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Comparative characterization of physicochemical properties and bioactivities of polysaccharides from selected medicinal mushrooms

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Abstract Mushroom polysaccharides have been known to possess various pharmacological activities. However, information on their chemical and biological differences between mushrooms remains limited. In this study, we aimed to examine the differences in physicochemical characteristics of polysaccharides prepared from *Antrodia cinnamomea* (AC-P), *Coriolus versicolor* (CV-P), *Grifola frondosa* (GF-P), *Ganoderma lucidum* (GL-P), and *Phellinus linteus* (PL-P), followed by evaluating their inhibitory effects on nitric oxide (NO) production in lipopolysaccharide (LPS)-stimulated RAW264.7 macrophages. Results showed that under similar conditions of preparation, the monosaccharide composition of polysaccharides varied between different mushrooms, and glucose was the predominant monosaccharide, followed by galactose and mannose. AC-P and GF-P contained the highest amount of (1,3;1,6)-β-D-glucans. The degree of branching of (1,3;1,6)-β-D-glucans in all polysaccharides ranged from 0.21 to 0.26, with the exception of GF-P (0.38). The molecular weights of different polysaccharides showed diverse distributions; AC-P, CV-P, and GF-P contained two major macromolecular populations (< 30 and >200 kDa) and possessed triple-helix conformation, whereas GL-P (10.2 kDa) and PL-P (15.5 kDa) only had a low molecular weight population without triple-helix structure. These polysaccharides showed different inhibitory potency on NO production in LPS-stimulated RAW264.7 cells.

Keywords Polysaccharides · β-Glucans · Physicochemical properties · Medicinal mushrooms

Introduction

Medicinal mushrooms have been popularly used as folk medicine and functional foods in Asia. The non-digestible or β-linked polysaccharides especially β-D-glucans are well-known mushroom bioactive components with various human health benefits (Cheung 2013; Giavasis 2014; Ruthes et al. 2015); they are large polymeric molecules that are resistant to digestion in the small intestine and fermentable in the large intestine. β-D-Glucans are polysaccharides of D-glucose monomers linked by β-glycosidic linkages. Studies have demonstrated that these polysaccharides possessed various biological activities including anti-tumor, immunomodulation, anti-virus, anti-inflammation, hypoglycemia, and hypolipidemia (Giavasis 2014; Zhang et al. 2007). Studies have also reported that the physicochemical characteristics of polysaccharides can significantly affect their bioactivities and mechanism of actions (El Enshasy and Hatti-Kaul 2013; Sletmoen and Stokke 2013). Although the immune-health properties of mushroom polysaccharides have received increasing attention in the scientific communities and the consumers, current scientific understanding of this class of ingredients is largely specific to an individual type of mushroom.

The macromolecular structure of non-digestible or β-linked polysaccharides is known to depend on both their sources and isolation methods. Studies have shown that the biological activity of these polysaccharides is affected by their primary structure, solubility, degree of branching,

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molecular weight, polymer charge, and others (Lam and Cheung 2013). In general, polysaccharides with higher molecular weights are believed to possess stronger immunomodulatory and anti-tumor activities than the lower one (El Enshasy and Hatti-Kaul 2013; Ren et al. 2012). For example, (1,3)-β-D-glucans of *Grifola frondosa* with high molecular weight (800 kDa) exhibited stronger anti-tumor and immunomodulatory activities than those with lower molecular weights (Adachi et al. 1990). Studies also indicated that polysaccharides and (1,3)-β-D-glucans of *Ganoderma lucidum* with high molecular weights (1086 and > 788 kDa, respectively) showed the best anti-tumor activity (Zhao et al. 2010). In another study, it revealed that among the four fractions of protein-bound polysaccharides from *Coriolus versicolor*, the fraction containing polysaccharides with the highest molecular weight (> 200 kDa) possessed the most potent immunomodulatory activity (Kim et al. 1990). Polysaccharides with high molecular weight characteristics were suggested to have an enhanced binding affinity to the receptors of immune cells, hence resulting in a stronger immunomodulatory activity than those with lower molecular weights (El Enshasy and Hatti-Kaul 2013).

(1,3)-β-D-Glucans having short (1,6)-β-linked branches and the degree of branching varying between 0.20 and 0.33 have been reported to have the strongest anti-tumor and immunomodulatory activities (Bae et al. 2013; El Enshasy and Hatti-Kaul 2013; Ruthes et al. 2015). The conformations of mushroom polysaccharides include single-helix, triple-helix, and random coils. A triple-helical form is usually more stable than other conformations, and numerous anti-tumor polysaccharides such as lentinan and schizophyllan have a triple-helix structure (Ooi and Liu 2000; Stachowiak and Reguła 2012). Lentinan having a triple-helix conformation was reported to possess higher in vivo anti-tumor activities than lentinan with single random-coil chains (Zhang et al. 2005). It was also found that the anti-tumor potency of schizophyllan was positively related to the ratio of the triple helix to the single-chain structure (Yanaki et al. 1983).

Previous studies on the physicochemical properties and bioactivities of mushroom polysaccharides have been mainly focused on a specific species of mushroom; comparative studies on their differences between mushroom species remain limited. In the market, polysaccharide products from mushrooms are mainly prepared by hot water extraction and marketed as products of relatively similar therapeutic claims. The functionalities of polysaccharides are dependent on their physicochemical properties; one of the main factors for causing the differences in these properties and the biological activities of polysaccharides is the extraction conditions. In this study, our objective was to examine the differences in the physicochemical properties of water-soluble polysaccharides including β-D-glucans of five selected popular medicinal mushrooms, namely *Antrodia cinnamomea*, *C. versicolor*, *G. frondosa*, *G. lucidum*, and *Phellinus linteus*, using a similar condition of preparation as used commercially; the inhibitory effects of these polysaccharides on NO production in LPS-stimulated RAW264.7 macrophage cells were also evaluated.

Materials and methods

Materials and chemicals

Dried fruiting bodies of five medicinal mushrooms including *A. cinnamomea*, *C. versicolor*, *G. frondosa*, *G. lucidum*, and *P. linteus* with collection number KJ-AC-14, KJ-CV-14, KJ-GF-14, KJ-GL-14, and KJ-PL-14, respectively, were provided by Kang Jian Biotech Co., Ltd. (Nantou, Taiwan). The authenticity of the species was confirmed by Dr. Min-Nan Lai and their culture specimens were deposited at Kang Jian Biotech Co., Ltd. Monosaccharide standards (arabinose, fucose, galactose, glucose, mannose, rhamnose, ribose, xylose, galacturonic acid, and glucuronic acid), dextran standards (5, 25, 150, 270, 670, 1100, and 1400 kDa), laminarin, aniline blue, Congo red, bovine serum albumin (BSA), lipopolysaccharide (LPS), α-amylase (*Aspergillus oryzae*, EC 3.2.1.1), and α-glucosidase (*Saccharomyces cerevisiae*, EC 3.2.1.20) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). 1-Phenyl-3-methyl-5-pyrazolone was purchased from Alfa Aesar Co. (Ward Hill, MA, USA). Gentiobiose was purchased from Tokyo Chemical Industry Co. (Tokyo, Japan). Endo-1,3-β-D-glucanase (*Trichoderma* sp., EC 3.2.1.39) and exo-1,3-β-D-glucanase (*Trichoderma virens*, EC 3.2.1.58) were purchased from Megazyme (Wicklow, Ireland). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and antibiotic (penicillin-streptomycin) were obtained from Gibco (Grand Island, NY, USA). All other chemicals used were of analytical grade.

Preparation of crude polysaccharides and water-soluble β-linked polysaccharides

The polysaccharides were prepared according to the procedure as described previously (Wu et al. 2013a). In brief, 3 g of mushroom powders were extracted with 150 ml of boiling water for 3 h. After filtration, fourfold volume of ethanol (95 %) was added to the filtrate slowly with stirring. The mixture was kept overnight at 4 °C and centrifuged to separate the supernatant and residue. The residue was washed successively with ethanol and redissolved in deionized water, followed by lyophilization to obtain the crude water-soluble polysaccharides.

Water-soluble β-linked polysaccharides (WSP) including β-D-glucans were prepared from crude polysaccharides. In brief, 0.2 ml of α-amylase (2 mg ml^{-1}; 60 U ml^{-1}) and

0.2 ml of α-glucosidase (1 mg ml^{-1}; 50 U ml^{-1}) were added to 100 ml of crude polysaccharide solution (0.5 mg ml^{-1}, dissolved in deionized water). The mixture was transferred to a dialysis tubing (molecular weight cutoff 12–14 kDa; Spectrum Laboratories Inc., Rancho Dominguez, CA, USA) and dialyzed against deionized water at 4 °C for 3 days, with water change every 8 h. The dialyzed solution was deproteinized with Sevag reagent (chloroform:n-butanol = 4:1, *v*/*v*) at a ratio of 3:1 (*v*/*v*) for three times, and then precipitated with fourfold volume of ethanol (95 %) and kept overnight at 4 °C. After centrifugation, the residue was washed successively with ethanol and redissolved in deionized water, followed by lyophilization to give WSP of *A. cinnamomea* (AC-P), *C. versicolor* (CV-P), *G. frondosa* (GF-P), *G. lucidum* (GL-P), and *P. linteus* (PL-P).

Determination of carbohydrate and protein contents

The content of carbohydrate in WSP was determined by the phenol-sulfuric acid method (Dubois et al. 1956) using glucose as the standard, and the absorbance at 490 nm was measured with a spectrophotometer (U-1800, Hitachi, Tokyo, Japan). The content of protein was determined by Bradford's method (Bradford 1976) using bovine serum albumin as the standard, and the absorbance at 595 nm was spectrophotometrically measured.

Analysis of monosaccharide composition

The analysis of monosaccharide composition was conducted according to the method described by Dai et al. (2010) with slight modification. Briefly, WSP sample (2 mg) was methanolyzed with 1 ml of anhydrous methanol containing 2 M HCl at 80 °C for 12 h. The solvent was then dried under reduced pressure, and the residue was hydrolyzed into component monosaccharides using 1 ml of 2 M trifluoroacetic acid (TFA) at 120 °C for 1 h. After cooling to room temperature, the hydrolyzed solution was co-evaporated with 2 ml of 50 % methanol to remove TFA; the procedure was repeated several times until the pH reached 7. The dried hydrolyzed product was dissolved in 0.4 ml of deionized water and filtered through a 0.45-μm membrane filter for subsequent derivatization.

The hydrolyzed products or monosaccharide standards (25 μl, dissolved in deionized water) were mixed with 25 μl of 0.6 M NaOH and 50 μl of 0.5 M 1-phenyl-3-methyl-5-pyrazolone (PMP, dissolved in methanol), and incubated at 70 °C for 100 min. After cooling to room temperature, the mixture was neutralized with 100 μl of 0.3 M HCl, followed by adding 500 μl of deionized water and 500 μl of chloroform, and then vigorously shaking until homogeneous. After removing the chloroform layer, another 500 μl of chloroform was added to the mixture; this extraction process was repeated three times. The aqueous layer was collected and filtered through a 0.45-μm membrane filter for HPLC analysis.

The resulting PMP derivatives were analyzed by an Agilent 1100 series HPLC system (Agilent Technologies, Palo Alto, CA, USA) consisting of a G1379A online degasser, a G1311A quaternary pump, an injection valve, and a G1314A UV-Vis detector set at wavelength 245 nm. The chromatographic data were recorded and processed with an Agilent Chemstation software. The separation of compounds was performed with a Mightysil RP-18 GP column (4.6 × 250 mm, 5 μm; Kanto Chemical, Tokyo, Japan) and a guard column at room temperature, with the mobile phase composed of 0.1 M phosphate buffer (pH 6.7) and acetonitrile in a ratio of 83:17 (*v*/*v*). The sample injection volume was 20 μl and the flow rate was 1 ml min^{-1}. The monosaccharides were identified by chromatographic comparisons with their respective authentic standards and quantified by the calibration curves obtained from known concentrations of monosaccharide standards (20–2000 μg ml^{-1}).

Determination of homogeneity and molecular weight

The homogeneity and molecular weight of WSP samples were evaluated and determined by high-performance gel-filtration chromatography (HPGFC) (Escarnot et al. 2011). The analysis was performed on an Agilent 1100 series HPLC system fitted with a TSKgel $GMPW_{XL}$ column (300 × 7.8 mm, 13 μm; Tosoh, Tokyo, Japan) and a G1362A refractive index detector. Sample solutions (2 mg ml^{-1}) were filtered through a 0.45-μm membrane filter and 20 μl was injected for each analysis. The mobile phase was 50 mM sodium nitrate solution containing 0.05 % sodium azide, and the flow rate was 0.6 ml min^{-1}. The temperature of the flow cell inside the RI detector was set at 35 °C. Dextran standards of 5, 25, 150, 270, 670, 1100, and 1400 kDa were used to establish a calibration curve for molecular weight estimation.

Analysis of β-D-glucan content and degree of branching of (1,3;1,6)-β-D-glucans

The relative content of 1,3-β-D-glucans was evaluated by aniline blue fluorescence method (Chang 2003). Briefly, WSP sample (2 mg) was dissolved in 4 ml of 0.3 M NaOH containing 0.5 M NaCl. After adjusting pH to 11.5 with 2 M HCl, the sample solution was diluted to a constant volume of 10 ml using 50 mM Na_2HPO_4-NaOH buffer (pH 11.5) containing 0.5 M NaCl. The diluted sample solution (200 μl) was transferred to a 96-well black plate and mixed with 20 μl of aniline blue solution (1 mg ml^{-1}, dissolved in deionized water). The mixture was kept at room temperature for 2 h, and the fluorescence intensity (Ex 395 nm, Em 495 nm) was measured by a microplate reader (Infinite M200 PRO, Tecan, Crailsheim, Germany). Laminarin (10–50 μg ml^{-1}, dissolved in the same

buffer solution) was used as reference standard to construct a calibration curve for estimating the relative content of 1,3-β-D-glucans in the polysaccharide samples.

The content of (1,3;1,6)-β-D-glucans and the ratio of (1,6)-β-D-glucosyl branches of (1,3;1,6)-β-D-glucans were determined by the enzymatic HPLC method described by Wang et al. (2014) with slight modification. In brief, WSP sample (5 mg) was dissolved in 2.5 ml of 0.5 M NaOH, and then stirred at room temperature overnight. After adding 0.2 ml of arabinose (10 mg ml^{-1}, as internal standard) and adjusting pH to 4.5 with 1 M HCl, the sample solution was diluted to a constant volume of 5 ml using 50 mM acetate buffer (pH 4.5). The diluted sample solution (1 ml) was reacted with exo-1,3-β-D-glucanase (1 U) and endo-1,3-β-D-glucanase (0.1 U) at 40 °C for 3 h with shaking. After heating in a boiling water bath for 15 min to stop the enzymatic reaction, samples were left to cool to room temperature, followed by slowly adding 4 ml of 95 % ethanol with stirring, samples were then kept at 4 °C for 2 h to precipitate the undigested polysaccharides. The supernatant was dried by rotary evaporation and then redissolved with 0.4 ml of deionized water. As glucose and gentiobiose were the digested products of (1,3;1,6)-β-D-glucans after enzymatic reaction, they were transformed to PMP derivatives and quantified by HPLC-UV.

The yield of (1,3;1,6)-β-D-glucan was calculated as (Wt-Glc × 0.9 + Wt-Gen × 0.95)/Wt-FB, and the (1,3;1,6)-β-D-glucan content in WSP was calculated as (Wt-Glc × 0.9 + Wt-Gen × 0.95)/Wt-PS; Wt-Glc and Wt-Gen are the weights of glucose and gentiobiose, respectively, and they are calculated by the calibration curves established with the authentic standards and internal standard (arabinose). The values of 0.9 (= 162.2/180.2) and 0.95 (= 324.3/342.3) are the factors for calculating the exact mass of glucosyl and gentiobiosyl groups in the polysaccharide chain, respectively. The values of 162.2 and 180.2 are the molecular weights of glucose in bound form and free form, respectively, whereas the values of 324.3 and 342.3 are the molecular weights of gentiobiose in bound form and free form, respectively. Both bound glucose and gentiobiose have one water molecule less than their respective free form. Wt-FB is the weight of original powder of mushroom fruiting bodies. Wt-PS is the weight of polysaccharides (determined by phenol-sulfuric acid method) in the original sample for enzymatic digestion.

The degree of branching in (1,3;1,6)-β-D-glucans was calculated as Gen/(Glc + Gen); Gen is the mmol of gentiobiose = Wt-Gen/342.3, and Glc is the mmol of glucose = Wt-Glc/180.2.

FT-IR spectroscopy

FT-IR spectra of WSP were recorded on a Nicolet 380 FT-IR spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) from 4000 to 400 cm^{-1}. Dried WSP (1 mg) and KBr powder (200 mg) were mixed and compressed into KBr disc for analysis.

Conformational analysis

The conformational structure of WSP in solution was determined by observing the shift of maximum absorbance wavelength (λ_{max}) of Congo red-polysaccharide complexes according to the method described by Wu et al. (2013b) with slight modification. In brief, 100 μl of WSP (3 mg ml^{-1}, dissolved in deionized water) were reacted with 100 μl of Congo red (100 μM) in a gradient of NaOH solutions (0, 0.025, 0.05, 0.075, 0.1, 0.15, 0.2, 0.3, 0.4, and 0.5 M). The absorbance in the range of 400–600 nm was measured with a microplate reader. The conformational transition of polysaccharides from triple-helical to single-stranded conformation was evaluated by comparing the λ_{max} at different concentrations of NaOH solutions. Laminarin was used as reference standard polysaccharides for comparison, and deionized water was used as control.

Cell culture

RAW264.7 (BCRC No. 60001), a murine macrophage cell line, was obtained from the Bioresource Collection and Research Center (BCRC) of the Food Industry Research and Development Institute (Hsinchu, Taiwan). Cells were cultured in DMEM containing 10 % FBS, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin at 37 °C in a humidified 5 % CO_2 atmosphere.

Measurement of nitric oxide

The nitric oxide (NO) production was determined by measuring the nitrite concentration in the culture media. RAW264.7 cells (5×10^5 cells ml^{-1}) were seeded in 96-well culture plates and incubated with or without samples at 37 °C for 24 h. The supernatant (100 μl) from each well was mixed with Griess reagent [100 μl, 1 % sulfanilamide, 0.1 % N-(1-naphthyl)-ethylenediamine dihydrochloride and 2.5 % phosphoric acid]. After 10 min incubation at room temperature, the absorbance at 540 nm was measured with a microplate reader, and the nitrite concentration was calculated from a sodium nitrite calibration curve.

Results

Yields and chemical properties

Table 1 summarizes the yield, total carbohydrate, and protein contents of WSP. The results showed that the yields of WSP from five selected medicinal mushrooms ranged from 1.66 to

Table 1 Chemical composition of the water-soluble polysaccharides from five selected medicinal mushrooms

Sample	Yield (%, DW)	Weight (%)	
		Total carbohydrate	Protein
Antrodia cinnamomea	1.66 ± 0.30	95.6 ± 5.4	ND
Coriolus versicolor	4.39 ± 0.29	87.5 ± 8.3	ND
Grifola frondosa	3.80 ± 0.68	82.6 ± 2.8	7.12 ± 0.33
Ganoderma lucidum	2.13 ± 0.37	78.5 ± 5.5	2.35 ± 0.24
Phellinus linteus	1.71 ± 0.39	69.8 ± 4.6	4.10 ± 0.77

Values are mean ± SD (n = 3)

DW dried weight, *ND* not detected

4.39 %. *C. versicolor* and *G. frondosa* contained markedly higher level of WSP than the others, while the lowest was noted in *A. cinnamomea* (1.66 %). A small amount of protein was found in WSP of *G. frondosa*, *G. lucidum*, and *P. linteus*, but not detected in that of *A. cinnamomea* and *C. versicolor*. The total carbohydrate content in WSP ranged from 69.8 to 95.6 %, which demonstrates the diverse constituent contents for different species.

Monosaccharide composition

Table 2 shows the monosaccharide composition of WSP from five selected medicinal mushrooms. Results showed that glucose was the predominant monosaccharide in all samples, with molar percentages ranging from 53.2 % (*P. linteus*) to 93.2 % (*C. versicolor*), and the second major monosaccharides were galactose (2.2–13.5 %) and mannose (1.3–14.9 %). Fucose (< 6.8 %) and xylose (< 2.4 %) were present in all samples with relatively low levels. *G. lucidum* and *P. linteus* were found to contain 2.0 and 1.4 % of galacturonic acid, respectively. A small amount of glucuronic acid (1.2–5.6 %) was detected in all samples except *A. cinnamomea*.

Homogeneity and molecular weight

As the low molecular weight components have been removed by dialysis (molecular weight cutoff 12–14 kDa), hence, the estimated molecular weights of all samples were larger than 10 kDa (Table 3). Results showed that two major macromolecular populations were detected in the WSP of *A. cinnamomea* (28.9 and 267.1 kDa), *C. versicolor* (10.4 and 402.3 kDa), and *G. frondosa* (19.6 and 722.7 kDa), indicating that these polysaccharides were heterogeneous, and their contents of low molecular weight populations were higher than high molecular weight populations. On the other hand, the WSP of *G. lucidum* and *P. linteus* contained mainly the low molecular weight populations (10.2 and 15.5 kDa, respectively), suggesting that they were homogeneous polysaccharides. Using the present procedure of preparation, WSP of *G. frondosa* was shown to have the highest molecular weight, followed by *C. versicolor* and *A. cinnamomea*, whereas no high molecular weight WSP was noted in *G. lucidum* and *P. linteus*.

β-D-Glucan content and degree of branching

Table 4 shows the yields, contents, and degree of branching of (1,3;1,6)-β-D-glucans and the relative contents of 1,3-β-D-glucans in the five selected medicinal mushrooms. Results showed that *A. cinnamomea* and *G. frondosa* contained high amount of (1,3;1,6)-β-D-glucans, which account for 26.0 and 13.2 % of the WSP, respectively. The degree of branching of (1,3;1,6)-β-D-glucans in WSP ranged between 0.21 and 0.38, and the highest degree of branching was noted in *G. frondosa*.

Table 2 Monosaccharide composition of the water-soluble polysaccharides from five selected medicinal mushrooms

Sample	Monosaccharide composition (molar %)									
	Arabinose	Fucose	Galactose	Glucose	Mannose	Rhamnose	Ribose	Xylose	GalA	GlcA
Antrodia cinnamomea	ND	2.0 ± 0.1	2.9 ± 0.2	84.0 ± 1.7	7.2 ± 1.9	1.4 ± 0.3	ND	2.4 ± 0.2	ND	ND
Coriolus versicolor	ND	1.2 ± 0.1	2.2 ± 0.2	93.2 ± 0.2	1.3 ± 0.1	ND	tr	tr	ND	1.4 ± 0.3
Grifola frondosa	ND	5.8 ± 0.4	10.5 ± 0.3	72.2 ± 0.3	7.8 ± 0.8	ND	2.2 ± 0.1	tr	ND	1.2 ± 0.4
Ganoderma lucidum	tr	3.3 ± 0.5	13.3 ± 0.2	64.7 ± 0.3	6.8 ± 1.0	2.0 ± 0.2	tr	1.3 ± 0.1	2.0 ± 0.1	5.6 ± 0.3
Phellinus linteus	tr	6.8 ± 1.0	13.5 ± 0.5	53.2 ± 0.7	14.9 ± 2.0	1.1 ± 0.1	tr	2.3 ± 0.1	1.4 ± 0.3	5.6 ± 0.3

Values are mean ± SD (n = 3)

ND not detected, *tr* trace (ratio < 1 %), *GalA* galacturonic acid, *GlcA* glucuronic acid

Table 3 Molecular weights of the water-soluble polysaccharides from five selected medicinal mushrooms

Sample	Molecular weight (kDa)	Relative content (%)
Antrodia cinnamomea	267.1 ± 2.3	32.2 ± 0.3
	28.9 ± 0.2	62.1 ± 0.4
Coriolus versicolor	402.3 ± 2.5	12.9 ± 1.2
	10.4 ± 0.0	87.1 ± 1.2
Grifola frondosa	722.7 ± 5.1	40.4 ± 5.2
	19.6 ± 0.0	54.9 ± 4.6
Ganoderma lucidum	10.2 ± 0.1	94.7 ± 0.4
Phellinus linteus	15.5 ± 0.2	94.6 ± 0.6

Values are mean ± SD ($n = 3$)

Based on the aniline blue fluorescence analysis, results showed that the order of 1,3-β-D-glucan content in WSP of the five selected medicinal mushrooms was *A. cinnamomea* > *G. frondosa* > *G. lucidum* > *P. linteus* > *C. versicolor*.

IR spectral characteristics

Results of IR spectra showed that the WSP from five selected medicinal mushrooms had similar absorption bands (Fig. 1). The wide and strong band around 3400 cm^{-1} was attributed to the O-H stretching vibration, and the band around 2930 cm^{-1} was derived from C-H stretching vibration. The bands between 1175 and 1000 cm^{-1} were due to the stretching and bending vibrations of C-O-C and C-O-H (Buranov and Mazza 2010). Characteristically, the intense signal around 1050 cm^{-1} indicated the presence of pyranose ring in polysaccharides.

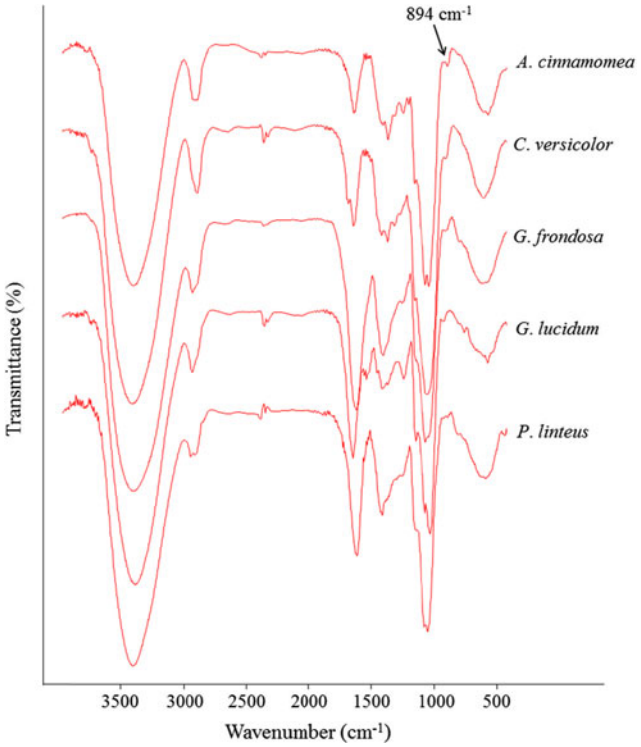

Fig. 1 FT-IR spectra of the water-soluble polysaccharides from five selected medicinal mushrooms

Conformational characteristics

Bioactive β-D-glucans were reported to have a triple helix conformation in aqueous or alkaline solution at pH < 13.2 (< 0.15 M NaOH), which can interact with Congo red (a dye) to form complexes and lead to bathochromic shift of the maximum absorbance wavelength (λ_{max}) (Ooi and Liu

Table 4 Yields, contents, and degree of branching of β-D-glucans in the five selected medicinal mushrooms

Sample	Yield of (1,3;1,6)-β-D-glucan (wt %, DW)	(1,3;1,6)-β-D-Glucan[a]/ polysaccharides (%)	Degree of branching of (1,3;1,6)-β-D-glucan[a]	1,3-β-D-Glucan relative content[b]/polysaccharides (%)
Antrodia cinnamomea	0.41 ± 0.02a	26.0 ± 1.4a	0.22 ± 0.01cd	97.0 ± 3.3a
Coriolus versicolor	0.13 ± 0.01b	3.4 ± 0.2e	0.26 ± 0.01b	4.5 ± 0.3e
Grifola frondosa	0.41 ± 0.02a	13.2 ± 0.5b	0.38 ± 0.01a	52.4 ± 2.1b
Ganoderma lucidum	0.13 ± 0.01b	7.9 ± 0.2c	0.23 ± 0.01c	20.5 ± 0.6c
Phellinus linteus	0.07 ± 0.00c	5.5 ± 0.2d	0.21 ± 0.00d	13.1 ± 0.1d

Values are mean ± SD ($n = 3$). Data in the same column with different letters are significantly different ($p < 0.05$) as analyzed by Duncan's multiple range test

DW dried weight

[a] Determined by endo- and exo-(1,3)-β-D-glucanase digestion and high-performance liquid chromatography with UV detection (HPLC-UV)

[b] Determined by aniline blue fluorescence method and laminarin was used as reference standard

2000; Zhang et al. 2010). As shown in Fig. 2, the values of λ_{max} of Congo red are largely shifted to longer wavelengths in the presence of WSP from *A. cinnamomea* (AC-P), *C. versicolor* (CV-P), *G. frondosa* (GF-P), and laminarin (reference β-D-glucan) in 0–0.15 M NaOH solutions, indicating the existence of triple-helix structures in these samples.

Inhibition on nitric oxide production

Figure 3 shows that the WSP from five selected medicinal mushrooms possessed inhibitory effects on the LPS-induced NO production in RAW264.7 cells; CV-P, GF-P, GL-P, and PL-P were shown to inhibit the NO production in a dose-dependent manner. Among the five WSP samples, GF-P, GL-P, and PL-P showed a stronger inhibitory activity than AC-P and CV-P did.

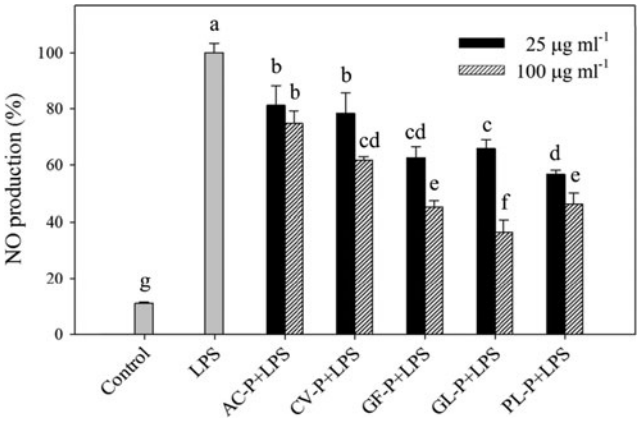

Fig. 3 Effects of water soluble polysaccharides from five selected medicinal mushrooms on nitric oxide (NO) production in LPS-stimulated RAW264.7 cells. Cells were treated with LPS (1 μg ml^{-1}) for 24 h in the absence or presence of samples at the concentrations of 25 and 100 μg ml^{-1}. Data are presented as mean ± SD (n = 3). The bars having different letters are significantly different ($p < 0.05$) as analyzed by Duncan's multiple range test

Discussion

Polysaccharides including β-D-glucan, major bioactive components of medicinal mushrooms, are well known to possess various pharmacological activities such as immunomodulation, anti-cancer, anti-virus, anti-inflammation, and others (Giavasis 2014; Zhang et al. 2007); the potency of these activities and their mechanism of actions were reported to depend on their physicochemical characteristics (El Enshasy and Hatti-Kaul 2013; Sletmoen and Stokke 2013). Under the similar conditions of preparation, this study demonstrates that the physicochemical properties of WSP from five selected medicinal mushrooms vary with the source of mushrooms. They

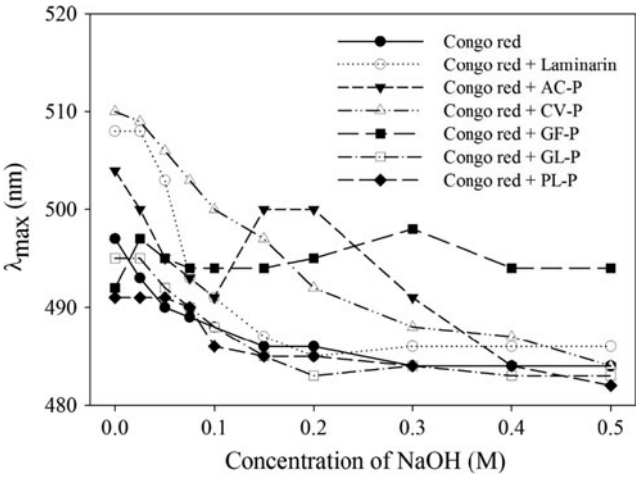

Fig. 2 The maximum absorbance wavelength (λ_{max}) of Congo red in the presence of the water soluble polysaccharides from five selected medicinal mushrooms at various concentrations of sodium hydroxide solution. Samples were the water-soluble polysaccharides of *A. cinnamomea* (AC-P), *C. versicolor* (CV-P), *G. frondosa* (GF-P), *G. lucidum* (GL-P), and *P. linteus* (PL-P)

were heteropolysaccharides and composed of six to ten different types of monosaccharides, with glucose as the major component. A small amount of protein was found in WSP of *G. frondosa*, *G. lucidum*, and *P. linteus*, but not detected in that of *A. cinnamomea* and *C. versicolor*. These proteins are probably bound to the soluble glucans, and the Sevag method was not able to separate them completely. These results clearly revealed that the WSP of different medicinal mushrooms prepared by the same conditions consisted of various molecular weight populations, which might lead to diverse bioactivities.

(1,3;1,6)-β-D-Glucans are part of the WSP and are considered to be the major bioactive polysaccharides in mushrooms (Cheung 2013; Ruthes et al. 2015). In this study, *A. cinnamomea* and *G. frondosa* were found to contain high amount of (1,3;1,6)-β-D-glucans, which account for 26.0 and 13.2 % of the WSP, respectively; this suggests that *A. cinnamomea* and *G. frondosa* may possess better immunomodulatory and anti-tumor activities than other mushrooms. Besides, the degree of branching of (1,3;1,6)-β-D-glucans in *A. cinnamomea*, *C. versicolor*, *G. lucidum*, and *P. linteus* ranged within 0.20 (1 branch in 5 backbone residues) and 0.33 (1 branch in 3 backbone residues), which is a general characteristic of bioactive glucans with potent immunomodulatory activity (El Enshasy and Hatti-Kaul 2013). The (1,3;1,6)-β-D-glucan of *G. frondosa* possessed a much higher degree of branching (0.38) than other mushroom samples, and this result was consistent with previous report that the bioactive β-D-glucan purified from *G. frondosa* had more β-1,6 linkages than the other common mushroom β-D-glucans (Nanba et al. 1987). This is the first study reporting on the contents and degree of branching of (1,3;1,6)-β-D-glucans in *A. cinnamomea*, and these results indicates that the fruiting

bodies of *A. cinnamomea* and *G. frondosa* might be good sources of (1,3;1,6)-β-D-glucan.

In this study, although laminarin [a (1,3;1,6)-β-D-glucan] was used as reference standard to calculate the 1,3-β-D-glucan contents, different β-D-glucans would exhibit a diverse fluorescence intensity at the same concentration (Ko and Lin 2004). Therefore, the estimated content of 1,3-β-D-glucan can only be used as reference values for preliminarily confirmation of the presence of 1,3-β-D-glucan in the samples. Among the five polysaccharide samples, the spectrum of *A. cinnamomea* showed a specific absorption peak at 894 cm^{-1}, indicating the existence of β-D-glycosidic linkages (Liu et al. 2012). The characteristic peak of β-D-glycosidic bonds in *A. cinnamomea* corresponded to its relative higher ratio of (1,3;1,6)-β-D-glucans to polysaccharides (26.0 %).

The λ_{max} of Congo red-AC-P and Congo red-CV-P complexes decreased substantially with the increase of alkaline concentrations (in the range of 0.15–0.50 M); this indicates the conformational transition of triple-helix structures into single chains (Zhang et al. 2010). As no apparent decrease of λ_{max} was observed in WSP of *G. frondosa* with increasing alkaline concentrations, this suggests that it may possess the ability to maintain a triple-helix conformation in the highly alkaline solutions (0.15–0.50 M). In addition, there was no obvious bathochromic shift of λ_{max} in the presence of WSP from *G. lucidum* (GL-P) or *P. linteus* (PL-P), suggesting that GL-P and PL-P did not exist as triple-helical form. This study indicates that the triple-helix conformation might only exist in the WSP containing higher molecular weight populations (AC-P 267.1 kDa, CV-P 402.3 kDa, and GF-P 722.7 kDa), which is consistent with previous studies indicating that the molecular weight of polysaccharides such as schizophyllan was positively related to the ratio of triple helix to single-chain structure (Yanaki et al. 1983; Zhang et al. 2013).

Nitric oxide (NO) is an inflammatory mediator that modulates immune responses. High levels of NO are produced in the presence of inflammatory stimuli such as LPS. Polysaccharides of five selected medicinal mushrooms demonstrated to have potent inhibitory activity on NO production in LPS-stimulated RAW264.7 cells; however, neither the (1,3;1,6)-β-D-glucan content nor the molecular weight of the WSP had correlation with the NO inhibitory potency, suggesting that the heteropolysaccharides other than (1,3;1,6)-β-D-glucan in WSP may have caused the variation of potency in inhibiting NO production.

In conclusion, this study has provided the fundamental information on the differences in physicochemical properties of the water-soluble bioactive polysaccharides of five selected popular medicinal mushrooms in Asia. The present results also indicate that under the similar conditions of commercial preparation, the physicochemical properties of WSP vary with the source of mushrooms, and hence, their functionalities in health food applications necessitate careful evaluation.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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