and then pressed into 1-mm pellets for FTIR measurement in the frequency range of 4000–400 cm^{-1}.[23]

2.7. Assay for antioxidant activities

2.7.1. Ferric-reducing antioxidant power (FRAP) assay

The total antioxidant potential of sample was determined using an iron(III) reducing ability of plasma (FRAP) assay as described by Benzie and Strain[24] as a measure of 'antioxidant power'. FRAP assay measures the change in absorbance at 593 nm owing to the formation of a blue-colored Fe(II)–tripyridyltriazine compound from the colorless oxidized Fe(III) form by the action of electron-donating antioxidants. The working FRAP reagent was prepared by mixing 10 vol of 300 mmol/L acetate buffer, pH 3.6, with 1 vol of 10 mmol/L TPTZ (2,4,6-tripyridyl-*s*-triazine) in 40 mmol/L HCl and with 1 vol of 20 mmol/L $FeCl_3$. Freshly prepared FRAP reagent (1.5 mL) was warmed to 37 °C, and a reagent blank reading was taken at 593 nm. Subsequently, 50 μL of sample and 150 μL of deionized water were added to the FRAP reagent. The final dilution of the sample in the reaction mixture was 1:34. The sample was

run in triplicate. After addition of the sample to the FRAP reagent, a second reading at 593 nm was performed after 8 min. The initial blank reading with the FRAP reagent alone was subtracted from the final reading of the FRAP reagent with the sample to determine the FRAP value of the sample. A standard curve was prepared using different concentrations (100–1000 μmol/L) of $FeSO_4{\cdot}7H_2O$. The final result was expressed as the concentration of antioxidants having a ferric reducing ability equivalent to that of 1 μmol/L $FeSO_4{\cdot}7H_2O$.

2.7.2. Effect of scavenging 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals

The free-radical scavenging capacity of the crude polysaccharides (PLs) and the purified polysaccharide (PLP) was analyzed by using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) test according to the method of Blois,[25] with some modifications. Vitamin C was used as reference material. Briefly, 0.2 mL of MeOH and 0.3 mL of various concentrations (50–800 μg/mL) of sample in MeOH were mixed in a 10-mL test tube. DPPH (2.5 mL of 75 μM in MeOH) was then added to achieve a final volume of 3 mL. The solution was kept at room temperature for 30 min, and the absorbance at 517 nm (A_{517}) was measured.

The DPPH scavenging effect was calculated as follows:

Scavenging effect (%) = $[A_0 - (A - A_b)/A_0] \times 100\%$,

where A_0:A_{517} of DPPH without sample; A:A_{517} of sample and DPPH; and A_b:A_{517} of sample without DPPH.

2.7.3. Superoxide radical assay

The superoxide radical assay was measured by the method of Liu et al.[26] with a minor modification. Samples were dissolved in distilled water at 0 (control), 50, 100, 200, 400, or 800 μg/mL. A 0.1-mL aliquot of each sample solution was mixed with 1 mL of 16 mM Tris–HCl (pH 8.0) containing 78 μM NADH, 1 mL of 16 mM Tris–HCl (pH 8.0) containing 10 μM PMS, and 1 mL of 16 mM Tris–HCl (pH 8.0) containing 50 μM NBT. After 5 min of incubation at 25 °C, the absorbance was measured at 560 nm. The superoxide radical effect was calculated as scavenging activity (%) = (1 − absorbance of sample/absorbance of control) × 100.

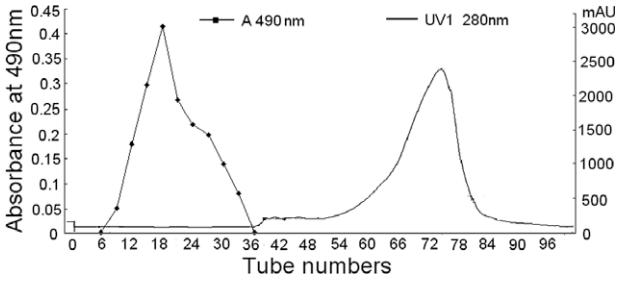

Figure 1. Elution curve of the crude polysaccharides from *Pteridium aquilinum* by a DEAE–Sepharose Fast-Flow column.

2.7.4. Self-oxidation of 1,2,3-phentriol assay

The scavenging abilities for the self-oxidation of 1,2,3-phentriol of all samples were determined according to the method of Marklund and Marklund[27] with a minor modification. Briefly, the samples were dissolved in distilled water at 0 (control), 20, 40, 60, 80, or 100 μg/mL. The sample solution (0.1 mL) was mixed with 2.8 mL of 0.05 M Tris–HCl buffer (pH 8.0) containing 1 mM EDTA and 1,2,3-phentriol (0.2 mL, 6 mM). The mixture was then shaken rapidly at room temperature. The absorbance of the mixture was measured at 325 nm per 30 s for 4 min against a blank, and a slope was calculated as absorbance/min. The scavenging ability for self-oxidation of 1,2,3-phentriol of different samples was calculated using the equation (1 − slope of sample/slope of control) × 100.

3. Results and discussion

3.1. Isolation and purification of polysaccharide

The crude polysaccharide (PL) extracted from *P. aquilinum* was obtained as a water-soluble dust-colored powder. Then PL was purified by anion-exchange chromatography on a DEAE-Sepharose column with the AKTA purifier system. The total sugar content of each tube was determined by the phenol–sulfuric acid assay, and the protein content was determined by a UV detector at a wavelength of 280 nm (Fig. 1). The polysaccharide fraction was collected and purified by gel chromatography on a Sephacryl S-400 HR (50 × 1.0 cm) column. A fraction was collected and named as PLP (purified polysaccharide). It showed only one symmetrical peak from gel-filtration chromatography, indicating that PLP was a homogeneous polysaccharide (Fig. 1). The weight-average molecular weight was calculated to be 458,000 Da, according to the calibration curve with standard dextrans.

The yield, average molecular weight, protein content, and sugar compositions of PLP were determined and are shown in Table 1. The total sugar content of the polysaccharide was determined to be 99.5%, using the phenol–sulfuric acid method.

3.2. Monosaccharide composition of PLP

The TLC results indicated that the PLP mainly consisted of D-glucose. Compared with standard saccharides (glucose, xylose, galactose, rhamnose, mannose, and arabinose), GC traces of the polysaccharide hydrolyzates showed the monosaccharide components of the samples (Fig. 2). The results indicated that glucose (relative mass 58.1%) was the predominant monosaccharide of PLP, along with low levels of galactose, mannose, rhamnose, and arabinose (relative mass 18.7%, 6.8%, 10.2%, and 6.1%).

3.3. FTIR spectroscopy

The IR spectrum of the PLP is shown in Figure 3. It shows a broadly stretched intense peak at around 3419 cm^{-1}, which is the characteristic absorption of hydroxyl groups, and a weak C–H band at around 2937 cm^{-1}. The relatively strong absorption peak at around 1614 cm^{-1} and some weak bands from 1400 cm^{-1} to

Table 1
Yields, protein contents, sugar contents, and M_w of PLP[a]

Sample	Yield (%)	Protein (%)	Carbohydrate (%)	*Mw* (Da)	Sugar component (%)				
					Glu[b]	Gal[b]	Man[b]	Ara[b]	Rha[b]
PLP	2.02	nd[c]	99.5	458,000	58.1	18.7	6.8	10.2	6.1

[a] Data are shown as mean (SD), $n = 3$.
[b] Glu, glucose; Gal, galactose; Rha, rhamnose; Man, mannose; Ara, arabinose.
[c] nd, Not detected.

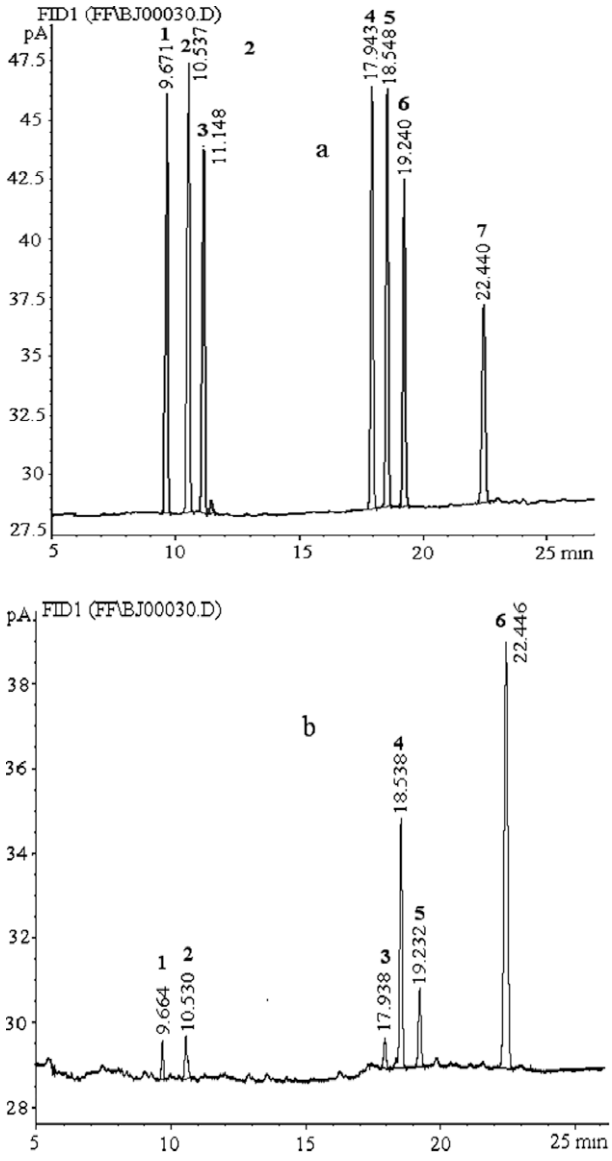

Figure 2. Chromatograms of monosaccharides (as their acetylated aldononitriles): (a) standard mixture (Peak identity: 1, Rhamnose; 2, Arabinose; 3, Xylose; 4, Mannose; 5, Glucose; 6, Galactose; 7, Internal standard); and (b) sample (peak identity: 1, Rhamnose; 2, Arabinose; 3, Mannose; 4, Glucose; 5, Galactose; 6, Internal standard).

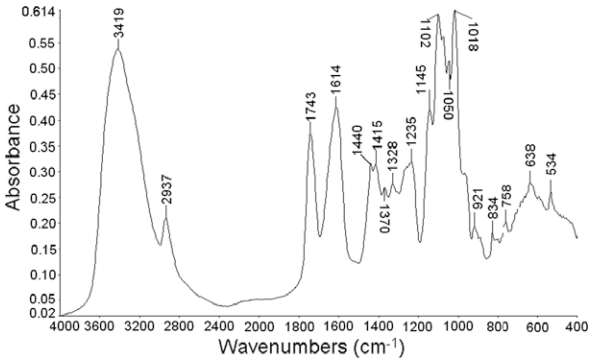

Figure 3. FTIR spectra of the polysaccharide of PLP.

1200 cm^{-1} are also characteristic IR absorptions of a polysaccharide. The polysaccharide has a specific band in the 1200–1000 cm^{-1} region. This region is dominated by ring vibrations overlapped with stretching vibrations of (C–OH) side groups and the (C–O–C) glycosidic band vibration. The IR absorption at about 834 cm^{-1} is characteristic of α-pyranoses.

3.4. Antioxidant activity analysis

3.4.1. Ferric-reducing antioxidant power

The FRAP (ferric-reducing antioxidant power) assay treats the antioxidants contained in the samples as reductants in a redox-linked colorimetric reaction, and the value reflects the reducing power of the antioxidants. The procedure is relatively simple and easy to standardize. This assay is also commonly used for the routine analysis of single antioxidants and total antioxidant activity of plant extracts.[28,29] The antioxidant potentials of different samples were estimated from their ability to reduce the TPTZ–Fe(III) complex to the TPTZ–Fe(II) complex. The antioxidant capacities of the purified polysaccharide are shown in Figure 4A and compared with vitamin C as a control standard.

Lower FRAP values could be a response to the lower production of reactive oxygen species (ROSs) or the result of an increase in ROSs that react with the antioxidants. Based on the FRAP value, the purified polysaccharide (PLP) was much stronger than the crude polysaccharides (PLs) in reducing power in a dose-dependent manner. Also, the results indicate that the purified polysaccharide has a strong potential antioxidant activity, which could bear comparison with that of vitamin C.

3.4.2. Scavenging activity to DPPH-radical

DPPH (1,1-diphenyl-2-picrylhydrazyl) is a free-radical compound that has been widely used to determine the free-radical scavenging ability of various samples.[30–32] DPPH is a stable free radical that shows maximum absorption at 517 nm in methanol. When DPPH encounters a proton-donating substance, for example, an antioxidant, the radical would be scavenged and the absorbance at 517 nm is reduced.[33] It is visually noticeable as a color change from purple to yellow. Based on this principle, the antioxidative activity of a substance can be expressed as its ability in scavenging the DPPH free radical. In this experiment, the DPPH free-radical scavenging effect of each sample was measured, and the results are shown in Figure 4B.

Both PL and PLP have strong antioxidative activity, and their scavenging effects are 50.3% and 83.1% at a dose of 800 μg/mL, respectively. These results indicate that the purified polysaccharide has a noticeable effect on scavenging free radicals, especially at high addition quantity. However, none of them have stronger activity than vitamin C of the same dose. The order of the antioxidative activity is vitamin C > PLP > PL.

3.4.3. Scavenging activity to superoxide radicals

Superoxide anion radicals are known to indirectly initiate lipid peroxidation as a result of H_2O_2 formation, creating precursors of hydroxyl radicals.[34] So its scavenging is extremely important to anti-oxidation work. In the PMS/NADH–NBT system, superoxide anion derived from dissolved oxygen by the PMS/NADH coupling reaction reduces NBT. The decrease of absorbance at 560 nm with antioxidants thus indicates the consumption of superoxide anion in the reaction mixture.[35] Fig. 4C shows the inhibitory effects of the crude polysaccharides (PLs) and purified polysaccharide (PLP) in a concentration-dependent manner by comparison with the same doses of vitamin C.

These results indicate that the purified polysaccharide has a stronger superoxide radical-scavenging activity than the crude polysaccharides, which could bear comparison with that of vitamin C. The percentage inhibition of superoxide generation by 800 μg/

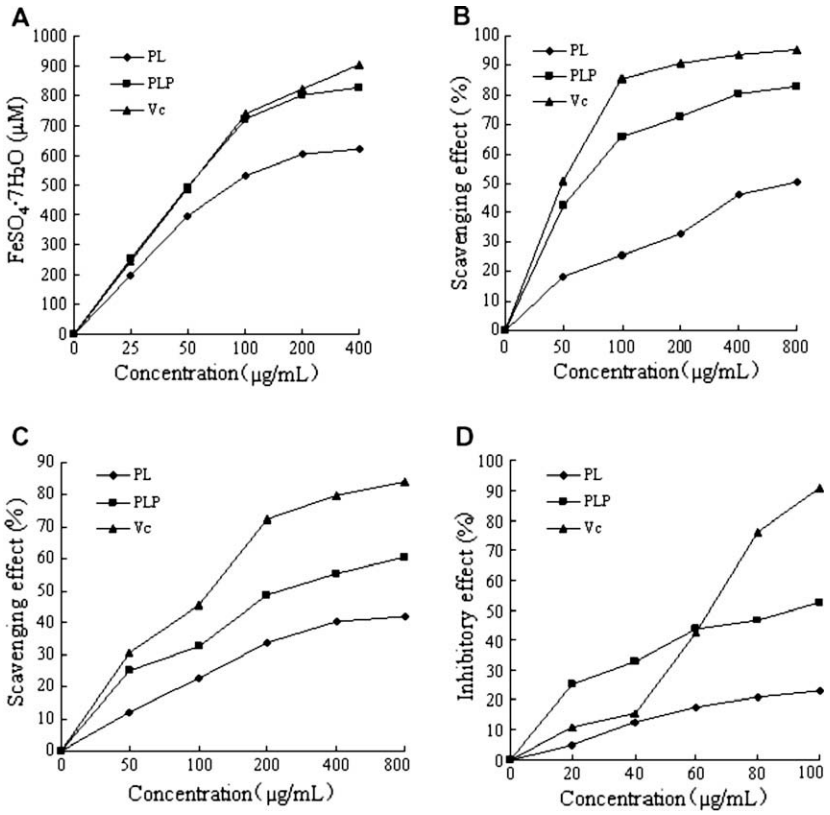

Figure 4. Antioxidant activity analysis of the polysaccharides from *Pteridium aquilinum* with various methods: (A) Ferric-reducing antioxidant power (FRAP) assay; (B) scavenging activity to DPPH radicals; (C) scavenging activity to superoxide radicals; (D) scavenging activity of the self-oxidation of 1,2,3-phentriol; data are presented as mean values ($n = 3$).

mL doses of the crude polysaccharides, the purified polysaccharide, and vitamin C was found as 42.2%, 60.5%, and 84.2%, respectively.

3.4.4. Scavenging activity of self-oxidation of 1,2,3-phentriol

Figure 4D depicts the scavenging power of the self-oxidation of 1,2,3-phentriol of the crude polysaccharides and the purified polysaccharide from *P. aquilinum* and vitamin C. The scavenging power of PLP is stronger than that of PL, but is a little weaker than the scavenging power of vitamin C at the same dose. The scavenging effects of the crude polysaccharides, the purified polysaccharide, and vitamin C on the self-oxidation of 1,2,3-phentriol concentration dependently increased and were 23.3%, 52.4%, and 90.7% at the dose of 100 μg/mL, respectively.

Because of the complex mechanism of antioxidant activity, one test is normally not enough to evaluate precisely the antioxidant activity of the potential antioxidant. Rather, a broad array of tests is required to evaluate the total antioxidant capacity. Superoxide anion is one of the precursors of the singlet oxygen and hydroxyl radicals, and indirectly initiates lipid peroxidation. Apart from that, the presence of superoxide anion can magnify the cellular damage because it produces other kinds of free radicals and oxidizing agents. Also, the scavenging activity of the DPPH radical and self-oxidation of 1,2,3-phentriol are used to evaluate the antioxidant activity of different samples.

We selected the FRAP assay to evaluate the antioxidant activities of the polysaccharides from *P. aquilinum* for the following reasons: None of the methods mentioned above can be treated as a total antioxidant capacity assay because that which they really measure is the capacity of antioxidants in scavenging specific radicals inhibiting the self-oxidation of 1,2,3-phentriol. The FRAP assay can be used to measure the total reducing capability of antioxidants based on the reaction principle described in Section 2. So we selected the above four methods to evaluate the antioxidant activities of the polysaccharides from *P. aquilinum*. All of them presented approximately an identical change in the trend of antioxidant activity. These results indicate that the purified polysaccharide has a noticeable effect on the scavenging free radicals, and has a high FRAP value, especially at high addition quantity.

The radical-scavenging activity of the purified polysaccharide was stronger than that of the crude polysaccharides used in this study. However, the radical-scavenging activity of the purified polysaccharide was lower than that of vitamin C. The scavenging powers of the samples and the standard both correlated well with increasing concentrations. The polysaccharides PL and PLP showed high FRAP antioxidant activity, and the order of FRAP activity of different samples is as follows: vitamin C > PLP > PL as in the case of DPPH, superoxide anion and the self-oxidation of 1,2,3-phentriol.

In our study, the purified polysaccharide (PLP) showed strong antioxidant capacity in antioxidation tests in vitro. Future research should be aimed at investigating the antioxidant activity of the purified polysaccharide (PLP) in experiments in vivo.

4. Conclusions

According to the above results, it was concluded that the water-extracted crude polysaccharides (PLs) were purified by DEAE–Sepharose FF and Sephacryl S-400 HR column chromatography, and the purified polysaccharide (PLP) prepared was confirmed of high purity. Antioxidation tests in vitro showed that PLP possessed a strong free-radical scavenging effect of the superoxide radical, and the DPPH radical showed an inhibition of the self-oxidation of 1,2,3-phentriol. Also, the PLP showed a higher FRAP value that may be comparable to that of vitamin C. The results suggest that

the water-soluble polysaccharide purified from *P. aquilinum* should be explored as a novel potential natural antioxidant.

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