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Physiochemical properties and antitumor activities of two α-glucans isolated from hot water and alkaline extracts of Cordyceps (Cs-HK1) fungal mycelia

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ABSTRACT

Two novel polysaccharides, WIPS and AIPS, were isolated and fractionated respectively from hot water and alkaline extracts of the mycelial biomass of a medicinal fungus *Cordyceps sinensis* (strain Cs-HK1). Through analytical and degradation experiments, WIPS and AIPS were characterized as α-D-glucans with a backbone of (1 → 4)-linked α-D-Glcp (>60%) and very similar molecular weights (M_w: WIPS 1180 kDa; AIPS 1150 kDa). WIPS had a short branch of (1 → 6)-linked α-D-Glcp (~14%), but AIPS was a linear glucan, distinctive from the branched structures of most glucans from medicinal fungi. In aqueous alkaline solutions, both AIPS and WIPS exhibited a random coil structure with the similar conformational parameters but significantly different polydispersity indexes, 0.19 versus 0.37. AIPS exhibited much more significant antitumor and immuno-stimulatory effects than WIPS in animal tests on melanoma tumor-bearing mice.

1. Introduction

Polysaccharides are identified as the major bioactive constituents of medicinal mushrooms (fungi) with notable antitumor, immunomodulatory activities and other valuable medicinal functions (Ooi & Liu, 2000; Wasser, 2002). The most common class of antitumor polysaccharides from medicinal fungi is comprised by the glucans with various glycosidic linkages, such as β-(1 → 3)-, β-(1 → 6)-, α-(1 → 3)-, and α-(1 → 4)-glucans. Most of these glucans are branched with various side chains attached at different positions and some are bounded with proteins or peptides as PSP complexes. The antitumor fungal polysaccharides documented in the literature and applied in commercial products are mostly extracted from mushroom fruit bodies or fungal mycelia, as intracellular polysaccharides (IPS) (Wasser, 2002), though a smaller number have also been isolated from the liquid media used for mycelial cultivation, as exopolysaccharides (EPS) (Zhang, Cui, Cheung, & Wang, 2007).

Cordyceps sinensis (Berk.) Sacc., generally called Cordyceps or DongChongXiaCao in Chinese, is a precious and highly acclaimed medicinal fungus in traditional Chinese medicine (TCM) with a broad spectrum of health promoting effects on the kidney, lung, liver and immune functions (Li & Tsim, 2004; Zhu, Halpern, & Jones, 1998). Since natural Cordyceps is very rare and not sufficient to meet the increasing demand, mycelial fermentation has become a major and more economical source of Cordyceps materials. Several bioactive IPS molecules have been isolated from the mycelial biomass of *C. sinensis* fungi and elucidated of the molecular structures (Kiho, Ookubo, Usui, Ukai, & Hirano, 1999; Kiho, Tabata, Ukai, & Hara, 1986; Wu, Sun, & Pan, 2006; Wu, Hu, Pan, Zhou, & Zhou, 2007; Wu, Zhang, & Leung, 2007). Our group has also established the mycelial culture of a fungus designated Cs-HK1, which was isolated from the fruiting body of a natural Cordyceps, and carried out liquid fermentation for the production of mycelial biomass and polysaccharides (Leung, Zhang, & Wu, 2006). So far our studies have been mainly devoted to the EPS isolated from the mycelial culture broth on their structures, properties and bioactivities (Leung, Zhao, Ho, & Wu, 2009; Wang, Cheung, Leung, & Wu, 2010; Yan, Li, Wang, & Wu, 2010), but the IPS extracted from the mycelial biomass of Cs-HK1 are still not well characterized.

The specific molecular structures and properties (e.g. molecular weight or size) of IPS retained from the mycelial biomass of a given fungus are dependent upon the methods and conditions of extraction, isolation and fractionation. Hot water and alkaline (water) are the two most common methods for extraction of IPS from fungi (Zhang et al., 2007). This work was to characterize the molecular structures and solution properties of two IPS molecules isolated from hot water and alkaline extracts of Cs-HK1 mycelia and their antitumor activity.

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2. Materials and methods

2.1. Fungal culture and mycelial biomass production

The Cordyceps fungus Cs-HK1 used in this study was originally isolated from the fruiting body of a natural Cordyceps and Cs-HK1 mycelial culture was maintained in a nutrient medium as reported previously (Leung et al., 2006). The mycelial biomass of Cs-HK1 fungus for the IPS extraction was produced in a stirred, aerated fermentor which was filled with over 1000 L liquid medium and operated at 25 °C for a period of 6–7 days. At the end of fermentation, the mycelial biomass was harvested from the fermentation broth by centrifugation, and the biomass was rinsed repeatedly with distilled water and spun down, and the final biomass was dried at 45–50 °C in an oven till constant weight. The dry biomass was packaged in plastic zipper bags and placed in a desiccator at room temperature before use.

2.2. Extraction and isolation of polysaccharides

The mycelial biomass was extracted in two ways, hot water and aqueous alkaline. For hot water extraction, each 40 g dry mycelium powder was mixed with 600 mL distilled water and heated at 90 °C in a water bath for 4 h, followed by centrifugation (6000 rpm, 30 min). The extraction was repeated once and the extract liquid collected was concentrated by evaporation under reduced pressure. The concentrated extract liquid was mixed with 4 volumes of 95% ethanol at 4 °C overnight for precipitation. The precipitate formed was collected after centrifugation (6000 rpm, 30 min) and then redissolved in distilled water and lyophilized, yielding the crude IPS fraction from hot water extract. For alkaline extraction, the mycelium powder was extracted with aqueous solution of 1.25 M NaOH and 0.04% $NaBH_4$ for three times (each at 10:1 (v/w) solvent to solid ratio) and the extract liquid was collected after centrifugation. The addition of 0.04% $NaBH_4$ to the aqueous alkaline solution for the extraction was to attain water soluble-polysaccharides from the fungus according to Wang, Xu, and Zhang (2008). The extract liquid was precipitated with 36% acetic acid (final concentration), followed by centrifugation. The supernatant was collected and concentrated by evaporation under reduced pressure and then precipitated with 3 volumes of cold acetone, followed by centrifugation at 8000 rpm for 30 min. The precipitate was collected, redissolved in distilled water and lyophilized, yielding the crude IPS fraction from the alkaline extract.

2.3. Purification of IPS

The crude IPS fractions isolated from hot water and alkaline extracts of the Cs-HK1 mycelia as described above were deproteinized by Sevag reagent (Staub, 1956) and decolorized with 30% H_2O_2. The IPS solutions were then dialyzed against tap water and distilled water for 3 days with 12–14 kDa MWCO membrane, respectively, and then concentrated and lyophilized. After these preliminary purification steps, the IPS fractions were applied to a DEAE-52 cellulose (anion) ion-exchange (IEC) column (2.6 × 60 cm, Cl^-; Whatman) and eluted with de-ionized water containing 0–0.5 M NaCl at a flow rate of 1.0 mL/min, following the gradient scheme as shown in Fig. 1. The eluate was monitored by the phenol–sulfuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956) and the major PS fractions detected were collected and concentrated by evaporation under reduced pressure, followed by dialysis against de-ionized water for 3 days with 12–14 kDa MWCO membrane. The PS fractions were concentrated and lyophilized, yielding the WIPS from the hot water extract and the AIPS from the alkaline extract, respectively.

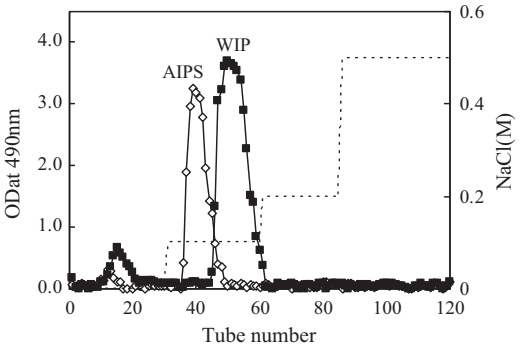

Fig. 1. Ion exchange chromatograms of two IPS fractions isolated from hot water and alkaline extracts respectively of Cs-HK1 mycelium and the elution scheme (on DEAE-52 cellulose column).

2.4. Structural analysis of IPS

All analytical methods mentioned in this section have been described in details by Yan et al. (2010). The total carbohydrate content of IPS fractions was determined by phenol–sulfuric acid method (Dubois et al., 1956) using D-glucose as a reference. Optical rotation was measured of the IPS samples dissolved in de-ionized water at 20 °C on a Perkin-Elmer 341 digital polarimeter at 589 nm. Molecular weight was measured by high pressure gel permeation chromatography (HPGPC) with the instruments and conditions as reported in Yan et al. (2010). The calibration equation for average molecular weight versus retention time in the HPGPC was prepared with T-dextran molecular weight standards ranging from 5 to 1400 kDa (Sigma, St. Louis, MO, USA). The monosaccharide constituents of IPS and Smith degradation products were analyzed by gas chromatography (GC) on an Agilent 6890N instrument with a HP-5 fused-silica capillary column (30 m × 0.25 mm × 1 μm) and a flame ionization detector (FID). Infrared (IR) spectra of IPS was recorded in the range of 4000–500 cm^{-1} on a Fourier transform infrared spectrophotometer (FTIR-800, Shimadzu, Japan) with the samples pressed into tablets with KBr. ^{13}C NMR was performed on a Bruker AV-400 spectrometer using 4,4-dimethyl-4-silapentane-1-sulfonic acid at δ 0.0 ppm as an external standard to determine the chemical shifts.

2.5. Chain conformation and particle size of IPS in aqueous solutions

Congo red test was performed of the IPS fractions as described previously (Wang et al., 2010) to detect the triple helix conformation or random coil structures of polysaccharide chains in an aqueous alkaline solution (Ogawa, Watanabe, Tsurugi, & Ono, 1972). In brief, the IPS sample was dissolved in de-ionized water at 1 mg/mL by vigorous agitation, together by drop-wise addition of 4 M NaOH to 0–0.5 M final concentrations. The IPS solution at each alkali concentration was mixed with an equal volume of 91 μM Congo red (Sigma), and the absorbance spectrum was recorded over 400–700 nm at room temperature on a spectrometer. Optical rotation of the alkaline IPS solution (without Congo red) was also measured for comparison.

Particle size distribution of IPS fractions was measured at 25 °C by dynamic light scattering (DLS) using a Malvern Zetasizer Nano (3000 SHA, Malvern Instruments Ltd., UK) at 632.8 nm and 90° scattering angle. The analysis of intensity autocorrelation function was performed with a Laplace inversion program (CONTIN). The sample solution (1.0 mg/mL) was centrifuged (20,000 rpm, 30 min) and filtered through a cellulose membrane (0.45 μm) prior to the measurement. Data was processed with the MAS OPTION software, and a total of five runs were performed for each measurement.

2.6. Animal test on antitumor and immuno-activities of IPS fractions

Antitumor activity of IPS fractions was tested against the growth of melanoma tumors in C57BL/6 mice induced by mouse melanoma B16 cells as reported previously (Wu, Hu, et al., 2007; Wu, Zhang, et al., 2007). The B16 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 mg/mL streptomycin at 37 °C in humidified atmosphere with 5% CO_2. Melanoma cells for inoculation of the mice were harvested from the culture flasks during exponential growth phase with 95% or higher cell viability (Trypan blue exclusion). The C57BL/6 mice (all male, 6–8 weeks old, and 18–22 g body weight) were obtained from Guangdong Provincial Experimental Animal Center (Guangzhou, China). Upon arrival the mice were maintained in the animal room for 3 days. On the 4th day, the mice were divided randomly into groups of 8 for various treatments, each being inoculated with 5×10^5 of the B16 cells by subcutaneous (SC) injection into the hypodermis of left forelimb armpit to induce tumors. The IPS samples and Cytoxan® (cyclophosphamide) (Jiangsu Hengrui Medicine Co. Ltd., Jiangsu, China) as a positive control drug were dissolved in 0.9% NaCl aqueous solution and filtered through 0.22 μm membrane. The drug solutions were administered to the mice at selected doses by intraperitoneal injection (i.p.) daily from day 1 to day 26 after tumor cell inoculation. A control group was also included which was administered with an equal volume of 0.9% NaCl solution. At the end of treatment, the mice were sacrificed by cervical dislocation and the tumors were removed and weighed. The antitumor effect of drug treatment was represented by percent reduction of tumor weight compared with the control. The statistical significance of all treatment effects was evaluated by Student's *t*-tests.

Lymphocyte proliferation assay was performed of the drug-treated mice to detect the treatment effects on the immune cells, following the protocol as reported previously (Lee et al., 2004) with modifications. In brief, spleens were aseptically collected from the sacrificed mice at the end of animal test, chopped into small pieces and sieved through a stainless steel mesh net. The spleen cells recovered were suspended in a lysis buffer (0.15 M NH_4Cl, pH 7.4) for 5 min to remove erythrocytes, followed by incubation for 1 h in Petri dishes at $\sim 5 \times 10^6$ cells/mL to remove adherent cells. The spleen cell suspension was decanted into a lymphocyte separation medium (GE Healthcare) in centrifuge tubes, spun down at 400 g for 20 min, and rinsed with the medium and spun down. The precipitated lymphocyte cells were collected and resuspended in RPMI-1640 medium, and the cell viability was determined by the MTT assay in 96-well plates. The absorbance was measured at 570 nm ($OD_{570\,nm}$) with an ELISA reader and used to represent the cell viability (relative value).

3. Results and discussion

3.1. Purification and chemical properties of IPS

Fig. 1 shows the elution profiles of two IPS fractions, one from the hot water extract and the other from the alkaline extract of Cs-HK1 mycelia on the DEAE-52 cellulose IEC column eluted stepwise with de-ionized water and 0.1, 0.2, and 0.5 M NaCl. With both fractions, the major polysaccharide peaks were eluted out at 0.1 M NaCl at a slightly different elution time, which were collected as WIPS (tube number 48–55) from hot water extract and AIPS (tube number 37–43) from alkaline extract, respectively. The lyophilized WIPS and AIPS exhibited a white color and fibrous morphology. Both WIPS and AIPS exhibited a single and symmetric peak on HPGPC (data not shown), indicating their homogeneity in molecular weight, corresponding to weight-average M_w of 1.18×10^6 Da and 1.15×10^6 Da, respectively. The slightly lower molecular weight of AIPS than WIPS was due probably to alkaline degradation of the polysaccharide during extraction.

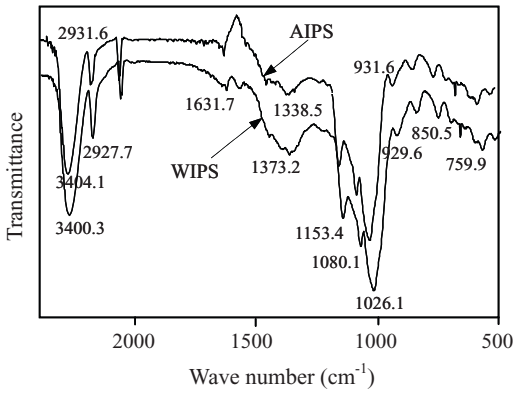

Fig. 2. FTIR spectra of WIPS-1 and AIPS-1.

Both WIPS and AIPS had a total carbohydrate content about 93% and a single monosaccharide D-glucose component. The optical rotation (c 0.20, H_2O) of AIPS was measured as +150, which was close to that of a α-D-glucan isolated from the mycelium extract of a *C. sinensis* fungus with hot phosphate buffer (0.05 M and pH 7.0 at 80 °C), [+146 (c 0.5, H_2O)] (Wu et al., 2006). The optical rotation of WIPS as +129 was close to that of a neutral mannoglucan isolated from the extract of same mycelium with hot acetate buffer (0.05 M pH 6.0 at 85 °C), [+126 (c 0.2, H_2O)] (Wu, Hu, et al., 2007; Wu, Zhang, et al., 2007). There was no absorption peak at 280 and 260 nm in their UV absorption spectra (data not shown), confirming the absence of protein and nucleic acid. These chemical data from our experiments and the literature suggest that the polysaccharides extracted from the *C. sinensis* fungal mycelia in various conditions all contained α-D-glucose and had higher optical rotation and water solubility. The different molecular weights ranging from $\sim 10^3$ to $\sim 10^6$ may be attributed to different degrees of degradation during the IPS extraction, isolation and purification processes.

3.2. Characterization of IPS structures based on spectral data

Fig. 2 shows the FTIR spectra of WIPS and AIPS. The intense broad peak at 3400.3 or 3404.1 cm^{-1} is characteristic of the hydroxyl groups with stretching vibration, and the peak at 2927.7 or 2931.6 cm^{-1} was ascribed to the weak C–H stretching vibration. No peak appearing around 1730 cm^{-1} indicates that uronic acids were absent in WIPS and AIPS. The three peaks at 1153.4 cm^{-1}, 1080.1 cm^{-1} and 1026.1 cm^{-1} on both spectra suggest the presence of C–O and C–C bands, and the peak at 1080.1 cm^{-1}, in particular, was due to the vibration of C–O at the C-4 position of glucose residue (Shingel, 2002). The absorption peak at 850.5 cm^{-1} was ascribed to α-type glycosidic linkage in polysaccharides, and the peak at 929.6 (or 931.6) cm^{-1} attributed to D-glucose in pyranose form (Barker, Bourne, Stacey, & Whiffen, 1954). The two peaks at 850.5 cm^{-1} and 929.6 (or 931.6) cm^{-1} were characteristic of α-(1 → 4)-glucans. Overall the IR spectral characteristics suggest that α-(1 → 4)-D-glucan was the main glycosidic linkage in the polymer structure of WIPS and AIPS. Table 1 shows the GC analysis results of Smith degradation products from WIPS and AIPS. The presence of erythritol in both WIPS and AIPS is suggestive of 1,4-linkage in the main chain. On the hand, the presence of glycerol but no D-glucose in WIPS suggests the existence of 1,6-linkage, and the 1.0:5.86 molar ratio of glycerol to erythritol suggests that the molar ratio of (1 → 6)-glycosyl and (1 → 4)-glycosyl residues was about 1: 6 in the repeating unit of WIPS.

Table 1
GC data of Smith degradation products of WIPS and AIPS.

Constituents	Retention time (min)			Molar ratio	
	Standard	WIPS	AIPS	WIPS	AIPS
Glycerol	4.19	4.19	N.D.[a]	1.00	N.D.
Erythritol	5.81	5.81	5.81	5.86	1.00
D-Glucose	8.68	N.D.	N.D.	N.D.	N.D.

[a] N.D.: not detectable.

Table 2
^{13}C NMR chemical shifts of WIPS and AIPS (δ, ppm).

Sugar residues	C-1	C-2	C-3	C-4	C-5	C-6
WIPS: (1 → 4)-α-D-Glcp (A)	99.21	71.00	72.71	76.10	70.61	59.88
(1 → 4,6)-α-D-Glcp (B)	98.65	71.00	72.30	76.10	69.71	65.93
α-D-Glcp (C)	98.65	70.61	72.71	68.75	70.61	59.88
AIPS:(1 → 4)-α-D-Glcp	99.28	71.02	72.76	76.22	70.62	59.94

Table 2 shows the chemical shifts of the major carbon atom signals on the ^{13}C NMR spectra of WIPS and AIPS, corresponding to the relevant sugar residues according to reference data (Agrawal, 1992; Funane et al., 2001; Uzochukwu, Balogh, Loefler, & Ngoddy, 2002). On the ^{13}C spectrum of AIPS, the anomeric carbon signal at δ 99.28 ppm is ascribed to the C-1 in (1 → 4)-D-glucan, which was another proof for a glucan with an α-glycosidic linkage (Chakraborty, Mondal, Pramanik, Rout, & Islam, 2004). The signal at 76.22 ppm is ascribed to C-4, and the signals at 71.02, 72.76, 70.62 and 59.94 ppm were attributed respectively to the C-2, C-3, C-5 and C-6 of (1 → 4)-α-D-Glcp residues. On the ^{13}C spectrum of AIPS, the signal peaks at δ 99.21 ppm and δ 98.65 ppm are assigned to C-1 of residues (1 → 4)-α-D-Glcp (A) and (1 → 4, 6)-α-D-Glcp (B), respectively, and the down-field shift assigned to C-4 (76.10 ppm) (Agrawal, 1992), indicating that residues A and B are both linked at C-4. In addition, the down-field shift for C-6 (65.93 ppm) in residue B suggests that B is also linked at C-6. Together with other signal values in A and B derived from the ^{13}C NMR experiment (Table 2), residues A and B are identified as (1 → 4)-α-D-Glcp and (1 → 4, 6)-α-D-Glcp, respectively. The signal at δ 98.65 ppm is ascribed to C-1 of residue α-D-Glcp (C); the signals from C-1 to C-6 of residue C match closely with the standard values of methyl glycosides (Agrawal, 1992), further indicative of an α-D-Glcp terminal.

According to all the above experimental and analytical results we conclude that AIPS is a linear, un-branched α-D-(1 → 4) glucan, and WIPS is a slightly branched linear (1 → 4)-α-D-glucan (~86%) with short (1 → 6)-α-D-glucose (~14%) side chains. The complete structures of AIPS and WIPS are represented respectively by,

AIPS: $[\rightarrow 4)$-α-D-Glcp $(1\rightarrow]_n$;

WIPS: $\{\rightarrow[4)$-α-D-Glcp $(1]_5\rightarrow 4)$-α-D-Glcp $(1\rightarrow\}_n$

6
↑
α-D-Glcp 1

Many of the antitumor polysaccharides isolated from mushrooms of basidiomycetes fungi are β-glucans, more commonly with a linear (1 → 3)-β-glycosidic linkage in the main chain and (1 → 6)-β branch points (Ooi & Liu, 2000; Wasser, 2002; Zhang et al., 2007). However, several polysaccharides isolated from mycelium of various *C. sinensis* fungi and extracted in various conditions including hot water, alkaline, acetate buffer or phosphate buffer were all built with a (1 → 4)-α-D-Glcp backbone in our present study and previous studies by Wu et al. (2006), Wu, Hu, et al. (2007) and Wu, Zhang, et al. (2007). *C. sinensis* is taxonomically classified as an Ascomycetes fungus. A unique and more notable feature of the AIPS isolated from the alkaline extract of Cs-HK1 mycelium was its linear structure, compared with the branched structures for most of the polysaccharides derived from *C. sinensis* and other mushroom fungi. Such a simple linear structure for AIPS may afford its potential use as a molecular standard for chemical modification at specific positions of the polysaccharide chain and for evaluation of the structure to biological function relationship. Moreover, the structure of AIPS was somewhat similar to that of amylose which is also a linear (1 → 4)-α-D-glucan. In this connection, AIPS can be an alternative biomaterial to amylose or cellulose for drug delivery and other biomedical applications (Liu, Jiao, Wang, Zhou, & Zhang, 2008). Some highly branched galactoglucomanns have been isolated from the mycelial hot water extracts of a *C. sinensis* fungus (Kiho et al., 1999, 1986), including a protein-containing galactomannan of 23 kDa molecular mass, which was highly branched and composed mainly of β-D-galactofuranosyl residues and a small proportion of α-D-galactopyranosyl residues, and a branched galactoglucomann about 15 kDa, composed of α-D-glucopyranosyl, β-D-galactofuranosyl and α-D-mannopyranosyl residues.

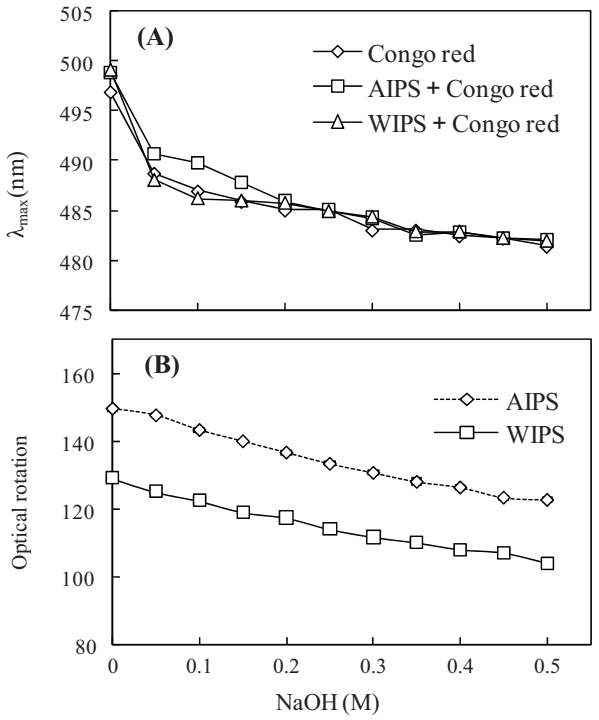

Fig. 3. Structural properties of WIPS and AIPS in aqueous solutions at various NaOH concentrations: (A) absorption maxima of Congo red, Congo red + WIPS, and Congo red + AIPS solutions; (B) optical rotation at 589 nm of WIPS and AIPS solutions.

3.3. Higher-order structures and properties of IPS polymer chains in solution

In the Congo red tests (Fig. 3A), the addition of WIPS or AIPS to the Congo red solution (as AIPS- or WIPS-Congo red) did not cause any notable change in the maximum absorption wavelength λ_{max} between 500 nm and 480 nm as compared with that of Congo red alone over the 0–0.5 M NaOH concentration range, suggesting that WIPS and AIPS chains existed as random coils instead of helical structures in the aqueous solution. Polysaccharide chains in an aqueous solution can form higher-order structures of triple helix conformation or random coils.

Fig. 3B shows the optical rotation trends of WIPS and AIPS versus NaOH concentration, both declining almost linearly with the increase in NaOH concentration. The variable optical rotation of

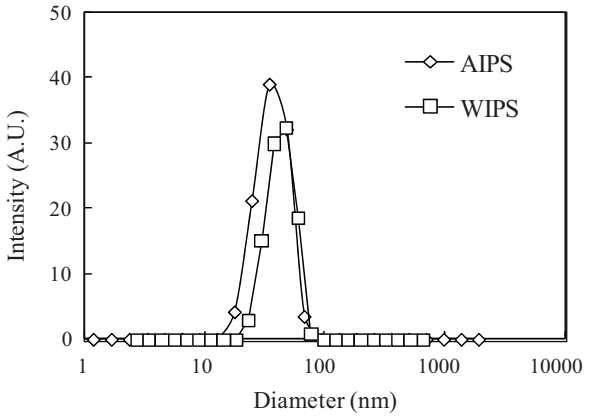

Fig. 4. Particle size distribution of WIPS and AIPS aggregates in water (each at 1.0 mg/mL).

Table 3
Molecular/aggregate size distribution of WIPS and AIPS dissolved/dispersed in water.

Sample	M_w (D_a)	*Z*-Avg diameter	PDI[a]	R_H (nm)	Φ_H (g/mL)[b]
WIPS	1.18×10^6	42.7 nm	0.187	21.4	0.047
AIPS	1.15×10^6	41.5 nm	0.370	20.8	0.050

[a] PDI: polydispersity index.
[b] Φ_H: average polymer density estimated by $\Phi_H = (M_w/N_A)/(4/3\ \pi R_H{}^3)^{-1}$ with the corresponding R_H and M_w values and N_A, the Avogadro's number = 6.02×10^{23}) (Akiyama et al., 2007).

polysaccharides in the alkaline solution is opposed to the characteristic of a helical conformation which retains a stable optical rotation at various NaOH concentrations. In other words, the optical rotation test also suggests that the two IPS fractions existed as random coils but did not form higher-order structures of helical conformation in solution. The presence of a random coil structure in aqueous solutions by both AIPS and WIPS molecules could be associated with their α-D-configuration, for several (1 → 3)-β-D-glucans, such as Lentinan isolated from *Lentinus edodes* and Schizophyllan from *Schizophyllum commune* mycelial culture broth, have been shown retaining a triple helix conformation in aqueous solutions (Ooi & Liu, 2000).

The average particle sizes of WIPS and AIPS aggregates in aqueous solution or dispersion were both distributed in a relatively narrow range around 44.2 and 37.9 nm, respectively (Fig. 4). Table 3 shows several other size parameters of the IPS polymer aggregates in water as determined by various methods. The particle size (Fig. 4) of AIPS as well as its molecular weight (M_w) and mean (*Z*-average) hydrodynamic diameter (R_H) (Table 3) was slightly smaller than that of WIPS. In comparison of the PDI values (Table 4), AIPS with a PDI of 0.370 was more homogenous in size than WIPS (PDI 0.187), since PDI is a quantitative indicator for the uniformity of particle size distribution (maximum 1 for mono-dispersed particles) (Gonçalves & Gama, 2008). WIPS and AIPS had nearly an equal average polymer density about 0.05 g/mL, which means 5.0 wt% of polysaccharide and 95% water in the particles, typical of a hydrogel. In view of all the size parameters in Table 4, WIPS and AIPS had very similar molecular properties except for the size distribution in an aqueous solution.

Table 4
Treatment effects of AIPS, WIPS and positive control drug Cytoxan (CTX) on melanoma tumor, animal growth and immune function in C57BL/6 mice.[a]

Drug (dose in mg/kg d)	Tumor weight (g)	Tumor inhibition (%)	Body weight gain (%)	T-cell viability ($OD_{570\,nm}$)
Control	5.93	0.0	27.2	0.342 ± 0.027
CTX (50)	0.52	91.23**	0.0	0.167 ± 0.032***
AIPS (200)	4.27	28.00*	21.2	0.466 ± 0.033***
WIPS (400)	5.21	12.14*	27.7	0.401 ± 0.030**

[a] Significance level, *$p < 0.05$; **$p < 0.01$; ***$p < 0.01$.

3.4. Antitumor activity

Table 4 shows the animal test results for the treatment effects of AIPS and WIPS against B16-melanoma tumor growth in mice. AIPS exhibited moderate antitumor activity with about 28% inhibition of the tumor growth, but WIPS exhibited a much lower activity with only 12% tumor inhibition. The positive control drug Cytoxan (CTX), a clinical drug for chemotherapy, exhibited a strong antitumor effect with more than 90% inhibition of the tumor growth, which was an adequate validation of the animal test protocol. However, CTX also caused strong inhibition of the animal growth, stopping completely the increase of body weight, while AIPS caused a slight and WIPS caused no inhibition of the animal growth, suggesting that the fungal polysaccharides were not or much less toxic than the chemical cancer drug CTX to animals.

Results from the lymphocyte proliferative assay (Table 4) showed that both AIPS and WIPS showed an enhancement effect on the T-cell proliferation or viability at high significance levels. The results suggest that AIPS and WIPS stimulated the host-mediated immunity, which may be attributable to the antitumor function. As biological response modifiers, the fungal polysaccharides exerted their antitumor action mainly via activation of the immune response of the host cells (Ooi & Liu, 2000; Wasser, 2002). In contrast, CTX had a significantly suppressing effect on the T-cell viability and its antitumor effect may be mainly attributed to the toxic effect.

4. Conclusions

Two polysaccharide molecules, WIPS and AIPS, were isolated respectively from hot water and alkaline extracts of the Cs-HK1 fungal mycelium. Both were characterized as homogeneous α-D-glucans and were similar in several molecular and solution properties including the molecular weight and high-order structures (random coil, particle size and distribution) in aqueous solutions. The major difference between the two was that AIPS was a linear and WIPS was a branched α-D-glucan. In addition, AIPS exhibited more significant antitumor and immuno-stimulating effects than WIPS in the animal test against melanoma tumor growth in mice. The highly linear structure of AIPS was distinctive as compared with the branched structures of most antitumor glucans from edible and medicinal fungi documented previously.

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