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Molecular properties and antioxidant activities of polysaccharides isolated from alkaline extract of wild *Armillaria ostoyae* mushrooms

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Abstract

This study aims to discover novel and bioactive polysaccharides (PS) from wild *Armillaria ostoyae*, a honey mushroom species. Two PS designated AkPS1V-1 (66.6 kDa) and AkPS1V-2 (15.3 kDa) were isolated and fractionated by anion ion exchange (IEC) and size exclusion chromatography (SEC) from the alkaline extract of *A. ostoyae* mushrooms. AkPS1V-1 was a glucan composed of solely glucose residues and AkPS1V-2 a heteropolysaccharide composed of glucose and galactose at 6:1 molar ratio. AkPS1V-2 exhibited higher antioxidant activities than AkPS1V-1 based on reducing power, radical scavenging and metal chelating assays. The structure of AkPS1V-2 was further analyzed and elucidated as a branched galactoglucan with a backbone composed of (1→6)-β-D-glucopyranosyl, (1→3)-β-D-glucopyranosyl, (1→3)-α-D-galactopyranosyl and (1→3,6)-β-D-glucopyranosyl residues at 3:1:1:1 ratio, and side chain of (1→3)-β-D-glucopyranosyl residue. This is the first report on a pure PS structure and its antioxidant activities from this mushroom species.

Keywords: *Armillaria ostoyae*; Polysaccharides; Extraction; Fractionation; Structure; Antioxidant activity.

1. Introduction

Edible fungi or mushrooms are nutritious, and healthy foods and many have notable medicinal properties and bioactivities. Polysaccharides (PS) represent a class of the most abundant biopolymers in edible fungi which can have a wide range of nutritional and medicinal functions such as antitumor, immunomodulatory, prebiotic and antioxidant activities (Stachowiak and Regula, 2012). Extraction is the first important step for acquirement of the PS from mushrooms. Most of the bioactive (non-starch) PS serve as the structural components of fungal cell wall. To make a tough cell wall, these PS form a rigid

and complex network. Both the tough cell wall and the rigid PS property create resistance to the extraction of PS from mushrooms. While hot water extraction (HWE) can retain the water-soluble PS, aqueous alkaline extraction is more effective to break the cell wall and to extract the PS from mushrooms (Latgé, 2007). Several of the well-known antitumor PS were extracted by alkaline water such as lentinan from *Lentinus edodes* (Chihara, Hamuro, Maeda, Arai & Fukuoka, 1970) and MD/D fraction from *Grifola frondosa* (Mayell, 2001).

The properties and bioactivities of PS depend on the structure characteristics such as monosaccharide composition, glycosidic bond, degree of branching, and molecular weight (MW). The most common structure form of antitumor and immunomoactive PS from mushrooms is β-(1→3) glucan with side chains, such as lentinan from *Lentinus edodes* and Schizophyllan from *Schizophyllum commune*, and some linked with protein/peptide as PSP complexes (Ooi & Liu, 2000; Zhang, Cui, Cheung & Wang, 2007). In addition to the well-known antitumor and immunomodulation, antioxidant activities have been widely evaluated in many recent studies on PS from various edible fungi (Kozarski et al., 2012; Lindequist, Niedermayer & Jülich, 2005 Vaz et al., 2011). However, most of the fungal PS tested for antioxidant activities were crude or partially purified PS fractions with complex or unknown chemical composition, and the actual activity of pure PS is uncertain.

Armillaria fungal species, generally called honey mushrooms for their yellow brown caps and sweet fragrance, belong to the family Tricholomataceae (Pegler, 2000; Muszynska, Sulkowska-Ziaja, Wolkowska, & Ekiert, 2011). This species grows symbiotically with *Gastrodia elata* (Tin Ma) plant which is a famous Chinese herbal medicine (Cha & Igarashi, 1995). Some *Armilliaria* species are edible, and consumed around the world, such as *A. mella* and *A. ostoyae* in Europe and China, *A. matsutake* S. in Japan and *A. ponderosa* in North America. *Armilliaria* mushrooms have significant nutritional and medicinal functions. Extract of *A. mellea* and *G. elata* has been formulated into herbal drug tablets called “Tian Ma Mi Huan Jun Pian” in China to treat various diseases such as headache, insomnia,

neurasthenia and infectious diseases (Gao, Li, Zhao & Wang, 2009; Tang & Eisenbrand, 1992). As the major bioactive constituents of *Armillaria*, PS have shown antitumor, immunodulating and antidiabetes effects (Kiho, Shiose, Nagai & Ukai, 1992; Lung & Chang, 2013; Vaz et al., 2011; Wu et al., 2012).

A. ostoyae is a popular edible mushroom in northeastern China and is widely distributed in the forests. However, there is no literature report on the molecular structure and antioxidant activity of PS from *A. ostoyae*. In this study, we applied a two-step extraction protocol, first by HWE to remove proteins and water soluble constituents, and next by alkaline extraction of wild *A. ostoyae* mushrooms collected from the Lesser Khingan Range forest in northeastern China. The crude PS isolated by ethanol precipitation were further purified and fractionated through a series of steps for further determination of molecular properties and antioxidant activities.

2. Materials and methods

2.1. Mushroom materials

Wild *A. ostoyae* mushrooms were collected from the Lesser Khingan Range forest in the Wuying region in China (Wuying Forestry Bureau) (altitude 320 m, coordinates 48°04' of Northern latitude and 129°15' of East longitude) in September 2009. The mushroom species was identified by comparing their morphological traits with reliable reference (Liu, 2004), and further confirmed by comparing the DNA sequence with the GenBank (National Center for Biotechnology Information website [http://www.ncbi.nlm.nih.gov]). The mushrooms were air dried, enclosed within plastic bags, and stored at 25^{o}C before use.

2.2. Extraction and isolation of PS

The mushrooms were dried completely in an oven at 50 ^{o}C to constant weight, and ground into powder with an electric mill. The mushroom power (100 g) was first defatted

with petroleum ether (PE) and then ethanol (EtOH) for 6 h each. The solid residue was then subject to hot water extraction (HWE) at 90^{o}C (~2 L distilled water) for the water soluble constituents. The residue collected after filtration was subject to alkaline extraction with 0.5 M NaOH and 0.05 M $NaBH_4$ in water (~2 L) at 4^{o}C for 6 h for three times. The alkaline extract was neutralized to pH 7 by acetic acid, and then concentrated by vacuum evaporation below 45^{o}C. The concentrated extract was subject to sequential ethanol precipitation in three steps, at 0.3:1, 1:1 and 5:1 EtOH (95%) volume ratio. The liquid mixture was kept at 4 ^{o}C for overnight in each precipitation step. The precipitate was collected by centrifugation and freeze-dried, yielding the crude PS, AkPS0.3V, AkPS1V and AkPS5V.

2.3. Purification of AkPS1V

The crude PS precipitated in the second step with 1:1 EtOH volume ratio, AkPS1V, was deproteinized by repeated treatment with Sevag regent (1:4 butanol to chloroform) until no precipitate formed, and then decolorized with 10% H_2O_2 at pH 8.5 and room temperature for 5 h. The AkPS1V solution was then dialyzed against distilled water through a 3500 Da MW cutoff membrane for 5 days and then lyophilized. The partially purified AkPS1V was fractionated by ion exchange chromatography (IEC) as follows. The AkPS1V was redissolved in 20 mM Tris-HCl (pH 7.4) and then loaded to 2.6 × 27 cm DEAE cellulose-52 IEC column (Sigma, St. Louis, MO, USA) which had been equilibrated with Tris-HCl buffer solution (20 mM, pH 7.4). The column was eluted sequentially with Tris-HCl (20 mM, pH 7.4) containing 0, 0.1, 0.3, 0.5 or 1 M NaCl at a flow rate of 1.5 mL/min. The eluate was collected with a fractional collector at 4 mL per tube. The carbohydrate content was monitored by anthrone test and the protein content monitored spectrophotometrically at 280 nm. The PS fraction eluted by the buffer from IEC was collected and further purified by size exclusion chromatography (SEC) on a 2.6 × 60 cm Superdex G-75 column (Sigma). The SEC column was loaded with 50 mg of PS sample and eluted with 0.3 M $(NH_3)_2CO_3$ at 0.3

mL/min. The selection of $(NH_3)_2CO_3$ as the mobile phase for SEC was according to literature (Siddiqui & Wood, 1971; Powell, Ahmed, Yates & Turnbull, 2010) and the specific conditions were set based on our preliminary trials. Two fractions abundant in sugar were collected, dialyzed, and freeze dried, yielding AkPS1V-1 and AkPS1V-2.

2.4. Analysis of AkPS1V-1 and AkPS1V-2

2.4.1. Intrinsic viscosity and molecular weight distribution

The intrinsic viscosity, [η], was determined by Huggins and Kraemer equations,

$$\eta_{sp}/c = [\eta] + k'[\eta]^2 c \quad (1)$$

$$(\ln \eta_r)/c = [\eta] - (1/2\text{-}k')[\eta]^2 c \quad (2)$$

where η_{sp}/c is reduced specific viscosity, $(\ln \eta_r)/c$ inherent viscosity, and k' is a constant depending on the molecular properties, solvent and experimental conditions. The viscosity of CPS solution in water was measured at 30°C with an Ubbelohde capillary viscometer (Huang, Siu, Wang, Cheung & Wu, 2013).

MW distribution of PS fractions was analyzed by high-pressure gel permeation chromatography (HPGPC) with two columns in series, Ultrahydrogel 250 and Ultrahydrogel 2000 (both 7.8 mm × 300 mm dimensions from Waters Co., Milford, MA, USA), with a Waters 1515 isocratic HPLC pump and a Waters 2414 refractive index detector, as described by Huang et al. (2013). Distilled water was used as the mobile phase at 0.6 mL/min and 50°C. PS samples were predissolved in distilled water at 5 mg/mL and centrifuged at 18,000 rpm for 25 min before injection. Dextran MW standards ranging from 1.0 to 670 kDa (Sigma) were used for calibration and the peak MW was computed with the Breeze V3.3 software.

2.4.2. FT-IR and NMR spectroscopy

Infrared (IR) spectrometry of AkPS1V-1 and AkPS1V-2 was performed in the 4000–500 cm^{-1} region on an Avatar 360 FTIR spectrometer (Thermo Nicolet, Cambridge, UK). Samples

were pressed into tablets with KBr and subjected to 64 scans at 4 cm^{-1} resolution in reference to air to obtain an average spectrum in the Spectrum 6.1 software.

NMR spectroscopy of AkPS1V-2 including ^{1}H NMR, ^{13}C NMR and HMBC was performed on a Bruker AVANCE III 600 spectrometer at 25^{o}C. The PS sample (5 mg) was lyophilized in deuterium twice and then dissolved in 500 μL 99.8% D_2O before analysis.

2.4.3. Monosaccharide composition

Monosaccharide composition of PS fractions was analyzed by HPLC after acid hydrolysis and 1-phenyl-3-methyl-5-pyrazolone (PMP) reaction as reported previously (Siu, Chen, & Wu, 2014). In brief, 5 mg of sample was hydrolyzed with 2 M trifluoroacetic acid (TFA) (2 mL) at 110^{o}C for 4 h. The hydrolysate was then dried under nitrogen in a hot water bath and re-dissolved in 2 mL water. The hydrolysate solution (450 μL) was mixed with 450 μL of 0.5 M 1-phenyl-3-methyl-5-pyrazolone (PMP) solution in methanol, and 450 μL of 0.3 M NaOH solution and then reacted at 70^{o}C for 30 min. The reaction was stopped by neutralizing with 450 μL of 0.3 M HCl, and the product was partitioned with chloroform three times. The aqueous layer was collected and filtered through a 0.45 μM membrane, and applied to HPLC. The HPLC was performed with an Agilent ZORBAX ECLIPSE XDB-C18 column (150 mm x 4.6 mm) on an Agilent 1100 instrument at 25^{o}C with potassium phosphate buffered saline (0.05 M, pH 6.9) containing 15% (solvent A) and 40% acetonitrile (solvent B) as mobile phases, and UV detection at 250 nm. Monosaccharide standards for the identification and quantification of the corresponding peaks were from Sigma.

2.4.4. Methylation analysis

AkPS1V-2 was subjected to methylation, acid hydrolysis, reduction and acetylation to give partially methylated alditol acetates (PMMAs). Methylation was performed according to documented method (Ciucanu and Kerek, 1984) with minor modifications. In brief, dry

AkPS1V-2 (5 mg) was predissolved in 2.5 mL anhydrous dimethyl sulfoxide (DMSO) with constant stirring for 3 h. Anhydrous sodium hydroxide (30 mg) was added to the AkPS1V-2 solution in nitrogen atmosphere with stirring for 30 min. Under nitrogen protection, methyl iodide (0.8 mL) was slowly added to the solution in an ice bath and stirred in dark at room temperature for 1 h. The reaction was stopped by adding 2.5 mL distilled water and the excess amount of methyl iodide was removed by vacuum evaporation at 40^{o}C. The partially methylated sample was extracted with dichloromethane. The dichloromethane solution was washed with deionized water three times to remove impurities. The partially methylated AkPS1V-2 was then evaporated to dryness under vacuum in a rotatory evaporator at room temperature. The methylation process was repeated three times for completion.

The partially methylated AkPS1V-2 was further hydrolyzed with 2 M TFA (1 mL) in a sealed tube at 110^{o}C for 6 h, and excess TFA was removed with a stream of nitrogen in a boiling water bath. The dried hydrolysate was redissolved in 1 mL ammonia saturated water and reduced with excess $NaBH_4$ at room temperature for 12 h. Excess $NaBH_4$ was reacted with acetic acid (till no bubbles appearing) and the borate acid formed was removed by co-distillation with methanol. The dried residue was acetylated with 1 mL acetic anhydride in a sealed tube at 110^{o}C for 2 h to form partially methylated alditol acetate (PMAA). The PMMA was evaporated to dryness under vacuum, and redissolved in chloroform, and washed three times with water. The products were analyzed with GC-MS using an Agilent 6890N GC and 5975 VL MSD through a fused silica capillary column (30 x 0.25mm ID, Agilent HP-5MS). The column temperature was fixed at 100^{o}C for 3 min, then increased to 250^{o}C at 3^{o}C/min and fixed at 250^{o}C for 10 min. The injector and the detector were fixed at 280^{o}C and 250^{o}C, respectively.

2.5. Antioxidant activity assays

The antioxidant activity of PS fractions was determined by three chemical assays,

Trolox equivalent antioxidant capacity (TEAC), ferric reducing ability of plasma (FRAP), and ferrous ion Fe^{2+} chelating as reported in detail previously (Siu et al., 2014). In all assays, the PS samples were predissolved in water into a series of dilution. For the TEAC assay in brief, $ABTS^{\bullet+}$ radicals were generated from 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) oxidation by potassium persulfate ($K_2S_2O_8$). The $ABTS^{\bullet+}$ solution (500 μl) was added into various concentrations of PS sample solution in water to attain an initial absorbance of 0.70 ± 0.02 at 734 nm. The TEAC activity of sample was expressed in μmol Trolox/g sample. For the FRAP assay, PS sample solution (120 μl) was mixed with 900 μl of freshly prepared FRAP reagent solution, and incubated at room temperature in dark for 2 h, followed by measurement of the absorbance at 593 nm using ferrous sulfate ($FeSO_4$) as a reference. FRAP activity was expressed in μmol $FeSO_4$/g sample. For the Fe^{2+} chelating assay, PS sample solution (1 ml) was mixed with 20 μl of 2 mM $FeCl_2$ for 1 min, followed by the addition of 40 μl of 5 mM ferrozine. The mixture was incubated at room temperature for 10 min, followed by measurement of absorbance at 562 nm. The chelating activity was expressed in μmol Trolox/g sample.

2.6. Statistical analysis

All experiments were performed in triplicates and the results were expressed as mean ± standard deviation (SD). Statistical significance of the antioxidant tests was determined by a one way analysis of variance (ANOVA) followed by Student's t-test. Difference was considered to be statistically significant at $p<0.05$.

3. Results and discussion

3.1. Yields and properties of crude PS from alkaline extract

Table 1 shows the yields, properties and antioxidant activities of the three crude PS fractions isolated from the alkaline mushroom extract by sequential EtOH precipitation with

0.3, 1 and 5 volume ratio of EtOH to the extract liquid. The highest yield was achieved at 0.3 EtOH volume ratio, and the yields were much lower in the later steps of precipitation, suggesting that the concentration of higher MW PS in the water extract was much higher. EtOH causes the precipitation of PS in an aqueous solution due to its high miscibility with water to compete for the water molecules bound to the PS surface. With increasing EtOH concentration in the PS solution, PS become unstable and form aggregate, and then precipitate. The crude PS with a higher MW is precipitated at a lower EtOH concentration. As predicted, the intrinsic viscosity (correlated to the average MW) of PS was highest at the lowest EtOH and vice versa. All crude PS fractions isolated from the alkaline extract were composed mainly of carbohydrate and protein plus a small portion of phenolic compounds. The crude PS fraction attained at 5 volume ratio of EtOH also had the highest protein and phenolic contents as well as the highest antioxidant activities.

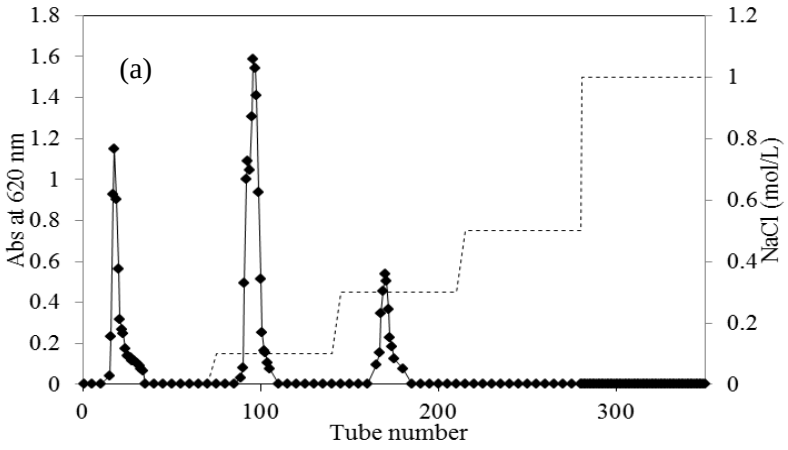
Table 1. Yields, chemical contents and antioxidant activities of crude PS fractions isolated from alkaline extracts of *A. ostoyae* mushrooms by differential ethanol precipitation with 0.3, 1 and 5 ethanol volume ratios to the extract liquid.

EtOH ratio	Yield (wt%)	Intrinsic viscosity (dL/g)	Total content (wt%)			Activity *	
			Sugar	Protein	Phenolics	TEAC	FRAP
0.3	3.30	0.25±0.01	45.8±2.3	34.7±1.9	1.37±0.1	39.9±3.9	14.4±1.3
1	1.36	0.20±0.01	53.3±2.6	10.7±0.1	1.51±0.1	38.1±2.1	26.7±1.9
5	0.35	0.03±0.00	29.1±1.4	43.5±0.7	2.86±0.3	115±8.7	53.8±2.7

*TEAC in μM Trolox/g, FRAP in μM Fe^{2+}/g, derived from the calibration curves generated by Trolox and ferrous sulphate, respectively.

3.2. MW profiles of purified PS fractions

Since AkPS0.3V was barely soluble in water and AkPS5V had a very low yield, AkPS1V, which also had the highest total sugar content, was collected for further fractionation (by IEC and SEC) and structural analysis. On the IEC elution profile of AkPS1V (Fig. 1a), the first peak eluted out with buffer solution was composed of protein-free PS, while the two later peaks eluted with 0.1 and 0.3 M NaCl in the buffer solution had absorbance at 280 nm for protein, and were probably PSPs (supplemental data Fig. 2). The first and second peaks were major fractions of AkPS1V accounting for 38% and 43% of the total mass, respectively. The protein-free PS fraction eluted out from SEC column exhibited two peaks (Fig. 1b), which were collected as AkPS1V-1 (tubes 23-29) and AkPS1V-2 (tubes 33-45), accounting for 37% and 63% of the total mass, respectively. AkPS1V-1 and AkPS1V-2 both exhibited a sharp, symmetric peak on HPGPC, indicating their MW homogeneity (supplemental data Fig. 3), and their average MWs were calibrated to 66.5 and 15.3 kDa, respectively. Acid hydrolysis of AkPS1V-1 resulted in glucose residues only while that of AkPS1V-2 contained glucose and galactose at 6:1 molar ratio according to HPLC analysis (supplemental data Fig. 4).



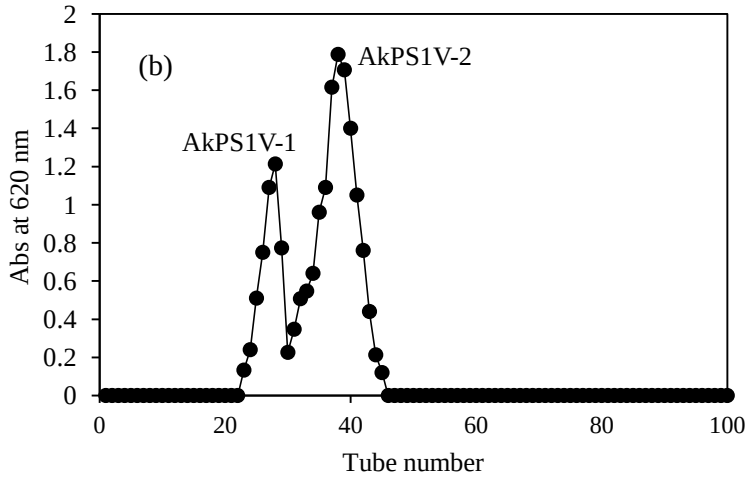
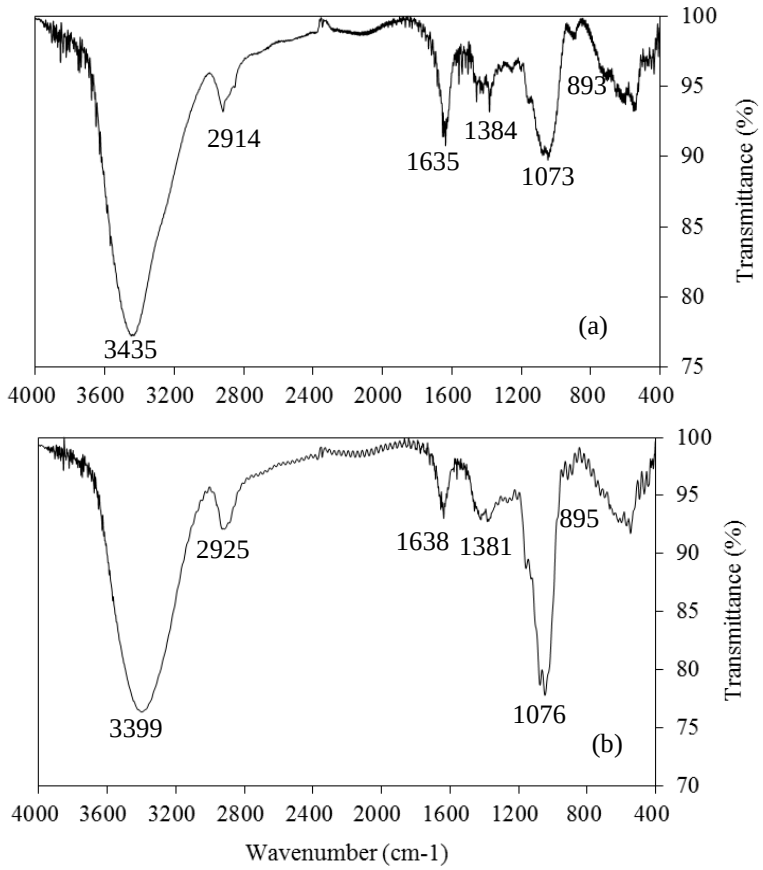


Fig. 1. Chromatographic profiles of AkPS1V fractions: (a) AkPS1V on a DEAE-cellulose 52 IEC column (eluted with different concentration of NaCl solution at 1.5 mL/min); (b) Neutral AkPS1V from (a) eluted out of the Superdex 75 SEC column with 0.3 M $(NH_3)_2CO_3$. Absorbance at 620 nm represents total carbohydrate content (relative) determined by anthrone test.

3.2. FT- IR spectral characteristics of AkPS1V-1 and AkPS1V-2

The FT-IR spectra of AkPS1V-1 (Fig. 2a) and AkPS1V-2 (Fig. 2b) were characteristic of PS structure. The broad intense peak at around 3400 cm^{-1} is attributed to the –OH group and the peak at around 1380 cm^{-1} to the –OH bending vibration. The peak at around 2920 cm^{-1} is assigned to the weak C-H stretching vibration and that at round 1635 cm^{-1} to the ring stretching of glucose. The peak at around 1075 cm^{-1} is attributed to the presence of pyranoside. The absence of absorption peak at around 1730 cm^{-1} confirms the absence of uronic acid.



278

279 **Fig. 2.** FT-IR spectra of (a) AkPS1V-1 and (b) AkPS1V-2.

280 Table 2 shows the fragments of AkPS1V-2 from methylation analysis including
281 2,3,4,6-Tetra-O-Me-Glcp, 2,4,6-Tri-O-Me-Glcp/Galp, 2,3,4-Tri-O-Me-Glcp and
282 2,4-Di-O-Me-Glcp in a relative molar ratio of 1:2:3:1. This set of results suggests that
283 AkPS1V-2 was composed of (1→3,6), (1→6) and (1→) glucopyranosyl and (1→3) linked
284 galactopyranosyl/glucopyranosyl residues.

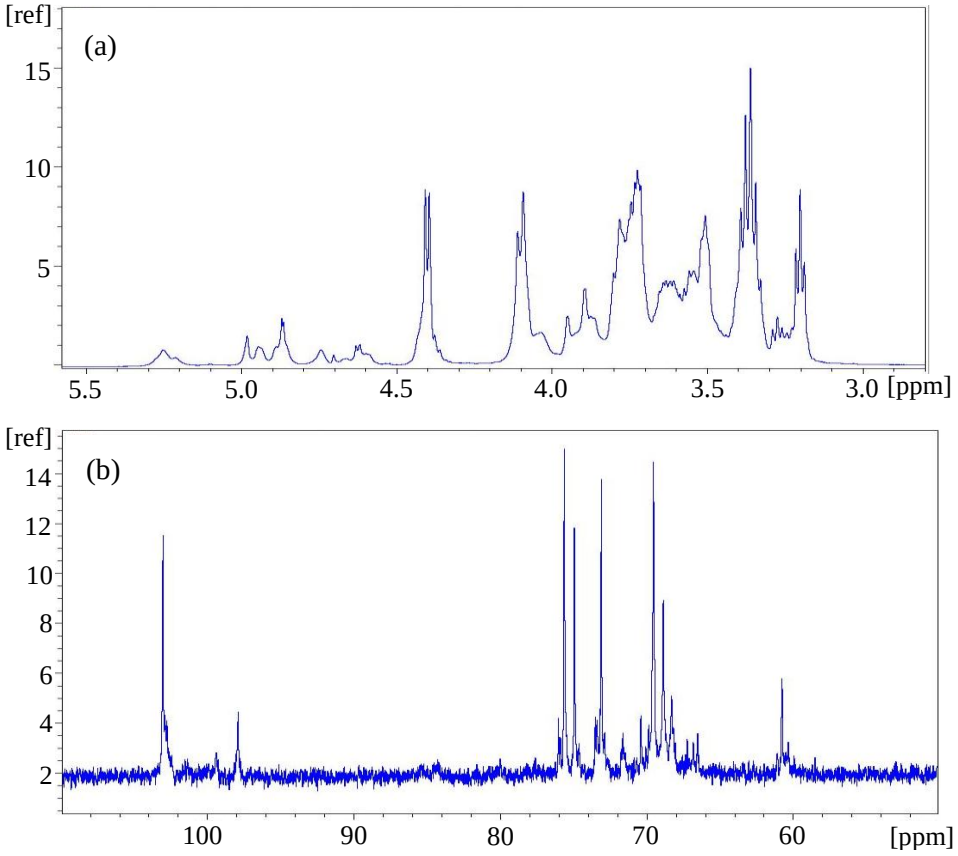
285 **Table 2.** GC-MS result of partially methylated alditol acetates of AkPS1V-2.

Methylated sugar	Mass Fragment (m/z)	Molar ratio	Linkage type
2,3,4,6-Tetra-O-Me-Glc*p*	59,71,87,101,117,129,145,161,205	1	1→
2,4,6-Tri-O-Me-Glc*p*/ 2,4,6-Tri-O-Me-Gal*p*	58,71,87,101,117,129,161,233	2	1→3
2,3,4-Tri-O-Me-Glc*p*	58,71,87,101,117,129,161,189,233	3	1→6
2,4-Di-O-Me-Glc*p*	58,74,87,101,117,129,139,159,173,189,233	1	1→3,6

The structural characteristics of AkPS1V-2 were further deduced from the NMR spectra (Fig. 3). On the ^{1}H NMR spectrum (Fig. 3a), the three overlapped peaks in the anomeric region (δ 4.41 ppm) are attributed to β-anomeric protons, and another peak at δ 4.9 ppm to α-anomeric proton. The chemical shifts from δ 3.2-4.1 ppm are assigned to protons of H-2 to H-6. The three β-configuration pyranose units and one α-configuration pyranose unit were further confirmed by the three anomeric carbon signals around δ 103 ppm on the ^{13}C NMR spectrum (Fig. 3b), which are ascribed to (1→3,6), (1→6) and (1→3)-D-glucopyranosyl, and one anomeric carbon signal in δ 98 ppm to (1→3)-D-galactopyranosyl. The relevant sugar residues were confirmed by labelling the C-1 to C-6 in ^{13}C NMR spectrum according to reference data (Table 3). The structural characteristics derived from NMR corroborate those from GC-MS.

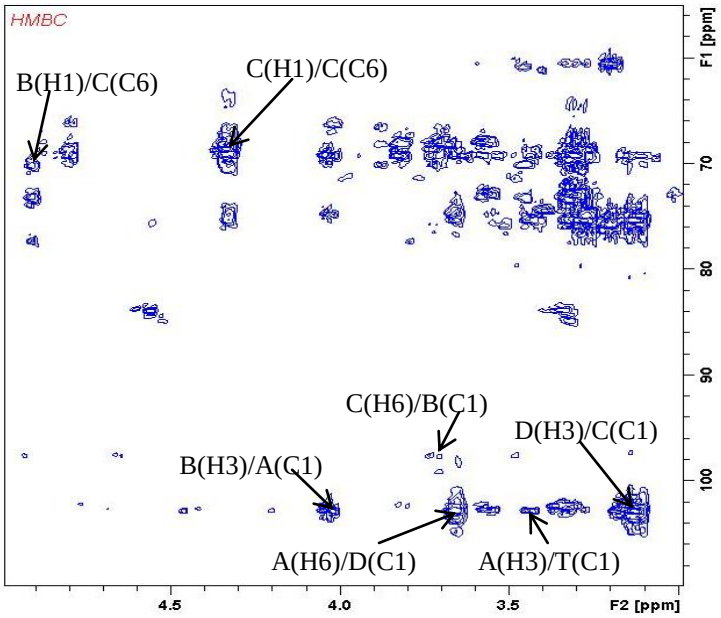
3.3. Linkage and structural characteristics of AkPS1V-2

Based on the ^{1}H-^{13}C HMBC spectrum (Fig. 4), the linkage sites and the sequence among residues were established (Table 3). A cross peak was observed between C-1 (δ 103 ppm) of residue A and H-3 (δ 3.66 ppm) of residue B [A(C1)/B(H3)], suggesting that C-1 of residue A was linked to H-3 of residue B. Similarly, the cross peaks at δ 97.9/3.66 ppm [B(C1)/C(H6)], δ 102.8/3.13 ppm [C(C6)/C(H1)], δ 103/3.42 ppm [C(C1)/D(H3)] and δ 102.9/4.02 ppm [D(C1)/A(H6)] suggest that the C-1 of residue B was linked to H-6 of residue C, C-1 of residue C to H-6 of another residue C, C-1 of residue C to H-1 of residue D, and C-1 of residue D to H-6 of residue A.



307

308 **Fig. 3.** (a) ^{1}H-NMR spectrum and (b) ^{13}C-NMR spectrum of AkPS1V-2.



318 **Fig. 4.** HMBC spectrum of AkPS1V-2.

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Table 3. Assignment of ^{1}H NMR and ^{13}C NMR chemical shifts of AkPS1V-2.

Sugar residue	Chemical shifts (ppm)					
	H1/C1	H2/C2	H3/C3	H4/C4	H5/C5	H6/C6
(A): →3,6)-β-D-Glc*p*(1→	4.41/	3.22/	3.42/	3.34/	3.51/	3.65, 4.09/
	102.8	73.4	84.2	69.7	74.6	70.1
(B): →3)-α-D-Gal*p*(1→	4.41/	3.94/	4.03/	4.1/	4.1/	3.6, 3.6/
	97.9	70.4	80.0	71.6	73.5	61.9
(C): →6)-β-D-Glc*p*(1→	4.41/	3.20/	3.38/	3.36/	3.50/	3.72, 4.09/ 68.9
	103.0	73.1	75.6	69.5	74.9	
(D): →3)-β-D-Glc*p*(1→	4.9/	3.25/	3.17/	3.21/	3.24/	3.33, 3.58/
	102.9	73.5	85.3	69.9	76.0	61.08

Based on all above analytical results, the following structural unit of AkPS1V-2 is established,

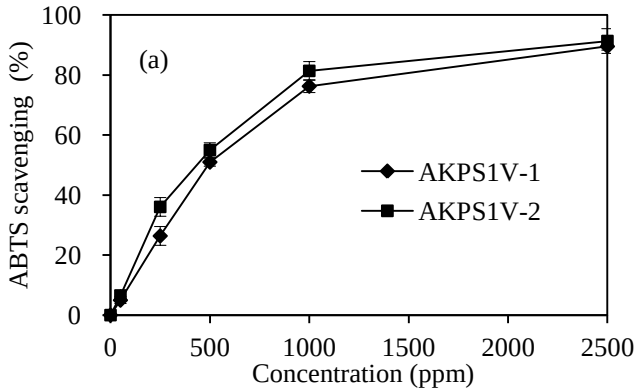
β-D-Glc*p*
1
↓
3

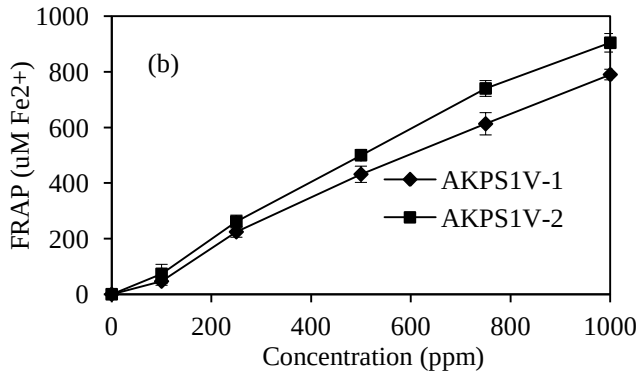
{6)-β-D-Glc*p*-(1→3)-α-D-Gal*p*-(1→[6)-β-D-Glc*p*-(1]$_3$→3)-β-D-Glc*p*-(1→}$_n$

Various polysaccharides with various molecular structures of have been isolated from *Armillaria* species, such as an α-(1→3) glucan isolated from *A. mellea* fruiting bodies (Sánchez , Garcia & Novaes-Ledieu, 1993) and an α-(1→6) glucan from *A. tabescens* mycelia (Luo, Xu, Yu, Yang & Zheng, 2008). Some glucans from *Armillaria* were in the form of PSP complexes, such as a glucan with β-(1→3) and β-(1→6) linkages and a glucan with α-(1→3) and α-(1→4) linkages from *A. mellea* fruiting bodies (Amar, Delaumèny, & Vilkas, 1976; Sánchez et al., 1993). In addition, various heteropolysaccharides such as xylomannan, heterogalactan have been isolated from *A. mellea* (Bouveng et al., 1967; Fraser & Lindberg, 1967; Sun, Liang, Zhang, Tong & Liu, 2009). Up to now, however, there is no literature report on a PS molecular structure from the *A. ostoyae* species.

3.4. Antioxidant activities of AkPS1V-1 and AkPS1V-2

Fig. 5 shows the antioxidant activities of AkPS1V-1 and AkPS1V-2 measured by (a) TEAC, (b) FRAP and (c) ferrous ion chelating assays, respectively, all in a dose-dependent manner. AkPS1V-2 showed a slightly higher activity than AkPS1V-1 in all three assays (Fig. 5 & supplemental data Table 1). As both PS had the similar chemical composition with glucose as the major constituent (>86%), the higher antioxidant activity of AkPS1V-2 (15.5 kDa) could be partially attributed to its lower MW than AkPS1V-1 (66.6 kDa). In comparison of the data in Table 1 and Supplemental data Table 1, the antioxidant activities of AkPS1V-1 and AkPS1V-2 were only significantly different from those of the crude PS, AkPS1V, from which these two were fractionated. This implies that the antioxidant activity of PS was not enhanced with the purification process and increase in the chemical purity of PS fractions.





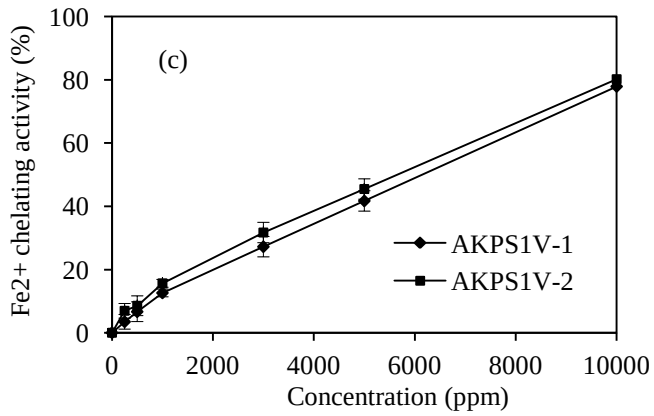


Fig. 5. Antioxidant activities of AkPS1V-1 and AkPS1V-2 measured by (a) TEAC, (b) FRAP, and (c) ferrous ion chelating assays (error bars for SD, n = 3).

Although rhamnose has been shown the most significant correlation with the antioxidant activity of PS among the monosaccharides (Lo, Chang, Chiu, Tsay & Jen, 2011), glucans as the major component of crude PS extracted from several medicinal mushrooms have also shown significant metal chelating and reducing power (Kozarski et al., 2012). Some purified glucans have also shown antioxidant activities such as the two glucans GLPL1 and GLPL2 from G. lucidum, both containing (1→3), (1→4) and (1→6) glucopyranosyl residues (Liu, Wang, Pang, Yao & Gao, 2010). The MW of PS is another factor affecting their bioactivities. In our present study, AkPS1V-2 with a lower MW (15.3kDa) showed higher radical scavenging, reducing and metal chelating abilities than AkPS1V-1 with a higher MW (66.6 kDa). The increasing antioxidant activity with MW reduction of PS has also been demonstrated with lower PS fractions derived from degradation of microbial PS with various methods such as acid hydrolysis (Yan et al., 2009), microwave radiation (Sun, Wang, Shi & Ma, 2009), and ultrasonication (Zhou, Yu, Zhang, He & Ma, 2012). Although it is quite uncertain about the active sites or functional groups responsible for the antioxidant activities of polysaccharides, the hydroxyl group has been regarded as the most possible group contributing to the antioxidant action by donating hydrogen atoms to stabilize the radicals and to terminate the radical chain reaction (Hu, Zhang, & Kitts, 2000). It has been suggested that, at an equal mass basis, PS with a lower MW may have more reductive hydroxyl terminals to react with the radical species and tend to have a higher antioxidant activity (Yan et al., 2009). However, we should not assume that MW and antioxidant activity follow a simple and proportional relationship. Although the MW difference between AkPS1V-2 and AkPS1V-1 was nearly four times, the differences in their antioxidant activities were very small (supplemental data Table 1). As stated early in this report, the bioactivities of PS can be

affected by several structure characteristics such as monosaccharide composition, glycosidic bond, degree of branching in addition to MW.

PS including intracellular PS and exopolysaccharides (EPS) from another *Armillaria* species, *A. mellea*, have also shown antioxidant activities such as radical scavenging, reducing power, metal chelating and protection against lipid peroxidation and DNA damage (Gao & Wang, 2012; Lung & Yu, 2013; Vaz et al., 2011). However, all these antioxidative PS were in crude form isolated from hot-water extract by ethanol precipitation without further purification. Such crude PS often contained significant amounts of proteins and low-MW pigments and are not able to show the actual activities of pure PS (Kozarski et al., 2012; Siu et al., 2014).

4. Conclusions

Two pure PS with homogenous MW distributions, AkPS1V-1 (66.6 kDa) and AkPS1V-2 (15.3 kDa), have been isolated from the alkaline extract of wild *A. ostoyae* mushrooms through a series of steps. AkPS1V-1 was composed of solely glucose residues and recognized as a glucan, and AkPS1V-2 was composed of glucose and galactose at 6:1 molar ratio. AkPS1V-2 exhibited a more significant antioxidant capacity due probably to its lower MW than AkPS1V-1. The molecule structure of AkPS1V-2 has been determined as a branched galactoglucan. This is a novel PS from this mushroom species. Further activity tests in animal models should be performed of the pure PS fractions for more reliable evaluation of their potential as natural antioxidants for human health.

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528 and analysis of structure and antioxidant activity of polysaccharide from *Porphyra*
529 *yezoensis* Udea. *Carbohydrate Polymers*, *87*(3), 2046-2051.

Table 1. Yields, chemical contents and antioxidant activities of crude PS fractions isolated from alkaline extracts of *A. ostoyae* mushrooms by differential ethanol precipitation with 0.3, 1 and 5 ethanol volume ratios to the extract liquid.

EtOH ratio	Yield (wt%)	Intrinsic viscosity (dL/g)	Total content (wt%)			Activity *	
			Sugar	Protein	Phenolics	TEAC	FRAP
0.3	3.30	0.25±0.01	45.8±2.3	34.7±1.9	1.37±0.1	39.9±3.9	14.4±1.3
1	1.36	0.20±0.01	53.3±2.6	10.7±0.1	1.51±0.1	38.1±2.1	26.7±1.9
5	0.35	0.03±0.00	29.1±1.4	43.5±0.7	2.86±0.3	115±8.7	53.8±2.7

*TEAC in μM Trolox/g, FRAP in μM Fe^{2+}/g, derived from the calibration curves generated by Trolox and ferrous sulphate, respectively.

Table 2. GC-MS result of partially methylated alditol acetates of AkPS1V-2.

Methylated sugar	Mass Fragment (m/z)	Molar ratio	Linkage type
2,3,4,6-Tetra-O-Me-Glc*p*	59,71,87,101,117,129,145,161,205	1	1→
2,4,6-Tri-O-Me-Glc*p*/ 2,4,6-Tri-O-Me-Gal*p*	58,71,87,101,117,129,161,233	2	1→3
2,3,4-Tri-O-Me-Glc*p*	58,71,87,101,117,129,161,189,233	3	1→6
2,4-Di-O-Me-Glc*p*	58,74,87,101,117,129,139,159,173,189,233	1	1→3,6

Table 3. Assignment of ^{1}H NMR and ^{13}C NMR chemical shifts of AkPS1V-2.

Sugar residue	Chemical shifts (ppm)					
	H1/C1	H2/C2	H3/C3	H4/C4	H5/C5	H6/C6
(A): →3,6)-β-D-Glc*p*(1→	4.41/	3.22/	3.42/	3.34/	3.51/	3.65, 4.09/
	102.8	73.4	84.2	69.7	74.6	70.1
(B): →3)-α-D-Gal*p*(1→	4.41/	3.94/	4.03/	4.1/	4.1/	3.6, 3.6/
	97.9	70.4	80.0	71.6	73.5	61.9
(C): →6)-β-D-Glc*p*(1→	4.41/	3.20/	3.38/	3.36/	3.50/	3.72, 4.09/ 68.9
	103.0	73.1	75.6	69.5	74.9	
(D): →3)-β-D-Glc*p*(1→	4.9/	3.25/	3.17/	3.21/	3.24/	3.33, 3.58/
	102.9	73.5	85.3	69.9	76.0	61.08

<Figure captions>

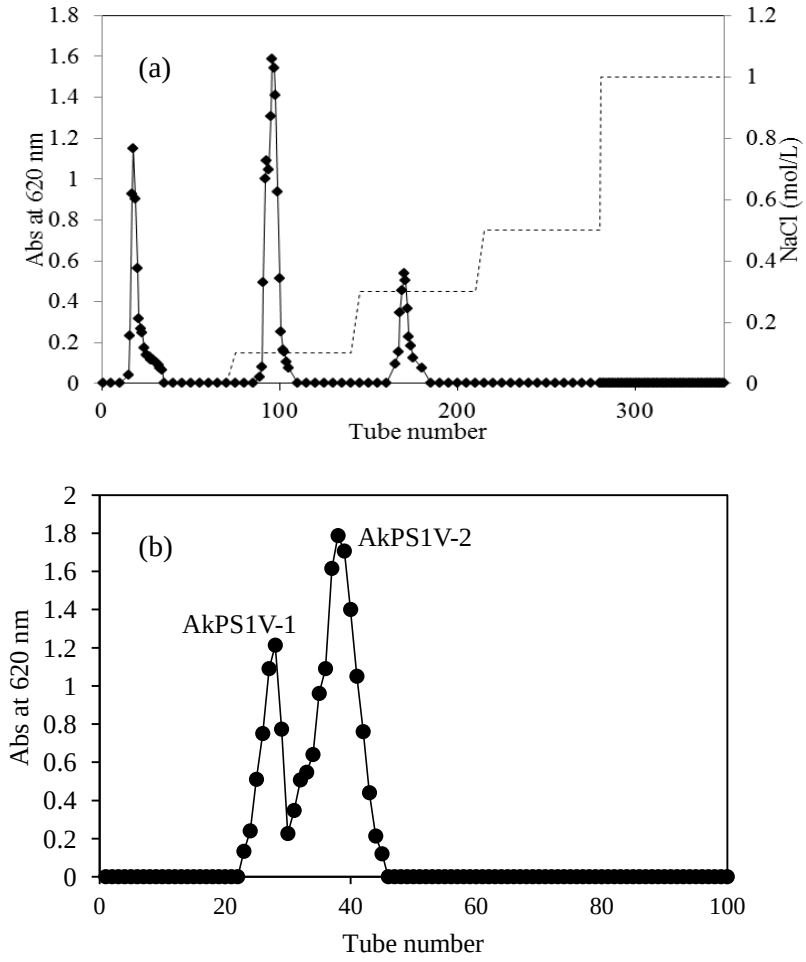
Fig. 1. Chromatographic profiles of AkPS1V fractions: (a) AkPS1V on a DEAE-cellulose 52 IEC column (eluted with different concentration of NaCl solution at 1.5 mL/min); (b) Neutral AkPS1V from (a) eluted out of the Superdex 75 SEC column with 0.3 M $(NH_3)_2CO_3$. Absorbance at 620 nm represents total carbohydrate content (relative) determined by anthrone test.

Fig. 2. FT-IR spectra of (a) AkPS1V-1 and (b) AkPS1V-2.

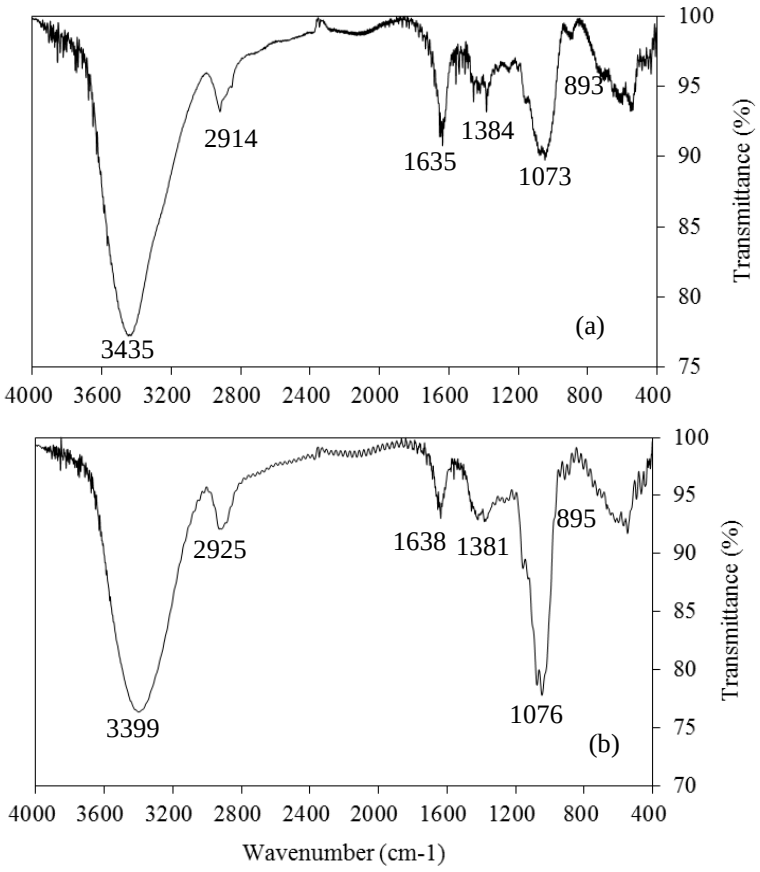
Fig. 3. (a) ^{1}H-NMR spectrum and (b) ^{13}C-NMR spectrum of AkPS1V-2.

Fig. 4. HMBC spectrum of AkPS1V-2.

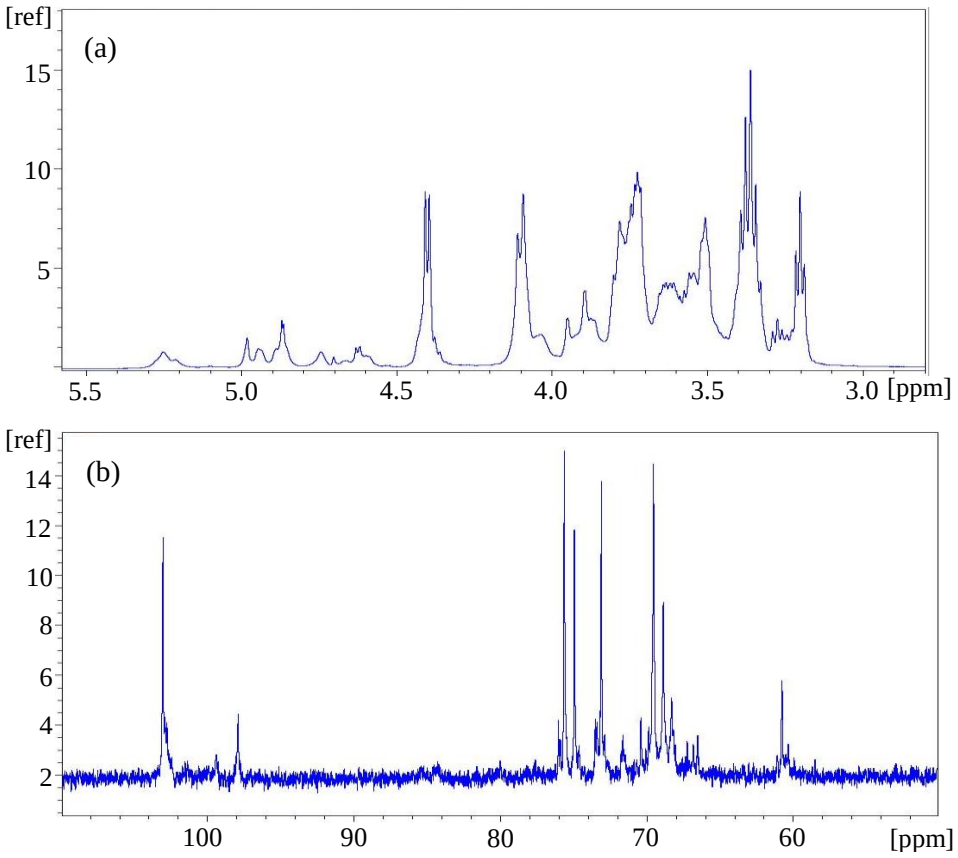
Fig. 5. Antioxidant activities of AkPS1V-1 and AkPS1V-2 measured by (a) TEAC, (b) FRAP, and (c) ferrous ion chelating assays (error bars for SD, $n = 3$).


(a)
Abs at 620 nm
NaCl (mol/L)
Tube number
(b)
AkPS1V-1
AkPS1V-2
Abs at 620 nm
Tube number

(Fig. 1.)


100
95
90
85
80
75
Transmittance (%)
3435
2914
1635
1384
1073
893
(a)
4000 3600 3200 2800 2400 2000 1600 1200 800 400
100
95
90
85
80
75
70
Transmittance (%)
3399
2925
1638
1381
1076
895
(b)
4000 3600 3200 2800 2400 2000 1600 1200 800 400
Wavenumber (cm-1)

(Fig. 2)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(a)
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10
5
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5.0
4.5
4.0
3.5
3.0
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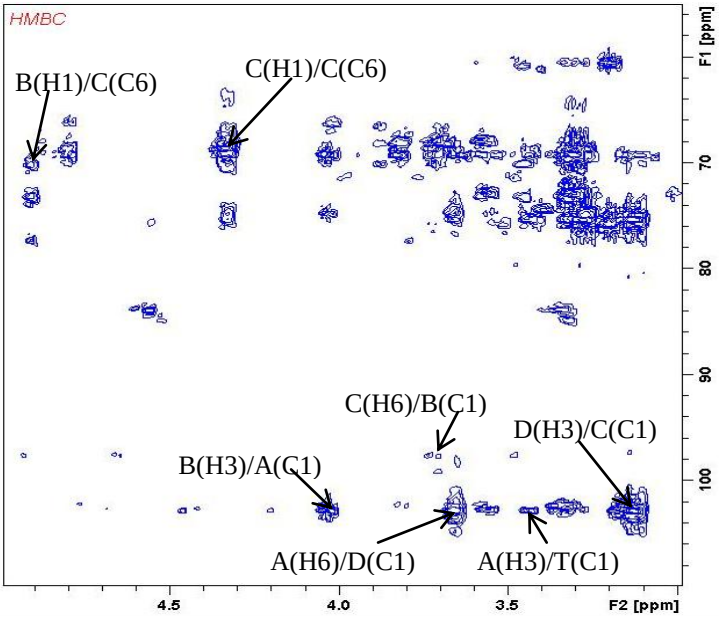
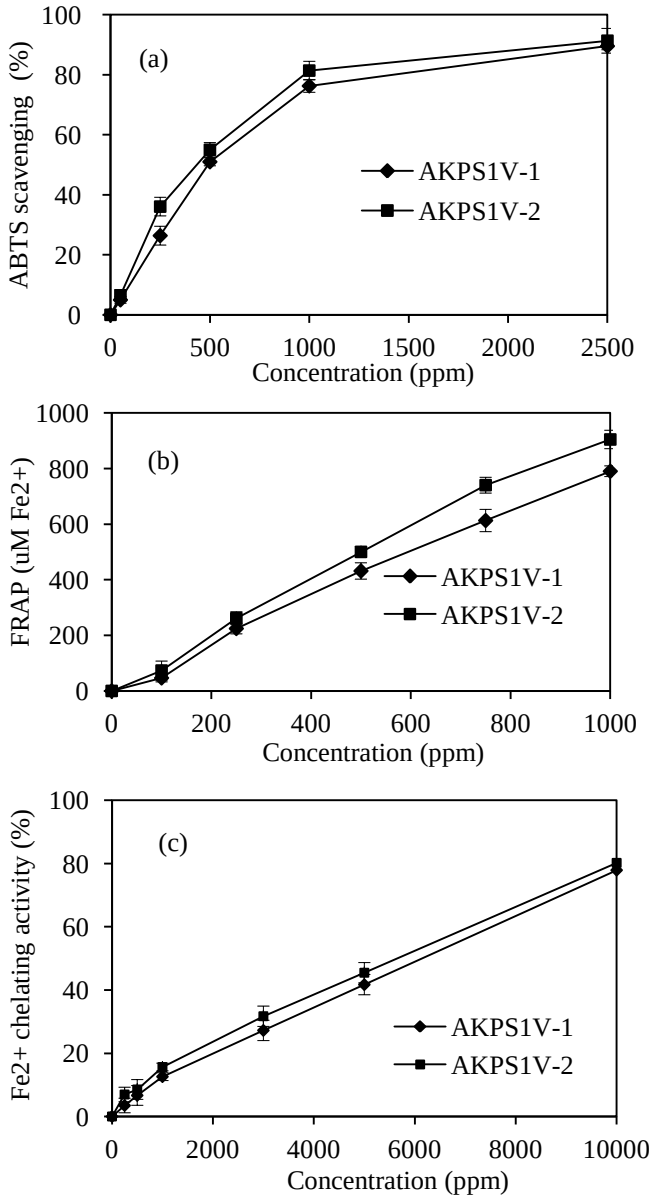
(b)
14
12
10
8
6
4
2
100
90
80
70
60
[ppm]

(Fig. 3)


HMBC
B(H1)/C(C6)
C(H1)/C(C6)
C(H6)/B(C1)
D(H3)/C(C1)
B(H3)/A(C1)
A(H6)/D(C1)
A(H3)/T(C1)
F1 [ppm]
70
80
90
100
4.5
4.0
3.5
F2 [ppm]

(Fig. 4)


(a)
ABTS scavenging (%)
100
80
60
40
20
0
0
500
1000
1500
2000
2500
Concentration (ppm)
AKPS1V-1
AKPS1V-2
(b)
FRAP (uM Fe2+)
1000
800
600
400
200
0
0
200
400
600
800
1000
Concentration (ppm)
AKPS1V-1
AKPS1V-2
(c)
Fe2+ chelating activity (%)
100
80
60
40
20
0
0
2000
4000
6000
8000
10000
Concentration (ppm)
AKPS1V-1
AKPS1V-2

(Fig. 5)