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Effect of extraction media on preliminary characterizations and antioxidant activities of *Phellinus linteus* polysaccharides

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ABSTRACT

Three partially purified polysaccharides were extracted from *Phellinus linteus* mycelia using hot water, 1% $(NH_4)_2C_2O_4$, and 1.25 M NaOH/0.05% $NaBH_4$, and the extracts were named PL-W, PL-A, and, PL-N respectively. PL-N mainly comprised xylose and arabinose with a high molecular weight (M_w) and the highest carbohydrate and uronic acid contents. PL-W and PL-A were mainly composed of glucose with high and low M_w fractions in various ratios. All three polysaccharides existed as compact coils in aqueous solutions and exhibited strong scavenging capacity and antioxidant activities in a concentration-dependent manner. The polysaccharides also had high uronic acid and carbohydrate contents and strong antioxidant activities. The M_ws, monosaccharide compositions, and chemical structures of the polysaccharides also affected their antioxidant activities. PL-A and PL-N had better antioxidant activities and could thus be developed as potential natural antioxidant agents for applications in food additives and biomedical industries.

1. Introduction

Oxidative stress, induced by free radicals, is believed to cause a variety of diseases, including cancer, heart disease, atherosclerosis, inflammation, carcinogenesis, Parkinson's and Alzheimer's diseases, and aging (Halliwell & Gutteridge, 1999). These pathological processes generally involve the oxidative alternation of physiologically critical molecules (e.g., proteins, lipids, carbohydrates, and nucleic acids), as well as the modulation of gene expression and inflammatory response (Kozarski et al., 2013). Humans have developed defense systems to promote antioxidation and repair oxidative damage. However, these systems are insufficient to completely deal with this oxidative damage. Meanwhile, the oxidative degradation of fats and oils in food is a common phenomenon that significantly affects the quality and safety of food because of the occurrence of secondary, potentially toxic compounds (Kozarski et al., 2013). Thus, developing and utilizing effective antioxidants for use in the pharmaceutical industry, food industry, and cosmetology to scavenge free radicals in the human body and prevent oxidative degradation processes is essential.

Exploring natural antioxidants, such as vitamins, proteins, glutathione, polyphenols and flavonoids, have received increasing interest because these substances protect the human body from oxidative damage, retard the progress of chronic diseases, preserve the color and taste of nutritious products, and avoid vitamin degradation (Nandita & Rajini, 2004). Polysaccharides represent a major class of bioactive molecules from edible and medicinal fungi which have notable antitumor, immunomodulatory, and antioxidant properties (Stachowiak & Reguła, 2012). Numerous studies have demonstrated that polysaccharides with antioxidant activities and radical-scavenging capacities are widely involved in mushrooms or fungi, including *Ganoderma lucidum*, *Lentinula edoeds*, *Grifola umbrellata*, *Schizophyllum commune*, and *Coriolus versicolor* (Kozarski et al., 2013; Wang et al., 2013). For example, Kozarski et al. (2011) have obtained partially purified polysaccharides from four medicinal mushroom species (*Agaricus bisporus*, *Agaricus brasiliensis*, *Phellinus linteus*, and *Ganoderma lucidum*), which exhibited relatively high antioxidant and immunomodulatory activities in vitro. Polysaccharides can thus be developed as new antioxidants or adaptogens to prevent oxidative damage in foods and in living organisms.

Phellinus linteus (*Berkeley* & *M. A. Curtis*) *Teng* (*P. linteus*) or Sanghuang is a species of mushrooms belonging to *Hymenochaetaceae Basidiomycetes* that is valuable in traditional Chinese medicine and is widely used in East Asia, especially

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Korea, China, and Japan (Hsieh, Wu, & Wu, 2013; Zhu, Kim, & Chen, 2008). Numerous pharmacological activities have been found in these species including antitumor, immunomodulatory, anti-inflammatory, anti-allergic, anti-angiogenic and antioxidant effects (Hsieh et al., 2013; Zhu et al., 2008). Polysaccharides represent a major class of bioactive constituents of *P. linteus* contributing to the health effects and pharmacological activities such as anticancer and antioxidation (Kozarski et al., 2011; Li et al., 2011). An increasing number of studies have been reported on the antioxidant activities of polysaccharides isolated from different *Phellinus* species, such as *P. linteus*, *P. nigricans*, and *P. baumii pilát* (Ge, Mao, Zhang, Wang, & Sun, 2013; Kozarski et al., 2011; Wang, Zhou, & Quan, 2014). In general, water-soluble polysaccharides were extracted by typical hot-water extraction and ethanol precipitation. However, the residue after this method was often discarded, leading to accumulation of biological wastes and loss of bioactive polysaccharides. Mizuno et al. have developed reliable procedures for effective extraction of polysaccharides from fruiting bodies or culture mycelia of medicinal mushrooms or fungi (Mizuno, 1999). To the best of our knowledge, little or no efforts have been made to effectively isolate bioactive polysaccharides from *P. linteus* by different extraction media.

In this study, three different extraction media, namely hot water, 1% ammonium oxalate [$(NH_4)_2C_2O_4$], and 1.25 M sodium hydroxide (NaOH)/sodium borohydride ($NaBH_4$) solutions were orderly used to extract water-soluble polysaccharides from *P. linteus* mycelia. The physiochemical properties, preliminary structures, and molecular characterizations of these polysaccharides were elucidated and compared. The antioxidant activities in vitro were also evaluated in order to better understand relationship between structure and functionality.

2. Materials and methods

2.1. Materials and chemicals

P. linteus mycelium was obtained from Jiangxi Kangdao Biology Co., Ltd., Nanchang, Jiangxi, China. The mycelium of *P. linteus* (Strain No. KCTC 6190) belonging to *Hymenochaetaceae* was obtained from KCTC (Korean Collection for Type Culture), Daejon, Korea. The mycelium powder was treated with refluxing petroleum ether twice for 6 h to remove lipids and pigments. The residue was air dried and sealed in airtight plastic bags before use. Congo red, hydrogen peroxide (H_2O_2), 2,4,6-tris(2-pyridyl)-s-triazine, 1,1-diphenyl-2-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2′-azinobis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). RPMI 1640 medium, fetal bovine serum, penicillin, and streptomycin (cell culture grade) were purchased from Gibco-BRL (Grand Island, NY, USA). All other chemicals and solvents were of laboratory grade and used without further purification.

2.2. Extraction and isolation of polysaccharides

P. linteus polysaccharides were successively extracted by hot water, 1% $(NH_4)_2C_2O_4$, and 1.25 M NaOH/0.05% $NaBH_4$ extraction media according to previous reports with minor modifications (Mizuno, 1999). The pretreated mycelium powder was extracted with distilled water at 95 °C twice for 8 h. After centrifuged, the residue was further extracted with 1% $(NH_4)_2C_2O_4$ solution (w/v) at 95 °C twice for 8 h. The residue obtained from 1% $(NH_4)_2C_2O_4$ extraction procedure was extracted with 1.25 M NaOH/0.05% $NaBH_4$ aqueous solution at room temperature twice for 3 h. Then the aqueous extracts were concentrated and precipitated with 4 volumes of 95% ethanol at 4 °C overnight, followed by centrifugation (5000 rpm, 10 min). Three precipitates were dissolved in distilled water, deproteinized using a Sevag reagent (Staub, 1956), dialyzed, and freeze-dried respectively to yield partially purified polysaccharides PL-W, PL-A and PL-N (Fig. 1).

2.3. Preliminary properties of polysaccharides

The total carbohydrate, uronic acid, and protein contents of polysaccharides were determined via the phenol–sulfuric acid method using glucose as a standard (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956), via sulfuric acid–carbazole method using glucuronic acid as a standard (Bitter & Muir, 1962), and via the Bradford method using bovine serum albumin (BSA) as a standard (Bradford, 1976), respectively. The polysaccharide yield (%) was calculated using the following formula: polysaccharide yield (%, w/w) = [weight of dried PLPS (g)/weight of raw materials (g)] × 100%. The intrinsic viscosity [η] of polysaccharides in water was determined at 25 ± 0.1 °C with an Ubbelohde capillary viscometer. The kinetic energy correlation was kept negligible.

2.4. Determination of molecular weight

The molecular weights (M_ws) of polysaccharides were determined by high pressure gel permeation chromatography (HPGPC) on an instrument comprising a Waters 1515 isocratic pump and a Waters 2414 refractive index detector with two ultrahydrogel columns 250 and 2000 (7.8 mm × 300 mm, Waters Corp., Milford, MA, USA) in series at 50 °C. Deionized water was used as the elution solvent flowing at 0.6 mL/min, and the samples were dissolved at this solvent at 2.0 mg/mL filtered through 0.45 μm membranes (Millipore, USA) prior to injection. The M_w at a given elution time was calculated from the calibration equation generated with dextran M_w standards ranging from 1 kDa to 1500 kDa (Sigma–Aldrich Chemical Co., St. Louis, MO, USA). The online Breeze software package (Waters Corp., Milford, MA, USA) was utilized for data collection and analysis.

2.5. Monosaccharide composition analysis

Five milligrams of the polysaccharide was hydrolyzed with 2 M H_2SO_4 at 100 °C for 8 h, followed by neutralization with $BaCO_3$ and centrifugation. The supernatant was evaporated to dryness. The dried residue was successively derivatized with 5 mg hydroxylamine hydrochloride and 0.5 mL pyridine at 90 °C for 0.5 h, followed by 0.5 mL Ac_2O at 90 °C for 0.5 h. The resulting nitrile acetates were analyzed by gas chromatography (GC) on an Agilent 5890 II instrument (HP-5 fused-silica capillary column, 30 m × 0.25 mm × 1 μm) with the conditions as reported before (Yan, Li, Wang, & Wu, 2010). D-Arabinose (D-Ara), D-glucose (D-Glu), D-galactose (D-Gal), D-mannose (D-Man), L-rhamnose (L-Rha), and D-xylose (D-Xyl) (all from Sigma–Aldrich Chemical Co., St. Louis, MO, USA) were used as monosaccharide standards.

2.6. FT-IR spectroscopy

Fourier-transform infrared (FT-IR) spectra of the polysaccharides were determined using a Nexus 670 FT-IR spectrometer (Thermo Nicolet Co., USA) in the wavenumber range of 500–4000 cm^{-1} with KBr pellets and referenced against air.

2.7. Dynamic light scattering (DLS)

Dynamic light scattering (DLS) using a Malvern Zetasizer Nano (3000 SHA, Malvern Instruments Ltd., UK) at 632.8 nm and 90°

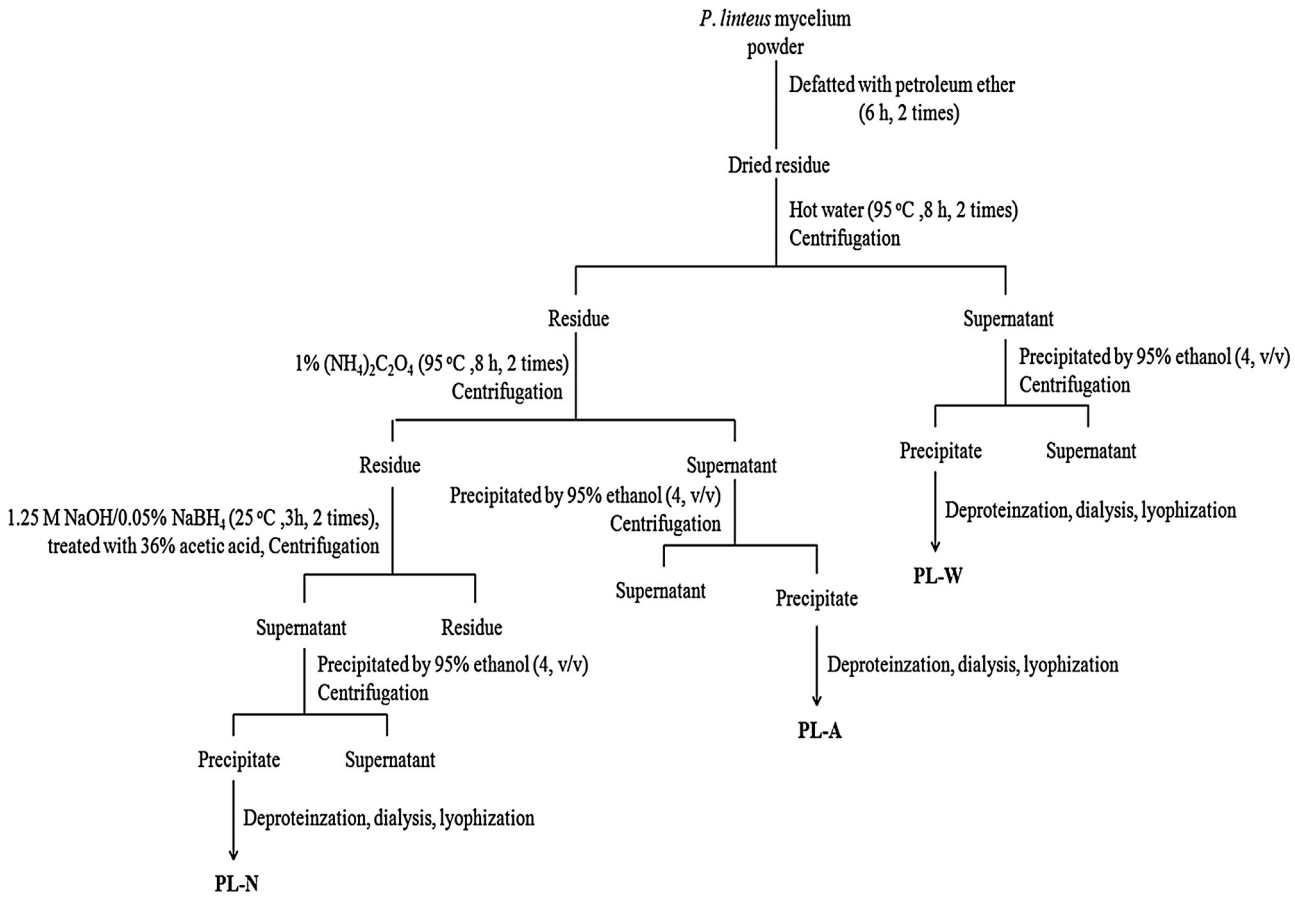

Fig. 1. Scheme for extraction and isolation of polysaccharides from the *P. linteus* myclia by different extraction media.

scattering angle was used to analyze the hydrodynamic radius (R_h) of polysaccharides in deionized water. The samples were dissolved with this solvent at a concentration of 2 mg/mL, and five runs were performed for each procedure. Data were processed with the MAS OPTION software.

2.8. Congo red test

Congo red test was performed to detect the chain conformation of polysaccharides in aqueous solution, in which a red shift in the light absorption maximum λ_{max} is attributed to the triple helices of polysaccharide chains (Yan, Wang, Li, & Wu, 2011). Sample aqueous solution at 1 mg/mL containing 91 μM Congo red was treated with 4 M NaOH at various concentrations ranging from 0 to 0.5 M. Visible light absorption spectrum was obtained over a 400–700 nm range at room temperature with a spectrometer and deionized water as the blank control.

2.9. Antioxidant activity assays in vitro

The antioxidant activities in vitro of polysaccharides were evaluated by calculation of the DPPH radical scavenging activity, Trolox equivalent antioxidant capacity (TEAC) assay, ferric reducing ability of plasma (FRAP) assay, and cytoprotection test in cell culture.

2.9.1. DPPH radical scavenging activity

DPPH radical scavenging activity was calculated according to the modified method of Konwarh, Pramanik, Kalita, Mahanta, and Karak (2012). The tested samples were previously dissolved in distilled water at various concentrations ranging from 0.05 mg/mL to 2 mg/mL. Three milliliters of the solution was added into 1 mL 0.1 mM DPPH solution in methanol. The reaction mixture was stirred and was incubated at room temperature (25 °C) for 30 min in the dark. The absorbance of the resulting solution was measured at 517 nm using a UV-1600 Spectrophotometer (Beijing Ruili Analytical Instrument Co., Ltd., China). The DPPH radical scavenging activity was calculated according to the following equation: scavenging activity (%) = $[1 - (A_1 - A_2)/A_0] \times 100\%$, where A_0, A_1, and A_2 are the respective absorbance values of the blank control (deionized water), the sample with DPPH solution, and the sample with deionized water. Vitamin C (V_c) was used as a positive antioxidant reference.

2.9.2. TEAC assay

The TEAC assay was performed to measure the eliminating or scavenging abilities of $ABTS^{\bullet+}$ free radical with Trolox as an antioxidant reference (Re et al., 1999). This compound is generated by reacting ABTS with potassium persulfate at room temperature in the dark. The polysaccharide solution (0.1 mL) was mixed with 3.9 mL diluted $ABTS^{\bullet+}$ solution at room temperature for 20 min. The absorbance of mixture was measured at 734 nm, and the $ABTS^{\bullet+}$ scavenging activity (in %) was given by $(1 - A/A_0) \times 100\%$, where A and A_0 represent the absorbance values of $ABTS^{\bullet+}$ solution with and without the test samples. The TEAC values were derived from the calibration curve obtained by Trolox in the 0–30 μM concentration range.

2.9.3. FRAP assay

FRAP assay measures the reducing capacity of a compound to transform ferric tripyridyltriazine complex into ferrous tripyridyltriazine at low pH (Benzie & Strain, 1996). The FRAP reagent was freshly prepared by mixing 3 M acetate buffer (pH 3.6), 0.1 M

2,4,6-tris (2-pyridyl)-s-triazine in 0.4 M HCl, and 0.2 M ferric chloride hexahydrate $FeCl_3{\cdot}6(H_2O)$ at 10:1:1 volume ratio. The FRAP reagent was warmed up to 37 °C, and each sample (905 μL) was added to 95 μL of this reagent. The mixture was incubated at room temperature for 15 min, and the absorbance was measured at 593 nm and was converted to a FRAP value (μmol Fe^{2+}/g sample) by calibration with ferrous sulfate with concentrations ranging from 0 to 30 μM.

2.9.4. Cell culture test

The cytoprotective activities of polysaccharides against H_2O_2-induced cell damages were tested in human neuroblastoma SH-SY5Y cell (ATCC) culture using the same procedure (Li et al., 2003). The polysaccharide samples were previously dissolved in phosphate buffered saline (PBS) at 10 mg/mL, and the 100 M H_2O_2 solution for the test was freshly prepared in PBS. The SH-SY5Y cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum in a CO_2 incubator at 37 °C. The cells were seeded into a 96 well-plate at 5×10^4 cells and were pretreated with polysaccharides at concentrations ranging from 10 μg/mL to 80 μg/mL for 24 h. Eighty micromolar of H_2O_2 (final concentration) was added to the wells for 24 h, with the control cells also incubated under the same condition. The cell viability was measured via MTT method and was expressed as a percentage of the value in control.

2.10. Statistical analysis

All treatments and assays were performed in triplicates and the data were represented by their mean ± standard deviation (SD). Statistical significance of differences between groups was analyzed by a one-way analysis of variance (ANOVA). Tests of significant differences were determined by Duncan's multiple range tests at $p = 0.05$ or independent sample T-test ($p = 0.05$). Results were processed by Origin 8.0 (OriginLab Corporation) and Prism 5.0 (GraphPad Software, Inc.).

3. Results and discussion

3.1. Extraction yields and chemical compositions

In this study, three water-soluble polysaccharides, namely, PL-W, PL-A, and PL-N, were orderly obtained from the culture mycelia of *P. linteus* by hot water, 1% $(NH_4)_2C_2O_4$, and 1.25 M NaOH/0.05% $NaBH_4$ extraction methods, as well as ethanol precipitation, deproteinization, dialysis, and lyophilization. Table 1 summarizes the yields, the chemical compositions, and the properties of these polysaccharides. The yields of crude polysaccharide obtained by hot-water extraction and 1% $(NH_4)_2C_2O_4$ were markedly higher than that obtained via 1.25 M NaOH/0.05% $NaBH_4$, suggesting that around 80% water-soluble polysaccharides were extracted from *P. linteus* mycelia by hot-water and acid extraction methods. In addition, the hot-water extraction method merely obtained about 43% water-soluble polysaccharides, and other active polysaccharides can be further extracted using other extraction media or assistive methods.

Table 1
Preliminary properties and compositions of polysaccharides extracted from *P. linteus* mycelia.

Sample	PL-W	PL-A	PL-N
Yield (%)	19.49	17.01	9.12
Carbohydrate (wt%)	64.46 ± 1.35	78.00 ± 1.32	84.92 ± 0.51
Protein (wt%)	1.53 ± 0.12	0.17 ± 0.06	0
Uronic acid (wt%)	11.12 ± 0.35	14.65 ± 1.51	16.92 ± 0.40
Water solubility[a]	Soluble	Soluble	Soluble
Sugar composition (molar ratios) (mol%)			
D-Glucose	8.0	8.0	1.8
D-Mannose	1.0	1.0	0
D-Galactose	0	0	1.0
D-Xylose	0	1.0	7.8
D-Arabinose	0	1.0	5.5
L-Rhamnose	0	0	0

[a] Evaluated at room temperature after dissolution in water under agitation at 10 g/L.

Analysis of the chemical compositions of these polysaccharides shows that the total carbohydrate content gradually increases from 64.5% to 84.9%, whereas the total protein content markedly decreases from 1.53% to 0%. In acidic and alkaline solutions, the cell walls of mycelia and mushrooms were more easily destroyed than that in hot water solution, which lead to more water-soluble polysaccharides extracted from these parts. The same result was observed by Klaus et al. (2011), who have extracted water-soluble polysaccharides from the *Schizophyllum commune* by hot alkali solutions with high carbohydrate contents. The protein content of PL-W remains at 1.53%, indicating that polysaccharide–protein complexes still reside in PL-W. Similarly, the uronic acid content evaluated in these polysaccharides gradually increased from 11.12% to 16.92%, which corresponded to the carbohydrate content. The uronic acid contents of polysaccharides are directly related to the scavenging ability and antioxidant activities (Ma, Chen, Zhu, & Wang, 2013). Higher uronic acid and carbohydrate contents result in stronger antioxidant activities of PL-A and PL-N.

3.2. Preliminary structural features

The monosaccharide compositions of PL-W, PL-A, and PL-N were analyzed by GC and Table 1 shows the results. Based on the monosaccharide standards, PL-W mainly comprised D-Glu and D-Man with a molar ratio of 8:1, whereas PL-A comprised D-Glu, D-Man, D-Xyl, and D-Ara with a molar ratio of 8:1:1:1, and PL-N comprised D-Ara, D-Xyl, D-Glu, and D-Gal with a molar ratio of 5.5:7.8:1.8:1. All polysaccharides isolated from *P. linteus* mycelia are identified as heteropolysacchairdes, which is consistent with the previous studies on *Phellinus* polysaccharides (Kozarski et al., 2011; Wang et al., 2014). In PL-W and PL-A, D-Glu is the dominant monosaccharide, whereas D-Xyl and D-Ara existed in PL-A but undetected in PL-W because of the difference of extraction medium. By contrast, PL-N with minor proportions of D-Glu (~11.2%) has much higher D-Ara and D-Xyl contents than that of PL-A. Notably, D-Gal was found only in PL-N.

Fig. 2 shows the FT-IR spectra of PL-W, PL-A, and PL-N between 500 and 4000 cm^{-1}. The profiles of PL-W, PL-A, and

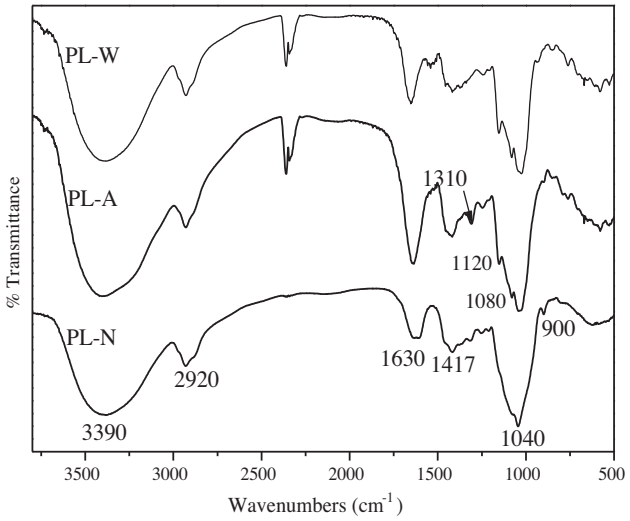

Fig. 2. FT-IR spectra of PL-W, PL-A and PL-N.

PL-N were similar, indicating that the polysaccharides extracted from *P. linteus* mycelia by different extraction media possessed similar structural characterizations. The bands around 3390 and 2920 cm^{-1} were attributed to the OH stretching vibration in hydrogen bonds and C—H stretching vibration, respectively. The two peaks corresponded to the characteristic absorption values of these polysaccharides. Two peaks at about 1630 and 1417 cm^{-1} were attributed to the absorption of the COO^- deprotonated carboxylic group (Manrique & Lajolo, 2002), indicating that the presence of uronic acid in these polysaccharides. The absorption bands at 1200–1000 cm^{-1} for each polysaccharide occurred from the stretching vibrations of C—OH side groups and C—O—C glycosidic band vibration. Three peaks located at about 1120, 1080, and 1040 cm^{-1} corresponded to C—O and C—C bands in the spectra of PL-W and PL-A, whereas only one peak at 1040 cm^{-1} was observed in the PL-N spectrum. The absorption peak at 1080 cm^{-1} indicated the presence of a β-glucosidic linkage. In addition, a characteristic absorption peak at 900 cm^{-1} was observed in the PL-N spectrum that indicated the β-configuration of the sugar units (Wang et al., 2014).

3.3. Molecular properties and chain conformation

Natural polysaccharides isolated from medicinal mushrooms or fungi are highly dispersed and have different molar masses ranging from ~10^3 Da to ~10^8 Da. The chemical compositions, M_ws, structures, and chain conformations of bioactive polysaccharides in aqueous solutions have significant roles in understanding the relationship between their structures and functions. However, the molecular properties and the chain conformations of water-soluble polysaccharides isolated from *P. linteus* and other *Phellinus* species are yet to be elucidated. In this study, the M_ws, size parameters, intrinsic viscosities, and chain conformations of these polysaccharides were demonstrated and compared.

Gel permeation chromatography (GPC) was employed to determine the M_ws of these polysaccharides and Fig. 3 shows the chromatographic profiles. For each polysaccharide, two Gaussian peaks were observed in the GPC chromatographs irrespective of the extraction medium. PL-W obtained by hot water extraction showed two main peaks with the weight average molecular weights (M_ws) of 320,000 kDa and 49.2 kDa, respectively, and the high fraction of ~63% was the dominant according to the peak area (Table 2). Two distinct groups exist for PL-A obtained by acid extraction, with M_ws of 975,000 kDa and 13.9 kDa. However, the low M_w fraction of ~60% was the main composition (Table 2). The polysaccharides in acidic solutions can be easily caused by degradation and transferred the high-M_w fraction into the low-M_w fraction, resulting to more amounts of low M_w fractions in PL-A compared with that in PL-W. This may be due to the destruction of hydrogen bonding interactions in acidic condition. The same phenomenon was also observed in our previous studies (Yan et al., 2009). However, PL-N obtained by alkali extraction exhibited a nearly single peak (~97%) at an M_w of 311,000 kDa from GPC (Table 2), which suggested that PL-N almost comprised high M_w compounds with high homogeneities. The slightly lower M_w of high M_w fractions in PL-N compared with that in PL-W was attributed to the alkaline degradation of the polysaccharide during extraction (Yan et al., 2011). In addition, the polydispersity indices M_w/M_n of the three polysaccharides lie between 1.6 and 8.9, and that of PL-A and PL-N were much lower than that of PL-W. The acid and alkali extraction methods have obtained narrower or more uniform M_w polysaccharide distributions. The utilization of different extraction media for isolating polysaccharides has led to a wide range of M_ws and size distributions from GCP results.

In dilute solutions, [η] is an important parameter that represents the hydrodynamic volume occupied by individual macromolecules and depends mainly on the dimension of the macromolecule chains. Table 2 lists the values of [η] for PL-W, PL-A, and PL-N. PL-N contained the highest carbohydrate content and higher M_w. This polysaccharide has also exhibited the highest value of [η] (127.5 ± 1.8 mL/g). Small [η] is usually associated with a relative compact coil, and large [η] with an extended rigid chain (Ma, Wang, & Zhang, 2008). The values of [η] for PL-W, PL-A, and PL-N were much lower than that of linear or stiff polymers having the same M_w, which suggested that these polysaccharides exhibited as compact coils in aqueous solutions. Table 2 shows that the R_h for PL-W (~32 nm), PL-A (~28 nm), and PL-N (~48 nm) measured by DLS in

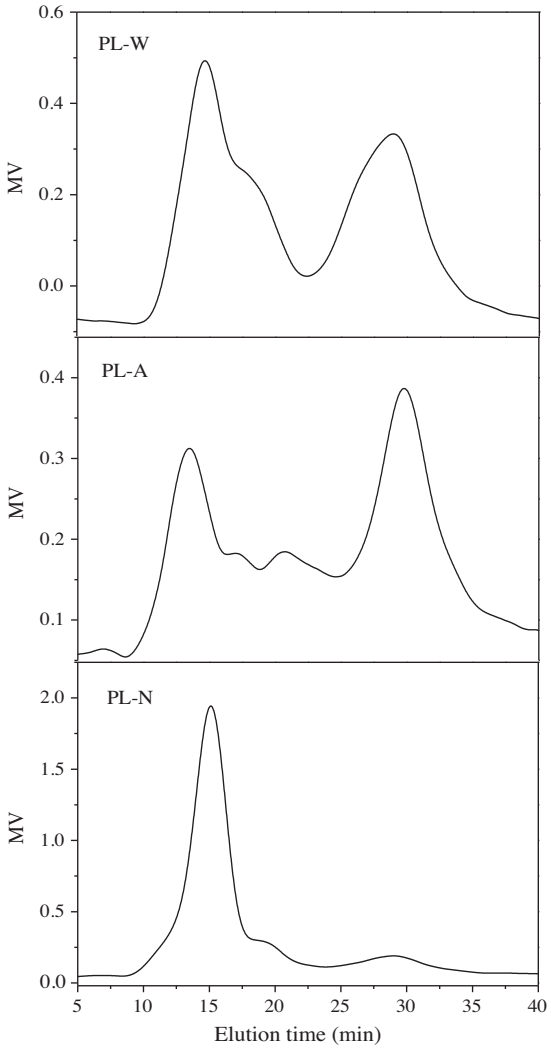

Fig. 3. Gel permeation chromatograms of PL-W, PL-A and PL-N.

Table 2
Molecular characterizations of polysaccharides extracted from *P. linteus* mycelia.

Sample	M_w (kDa)[a]	M_n (kDa)[b]	M_w/M_n [c]	%/area	R_h (nm)	[η] (mL/g)
PL-W	320,000	35,900	8.9	62.6	32.2	36.44 ± 1.12
	49.2	9.3	5.3	37.4		
PL-A	975,000	517,000	1.9	39.7	28.2	36.15 ± 1.86
	13.9	4.9	2.8	60.3		
PL-N	311,000	160,000	1.9	97.0	47.8	127.47 ± 1.78
	15.8	9.8	1.6	3.0		

[a] Weight average molecular weight.
[b] Number average molecular weight.
[c] Polydispersity index.

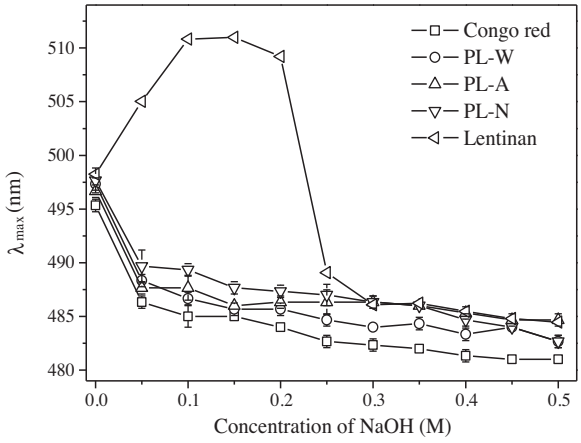

Fig. 4. Changes in absorption maximum (λ_{max}) of Congo red, Congo red + PL-W, Congo red + PL-A, Congo red + PL-N, and Congo red + lentinan at various NaOH concentrations. Each value is expressed as mean ± SD ($n = 3$).

water showed a similar trend with the values of M_w and [η], which implied their compact coil structures.

Fig. 4 shows the changes in maximum absorbance (λ_{max}) of Congo red–polysaccharide complexes with alkaline concentrations ranging from 0 to 0.5 M. Lentinan adopts a triple helix conformation at low alkaline concentrations, thereby exhibiting a large red shift in λ_{max} compared with the Congo red solution. As the alkaline concentration increases, lentinan undergoes a helix–coil transition and λ_{max} approaches that of the Congo red solution. For the three polysaccharides, the Congo red–polysaccharide complexes resulted in a blue shift in λ_{max} (~15 nm) from 497 nm to 482 nm in a NaOH concentration range of 0–0.5 M. This shift was different from that of lentinan, indicated that the three polysaccharides had random coil conformations in aqueous solution. This difference is attributed to the larger molecular weights and monosaccharide compositions of these polysaccharides. Mao, Hsu, and Hwang (2007) have pointed out the inability of a heteropolysaccharide to form a triple-helix structure. All three polysaccharides were heteropolysaccharides based on the monosaccharide compositions, thereby as compact random coils. This result was also consistent with the finding about the conformation analysis by [η] in dilute solution.

3.4. In vitro antioxidant activity

3.4.1. DPPH radical scavenging activity

As a stable free radical, DPPH has been widely employed to evaluate the free radical-scavenging capacity of natural compounds by donating hydrogen to form a stable DPPH molecule (Konwarh et al., 2012). Fig. 5(a) shows the scavenging activity of PL-W, PL-A, and PL-N on the DPPH radical compared with V_c. Among all the samples, PL-N possessed higher DPPH radical-scavenging capacity compared with PL-W and PL-A in a concentration-dependent manner. At the concentration of 2.0 mg/mL, the scavenging effects of PL-W, PL-A, and PL-N on DPPH radical were 58.6%, 65.5%, and 72.1%, respectively, but lower than V_c (94.1%, 0.1 mg/mL). PL-A and PL-N obtained by acid and alkali extraction methods have exhibited marked DPPH radical-scavenging capacities, and were stronger than that of the previous studies on polysaccharides isolated from *Phenillus* species by hot water extraction (Ge et al., 2013; Kozarski et al., 2011; Wang et al., 2014). More bioactive polysaccharides may be extracted using acid or alkali solution than hot water. In addition, PL-A with higher amounts of low M_w fractions and uronic acid contents showed stronger a DPPH radical-scavenging capacity than PL-W. Xing, Liu, Guo, and Yu (2005) have reported that these

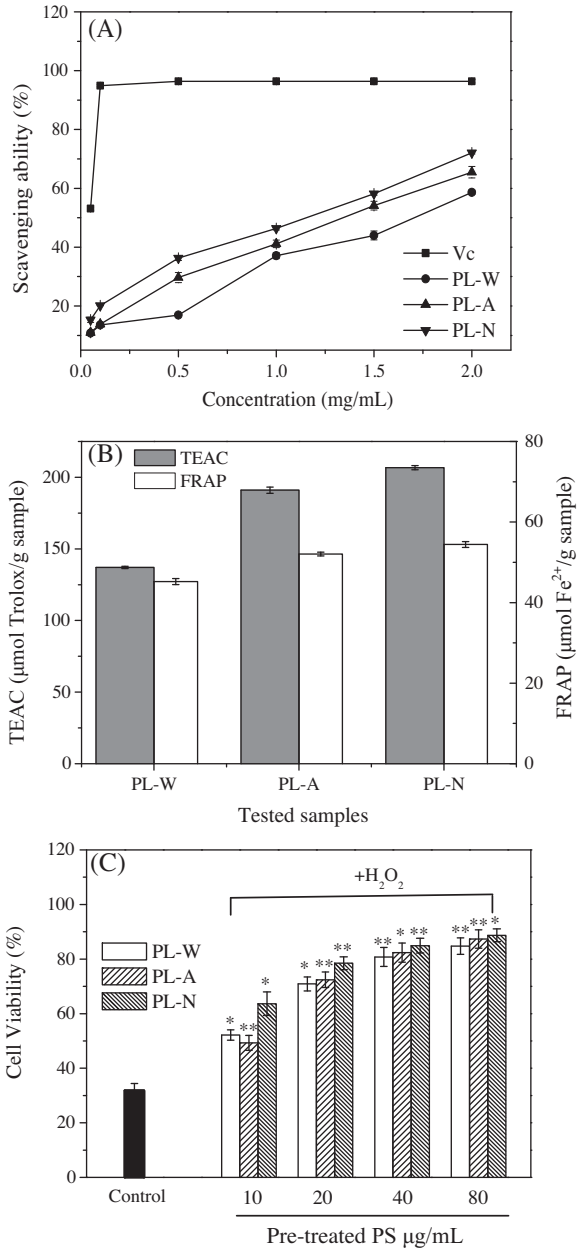

Fig. 5. Antioxidant activities of PL-W, PL-A and PL-N by (A) scavenging ability on DPPH; (B) TEAC and FRAP assays; and (C) cytoprotective effects at various concentrations against H_2O_2-induced SH-SY5Y cell injury, and each value is expressed as means ± SD ($n = 6$), where $^*p < 0.05$ and $^{**}p < 0.01$.

capacities for low M_w polysaccharides were more pronounced than that of high M_w polysaccharides because of the intra-molecular hydrogen bond effect. Furthermore, the DPPH radical scavenging capacities of polysaccharides are related to the monosaccharide components and chemical structures.

3.4.2. TEAC and FRAP assays

Fig. 5(b) shows the in vitro antioxidant activities of PL-W, PL-A, and PL-N through TEAC (on $ABTS^{\bullet+}$ radical scavenging activity) and FRAP assay (on ferric reducing ability), which both exhibit a similar trend with the increase in carbohydrate and uronic acid contents. PL-N was obtained by alkali extraction markedly showed the highest antioxidant activity with a TEAC value of 206.7 μmol Trolox/g sample and a FRAP value of 54.4 μmol Fe^{2+}/g sample, suggesting that the extraction medium has an important impact on

the antioxidant activities of polysaccharides. The polysaccharides isolated from *P. linteus* mycelia by acid or alkali extraction had higher carbohydrate and uronic acid contents (Table 1), which led to enhanced antioxidant activities and was consistent with that of the DPPH radical-scavenging activity. The antioxidant effects were attributed to their hydrogen-donating abilities (Wang et al., 2010). The presence of uronic acid groups in the polysaccharides could activate the hydrogen atom of the anomeric carbon. The higher activated capacity of the group implies a stronger hydrogen atom-donating capacity. PL-N with higher uronic acid content showed the best antioxidant activity.

3.4.3. Cytoprotective activity

MTT method was developed to evaluate the cytoprotective effect of polysaccharides on the neurotoxicity induced by H_2O_2 in SH-SY5Y cells. The cells were pretreated with polysaccharides for 24 h prior to H_2O_2 exposure. Fig. 5(c) shows the cytoprotective effects of PL-W, PL-A, and PL-N on H_2O_2-induced neurotoxicity in SH-SY5Y cells at various concentrations. The cell viability significantly decreased after H_2O_2 exposure, but showed a significant increase when pretreated with these polysaccharides in a dose-dependent manner from 10 μg/mL to 80 μg/mL compared with H_2O_2 alone. PL-A and PL-N possessed stronger protective effects against H_2O_2 and exhibited better antioxidant activities. At a concentration of 80 μg/mL, the viability of cells pretreated with PL-W, PL-A, and PL-N reached 84.7%, 87.4%, and 88.7%, respectively. Results from the cell culture tests agreed with those from the chemical assays.

4. Conclusions

In this study, three water-soluble polysaccharides (PL-W, PL-A, and PL-N) were obtained from *P. linteus* mycelia by hot water, 1% $(NH_4)_2C_2O_4$, and 1.25 M NaOH/0.05% $NaBH_4$ extraction methods in sequence. Physicochemical characterizations, molecular properties, and chain conformation were analyzed and compared. The in vitro antioxidant activities showed that these polysaccharides exhibited antioxidant activities in a dose-dependent manner. PL-N and PL-A showed stronger scavenging capacities and antioxidant activities compared with those of PL-W, which could be explored as novel natural antioxidants for use in foods or in medicine. Further analyses are necessary to purify and characterize the two active polysaccharides.

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