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Structure and antioxidant activity of an extracellular polysaccharide from coral-associated fungus, *Aspergillus versicolor* LCJ-5-4

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ABSTRACT

An extracellular polysaccharide AVP was isolated from the fermented broth of coral-associated fungus *Aspergillus versicolor* LCJ-5-4. AVP was a mannoglucan with molecular weight of about 7 kDa, and the molar ratio of glucose and mannose was 1.7:1.0. On the basis of detailed one- and two-dimensional nuclear magnetic resonance (1D and 2D NMR) spectroscopic analyses, the backbone of AVP was characterized to be composed of (1 → 6)-linked α-D-glucopyranose and (1 → 2)-linked α-D-mannopyranose units. The mannopyranose residues in the backbone were substituted mainly at C-6 by the side chain of (1 → 2)-linked α-D-mannopyranose trisaccharides units. The antioxidant activity of AVP was evaluated with the scavenging abilities on 1,1-diphenyl-2-picrylhydrazyl (DPPH), superoxide and hydroxyl radicals in vitro, and the results indicated that AVP had good antioxidant activity, especially scavenging ability on superoxide radicals. AVP was a novel extracellular polysaccharide with different structural characteristics from other extracellular polysaccharides and could be a potential source of antioxidant.

1. Introduction

Marine microorganisms often produce extracellular polysaccharides with novel structures and diverse biological activities due to their specific marine environment (Arena et al., 2009; Mancuso Nichols, Garon, Bowman, Raguénès, & Guézennec, 2004; Roger, Kervarec, Ratiskol, Colliec-Jouault, & Chevolot, 2004; Rougeaux, Pichon, Kervarec, Raguenes, & Guezennec, 1996; Sun et al., 2009). The novel active extracellular polysaccharides from marine microorganisms hold a great potential application in biology and pharmacology (Du et al., 2009; Kanekiyo et al., 2005; Kimura, Sumiyoshi, Suzuki, & Sakanaka, 2006; Umezawa et al., 1983).

Symbiotic and epiphytic microorganisms of large marine organisms have close relationship with the hosts (Li, 2009). Recently, some investigations indicated that symbiotic and epiphytic microorganisms may be involved in organism biosynthesis, metabolism and other life activities of large hosts (Croft et al., 2005; Dobretsov & Qian, 2004; Osinga et al., 2001). Interest in extracellular polysaccharides produced by symbiotic and epiphytic microorganisms is increasing because of their unique structures and specific properties. The marine bacterium *Shewanella colwelliana* separated from *Crassostrea virginic* could produce a kind of acidic polysaccharide, with mannose, glucose, galactose and pyruvic acid (Sledjeski & Weiner, 1993). *Aeromonas* sp. HYD154 from the stratum corticale of *Alvinella pompejana* secreted an acidic polysaccharide, which contained glucose, galactose, glucuronic acid, galacturonic acid and 4,6-pyruvic acidic galactose (Cambon-Bonavita, Raguenes, Jean, Vincent, & Guezennec, 2002). *Flavobacterium uliginosum* from the surface of the marine algae produced a neutral polysaccharide with antitumor activity, and the polysaccharide was composed of glucose, mannose and fucose (Vincent et al., 1994). With today's interest in new renewable sources of chemicals and polymers, the extracellular polysaccharides isolated from marine microorganisms represent potential source to be explored. In the paper, the structure and antioxidant activity in vitro of an extracellular polysaccharide isolated from the fermented broth of coral-associated fungus *Aspergillus versicolor* LCJ-5-4 were investigated.

2. Material and methods

2.1. Materials

Monosaccharides (D-glucose, L-rhamnose, D-xylose, L-arabinose, D-mannose, L-fucose, D-galactose, D-glucuronic acid, D-galacturonic acid, D-mannuronic acid, *N*-acetyl-β-D-glucosamine), 1,1-diphenyl-2-picrylhydrazyl, trifluoroacetic acid,

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thiobarbituric acid, trichloroacetic acid, 1-phenyl-3-methyl-5-pyrazolone, ascorbic acid and ferrozine were from Sigma–Aldrich (St. Louis, MO, USA). Dextran T-series standards (*M*w: 41.1, 25.5, 12.5, 5.7, 3.65 and 2.5 kDa) were from National Institutes for Drugs and Biological Products (Beijing, China). Dialysis membranes (flat width 44 mm, molecular weight cut off 3500) were from Lvniao (Yantai, China). Q Sepharose Fast Flow and Superdex 75 were from Amersham Biosciences (Uppsala, Sweden).

2.2. Microorganism and culture media

A. versicolor LCJ-5-4 was isolated from coral *Cladiella* sp. collected from Lingao, Hainan province of China. It was identified according to its morphological characteristics and 18S rRNA sequences by Professor Fang, C. X. in China Center for Type Culture Collection. The voucher specimen is deposited in Professor Zhu's laboratory at −80 °C. The producing strain was prepared on Potato Dextrose agar slants at 3.33% salt concentration and stored at 4 °C. *A. versicolor* LCJ-5-4 was grown under static conditions at 20 °C for 30 days in the liquid medium. The liquid medium composed of sorbitol (20 g/L), maltose (20 g/L), monosodium glutamate (10 g/L), KH_2PO_4 (0.5 g/L), $MgSO_4{\cdot}7H_2O$ (0.3 g/L), tryptophane (0.5 g/L), yeast extract (3 g/L), and sea salt (33.3 g/L), after adjusting its pH to 6.5. The fermented whole broth (about 90 L) was obtained (Zhuang et al., 2011).

2.3. Isolation and purification of the extracellular polysaccharide from coral-associated fungus A. versicolor LCJ-5-4

The fermented broth was filtered through cheesecloth to separate into filtrate and mycelia. The filtrate was concentrated under reduced pressure to about a quarter of the original volume and then extracted three times by adding same volume of ethylacetate (1.0 h) to remove the liposoluble compounds. The aqueous layer was concentrated under reduced pressure at 40 °C and a threefold of the volume of 95% (v/v) ethanol was added. The resulting precipitate was recovered using centrifugation at 8000 rpm for 10 min, and was dialyzed in a cellulose membrane tubing (molecular weight cut off 3500) against distilled water for 48 h. The retained fraction was dried, and the protein in the fraction was removed by the method of Sevag (Matthaei, Jone, Martin, & Nirenberg, 1962). The crude polysaccharide was fractionated by a Q Sepharose Fast Flow column (300 mm × 30 mm) coupled to an AKTA FPLC system, eluted with a step-wise gradient of 0, 0.3 and 2.5 mol/L NaCl. The fractions were assayed for carbohydrate content by the phenol–sulfuric acid method. The fractions (48 mL) eluted with 0.3 mol/L NaCl were pooled, dialyzed and further purified on a Superdex 75 column (70 cm × 2 cm) eluted with 0.2 mol/L NH_4HCO_3 at a flow rate of 0.3 mL/min. The major polysaccharide fractions were pooled and freeze–dried.

2.4. General analysis

Total sugar content was determined by the phenol–sulfuric acid method using glucose as the standard (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). Protein content was measured by the method of Bradford (1976). Sulfate ester content was estimated according to the method reported by Therho and Hartiala (1971). Uronic acid content was determined by the carbazole–sulfuric acid method (Bitter & Muir, 1962).

2.5. Determination of purity and molecular weight

Purity and molecular weight were determined by high performance gel permeation chromatography (HPGPC) with a TSKgel G3000PW_{XL} column (7.8 mm × 30.0 cm, Tosoh, Japan) and a refractive index detector (Agilent 1100 Series), eluted with 0.2 mol/L Na_2SO_4 at a flow rate of 0.5 mL/min. 20 μL of 1% sample solutions in 0.2 mol/L Na_2SO_4 was injected. The molecular weight was estimated by reference to a calibration curve made by a set of dextran T-series standards (*M*w: 41.1, 25.5, 12.5, 5.7, 3.65 and 2.5 kDa) (Mao et al., 2008).

2.6. Analysis of monosaccharide composition

Monosaccharide composition was determined by reversed-phase high performance liquid chromatography (HPLC) after pre-column derivatization and UV detection (Sun et al., 2009). Briefly, polysaccharide was hydrolyzed with 2 mol/L trifluoroacetic acid at 100 °C for 6 h in a sealed tube. Excess acid was removed by co-distillation with methanol for four times after the hydrolysis was completed. 1 mg of dry hydrolysate was dissolved in 100 μL of 0.3 mol/L NaOH, and then added to 120 μL of 0.5 mol/L methanol solution of 1-phenyl-3-methyl-5-pyrazolone (PMP) at 70 °C for 1 h. Finally, the mixture was added 100 μL of 0.3 mol/L HC1 solution and vigorously shaken and centrifuged for 5 min. The supernatant, containing the labeled carbohydrates, was finally filtered through 0.22 μm nylon membranes (Westborough, MA, USA) and 10 μL of the resulting solution was injected into the XDB-C_{18} column (4.6 mm × 250 mm) fitted with Agilent XDB-UV detector. The mobile phase was a mixture of 0.1 mol/L KH_2PO_4 (pH 10)–acetonitrile (83:17). The flow rate was 1.0 mL/min and column temperature was 30 °C. Sugar identification was done by comparison with reference sugars (D-glucose, L-rhamnose, D-xylose, L-arabinose, D-mannose, L-fucose, D-galactose, D-glucuronic acid, D-galacturonic acid, D-mannuronic acid and *N*-acetyl-β-D-glucosamine). Calculation of the molar ratio of the monosaccharide was carried out on the basis of the peak area of the monosaccharide.

2.7. IR spectroscopy analysis

The polysaccharide was mixed with KBr powder, ground and then pressed into a 1 mm pellets for Fourier-transform infrared (FT-IR) spectrum measurement in the frequency range of 4000–500 cm^{-1}. FT-IR spectrum was measured on a Nicolet Nexus 470 spectrometer.

2.8. Methylation analysis

Methylation analysis was performed by the method of Hakomori (1964). In the assay, 2 mg of polysaccharide was dissolved in 1 mL of dimethyl sulfoxide, and then 200 mg of anhydrous NaH was added. The mixture was stirred at room temperature for 1.5 h. 0.5 mL of iodomethane was then added to the mixture and stirred for a further 1.5 h. The reaction was terminated with addition of water, and the residue was extracted with $CHCl_3$. The extract was washed with distilled water and evaporated to dryness. The completion of methylation was confirmed by FT-IR spectroscopy as the disappearance of OH bands. After hydrolysis with 2 mol/L trifluoroacetic acid at 105 °C for 6 h, the methylated sugar residues were converted to partially methylated alditol acetates by reduction with $NaBH_4$, followed by acetylation with acetic anhydride. The derivatised sugar residues were extracted into dichloromethane and evaporated to dryness, dissolved again in 100 μL dichloromethane. The products were analysed by gas chromatography–mass spectrometric (GC–MS) on DB 225 using a temperature gradient: first 100–220 °C with a rate of 5 °C/min, then keeping at 220 °C for 15 min. GC–MS was performed on an HP6890II instrument. The methylated sugar linkages were

identified by the retention time and fragmentation pattern (Mao et al., 2008).

2.9. NMR spectroscopy analysis

^{1}H nuclear magnetic resonance (NMR) and ^{13}C NMR spectra were recorded at 23 °C using a JEOL JNM-ECP 600 MHz spectrometer. 70 mg of polysaccharide was dissolved in 1 mL of 99% D_2O followed by centrifugation and freeze–dried. The process was repeated twice, and the final sample was dissolved in 1.0 mL of 99.98% D_2O. Chemical shifts are expressed in ppm using acetone as internal standard at 2.225 ppm for ^{1}H and 31.07 ppm for ^{13}C. ^{1}H–^{1}H correlated spectroscopy (COSY), ^{1}H–^{1}H total correlation spectroscopy (TOCSY), ^{1}H–^{13}C heteronuclear multiple quantum coherence spectroscopy (HMQC) and ^{1}H–^{13}C heteronuclear multiple bond correlation spectroscopy (HMBC) experiments were also carried out using the pulse programs supplied with the Bruker manual.

2.10. Analysis of antioxidant activity

Scavenging ability of 1,1-dihpenyl-2-picrylhydrazyl (DPPH) radicals was measured according to the method described by Shimada, Fujikawa, Yahara, and Nakamura (1992). Briefly, 1 mL of sample solution at different concentrations (0, 0.25, 0.5, 1.0, 2.0, 4.0, and 8.0 mg/mL) was added to 3 mL of 0.004% ethanol solution of DPPH. Absorbance at 517 nm was measured after 30 min. The scavenging ability was calculated according to the equation below: scavenging ability (%) = $(1 - A_{sample}/A_{control}) \times 100$, where $A_{control}$ is the absorbance of control without the tested samples, and A_{sample} is the absorbance in the presence of the tested samples. Ascorbic acid was used as positive control in all antioxidant assays.

Scavenging ability of superoxide radicals was assessed according to the method reported by Marklund and Marklund (1974). Briefly, 3 mL of 0.05 mol/L Tris–HCl buffer (pH 8.2) and 1 mL of samples solution at different concentrations (0, 0.25, 0.5, 1.0, 2.0, 4.0, and 8.0 mg/mL) were incubated at 25 °C for 10 min, and then 200 μL of pyrogallol at the same temperature were added to the mixture and the reaction was proceed at 25 °C for 4 min. Finally, the reaction system was terminated by the addition of 0.5 mL of HCl. The absorbance of the mixture was measured at 320 nm against the blank. The scavenging ability was calculated according to the equation: scavenging ability (%) = $(1 - A_{sample}/A_{control}) \times 100$, where $A_{control}$ is the absorbance of control without the tested samples, and A_{sample} is the absorbance in the presence of the tested samples.

Scavenging ability of hydroxyl radicals was determined according to the method of Smirnoff and Cumbes (1989). In the assay, 0.3 mL of 5 mmol/L orthophenanthroline, 0.8 mL of 0.75 mol/L phosphate buffer (pH 7.4) and 0.3 mL of 7.5 mmol/L FeSO4 were added to 0.5 mL of sample solution at different concentrations (0, 0.25, 0.5, 1.0, 2.0, 4.0, and 8.0 mg/mL). Finally, 0.2 mL of 1% H_2O_2 was added, and the reaction mixture was then incubated at 37 °C for 60 min. The absorbance of the resulting solution was measured at 532 nm. The scavenging ability was calculated according to the equation: scavenging ability (%) = $(1 - A_{sample}/A_{control}) \times 100$, where $A_{control}$ is the absorbance of control without the tested samples, and A_{sample} is the absorbance in the presence of the tested samples.

All bioassay results were expressed as means ± standard deviation (*SD*). The experimental data were subjected to an analysis of variance for a completely random design, and three samples were prepared for assays of every antioxidant attribute.

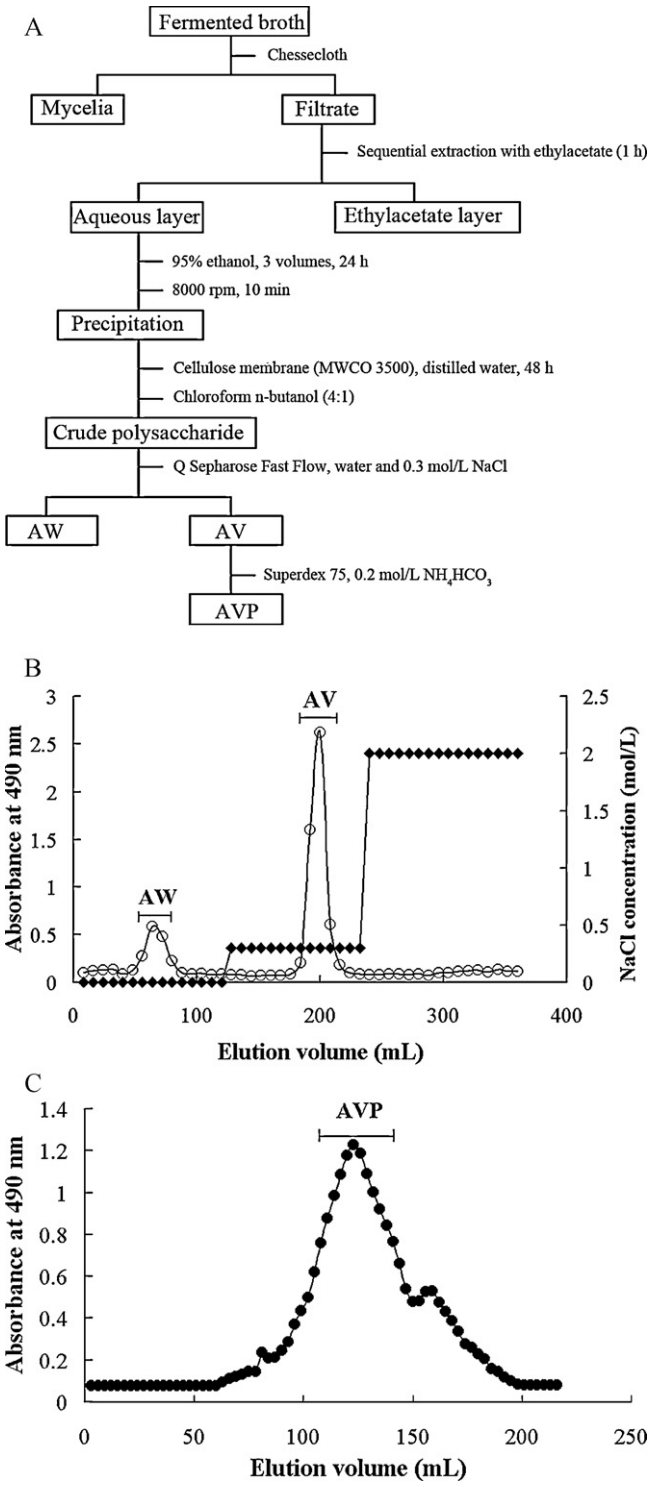

Fig. 1. Isolation and purification of the extracellular polysaccharides produced by coral-associated fungus *A. versicolor* LCJ-5-4. (A) Scheme for the isolation and purification of the extracellular polysaccharide; (B) the crude polysaccharides were applied to a Q Sepharose Fast Flow column. The fraction eluted with 0.3 mol/L NaCl was pooled and named as AV; (C) AV was applied to a Superdex 75 column and eluted with 0.2 mol/L NH_4HCO_3. The peak fractions containing the polysaccharides were pooled and named as AVP.

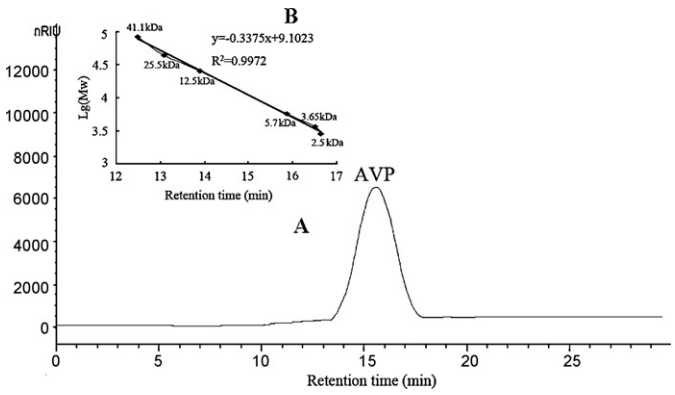

Fig. 2. HPGPC chromatogram of the extracellular polysaccharide AVP and the standard curve of molecular weight. (A) HPGPC chromatogram of AVP on TSKgel G3000PW$_{XL}$ column (7.8 mm × 30.0 cm); (B) the standard curve of molecular weight.

3. Results and discussion

3.1. Isolation and composition analysis of the extracellular polysaccharide

The procedures used for the isolation and purification of the extracellular polysaccharide from the fermented broth of coral-associated fungus *A. versicolor* LCJ-5-4 were shown in Fig. 1A. The crude extracellular polysaccharides from the fermented broth were purified on a Q Sepharose Fast Flow column (Fig. 1B). The polysaccharide fraction AV, eluted with 0.3 mol/L NaCl, contained abundant total sugar, and was the major component of the crude polysaccharides. The fraction AV was further purified by a Superdex 75 column, and a polysaccharide fraction AVP was obtained (Fig. 1C). The yield of AVP from crude polysaccharides (0.24 g/L) was about 60%. AVP contained 92.4% total carbohydrate and minor amounts of protein (0.94%), and did not have any sulfate ester. Moreover, AVP gave a single and symmetrically peak in HPGPC chromatogram (Fig. 2A), and its average molecular weight was estimated to be about 7 kDa (Fig. 2B). Monosaccharide composition analysis by reversed-phase HPLC showed that AVP consisted of glucose and mannose with a molar ratio of 1.7:1.0 (Fig. 3A and B). No acidic sugar and amino sugar were detected in AVP.

3.2. Analysis of IR spectroscopy

As shown in Fig. 3C, the broad and intense band at 3322 cm^{-1} was the stretch vibration of hydroxyl groups. The signal at 2932 cm^{-1} was attributed to the stretch vibration of the C–H bond. The band at 1651 cm^{-1} was assigned to the bending vibration of O–H, and the signal at 1537 cm^{-1} was attributed to the vibration of C–O. The band at 1390 cm^{-1} was attributed to the bending vibration of C–H, and the signal at 1052 cm^{-1} was due to the stretch vibration of C–O–C linkages. The characteristic absorption band at 855 cm^{-1} suggested the presence of α-anomeric configuration. In addition, the strong characteristic absorption at 812 cm^{-1} indicated the presence of D-mannopyranose units (Mathlouthi & Koenig, 1986; Shingel, 2002).

3.3. Methylation analysis

Methylation analysis could provide important information for the linkage position assignments of each monosaccharide. A large amount of 1,5,6-tri-O-acetyl-2,3,4-tri-O-methyl-D-glucitol, which originated from the (1 → 6)-linked glucose residue, was found in AVP (Table 1). 1,2,5-tri-O-acetyl-3,4,6-tri-O-methyl-D-mannitol and 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-mannitol

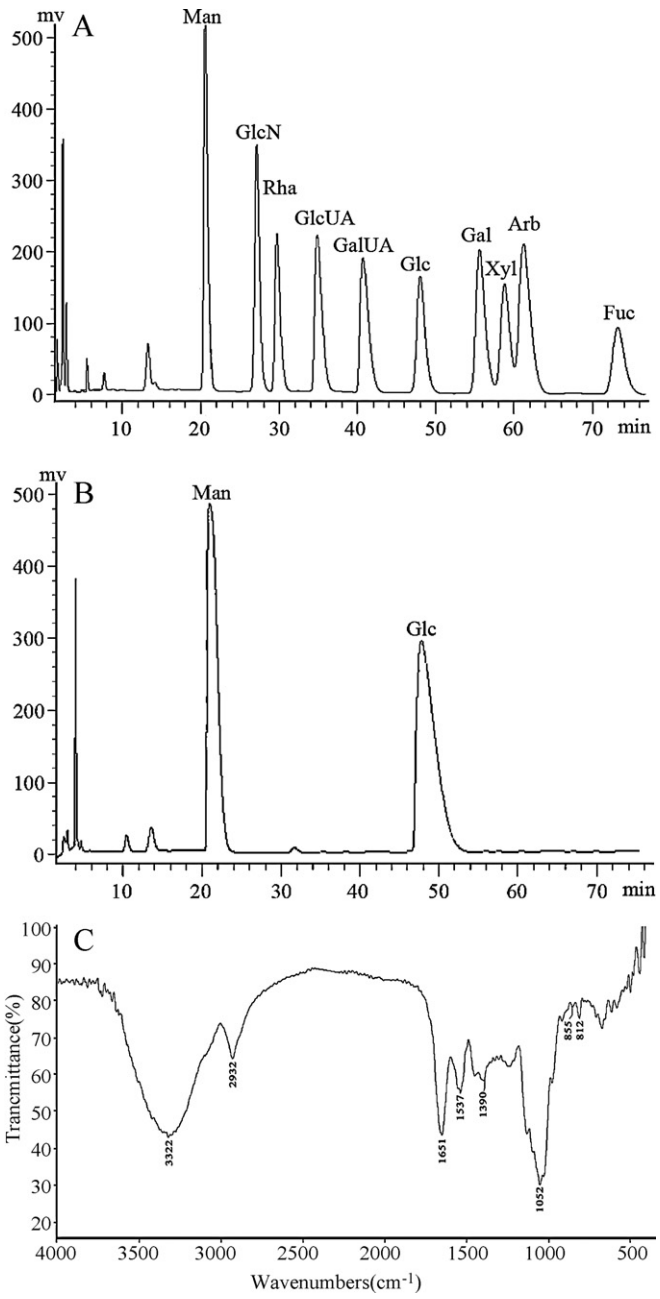

Fig. 3. HPLC chromatography and IR spectrum of the extracellular polysaccharide AVP. (A) HPLC chromatography of monosaccharide standards; (B) HPLC chromatography of AVP; (C) IR spectrum of AVP.

were also detected, indicating the presence of (1 → 2)-linked mannose and (1→)-linked mannose residues. In addition, AVP should have a partially branched structure because of the presence of 1,2,5,6-tetra-O-acetyl-3,4-di-O-methyl-D-mannitol, which originated from the (1 → 2,6)-linked mannose residues. The molar ratios of (1 → 6)-linked glucopyranose, (1 → 2)-linked mannopyranose, (1 → 2,6)-linked mannopyranose and (1→)-linked mannopyranose residues were about 6.8:1.91:1.12:1.0. The result suggested that AVP was a mannoglucan with branch. The main chain of the polysaccharide may be composed of (1 → 6)-linked glucopyranose residues, and the side chains attached to (1 → 2,6)-linked mannopyranose. However, it could not be determined that (1 → 2)-linked mannopyranose was in the main chain or in the side chain.

Table 1
Identification of the O-methylated alditol acetates derived from AVP.

Methylated alditol acetate	MS (*m*/*z*)	Molar ratio	Linkage type
1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-mannitol	87, 101, 117, 145, 161, 205	1.0	Man*p*-(1→
1,2,5-tri-O-acetyl-3,4,6-tri-O-methyl-D-mannitol	129, 161, 189	1.91	→2)-Man*p*-(1→
1,5,6-tri-O-acetyl-2,3,4-tri-O-methyl-D-glucitol	87, 101, 117, 129, 161, 189, 233	6.8	→6)-Glc*p*-(1→
1,2,5,6-tetra-O-acetyl-3,4-di-O-methyl-D-mannitol	129, 189	1.12	→2,6)-Man*p*-(1→

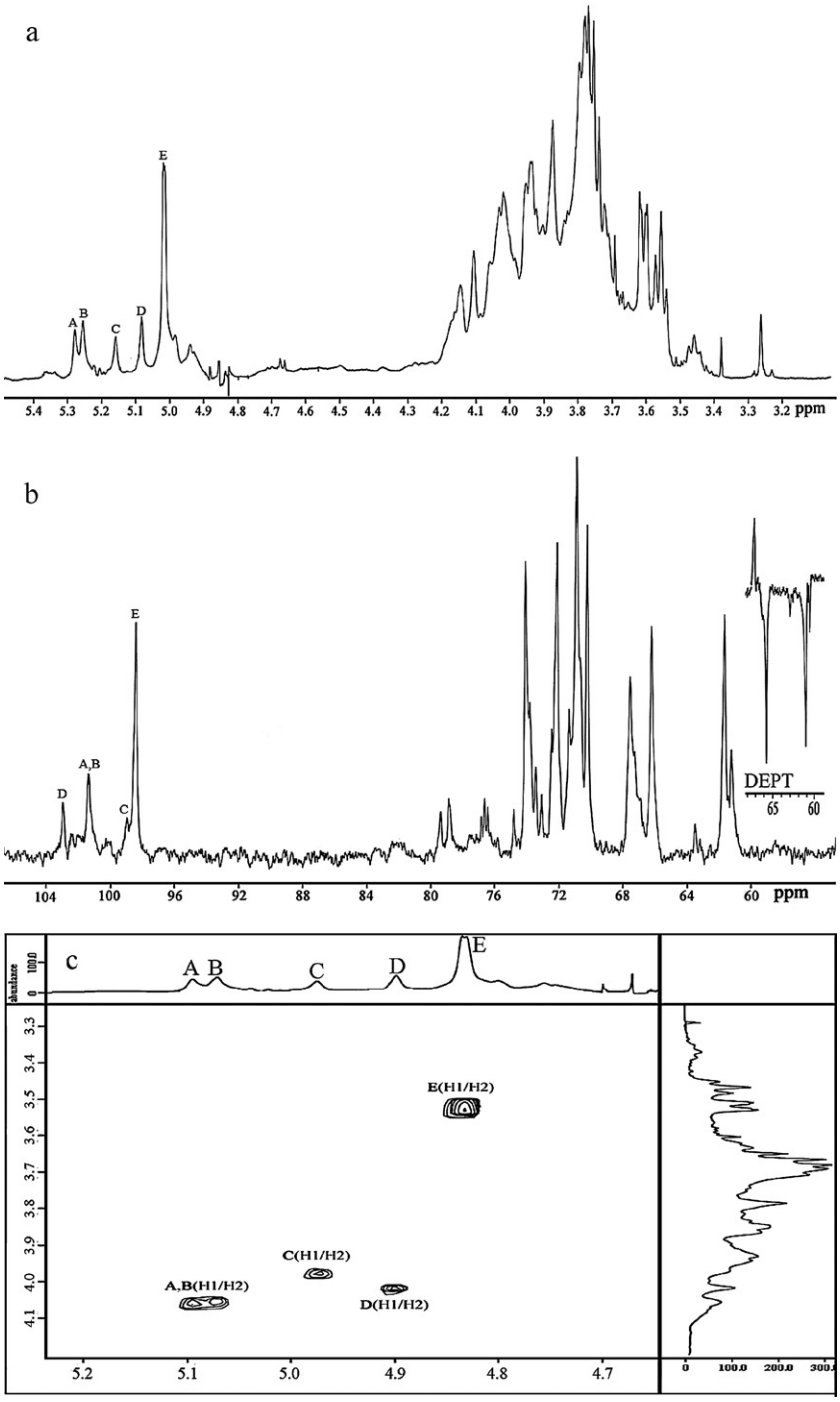

Fig. 4. NMR spectra of the extracellular polysaccharide AVP. Spectra were performed at 23 °C on a JEOL ECP 600 MHz spectrometer using acetone as internal standard. (a) ^{1}H NMR spectrum; (b) ^{13}C NMR and DEPT spectra; (c) ^{1}H–^{1}H COSY spectrum; (d) ^{1}H–^{1}H TOCOSY spectrum; (e) ^{1}H–^{13}C HMQC spectrum; (f) ^{1}H–^{13}C HMBC spectrum. A–E correspond to (1 → 2)-α-D-Man*p*, (1 → 2)-α-D-Man*p*, (1 → 2, 6)-α-D-Man*p*, (1→)-α-D-Man*p*, and (1 → 6)-α-D-Glc*p*, respectively. Glc*p*: glucopyranose, Man*p*: mannopyranose.

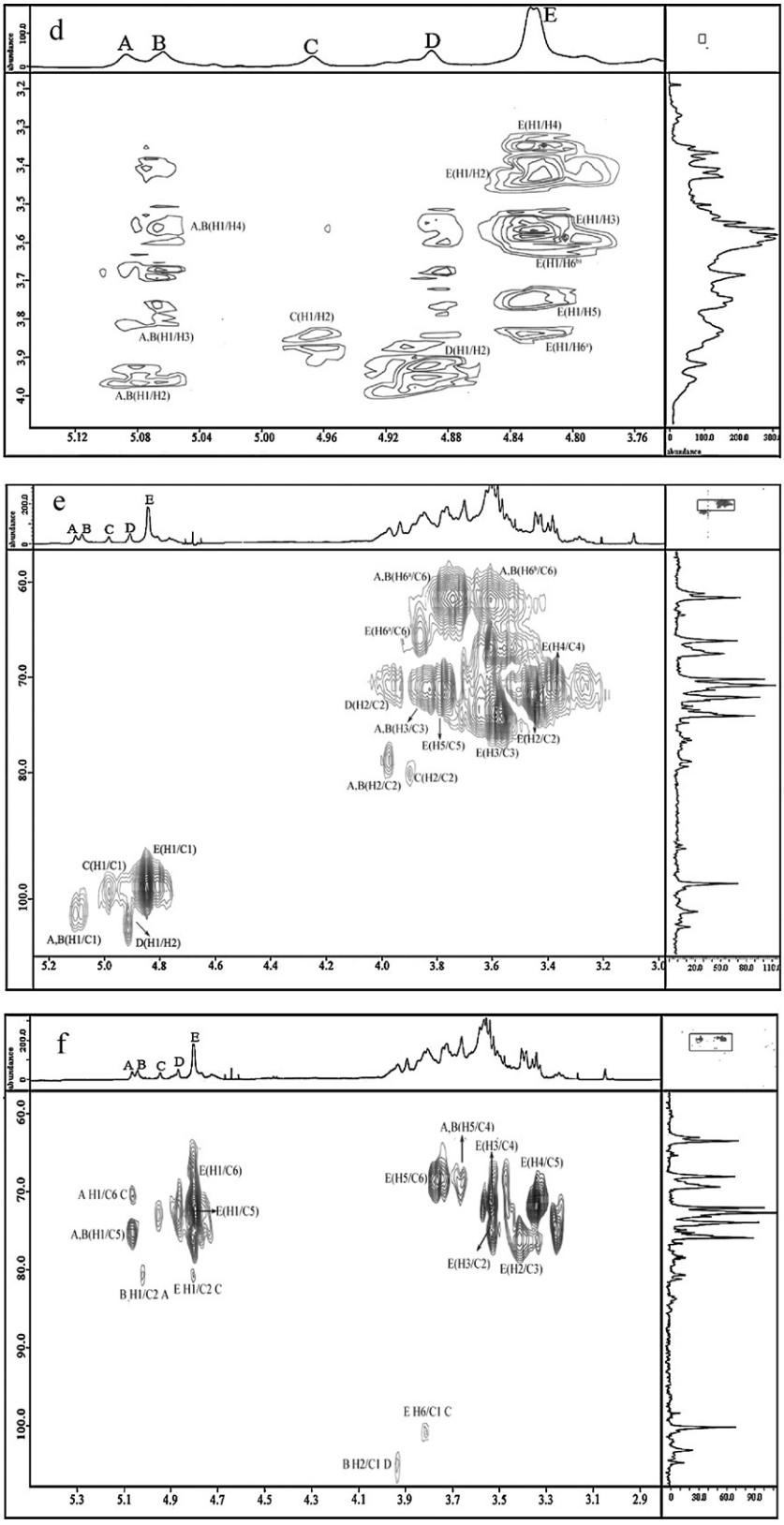

Fig. 4. (*Continued*)

3.4. Analysis of NMR spectroscopy

From the ^{1}H NMR spectrum of AVP (Fig. 4a), the anomeric proton signals at δ 5.28, 5.26, 5.16, 5.08 and 5.02 ppm were attributed to α-configuration pyranose units, and had relative integrals of 1.0:1.13:1.2:0.95:6.58. The chemical shifts from δ 3.4 to 4.2 ppm were assigned to protons of C-2–C-6 of hexosyl glycosidic ring. In the anomeric region of the ^{13}C NMR spectrum, four anomeric carbon signals occurred at δ 102.27, 100.67, 98.30 and 97.72 ppm (Fig. 4b). As shown in the DEPT spectrum, two CH_2OH groups

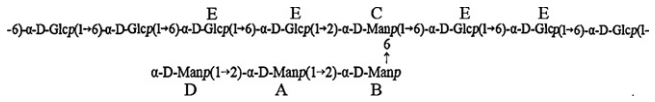

Fig. 5. Proposed structure of the extracellular polysaccharide AVP. A–E correspond to (1 → 2)-α-D-Man*p*, (1 → 2)-α-D-Man*p*, (1 → 2, 6)-α-D-Man*p*, (1→)-α-D-Man*p*, and (1 → 6)-α-D-Glc*p*, respectively. Glc*p*: glucopyranose, Man*p*: mannopyranose.

had the carbon signals at δ 60.9 and δ 65.49 ppm. The signal at δ 65.49 ppm was assigned to the substituted C-6 mannose units, while signal at δ 60.9 ppm was correlated to the C-6 non-substituted mannose residues. The result confirmed the presence of the substituted C-6 linkage patterns, which was in agreement with methylation results.

The ^{1}H NMR spin systems of the polysaccharide were assigned by the ^{1}H–^{1}H COSY spectrum (Fig. 4c) and the ^{1}H–^{1}H TOCSY spectrum (Fig. 4d). The direct C–H coupling was determined by the ^{1}H–^{13}C HMQC spectrum (Fig. 4e). Combined with the analysis of the ^{1}H–^{13}C HMQC spectrum and the comparison with the chemical shift data of similarly substituted sugar residues (Kobayash et al., 1995; Shibata, Suzuki, Kobayashi, & Okawa, 2007), the assignment of the main signals of the five sugar residues could be completed (Table 2). The H-1 of **A** and **B** was related to the same H-2 in the ^{1}H–^{1}H COSY spectrum and the same C-1 in the ^{1}H–^{13}C HMQC spectrum. Thus, **A** and **B** were suggested to be α-Man*p* with a similar substitution pattern. The ^{1}H–^{13}C HMQC spectrum revealed the substitution of **A** and **B** at C-2 due to the downfield shift (δ 78 ppm) of the resonance of C-2 in comparison with that of the parent α-Man*p* (δ 70–72 ppm). Thus, **A** and **B** were both (1 → 2)-α-D-Man*p* residues. The slight differences of anomeric proton signals between **A** and **B** could be due to the effect of linked different residues. As the same, **C** was considered to be (1 → 2, 6)-α-D-Man*p* residues because of the downfield shifts of the C-2 (δ 78 ppm) and C-6 (δ 66 ppm) as compared with that of the parent α-D-Man*p* (C-2, δ 70-72 ppm; C-6, δ 61 ppm). **D** was proposed to be a (1→)-α-D-Man*p*. Combined with ^{1}H NMR spectrum, methylation analysis and the chemical shift data of similarly substituted sugar residues, **E** was assigned to (1 → 6)-α-D-Glc*p*. The chemical shift of C-6 in **E** also changed from δ 60.5 to 66.9 ppm in comparison with the parent α-D-Glc*p* (Luo, Xu, Yu, Yang, & Zheng, 2008; Purama, Goswami, Khan, & Goyal, 2009).

Based on the analysis of the ^{1}H–^{13}C HMBC spectrum (Fig. 4f), the repeating units of the monosaccharide sequences in the polysaccharide chain were established. The presence of strong cross peak H-1/C-6 in **E** confirmed that (1 → 6)-α-D-Glc*p* was the main pattern of linkage. The weak inter-residue cross peaks H1(**E**)/C2(**C**) and H6(**E**)/C1(**C**) indicated that (1 → 6)-α-D-Glc*p* residue linked to the C-2 of (1 → 2, 6)-α-D-Man*p* residue, and the (1 → 2, 6)-α-D-Man*p* residue linked to the C-6 of (1 → 6)-α-D-Glc*p*. The results suggested the presence of segment 6)-α-D-Glc*p*(1 → 2,6)-α-D-Man*p*(1 → 6)-α-D-Glc*p*(1-. It was proposed that (1 → 2, 6)-linked-α-D-Man*p* occupied the branching point of the backbone. On the basis of the relative amount, the ratio of the branch and backbone was estimated at 12% by mole equivalent to one branching point for every 8 residues in the backbone. From the cross signal of H1(A)/C6(C), it was presumed that (1 → 2)-α-D-Man*p* linked to C-6 of the branched (1 → 2,6)-α-D-Man*p*. The cross signals at δ 5.26/78.2 could be assigned to H1(B)/C2(A) though the similar chemical shifts of A and B residues. Combined with the cross signal H2(B)/C1(D), it was concluded that the major branch chain was composed of α-D-Man*p*(1 → 2)-α-D-Man*p*(1 → 2)-α-D-Man*p*(1-. The possible repeating structure of AVP was showed in Fig. 5.

Generally, polysaccharides with glucose and mannose residues were mostly glucomannans, and their main chains were mainly composed of mannose. The glucomannans were common in the *Amorphophallus* tubers called konjac glucomannans (Dave & McCarthy, 1997). However, the mannoglucan which had mainly glucose backbone were uncommon. Inoue, Kawamoto, and Kadoya (1983) reported an antitumor mannoglucan from the culture filtrate of *Microellobosporia grisea* which had a tetrasaccharide (1 → 4)-β-D-Glc*p* backbone, a single α-D-mannose located at both C-3 and C-6 of every other glucosyl residues. Wu, Hu, Pan, Zhou, and Zhou (2007) isolated a mannoglucan from edible *Cordyceps sinensis* mycelium, and it had a α-D-glucan backbone with (1 → 4)- and (1 → 3)-linkages, and the side chains of (1 → 6)-α-D-Man*p* were attached to the backbone by C-6 of (1 → 3)-α-D-Glc*p* residues. A mannoglucan from *Cistanche deserticola* contained (1 → 6)-α-D-Glc*p* and (1 → 6)-β-D-Man*p* backbone, and mannoglucan branches (Wu & Tu, 2004). The extracellular polysaccharide AVP from coral-associated fungus *A. versicolor* LCJ-5-4 had different structural characteristics from other extracellular polysaccharides. The results suggested that marine microorganisms could be a potential source of polysaccharides with novel structures.

3.5. Assay of antioxidant activity

The antioxidant activity of the extracellular polysaccharide AVP was investigated by the assays of the scavenging abilities on DPPH, superoxide and hydroxyl radicals. Superoxide radical was a weak oxidant in most organisms, but it could produce hydrogen peroxide and hydroxyl radical through dismutation and other types of reaction and was the source of free radicals formed in vivo (MacDonald, Galley, & Webster, 2003). Hydroxyl radical is one of the reactive oxygen species generated in the body, and removing hydroxyl radicals is important for antioxidant defense in living cell systems (Aruoma, 1998). DPPH is a useful reagent to evaluate the free radical scavenging ability of the hydrogen donating antioxidant, which can transfer hydrogen atoms or electrons to DPPH radicals (Braca et al., 2001).

As shown in Table 3, the antioxidant activity of AVP was concentration-dependent. At 4.0 mg/mL, the scavenging ability of AVP on superoxide radicals was up to 90.8%. Moreover, AVP showed strong scavenging ability on superoxide radicals as evidenced by its low EC_{50} value (<1.5 mg/mL). The results suggested that AVP was effective scavenger for superoxide radicals, and could be better advantageous for preventing injury induced by superoxide radicals in pathological conditions. In addition, it was found that AVP had a more noticeable scavenging ability on superoxide radicals than the exopolysaccharide As1-1 produced by marine fungi *Aspergillus* sp. Y162, and the EC_{50} value of As1-1 was 3.4 mg/mL (Chen et al., 2011). The scavenging ability of AVP on superoxide radicals was lower than that of ascorbic acid, and EC_{50} value of ascorbic acid was 0.13 mg/mL.

At 8.0 mg/mL, the scavenging ability of AVP on DPPH radicals was near to 80%, and its EC_{50} value was 2.05 mg/mL, indicating that AVP also was good effectiveness in the antioxidant attribute. The scavenging ability of AVP on DPPH radicals was stronger than the exopolysaccharides PS2-1, PS1-2 and PS1-1 produced by marine fungus *Penicillium* sp. F23-2 (Sun et al., 2009), and the EC_{50} values of the three exopolysaccharides were in the range of 2.53–6.81 mg/mL. In addition, AVP showed scavenging ability on hydroxyl radicals, but was moderate effect in the antioxidant attribute. The EC_{50} value of scavenging ability of AVP on hydroxyl radicals was 4.0 mg/mL.

The present result suggested that the extracellular polysaccharide AVP isolated from coral-associated fungus *A. versicolor* LCJ-5-4 could be beneficial to help human body alleviate oxidative damages in cell induced by oxygen radicals, and effectively used as potential antioxidants. The antioxidant activity of AVP may be attributed to the polysaccharide can connect with radicals and terminate the radical chain reaction. The antioxidant mechanisms of polysaccharides are complex and required further investigation.

Table 2
^{1}H and ^{13}C chemical shifts for the extracellular polysaccharide AVP.

Residues[b]	Chemical shifts (ppm)[a]					
	H1/C1	H2/C2	H3/C3	H4/C4	H5/C5	H6/C6
A	5.28/100.7	4.15/78.2	3.99/70.2	3.69/66.9	3.84/73.2	3.92, 3.77/61.0
B	5.26/100.7	4.15/78.2	3.99/70.2	3.69/66.9	3.84/73.2	3.92, 3.77/61.0
C	5.16/98.3	4.06/78.7	3.72/70.5	3.84/nd[c]	3.64/nd[c]	3.96, 3.70/66.6
D	5.08/102.3	4.11/70.7	3.87/nd[c]	3.78/nd[c]	3.71/72.2	3.69, 3.62/60.6
E	5.01/97.7	3.62/71.4	3.74/73.4	3.56/69.5	3.94/70.2	4.02, 3.80/66.9

[a] The spectra were recorded using a JEOL JNM-ECP 600 MHz spectrometer. Chemical shifts are referenced to internal acetone at 2.225 ppm for ^{1}H and 31.07 ppm for ^{13}C.
[b] A–E correspond to (1 → 2)-α-D-Man*p*, (1 → 2)-α-D-Man*p*, (1 → 2, 6)-α-D-Man*p*, (1→)-α-D-Man*p*, and (1 → 6)-α-D-Glc*p*, respectively. Glc*p*: glucopyranose, Man*p*: mannopyranose.
[c] Not detected.

Table 3
Analysis of antioxidant activity for the extracellular polysaccharide AVP.

Antioxidant activity	Sample	Concentration (mg/mL)					
		0.25	0.5	1.0	2.0	4.0	8.0
Scavenging ability on DPPH radicals (%)	AVP	22.5 ± 3.2	29.0 ± 3.6	37.3 ± 2.8	48.0 ± 3.2	63.5 ± 1.4	77.7 ± 2.7
	Ascorbic acid	94.0 ± 2.2	95.4 ± 3.3	96.5 ± 2.6	96.6 ± 2.1	96.8 ± 1.2	96.8 ± 2.8
Scavenging ability on Superoxide radicals (%)	AVP	5.4 ± 1.2	16.9 ± 3.0	36.9 ± 2.2	67.5 ± 2.5	88.6 ± 2.7	90.8 ± 3.1
	Ascorbic acid	89.9 ± 2.1	93.6 ± 3.3	95.8 ± 3.1	96.2 ± 2.5	96.7 ± 2.2	96.6 ± 3.2
Scavenging ability on Hydroxyl radicals (%)	AVP	1.5 ± 1.4	7.1 ± 1.9	18.3 ± 2.6	32.8 ± 3.2	49.2 ± 3.5	65.6 ± 2.0
	Ascorbic acid	7.3 ± 1.2	19.9 ± 1.8	45.0 ± 2.0	69.3 ± 2.8	85.9 ± 3.0	93.8 ± 2.2

4. Conclusion

The water-soluble extracellular polysaccharide AVP with antioxidant activity was successfully obtained from the fermented broth of coral-associated fungus *A. versicolor* LCJ-5-4. Based on detailed 1D, 2D NMR spectroscopic analyses, the main chain of AVP was characterized to consist of (1 → 6)-α-D-glucopyranose and (1 → 2)-α-D-mannopyranose units. On average there was one branch point for every 8 sugar residues in the backbone, and the side chain consisted of (1→)-α-D-mannopyranose and (1 → 2)-α-D-mannopyranose which was linked to C-6 of the (1 → 2)-α-D-mannopyranose in the backbone, which makes it a novel mannoglucan distinguishing it from other extracellular polysaccharides. AVP possessed a good scavenging ability on superoxide radicals and could be a potential source of antioxidant. An in-depth antioxidant property study on extracellular polysaccharides with different structural characterization will play an important role in the understanding of the mechanism of antioxidant activity.

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