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ORIGINAL PAPER

Characterisation and bioactivity of protein-bound polysaccharides from submerged-culture fermentation of *Coriolus versicolor* Wr-74 and ATCC-20545 strains

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Abstract The protein-bound polysaccharides of *Coriolus versicolor* (CPS) have been reported to stimulate overall immune functions against cancers and various infectious diseases by activating specific cell functions. A New Zealand isolate (Wr-74) and a patented strain (ATCC-20545) of *C. versicolor* were compared in this study. The fruit bodies of both strains were grown for visual verification. Both strains were grown in submerged-culture using an airlift fermentor with milk permeate as the base medium supplemented with glucose, yeast extract and salt. Metabolic profiles of both strains obtained over 7-day fermentation showed very similar trends in terms of biomass production (8.9–10.6 mg/ml), amounts of extracellular polysaccharide (EPS) from the culture medium (1150–1132 μg/ml), and intracellular polysaccharide (IPS) from the mycelium (80–100 μg/ml). Glucose was the dominant sugar in both EPS and IPS, and the polymers each consisted of three molecular weight fractions ranging from 2×10^6 to 3×10^3 Da. Both the EPS and IPS were able to significantly induce cytokine production (interleukin 12 and γ interferon) in murine splenocytes in vitro. Highest levels of interleukin 12 (291 pg/ml) and γ interferon (6,159 pg/ml) were obtained from samples containing Wr-74 IPS (0.06 μg/ml) and ATCC 20545 IPS (0.1 μg/ml), respectively. The results indicated that lower levels of EPS and IPS generally resulted in higher immune responses than did higher polymer concentrations.

Keywords *Coriolus versicolor* · Fruiting · Polysaccharides · Bioactive · Cytokines · Optimum dose · Molar mass · Milk permeate

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Introduction

The biopolymers produced by *Coriolus versicolor* (also known as *Yun Zhi*) are protein-bound polysaccharides (denoted as Coriolus polysaccharides, CPS), which are known to display a wide range of biological activities. CPS are used as anti-cancer agents, immunopotentiators and biological response modifiers [2, 3, 8, 9, 15, 20, 25, 30]. Although the exact anti-tumor action is still not completely clear, some studies have suggested action through the ability of this biopolymer to activate overall immune function by enhancing proliferation of T- and B-lymphocytes and the cytotoxic activity of NK cells to inhibit attacks from pathogens or growth of various cancers [3, 6, 10, 14, 17, 19, 24, 25]. Over the last three decades, scientific and medical research carried out in Japan, China, Korea and more recently in the USA, have demonstrated the distinct physiological activities of the fungal polysaccharides [5, 26].

Belonging to the higher class of fungi-*Basidiomycetes*, *C. versicolor* has long been treasured in the East for its

medicinal value. Of more than 200 medicinal mushrooms, those few yielding CPS are commercially the most established. During the 1970s, Japanese scientists began extensive controlled clinical research on the CPS. The first commercial CPS preparation known as PSK was obtained from CM-101 strain (ATCC 20547), produced through submerged-culture fermentation by Japanese Kureha Chemical Industries Co. Ltd in 1977. PSK was subsequently approved for human use in Japan by the Japanese government Health and Welfare Ministry as a chemoimmunotherapy agent in the treatment of cancer [30]. By 1985, it ranked nineteenth on the list of the World's most commercially successful drugs [30]. Another well documented patented biopolymer (known as polysaccharopeptide or PSP) of *C. versicolor* was obtained from the *Cov-1* strain [16, 29]. Cui and Christi [5] comprehensively reviewed the physiological activity, uses and production.

In spite of the long history of the recognition for its efficacy, application of CPS beyond the pharmaceutical arena has not been fully explored. With the increasing concern for longer lifespan and better quality of life, CPS may find new application in the food industry as a novel bioactive ingredient.

The aim of this study was to characterize the physicochemical properties and bioactivities of CPS produced through submerged-culture fermentation by Wr-74 strain isolated in New Zealand. The CPS for Wr-74 was compared to those of ATCC-20545 strain (also known as CM-103, one of the strains used in commercial production of PSK) in terms of molecular weight; monosaccharide and amino acid compositions; and its physiological activity measured as cytokine production. The CPS assayed may be obtained from the culture medium, denoted as extracellular polysaccharides (EPS), and from the mycelium, denoted as the intracellular polysaccharides (IPS). The base medium used in this study was ultrafiltration permeate of milk. This is a food material available in high quality and quantity on several industrial sites in New Zealand.

Materials and methods

Organisms

The *C. versicolor* strain CM-103 (deposited by Kureha Chemi. Ind. Co., Ltd, Japan) was obtained from the American Type Culture Collection (Rockville, MD, USA) and denoted as ATCC-20545. A strain isolated in New Zealand by the New Zealand Forest Research Institute (Rotorua, New Zealand) was denoted as White rot 74 (Wr-74).

Submerged-culture fermentation

A modified 7-l airlift bioreactor (46 cm in height and 14.5 cm in diameter), with two impellers was used for the submerged-culture fermentation of the mycelia (see Fig. 1). One of the impellers was a two-blade helical impeller mounted on a shaft approximately 5 cm above the vessel base which assisted the uplift of thick culture medium during the advanced stage of fermentation. The other impeller, a six-blade Rushton turbine was mounted on the same shaft approximately 9 cm above the helical impeller. This impeller helped to reduce the size of the air bubbles to increase the level of dissolved oxygen in the broth. Both impellers were housed in a 20 cm (length) by 9 cm (diameter) central draught-tube with annular liquid downflow to achieve effective broth circulation. Filtered air (filtered using 0.22 µm filter, Millipore Corp. Bedford, MA, USA) was sparged through sintered glass from the base of the vessel below the draught tube.

The growth medium consisted of 100 g glucose (Merck, Darmstadt, Germany), 30 g yeast extract (Benton Dickson, MI, USA), 15 g $(NH_4)_2SO_4$, 10 g KH_2PO_4, 2.5 g $MgSO_4 7H_2O$ and 2.5 g KCl dissolved in water (2.5 l) plus milk permeate (2.5 l, Fonterra Cooperative Ltd, Longburn, New Zealand). The medium was sterilized at 121°C for 15 min. The inoculum (500 ml) was prepared in the same medium in 500 ml shake flasks (each flask contained 100 ml medium) with an agitation speed of 180 rpm at 27°C for 5–7 days in a rotational shaker (Environ Shaker,

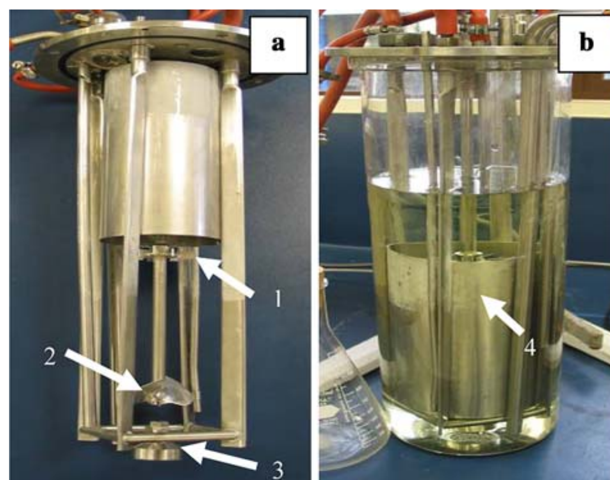

Fig. 1 **a** Internal construction of the modified air lift fermenter. *1* 6-blade Rushton turbine, *2* two blade helical impeller, *3* air sparged through sintered glass; **b** Fermenter with growth medium ready for inoculation. *4* draught-tube to promote broth circulation

Lab-line Instruments, IL, USA). Fermentation in the airlift bioreactor was carried out in batch mode at an agitation speed of 145 rpm and an air flow rate of 4 l/min for approximately 7 days at 27°C. Sterilized water was added to the fermented broth to compensate for the loss of moisture every 24 h and maintained a 5.0 l fill level.

The relative viscosity (η_r) of the fermented broth at the end of the fermentation was measured using a Cannon–Fenske capillary viscometer (Viscometer no. 100, 821 K, Cannon Instrument Co., PA, USA). The mycelium was removed from the broth by centrifugation (4,000*g*, 10 min, 20°C) prior to viscosity measurement. Each sample (5 ml) was loaded into the capillary viscometer and allowed to equilibrate for ~5 min before measuring the efflux time. Density of the broth was measured using a pycnometer (Brand, Wertheim, Germany) at 20°C. The η_r of the sample was measured at 20°C using water at 20°C as a reference sample:

$$\eta_r = \frac{t\rho}{t_s\rho_s},$$

where t, t_s ρ and ρ_s, are the efflux time and densities of the sample and the water, respectively.

The amount of reducing sugar utilized during fermentation was determined using the dinitrosalicylic acid (DNS) method [21] measured at 272 nm against a glucose standard curve.

The biomass was collected by filtration of the fermented broth (50 ml) using a Buchner funnel and Whattman filter paper (Whattman, FL, USA; No. 541). The biomass was washed thoroughly with distilled water and dried at 105°C for 5 h to a constant weight. This was carried out in replicate.

Fruit bodies

To verify that strains were *C. versicolor*, they were cultivated to form fruit bodies for visual examination. The fungi were cultivated singly in bags containing 3 kg wood chips of *Photinea*, supplemented with the 300 g maize starch, 600 g corn meal and 4 kg water. After sterilization at 121°C for 30 min and cooling, the bags were inoculated with the stock cultures of ATCC-20545 or Wr-74 and incubated for 10–15 days at 25°C to allow the mycelia to grow and cover the entire solid substrate. The bags were then ripped open and transferred to a moist external environment with a temperature ranging from 15 to 20°C. The formation of fruit bodies took place after approximately 30 days. Morphological characteristics of the fruit bodies were examined.

Isolation of extracellular and intracellular polysaccharides

Mycelial biomass from the fermentation broth was separated by centrifugation (2,100*g*, 10 min). The supernatant was added to four volumes of 95% (v/v) ethanol to precipitate the extracellular polysaccharide (EPS). The EPS was recovered by centrifugation (2,100*g*, 20 min) and freeze-dried. The mycelial biomass pellet was heated in water (90°C) for 2 h to release the intracellular polysaccharide (IPS). The mixture was filtered through Whatman No. 541 filter paper (Whatman, FL, USA), in a Buchner funnel by suction and the extract collected as filtrate from the biomass. The IPS in the extract was recovered from this filtrate using the same procedure as the EPS recovery. EPS and IPS levels were quantified by the phenol/sulphuric acid method [7] using glucose as a standard curve. The key steps involved in EPS/IPS isolation and quantification are shown in Fig. 2.

Amino acid composition

Quantitative amino acid analysis was performed by the AOAC method [1] using a Waters^{TM} HPLC system and Waterton^{TM} cation exchanger amino acid analysis column (Waters Corporation, MA, USA). A mixture of standard amino acids was used for comparison.

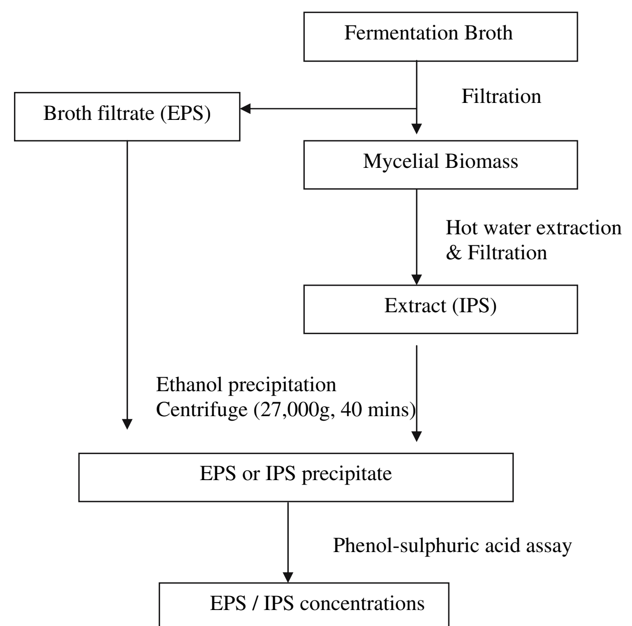

Fig. 2 Key steps involved in isolating and quantifying EPS and IPS from the fermentation broth

Sugar composition

Freeze dried samples of EPS and IPS (~5 mg each) were redispersed in 2 M aqueous trifluoroacetic acid (Sigma-Aldrich, St Louis, MO, USA; 2 ml) and heated in a sealed tube at 100°C for 20 h. After cooling, the hydrolysed samples were evaporated to dryness using a rotary evaporator. The sample was then redissolved in 2 ml Milli-Q water (water purified by treatment with a Milli-Q apparatus, Millipore Corp. Bedford, MA, USA) and filtered through a 0.2 μm membrane filter (Millipore Corp. Bedford, MA, USA). Sugar composition of the polysaccharides was determined using a Waters™ 2690 Separation module HPLC platform (Waters Corporation, MA, USA) and the Aminex HPX-87H column (Bio-Rad Laboratories, CA, USA). The monosaccharides were separated isocratically using a mobile phase containing 0.018 M sulphuric acid was and operated at a flow rate 0.6 ml/min. Separate standard solutions of glucose, galactose, rhamnose, mannose, xylose, fructose and arabinose (7.5 g/l each) in Milli-Q water were prepared and filtered through a Millipore filter (0.2 μm) prior to sample injection.

Determination of molar mass

The weight-average molecular weights (M_w) of the polysaccharides (i.e. EPS, IPS and the mushroom extract) were determined using size-exclusion chromatography (SEC) coupled to a multi-angle laser light scattering (SEC-MALLS) device. A size-exclusion chromatography column (Shodex SB-806 HQ HPLC column, Shoko Co., Tokyo, Japan) was used in a HPLC system (GBC Scientific Equipment Ltd, VIC, Australia). The mobile phase consisted of filtered (0.025 μm, Millipore Corp. Bedford, MA, USA) Mili-Q water containing 0.02% w/v sodium azide (Serva, Heidelberg, Germany). The mobile phase was degassed and pumped through the HPLC column at a flowrate of 0.5 ml/min at 20 psi. The eluant from the SEC column passed through the UV detector set at 280 nm, the DAWN DSP MALLS (Wyatt Technology, Santa Barbara, CA, USA) photometer (fitted with 16 photo detectors at different angles, a helium neon laser with an operating wavelength of 632.8 nm, and a K-5 flow cell) and then through the differential refractive index (DRI) detector (Waters, model R401, Milford, MA, USA). Toluene (Riedel-deHaën, Hannover, Germany) was used as a reference with a Rayleigh ratio of 2.34×10^{-5} cm^{-1}. Dextran solution (1%, w/v, M_w: 9,300 Da, Sigma-Aldrich, St Louis, MO, USA) was used for normalization before introducing the samples. The polysaccharide sample (20 μl) was loaded and separated at 25°C over approximately 40 min elution time. A dn/dc value of ~0.14 ml/g was obtained for both EPS and IPS samples. Astra software (version 4.5, Wyatt Technology Corp., Santa Barbara, USA) was used to analyse the data using the Zimm fit method to determine the M_w of the polymers. The Zimm method [28] of data fitting was carried out by plotting Kc/R_θ versus $\sin^2(\theta/2)$ as:

$$\frac{Kc}{R_\theta} = \frac{1}{M_w P_\theta} + 2A_2 c,$$

where $R_{(\theta)}$ is the Rayleigh excess scattering, K is the optical constant, c is the solute concentration (g/ml), M_w is the weight-average molar mass and P_θ is the scattering function.

Physiological test based on cytokine production by murine splenocytes

The spleens from four female mice, 6–8 weeks old, served as the source of murine splenocytes for cytokine production. The mice were euthanised. Their spleens were removed and washed with a tissue culture medium, which was made up of RPMI 1640 medium (Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% (v/v) foetal bovine serum (Gibco-BRL, Paisely, UK), 100 mM L-glutamine, 2 μM 2-mercaptoethanol, 100 μg/ml penicillin-G and 100 μg/ml streptomycin sulphate. The spleens were cut into small pieces and suspended in 5 ml ACK lysis buffer (150 mM NH_4Cl; 10 mM $KHCO_3$; 0.1 mM EDTA; BDH, Poole, UK) and left for approximately 5 min to allow lysis of the red blood cells. The spleen cells were washed twice in suspension with phosphate buffered saline (PBS, Gibco-BRL, Paisely, UK) and centrifuged after each washing at 500g for 10 min. The cell pellet was resuspended in the tissue culture medium (3 ml) and the number of cells was counted using a FACS Calibur flow cytometer (Becton Dickinson, CA, USA). Final cell concentration was adjusted to 2×10^6 cells/ml.

The aqueous solutions of EPS and IPS of ATCC-20545 and Wr-74 were sterilized using cobalt-60 gamma irradiation. Each of the samples was serially diluted using the tissue culture medium to obtain four different concentrations. The polysaccharide samples (each in triplicate) were transferred into a 96-well plate (Corning, Acton, MA, USA). Each well contained 50 μl sample, 50 μl cell suspension and 50 μl stimulant solution. The positive control contained the tissue culture medium (50 μl) instead of the aqueous sample whereas the negative control contained only the cell suspension (50 μl) and the culture medium (100 μl).

For interleukin-12 (IL-12) induction, the stimulant used in one of the 96-well plate was *Staphylococcus aureus* strain Cowan (*SAC*, Sigma-Aldrich, St Louis, MO, USA; 1.5 μg/ml). This plate was incubated at 37°C for 20 h. In another 96-well plate, *Concanavaline* A (*Con* A, Sigma-Aldrich, St Louis, MO, USA; 2.5 μg/ml) was used as stimulant in the induction of gamma-interferons (IFN-γ). This plate was incubated at 37°C for 48 h in a 5% CO_2 atmosphere. After incubation, the supernatant from each well was collected for the quantification of IL-12 and IFN-γ using the ELISA technique (Greiner Bio-one, Gloucestershire, UK).

Results

Strains for producing EPS/IPS and their metabolic patterns

There are 39 species of *C. versicolor* recorded in the American Type Culture Collection (ATCC) organization. Of the 16 strains deposited in ATCC by Kureha Chemical Industry Ltd, the CM-103 strain (ATCC-20545) has been widely reported to produce high levels of CPS [11]. In the strain screening study, a high EPS producing strain, denoted as Wr-74, was obtained from the New Zealand Forest Research Institute (Rotorua, New Zealand). The culture was isolated from New Zealand by this institute.

Submerged-culture fermentations of ATCC-20545 and Wr-74 were carried out over 7-day duration in the modified air-lift fermentor. All measurements were carried out in duplicate. Both fermented broths appeared viscous with relative viscosity values reaching ~43 and ~65, respectively. The metabolic profiles of ATCC-20545 and Wr-74 strains were fairly similar over the duration of the fermentation (Fig. 3). The Wr-74 strain utilized a higher amount of reducing sugar from the medium than ATCC 20545 (~51% utilized) with very similar pH (Fig. 3a) at the end of the 7-day fermentation. The biomass profiles for both cultures increased steadily throughout the fermentation, with ATCC-20545 achieving a higher level of biomass (~10.6 mg/ml) compared to Wr-74 (~8.9 mg/ml). Both levels are higher than biomass yields reported recently where values ranged from 2.3 to 4.2 mg/ml [23].

Figure 3b shows that EPS and IPS production by both ATCC 20545 and Wr-74 continued throughout fermentation. EPS and IPS levels increased almost linearly for 162 h despite the pH having bottomed out at 114 hrs. In contrast to the relative biomass levels, Wr-74 gave higher product titres than ATCC-20545. This is shown graphically in Fig. 3c where Wr-74 gave consistently the higher yield of IPS per unit biomass ($Y_{IPS/x}$). Furthermore, ATCC-20545 appears to produce a relatively constant $Y_{EPS/x}$ throughout fermentation in marked contrast to Wr-74 where $Y_{EPS/x}$ continues to climb (Fig. 3c). At all stages ATCC-20545 produced the superior yield of EPS on sugar utilized by virtue of its lower utilization of the sugar available (Fig. 3d).

The levels of EPS obtained in this study were higher than those reported earlier by Tavares et al. [23] who observed maximum EPS levels ranging between 274 and 700 μg/ml from various strains of *C. versicolor*.

Other differences between ATCC-20545 and Wr-74 were noted in the appearance of the fermented broths, with Wr-74 showing less yellowness in color and a lower intensity of a ‘‘sweet’’ odor.

It is known that the culture medium plays an important role in both EPS production and mycelial growth. These results show that milk permeate, sometimes considered an industrial waste, can be a useful base medium for the fermentation of *C. versicolor* for EPS/IPS production.

Morphological examination of the fruit bodies

The fruit bodies formed had smooth and velvety brackets with variously coloured zonation. Fruit bodies of both strains grown on the logs were densely overlapped without any stalks. The brackets were semicircular, flattened, thin and tough. The appearance was consistent with the description of typical *C. versicolor* [18]. The surface of ATCC-20545 fruit body appeared dark brown while that of Wr-74 fruit bodies had lighter brown zonal patterns on the brackets. Both colors were included in the description of the *C. versicolor* fruit bodies by Soothhill and Fairhurst [22].

Composition of EPS and IPS

The monosaccharide compositions of the EPS and IPS samples were analyzed using HPLC. Glucose was clearly separated from the other sugars and was the dominant sugar present in both EPS and IPS samples (~93.9% in Wr-74 EPS, ~79.1% in Wr-74 IPS; ~86.3% in ATCC-20545 EPS, ~72.2% in ATCC-20545 IPS), with a higher proportion found in EPS than in IPS. (Note that the percentage values obtained were based on the amounts of total carbohydrate in EPS and IPS, respectively.) The proportion of glucose in IPS was also found to be very close to the reported value of ~75% obtained from the fruit body extract [12] while Wang et al. [25] reported that the EPS isolated contained only glucose.

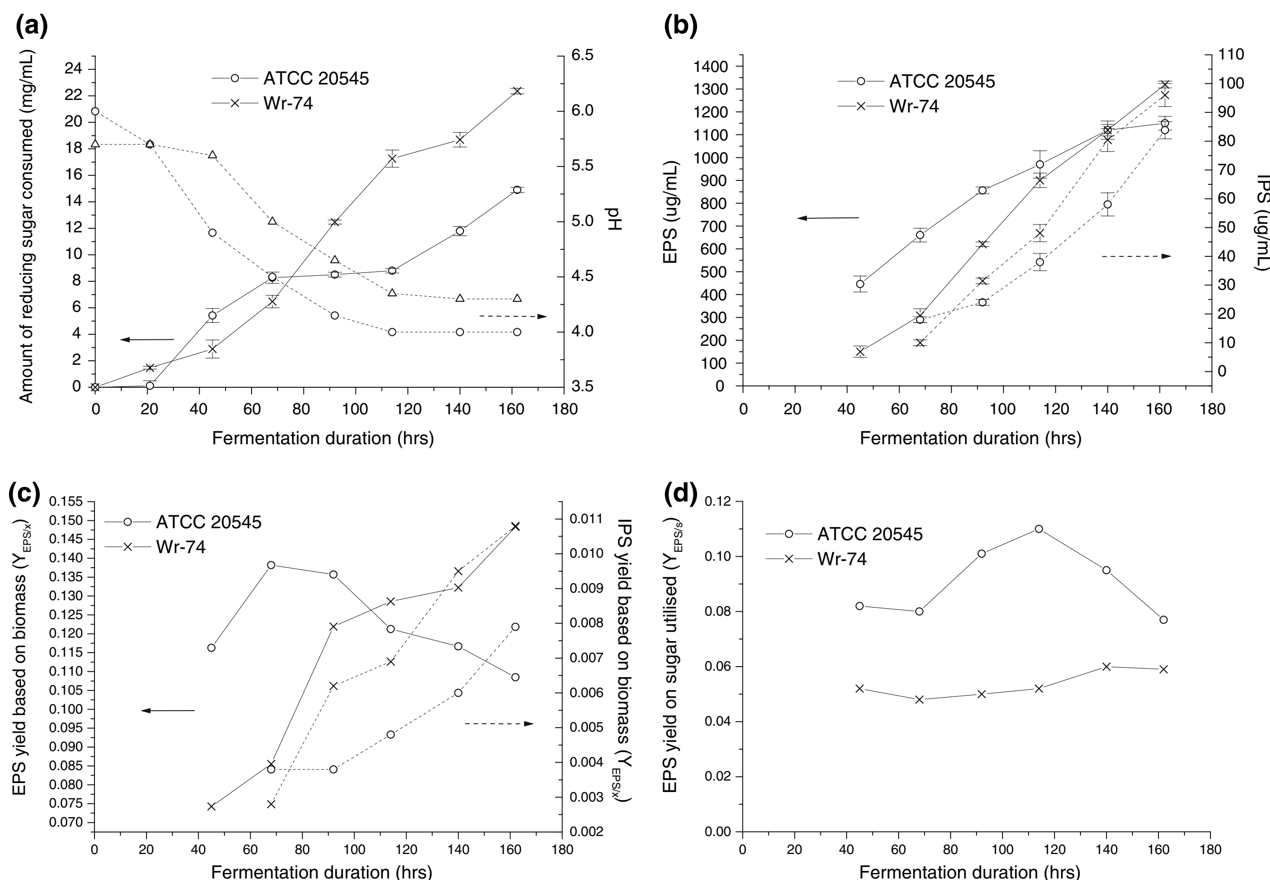

Fig. 3 Comparison of the metabolic profiles of strains ATCC-20545 and Wr-74 in terms of the **a** amount of reducing sugar utilized and changes in pH of the growth media during fermentation; **b** amount of EPS and IPS produced; **c** EPS yield ($Y_{EPS/x}$) and IPS yield ($Y_{IPS/x}$) based on biomass; and **d** EPS yield based ($Y_{EPS/s}$) on sugar utilized (*error bar* indicates mean ± standard error, $n = 2$)

The other sugars were difficult to separate, but appeared to include galactose, mannose, rhamnose and xylose. Minor sugars reported by other workers included mannose, xylose, galactose, rhamnose, arabinose and fucose [12, 15]. The variation in the results could be due to the different fungal strains which were reported to have slightly different monosaccharide composition [11, 30]. In addition, the different purification procedures used to obtain the polysaccharide samples could include contaminants from cellular materials and/or medium components.

Molecular weights of EPS and IPS

The absolute weight-average molecular weights (M_w) of the EPS and IPS from ATCC-20545 and Wr-74 were analyzed using SEC-MALLS. The SEC-MALLS chromatograms showed three prominent fractions denoted as Peak 1, 2 and 3 (Fig. 4). The shape of the chromatograms (light scattering, UV and RI signals) for both strains especially between EPS of Wr-74 and ATCC-20545 were very similar (Fig. 4a, c). In addition, the weight-average molar mass of each of the three fractions (Table 1) were almost identical between the two strains. It must be noted that very similar molar mass range does not imply similar molecular structure and molecular conformation. The chromatograms between the IPS samples from the two strains were also quite similar (Fig. 4b, d). The molecular weight fractions obtained in Peak 1 and Peak 2 had very close molecular weight range except for Peak 3 where the IPS of Wr-74 contained a higher molar mass fraction than that of ATCC-20545.

From the RI signals of all the chromatograms, it is evident that the largest molecular weight fraction (~2×10^6 Da, Peak 1) was present at a very low concentrations (2–3.8%) compared to that of the intermediate molecular weight fraction (13–30%). The smallest molecular weight fraction was present in the higher concentrations (66–85%) in the EPS and IPS samples of both cultures. The UV signal appearing in parallel with Peak 2 suggest the presence of proteins.

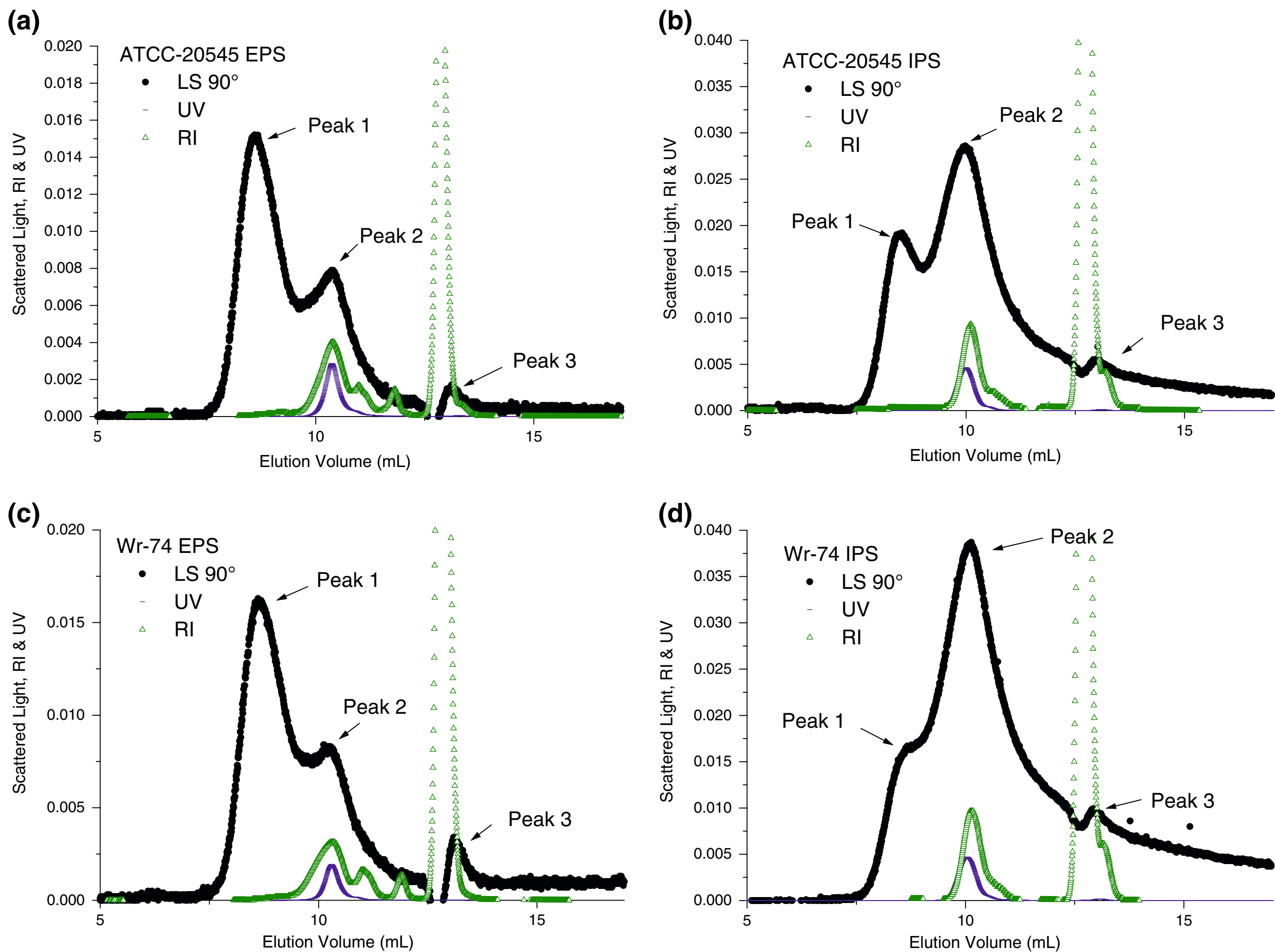

Fig. 4 Chromatogram obtained from SEC-MALLS showing light scattering signal at 90° (*circle*), UV signal (*line*) and DRI signal (*triangle*) plotted as a function of elution volume for **a** ATCC-20545 EPS (area under the RI chromatogram for Peak 1 = 3.8%, Peak 2 = 30%, Peak 3 = 66.2%), **b** ATCC-20545 IPS (area under the RI chromatogram for Peak 1 = 3.5%, Peak 2 = 15%, Peak 3 = 81.5%), **c** Wr-74 EPS (area under the RI chromatogram for Peak 1 = 2%, Peak 2 = 16.8%, Peak 3 = 82%) and **d** Wr-74 IPS (area under the RI chromatogram for Peak 1 = 2%, Peak 2 = 13%, Peak 3 = 85%) solutions at a concentration of 2.58, 1.45, 2.69 and 1.02 mg/ml, respectively

Table 1 Weight-average molecular weights of EPS and IPS fractions separated by size exclusion column

	Molecular weight (Da)		
	Peak 1	Peak 2	Peak 3
EPS			
ATCC-20545	2.1×10^6	2.4×10^4	2.7×10^3
Wr-74	2.3×10^6	2.6×10^4	3.0×10^3
IPS			
ATCC-20545	2.0×10^6	1.4×10^5	7.1×10^3
Wr-74	2.3×10^6	1.5×10^5	1.2×10^4

The three molecular weight fractions (of both IPS and EPS) within each strain were similar and could imply that the molecular species excreted into the medium were synthesized within the cells. The only exception was a slightly higher molar mass in the IPS fraction (Peak 3) of Wr-74 strain as compared to Peak 3 of the EPS fraction.

Amino acid composition of the protein fraction of EPS/IPS

The amino acid compositions of the protein content of the EPS and IPS (Table 2) indicated that the IPS samples from ATCC-20545 and Wr-74 contained higher levels of amino acid than the EPS samples. The amino acids in IPS (in decreasing amounts) included aspartic acid, glutamic acid, glycine, alanine, methionine, tyrosine, phenylalanine, histidine, lysine and arginine. Large amounts of aspartic acid and glutamic acid have also been found for PSP [15]. The results also showed that IPS contained a relatively high level of

tryptophan, which was absent in EPS. One possible reason for the abundance of amino acids in IPS can be explained if part of the intracellular functional or structural proteins or peptides might be released together with IPS from the mycelia during the hot water extraction. The acidic amino acids and the neutral amino acids are predominant in IPS as also reported by Hotta et al. [11]. The amount of acidic and neutral amino acid in IPS obtained from ATCC-20545 and Wr-74 accounted for 72 and 76% on total amino acids basis, respectively (Table 2). The EPS samples from ATCC-20545 and Wr-74 contained higher proportions of acidic and neutral amino acids (96 and 95 %, respectively).

Effects of EPS and IPS on cytokine production

The effects of the EPS and IPS on the murine splenocytes to produce cytokines IL-12 and IFN-γ were investigated using the ELISA technique. The dose-dependence curve shown in Fig. 5 indicates that amount of IL-12 produced by SAC-stimulated murine splenocytes increased markedly after 48-h incubation with the EPS and IPS at various concentrations. Interestingly, in the general trend shown in Fig. 5, significantly higher

Table 2 Amino acid composition of EPS and IPS from Wr-74 and ATCC-20545 strains

Amino acids	ATCC-20545 IPS	ATCC-20545 EPS	Wr-74 IPS	Wr-74 EPS
Acidic AA (%, w/w)				
Aspartic acid	2.130	0.469	2.535	0.228
Threonine	0.864	0.384	0.863	0.149
Serine	1.069	0.406	1.378	0.147
Glutamic acid	1.882	0.313	1.906	0.157
Proline	0.623	0.305	0.600	0.100
Neutral AA (%, w/w)				
Glycine	1.266	0.360	1.119	0.147
Alanine	0.983	0.282	0.953	0.125
Cysteine	0.027	0.158	0.023	0.035
Valine	0.660	0.437	0.617	0.157
Methionine	0.132	0.016	0.132	0.013
Isoleucine	0.378	0.259	0.367	0.101
Leucine	0.675	0.420	0.692	0.170
Tyrosine	0.372	0.024	0.479	0.021
Phenylalanine	0.389	0.079	0.403	0.073
Basic AA (%, w/w)				
Histidine	0.361	0.051	0.332	0.025
Tryptophan	1.184	–	0.924	–
Lysine	1.595	0.127	1.473	0.048
Arginine	1.375	0.045	1.210	0.038
Total amino acids (%, w/w)	15.965	4.135	16.006	1.734

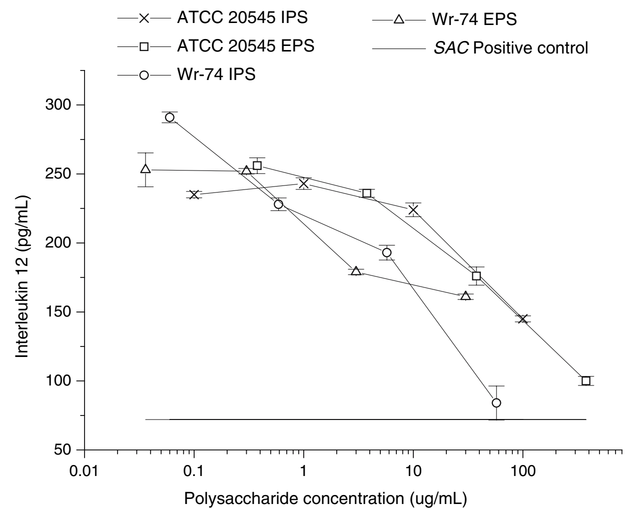

Fig. 5 Comparisons of IL-12 levels produced by murine splenocytes after co-cultured with EPS or IPS from ATCC-20545 or Wr-74 at different polysaccharide concentrations. The level of IL-12 in the positive control is represented by the horizontal line parallel to the X-axis (72 pg/ml). *Error bar* represents mean ± standard error, $n = 3$

IL-12 levels were induced at lower EPS/IPS concentrations (<10 μg/ml). This could imply a complex response and indicates the importance of applying the polysaccharide at an optimal concentration for maximum modulation of the immune response. The results based on analysis of variance (ANOVA, P value < 0.05) also indicated that Wr-74 IPS could induce significantly higher levels of IL-12 than ATCC-20545 IPS at the lowest concentration of Wr-74 IPS. Based on the increasing levels of IL-12 with decreasing concentration of Wr-74 IPS, higher levels of IL-12 could possibly be achieved if Wr-74 IPS were dosed at an even lower concentration (i.e. <0.06 μg/ml). For ATCC-20545 IPS, at concentration between 10 and 0.1 μg/ml, the IL-12 levels appeared to plateau which could suggest that it had reached its optimal concentration and was less effective than Wr-74 IPS in stimulating IL-12 production. In the case of EPS, both Wr-74 and ATCC-2055 at ~0.30 μg/ml induced very similar levels of IL-12.

Figure 6 shows the levels of IFN-γ produced by Con A-stimulated murine splenocytes after 48 h incubation with the EPS and IPS. Again, higher levels of IFN-γ were induced at lower polysaccharide concentrations. ATCC-20545 IPS was found to induce higher levels of IFN-γ than Wr-74 IPS at IPS concentrations of 0.1 μg/ml and 0.06 μg/ml, respectively. In the case of EPS, both Wr-74 EPS and ATCC-2055 EPS induced very similar levels of IFN-γ concentrations (~4,400 pg/ml) at their optimal concentrations of 0.036 and 0.37 μg/ml, respectively.

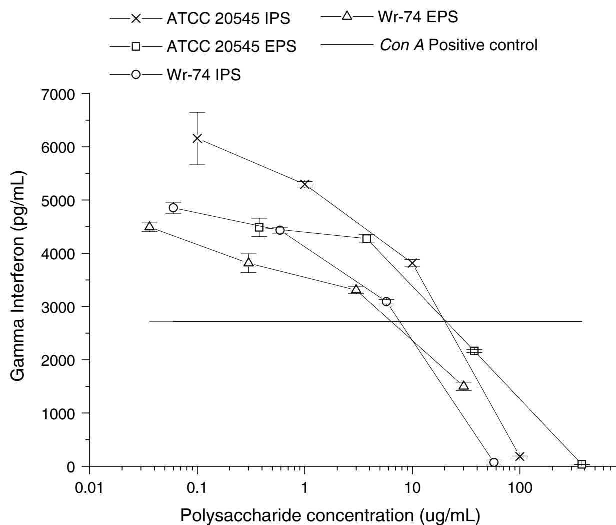

Fig. 6 Comparisons of IFN- γ levels produced by murine splenocytes after co-cultured with EPS or IPS from ATCC-20545 or Wr-74 at different polysaccharide concentrations. The level of IFN-γ in the positive control is represented by the horizontal line parallel to the X-axis (1,874 pg/ml). *Error bar* represents mean ± standard error, $n = 3$

Discussion

High yields of EPS and IPS were produced by the submerged-culture air-lift fermentation of *C. versicolor* using milk permeate as a base medium. The metabolic profile (biomass, EPS, IPS, pH), sugar and amino acid compositions of EPS and IPS, weight-average molar mass distributions and biological activities of Wr-74 strain were comparable to ATCC-20545 strain. This study shows that the amount of EPS recovered was approximately 10 times higher than IPS obtained from the mycelium for both strains.

The isolated IPS and EPS consisted of three molecular weight fractions ranging from ~2.0×10^6 to 2.7×10^3 with a large proportion (66–85%) belonging to the smallest fraction. Glucose was found to be the dominant sugar present in both IPS and EPS and could imply that the biopolymers were mainly glucan. The polymers also contain proteins which appeared in the intermediate molecular weight fraction. This fraction could be the protein-bound polysaccharide reported in the extracts of all strains of *C. versicolor* reported so far [11, 30]. In relation to another recent study, three polysaccharide fractions were also obtained from the hot water extract of the fruit bodies of *C. versicolor* [12]. The molecular weights of these fractions were reported to be 1.2×10^6, 1.5×10^5 and 1.5×10^3 Da obtained by gel permeation chromatography and the smallest fraction was also present as the highest concentration [12]. Some earlier studies appeared to have reported the presence of molecular species approximately 1.0×10^5 Da as was in the case of PSP obtained from the mycelia (i.e. IPS) of *C. versicolor Cov-1* strain [16]. Sakagami et al. [20] reported four sub-fractions found in PSK separated via membrane filters and reported the largest fraction to be $>2.0 \times 10^5$ Da. The authors reported that this fraction exhibited significant antimicrobial activity in mice. Tavares et al. [23] found the molar mass of the EPS obtained by gel permeation chromatography was ~6.7×10^4 Da. Wang and workers [25] compared EPS with IPS using a gel permeation column. They found that IPS and EPS contained two high molecular weight fractions (2.8×10^4 Da from IPS; 1.5×10^4 Da from EPS) and a lower molecular fraction of ~3.5×10^3 Da. The difference in the molar mass appeared to differ considerably in other studies, which could be due to the different fungal strains and/or the method used to determine molar mass.

Most studies reported to date have examined the intracellular polysaccharides obtained from either the extracts of mycelia or fruit bodies from solid phase fermentation. However, few studies on EPS obtained from the media of submerged-culture fermentation have been reported [13, 23]. In this study, it was found that the ability of the EPS and IPS to induce cytokines was dose dependent with higher levels of cytokines induced at low EPS/IPS concentrations. This study indicated that choosing the right EPS/IPS concentration is very important in maximizing immune response in vitro. Use of an excessively high concentration of polysaccharide could have a counter effect as seen in the case of IFN-γ production and may help explain the negative results obtained in the prevention of intestinal cancer using 2% dosage (mushroom extracts) in mice reported recently [4]. One study on human trials used 50 mg/kg body weight and reported enhancement of immunity without any adverse effects [27]. The modulation of immune response is a highly complex matter particularly when employing a complex natural product. Obtaining the optimum dose for the desired immune response will require considerable research in in vivo using both animal and human studies. The application of the *C. versicolor* polysaccharides in food as a bioactive ingredient is not envisaged to occur in the near future until we have a clear understanding of its dose dependence efficacy in human subjects.

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