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Antioxidation and antiproliferation properties of polysaccharide-protein complex extracted from *Phaeogyroporus portentosus* (Berk. & Broome) McNabb

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Phaeogyroporus portentosus is widely consumed as food in Thailand, but its potential bioactivities remain largely unknown. Here, the bioactivities of the hot water-extractable polysaccharide-protein complex (PPC) were evaluated after precipitating with ethanol. This crude PPC extract was then fractionated by DEAE-cellulose ion exchange column chromatography, and subsequently further purified by Superdex G-200 gel filtration column chromatography, giving an abundant polysaccharide fraction (PPC-P11) and a much less common fraction (PPC-P12) that was obtained in an insufficient amount for further analysis. The PPC-P11 fraction was characterized for its molecular weight and its functional groups were determined by ¹³C and ¹H NMR spectroscopy, FT-IR spectroscopy and gel permeation chromatography (GPC) respectively. It contained a high amount of glucose with an average molecular weight of 294.7 kDa (as compared to < 21.5 kDa by reducing SDS-PAGE analysis). The NMR signal observed at 103.5 ppm was assigned to the C1 of beta-glucose, and those between 20 and 40 ppm suggest the presence of a glucan-protein compound. PPC-P11 was found to display a dose-dependent antioxidant capacity in each of the four complementary test systems assayed (hydrogen peroxide, DPPH, ABTS and nitric oxide free radical scavenging) with quite low IC₅₀ values (< 100 µg/mL). Moreover, a relatively strong *in vitro* antiproliferative effect was found against five human cell lines, with IC₅₀ values ranging from 1.178 ± 0.129 µg/mL (breast cancer; BT474) to 5.183 ± 0.229 µg/mL (hepatoma cancer; HEP-G2). PPC-P11 from *P. portentosus* is, therefore, a potential antioxidant source for both health medicines and the food industry.

Key words: Antioxidation, antiproliferation, polysaccharide-protein complex, *Phaeogyroporus portentosus*.

INTRODUCTION

Polysaccharides from natural sources have gained

considerably interest among several biotechnological

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products and have a large range of industrial and commercial applications. Some can show strong antigenic and pathogenic activities and so are employed successfully by the pharmaceutical industry for the formulation of vaccines, whilst other polysaccharides are used as industrial food additives because of their chemical-physical properties (Arena et al., 2006). Due to their unusual multiplicity and structural complexity, polysaccharides are able to contain many biological messages and accordingly perform several functions. Moreover, these biopolymers have the ability to interact with other polymers, such as proteins and lipids, as well as other polysaccharides. As a consequence, this enormous potential variability in polysaccharide structure allows for the flexibility necessary for the precise regulatory mechanisms of various cell-cell interactions in higher organisms (Sharon and Lis, 1993; Chang, 2002).

Numerous polysaccharides and polysaccharide-protein complexes (PPCs) have been isolated from the fruiting bodies of fungi (mushrooms) as well as other fungi, yeasts, algae, lichens and plants, and used as a source of therapeutic agents. The most promising biopharmacological activities of these biopolymers are their immunomodulation and anti-cancer effects (Ooi and Liu, 2000). They are mainly present as glucans with different types of glycosidic linkages, such as (1→3), (1→6)- β -glucans and (1→3)- α -, and as true heteroglycans, while others mostly bind to protein residues as PPCs (Kidd, 2000; Wasser, 2002). Unique anticancer preparations have been developed from mushrooms, such as the polysaccharide lentinan from *Lentinus edodes* (Sasaki and Takasuka, 1976; Chihara et al., 1989), PSK and Krestin from *Coriolus versicolor* (Sakagami et al., 1991), and Schizophyllan from *Schizophyllum commune* (Komatsu et al., 1969). Medicinal mushrooms useful against cancer are known in China, Russia, Japan and Korea as well as in the USA and Canada. The most significant medicinal effect of mushrooms that has attracted significant attention in the recent years is their reported antitumor property. The scientific and clinical research on medicinal mushrooms is rapidly expanding. Medicinal mushroom extracts are ideally suited for improving general health and they are a fast growing commodity in the field of nutraceuticals. Although the mechanism of their antitumor action is still not completely clear, these polysaccharides and PPCs are suggested to enhance cell-mediated immune responses *in vivo* and *in vitro* and act as biological response modifiers (Mizuno, 1999).

Edible mushrooms are considered a rich food because they contain protein sugar, lipid, vitamins, amino acids and crude fiber (Chang, 1996). They also contain important minerals required for the normal functioning of the body (Gbolagade et al., 2006). Recently, mushrooms have become attractive not only as food (nutrition, flavor and texture) but also as a functional food and a source of physiologically beneficial medicines, while being devoid of undesirable side effects (Sadler, 2003).

Phaeoggyroporus portentosus (Berk. & Broome) McNabb, or the black bolete, a mycorrhizal mushroom in the Order *Boletales* and the Family *Gyrodontaceae*, is a popular edible mushroom in Thailand, especially in Northern Thailand where after harvesting from the wild in the rainy season it commands a relatively high price. In recent years, mushroom polysaccharides and PPCs have been demonstrated to play an important role as free radical scavengers and antioxidants for the prevention of oxidative damage in living organisms. Accordingly, due to the increased demand as a health food, mushroom production in Thailand has increased dramatically. Although *P. portentosus* has been used traditionally as food in Thailand for long time, its medicinal value has hardly been studied with only limited data in the literature on its β -glucan content. Therefore, the aims of this study were to purify and characterize the PPCs from the dried mycelia and to assay them for *in vitro* antioxidation activity and also for any *in vitro* cytotoxic/anti-proliferative activity against human malignant cell lines.

MATERIALS AND METHODS

Chemical and biological materials

Ammonium ferrous sulfate, 2, 2-azino-bis (3-ethylbenzothiazoline-6-sulfonate) (ABTS), butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), curcumin from *Curcuma longa* (Turmeric), 1,1-Diphenyl-2-picrylhydrazyl (DPPH), MTT (3-[5,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide), naphthylethylenediamine dihydrochloride (NED), potassium persulphate, sodium nitroprusside (SNP), α -tocopherol and xylenol orange were purchased from Sigma. All other unlabeled chemicals and reagents were of analytical grade. The five human tumor cell lines, BT474 (breast), CHAGO (lung), HEP-G2 (hepatoma), KATO-3 (gastric) and SW620 (colon), were obtained from the Institute of Biotechnology and Genetic Engineering, Chulalongkorn University, Bangkok, Thailand.

Microorganism and maintenance

P. portentosus was obtained from Mushroom Research and Development Group, Biotechnology Research and Development, Department of Agriculture, Bangkok, Thailand, and was maintained on a potato dextrose agar (PDA; 20% (w/v) potato, 2% (w/v) dextrose, 1.5% (w/v) agar) slant at 4°C and subcultured every 2 months. The mycelia of *P. portentosus* were grown in potato dextrose broth (PDB; as per PDA without the agar) liquid media. Mycelial plugs of *P. portentosus* grown in PDB media for 20 days at 25°C were seeded to liquid media under aseptic conditions. After 20 days of incubation, mycelia were collected by filtering through Whatman filter paper No. 42 and then washed three times with the same volume of distilled water. Collected mycelia were freeze-dried.

Extraction and enrichment of the crude PPC from *P. portentosus* mycelia

Extraction of PPC

Dried mycelia of *P. portentosus* were homogenized in liquid nitrogen with a pestle. A 500 g portion of the dried mycelium powder of *P. portentosus* was suspended in distilled water at 5% (w/v) and stirred at 95°C for 3 h, cooled to room temperature and overnight at 4°C. After that, it was clarified by centrifugation for 30 min, at 15,000

$\times g$ at 4°C. The supernatant was harvested, concentrated in a rotary evaporator under reduced pressure at 50°C and filtered. The filtrate was precipitated with 4 volumes of 95% (v/v) ethanol and the suspension was kept overnight at 4°C. The precipitate was harvested by centrifugation for 45 min, $15,000 \times g$ at 4°C, washed with ethanol, and then air. The supernatant was dialyzed against distilled water for 72 h and precipitated by 4 volumes of 95% (v/v) ethanol and kept overnight at 4°C. After centrifugation for 45 min, at $15,000 \times g$ and at 20°C to harvest the crude PPC as the pellet, it was washed by 95% (v/v) ethanol and freeze dried for 24 h, giving the dried crude PPC.

Enrichment of the crude PPC extract

The crude PPC preparation was dissolved in distilled water at 1.5 mg/mL and then 10 mL of solution and was injected into a DEAE-cellulose anion exchange column (1.6 cm i.d. and 20 cm long). The column was initially eluted with 200 mL distilled water at 1.0 mL/min collecting 10 mL fractions, and then followed by 500 mL of 0.5 M NaCl as a gradient elution at the same flow rate and fraction collection volume. The major PPC fractions, as determined by the phenol sulfuric acid method, were then purified by Superdex G-200 gel filtration column chromatography (1.6 cm i.d. and 60 cm long), eluted with 1,000 mL of distilled water at a flow rate of 0.5 mL/min and 10 mL fractions was collected. The polysaccharide fractions were determined by the phenol sulfuric acid method before being pooled and dried.

Determination of the protein and carbohydrate content

The protein concentration was determined following the standard Bradford assay (Bradford, 1976), with 3 dilutions of a known concentration of bovine serum albumin (2.5-20 mg/ μ L) as the standard. Absorbance was measured at 595 nm. During the column chromatographic separations, the elution profiles of proteins were determined by measuring the absorbance at 280 nm. Total neutral carbohydrate content was estimated by the method of phenol sulfuric acid method, as reported previously (Dubois et al., 1979), using D-glucose as the standard.

Protein pattern analysis by SDS-PAGE

Discontinuous reducing SDS-PAGE gels were prepared with 0.1% (w/v) SDS in 12.5% and 5% (w/v) acrylamide separating and stacking gels, respectively, with Tris-glycine buffer pH 8.3 containing 0.1% (w/v) SDS as the electrode buffer, according to the procedure of Laemmli (1970). Samples to be analyzed were treated with reducing sample buffer and boiled for 5 min prior to application to the gel. Electrophoresis was performed at a constant current of 20 mA per slab at room temperature in a Mini-Gel Electrophoresis unit. Molecular weight standards were co-resolved in each gel alongside the samples to determine the subunit molecular weight of the purified protein(s). After electrophoresis, proteins in the gel were visualized by coomassie Blue R-250 staining (0.1% (w/v) coomassie Blue R-250 in 10% (v/v) acetic acid and 45% (v/v) methanol) and several changes of destaining solution (10% (v/v) acetic acid and 45% (v/v) methanol) until the background was clear.

Structure elucidation

Attenuated total reflectance-fourier transform infrared spectrophotometry (ATR-FT-IR)

The ATR-FT-IR spectra were recorded on a Nicolet Impact 410 Fourier Transform Infrared Spectrophotometer. Solid samples were generally examined by incorporating the sample with potassium bromide to form a pellet. Spectra of liquid samples were recorded as

thin film on a sodium chloride (NaCl) cell.

¹³C and ¹H nuclear magnetic resonance spectroscopy (NMR)

The ¹H-NMR, and ¹³C-NMR spectra were recorded on a Varian Spectrometer operated at 400 MHz for ¹H nuclei and at 100 MHz for ¹³C nuclei, with the sample dissolved in D₂O.

Gel permeation chromatography (GPC)

The molecular weight of the enriched PPC (PPC-P11) was determined by gel permeation chromatography on a Waters model 600E composed of a Waters Ultra-hydroseal linear 1 column and a refractive index detector. The purified polysaccharide solution at 2 mg/mL (20 μ L) was injected into the column and eluted with 0.05 M sodium acetate buffer (pH 5) as the mobile phase at a flow rate of 0.6 mL/min. The retention times under the same conditions of an eight size standard Pullulan was used as a molecular weight (M_w) standard (M_w) of 5.8, 12, 24, 48, 100, 180, 380 and 750 kDa) to calibrate the column.

In vitro antioxidant activity

DPPH radical scavenging assay

The DPPH free radical scavenging activity of the enriched PPC (PPC-P11) was determined by the method of Mohsen and Ammar (2009), with slight modification. 1 mL of the tested samples at various concentrations (0 to 500 μ g/mL) was added to ethanolic DPPH solution (3 mL, 0.1 mM). Discoloration was measured spectroscopically at an absorbance of 517 nm after incubation for 30 min at 30°C in the dark. BHT was used as the positive control. The DPPH scavenging effect was calculated as follows:

$$\text{DPPH scavenging effect (\%)} = [1 - (A_{\text{sample}} - A_{\text{blank}}) / A_{\text{control}}] \times 100 \quad (1)$$

Where, A_{sample} , A_{blank} and A_{control} were defined as the absorbance of the sample with DPPH, the sample alone and DPPH alone, respectively.

ABTS radical scavenging assay

The ABTS free radical assay was carried out according to the method of Cai et al. (2004). The ABTS cation radical solution was prepared by mixing 7 mM ABTS and 2.45 mM potassium persulphate and incubating in the dark at room temperature for 12 h. The ABTS cation radical solution was then diluted with water to obtain an absorbance of 0.70 ± 0.02 at 734 nm and 3 mL of this dilution was added to the test sample (0.1 mL total, prediluted to various concentrations between 0-200 μ g/mL) and mixed vigorously. The absorbance was measured at 734 nm after standing for 6 min. BHT (0-200 μ g/mL) was used as the positive control. The ABTS scavenging effect was calculated as follows:

$$\text{ABTS scavenging effect (\%)} = [1 - (A_{\text{sample}} - A_{\text{blank}}) / A_{\text{control}}] \times 100 \quad (2)$$

Where, A_{sample} , A_{blank} and A_{control} were defined as the absorbance of the sample plus ABTS, the sample alone and ABTS alone, respectively.

Nitric oxide (NO) radical scavenging

At physiological pH, nitric oxide (NO) generated from an aqueous SNP solution interacts with oxygen to produce nitrite ions, which may be quantified by the Griess Illosvoy reaction (Govindarajan et al., 2003). The reaction mixture contained 10 mM SNP, phosphate buffered saline pH 7.4 (PBS) and various doses (0-200 μ g/mL) of

Table 1. The yield of polysaccharide-protein complex (PPC) fractions obtained from 500 g of dried *P. portentosus* mycelium powder.

Fraction	Amount of polysaccharide (mg)	Yield (from 500 g of dried mycelium) (%)
Crude PPC	5,684.45 ± 0.05	1.1369
PPC-P1	435.28 ± 0.19	0.0871
PPC-P2	74.89 ± 0.002	0.0149
PPC-P11	245.08 ± 0.11	0.0492
PPC-P12	0.47 ± 0.0002	0.000094

Data are shown as the mean ± SD and are derived from three independent repeats.

the test solution in a final volume of 3 mL. After incubation for 150 min at 25°C, 1 mL sulfanilamide (0.33% (w/v) in 20% (v/v) glacial acetic acid) was added to 0.5 mL of the incubated solution and allowed to stand for 5 min. Then 1 mL of 0.1% (w/v) NED was added and the mixture was incubated for 30 min at 25°C. The pink chromophore generated during the diazotization of nitrite ions with sulphanilamide and subsequent coupling with NED was measured spectrophotometrically at an absorbance wavelength of 540 nm against a blank sample. All tests were performed three times. Curcumin (0-200 µg/mL) was used as a standard (Hazra et al., 2009).

Hydrogen peroxide (H₂O₂) scavenging

The hydrogen peroxide (H₂O₂) scavenging activity was determined as previously described (Soares et al., 2009) with minor changes. An aliquot of 50 mM H₂O₂ and various concentrations (0-100 µg/mL) of samples were mixed (1:1 (v/v)) and incubated for 30 min at room temperature. After incubation, 90 µL of the H₂O₂-sample mixture was mixed with 10 µL methanol and 0.9 mL FOX reagent (3.96 mM BHT, 0.1 mM xlenol orange, 0.256 mM ammonium ferrous sulfate in 90% (v/v) methanol, 0.025 M H₂SO₄) and then vortexed and incubated at room temperature for 30 min. The absorbance of the ferric-xlenol orange complex was measured at 560 nm. All tests were carried out three times and sodium pyruvate was used as the reference compound (Floriano-Sánchez et al., 2006).

Anti-proliferation /cytotoxicity assay for human malignant cell lines

The bioassay for the combined *in vitro* antiproliferative and cytotoxic activities (without discrimination of the two activities) towards five human malignant cell lines, BT474, CHAGO, HEP-G2, KATO-3 and SW620, was performed in routine tissue culture (Kheeree et al., 2010). Cells were maintained in complete media (CM; RPMI-1640 supplemented with 10% (v/v) fetal calf serum (FCS) and 2.0 mM L-glutamine) at 3 °C under a 5% (v/v) CO₂ atmosphere. Cells were trypsinized, aspirated and washed before being seeding at a final density of 5 × 10³ cells/µL in 200 µL of CM per well in a 96 well plate and cultured for 24 h. After that, serial dilutions of the PPC were added (0-25 µg/mL final concentration in a total volume of 200 µL CM) into each well, mixed and the cultures incubated for 72 h. Next, 10 µL of MTT solution (5 mg/mL) was added to each well and incubated for a further 4 h before the media was carefully aspirated off and the adhered cells gently washed with RPMI-1640 to remove all remaining media prior to adding 150 µL DMSO per well and leaving for 30 min. The cell remnants and solution were then aspirated to ensure all the cells were lysed and the crystals dissolved, and the absorbance at 540 nm was measured using a microtiter reader. Each assay was performed with triplicate wells with 10 µg/L doxorubicin and the CH-Liver cell line as the positive control and all cell lines, w/o PPC as negative controls.

RESULTS AND DISCUSSION

Basidiomycete mushrooms, which along with the Ascomycota form the “higher fungi” subkingdom Dikarya, have been used in folk medicine throughout the world since ancient times. In the last decade, several medicinally active Basidiomycete species have been commercially developed (Wasser and Weis, 1999a) and it is now well established that mushrooms represent a source material for the development of drugs (Mizuno, 2000). The hot water soluble PPC was extracted, and enriched from the mycelium of *P. portentosus* as described below, and then its bioactivity in terms of its *in vitro* antioxidant and cytotoxic /antiproliferative activities were determined.

Enrichment of the PPC from *P. portentosus*

A hot-water soluble crude PPC preparation from *P. portentosus* was obtained from the mycelium by drying, grinding and extracting the dry powder by hot water and subsequent precipitation by ethanol, as detailed in the material and methods section, and is summarized in Table 1. The subsequent extraction and purification steps of the crude PPC, detailed in the material and methods section, are schematically summarized in Figure 1. DEAE-cellulose anion exchange column chromatography separated the crude PPC preparation into two peaks; an unbound fraction (PPC-P1) and a bound fraction (PPC-P2) that accounted for 7.66 and 1.32% of the total crude PPC (as carbohydrate) (Figure 2A and Table 1). Since PPC-P1 was eluted by water, it is likely to be a neutral PPC, although a cationic (positively charged, basic) PPC is possible, whereas since PPC-P2 was eluted by 0.5 M NaCl, it is likely to be an anionic (negatively charged, acidic) PPC. The protein contents in each polysaccharide fraction were monitored by direct reading of the absorbance at 280 nm (as opposed to say Lowry or Bradford assays). In contrast to PPC-P1, where the protein and carbohydrate levels followed each other in the respective fractions, those fractions eluting as PPC-P2 showed a very low absorbance at 280 nm, consistent with a low protein yield, but this absorbance was much lower than the carbohydrate levels in the same fractions (Figure 2A). Although the direct photometric reading of the absorbance

Figure 1. Schematic diagram summarizing the main stages in the extraction and enrichment of the polysaccharides-protein complexes (PPC), including PPC-P11, from the dry mycelium of *P. portentosus*.

at 280 nm has long been used for determination of proteins, largely due to the logistics of its ability to non-destructively continuously monitor column eluates, only the minor amino acids tryptophan, tyrosine, and phenylalanine are actually responsible for the spectrometric absorbance at this wavelength. In essence, the absorbance at 280 nm then determines the true amount of only these three amino acid contents, rather than the whole protein. Naturally, one would expect a more intense absorbance for the same mass of protein in those proteins with a higher content of these three amino acids than those totally without or merely with minute amounts of them. Thus, one can anticipate nearly zero absorbance even though there should be a tremendous quantity of proteins present. Therefore, the actual carbohydrate to protein level of PPC-P2 is unknown and alternative

methods for the analysis of the protein content are obviously required, such as Bradford or Lowry analysis.

Fraction PPC-P1 was then subjected to further purification using Superdex G-200 gel filtration column chromatography, resulting in two peaks being separated, PPC-P11 and PPC-P12 at fraction numbers 5-7 and 10, respectively (Figure 2B). Due to the low yield attained for PPC-P12 (Figure 2B and Table 1), this fraction was not analyzed any further in this study. Protein patterns were identified on SDS-PAGE gels stained with Coomassie brilliant blue R-250, with the numerous protein bands being visualized on a polyacrylamide gel. PPC-P11 had two major protein bands, which had molecular weights lower than 21.5 kDa and around 12-15 kDa, respectively (Figure 2C).

Various methods have been used for the isolation of

Figure 2. (A) DEAE-cellulose ion exchange column chromatogram showing the separation profile of crude PPC, giving fractions PPC-P1 and PPC-P2 from distilled water and 0.5 M NaCl elution, respectively. Open and closed circles represent the A_{280} (protein) and A_{490} (carbohydrate, following phenol/ H_2SO_4) respectively. (B) Superdex G-200 gel filtration column chromatogram for fraction PPC-P1 from the distilled water elute giving fractions PPC-P11 and PPC-P12. Open and closed circles represent the A_{280} (protein) and A_{490} (carbohydrate, following phenol/ H_2SO_4), respectively. (C) SDS-PAGE of PPC-P11 from *P. portentosus*. Lane 1, molecular weight markers; Lane 2, 15 μ g of PPC-P11.

Figure 3. ATR-FT-IR spectroscopy of PPC-P11 from *P. portentosus*.

fungus polysaccharides and PPCs. Repeated extraction with hot water and cold NaOH has been used for isolating polysaccharides from *Agaricus blazei* and *Sparassis crispa* (Ohno et al., 2000; Ohno et al., 2001) followed by fractionation by ion-exchange chromatography on DEAE-Sephadex A-25. Other investigators have used similar protocols involving hot water extraction, gel filtration, and ion-exchange chromatography for isolating polysaccharides from *Sarcodon aspratus* (Mizuno et al., 2000), *Agaricus blazei* (Mizuno et al., 1999), *Omphalia lapidescens* (Ohno et al., 1992) and *Phellinus linteus* (Song et al., 1995). An additional affinity chromatography step was used by Mizuno et al. (1995) to isolate polysaccharides from *Tricholoma giganteum*, and by Zhang et al. (1994) to isolate polysaccharides from *Ganoderma tsugae*. Chromatofocusing purification steps have been used to obtain a proteoglycan from *Agaricus blazei* (Fujimiya et al., 1998). Polysaccharides have been isolated from *Hehenbuehelia serotina* (Ma et al., 1991) by a procedure where the initial extraction is followed by ethanol extraction and then ion-exchange chromatography, gel filtration, and affinity chromatography.

Characterization of the PPC

The major enriched PPC component obtained from the mycelia of *P. portentosus* (PPC-P11) following hot-water extraction, DEAE-cellulose and Superdex G-200 column chromatography was characterized in terms of evaluating

its functional groups using ATR-FT-IR and NMR spectroscopy, and determination of its M_w using GPC. The results are shown below.

Functional group analysis of PPC by ATR-FT-IR spectroscopy

The results from the ATR-FT-IR spectroscopic analysis were used to confirm that PPC-P11 was a polysaccharide (Figure 3). The intensity of the bands between 3,600–3,200 cm^{-1} are ascribed to O-H stretching in the constituent sugar residues of polysaccharides, whilst the absorbance at $\sim 2,992 \text{ cm}^{-1}$ represent C-H stretching in the sugar ring. PPC-P11 has residual water bands around $\sim 1,635 \text{ cm}^{-1}$, whilst bands around 1364 cm^{-1} represent the C-H and O-H deformation vibrations, and those around 888.1 cm^{-1} the C-H deformation vibrations. The band at $\sim 1052 \text{ cm}^{-1}$ indicates the C-O-C and O-H residues in a pyran structure.

^{13}C and ^1H NMR spectroscopy of the PPC

The ^{13}C NMR spectroscopic analysis of PPC-P11 was relatively simple (data not show), due to the homopolysaccharide type present in these organisms. The absence of signals at 180–120 ppm shows that this sample was not contaminated by phenolic compounds. The presence of glucose was observed through a signal

Figure 4. The elution profile of PPC-P11 from the gel permeable chromatography column, used to determine the molecular weight by reference of the retention time to that of the eight known Pullulan size standards.

at 103.5 ppm, characteristic of the α -configuration. Other important signals in the spectra are those in the area of 60-80 ppm, where they are related to the C2, C3, C4, C5 and C6 of carbohydrate. In addition, the signals in the area between 28.5 and 33.0 ppm are in accord with a characteristic glucan-protein compound structure (Gonzaga et al., 2005). The ^1H spectra showed a chemical shift in the anomeric region at 4-6 ppm. Signals of 4.1 and 4.6 were obtained in this spectrum, corresponding to the ppm of β -glucan (data not show) (Gonzaga et al., 2005; Kawagishi et al., 1990). We also observed signals in the 1.4- to 2.5-ppm region that are related to the glucan-protein structure.

The molecular weight of PPC as determined by GPC

The GPC analysis indicated that PPC-P11 had an average M_w of 294.7 kDa (Figure 4). The fact that PPC-P11 was eluted by distilled water (unbound fraction) in DEAE-cellulose anion exchange chromatography and that PPC-P11 had prominently neutral or positive charges on its molecule indicated the PPC-P11 is an cationic polysaccharide and so can be eluted by sodium acetate buffer (pH 5) from the GPC column.

In vitro antioxidant activity

Free radicals are chemical entities that can exist separately with one or more unpaired electrons. As such they

are highly reactive (oxidative) and can cause extensive tissue damage. Lipids, proteins and DNA are all susceptible to attack by free radicals (Halliwell et al., 1995). Antioxidants may offer resistance against such free radical based oxidative stress by scavenging the free radicals. Thus, when the balance between reactive oxygen species (ROS) production and oxidant defenses is lost, then oxidative stress through a series of events, deregulates the cellular functions and leads to various pathological conditions, such as AIDS, ageing, arthritis, carcinogenesis, cardiovascular dysfunction, cataract, diabetes, liver disorders, Parkinson's dementia, Alzheimer's disease, retinopathy and rheumatism (Tiwari, 2001; Droge, 2002; Willcox et al., 2004).

DPPH free radical scavenging assay

Unlike other free radicals, such as the hydroxyl radical and superoxide anion, DPPH has the advantage of being unaffected by certain side reactions, such as metal ion chelation and enzyme inhibition (Amarowicz et al., 2004). A freshly prepared DPPH solution exhibits a deep purple color with an absorption maximum at 517 nm that fades or disappears when an antioxidant (reductant) is present in the medium. Thus, antioxidant molecules can quench DPPH free radicals (that is by providing hydrogen atoms or by electron donation, conceivably via a free-radical attack on the DPPH molecule) and convert them to a colorless product (that is 2, 2-diphenyl-1-hydrazine, or a

Figure 5a, b. Anti-oxidant activity dose response curves of PPC-P11 isolated from *P. portentosus* as determined by the (A) DPPH radical scavenging activity, (B) ABTS cation scavenging activity, ($\circ$) PPC-P11 and the three standards; ($\bullet$) α -tocopherol; ($\blacktriangle$) BHT; and ($\blacksquare$) BHA).

substituted analogous hydrazine), resulting in a decrease in the absorbance at 517 nm. Hence, the more rapidly the absorbance decreases, the more potent the antioxidant activity of the extract. This test is a commonly employed assay in antioxidant studies of specific compounds or extracts across a short time scale. The principle advantage of the DPPH assay is that its reduction can be measured directly in the reaction medium by a continuous spectrophotometric assay. In addition, the DPPH assay is known to give reliable information concerning the antioxidant ability of the tested compounds. Reasonable free radical scavenging capacities of PPC-P11, as measured by the DPPH assay, were found in a dose-dependent manner (Figure 5A).

However, the 50% inhibition value (IC_{50} value) for PPC-P11 ($\text{IC}_{50} = 72.5 \pm 0.13 \mu\text{g/mL}$) was significantly (6.5- to 18.0-fold) higher than that of the commonly used synthetic antioxidants α -tocopherol, BHT, and BHA (IC_{50} values of 4.02 ± 0.02 , 10.5 ± 0.025 , and $10.7 \pm 0.011 \mu\text{g/mL}$ respectively), whilst PPC-P11 only attained a maximal DPPH scavenging effect of ca. 78% compared to ~95% for the other three standard compounds.

ABTS radical scavenging assay

The reactions with $\text{ABTS}^{+\bullet}$ radicals involve a single-electron transfer process. Bleaching of a preformed solution of the blue-green radical cation $\text{ABTS}^{+\bullet}$, which has an ab-

absorption maximum at 734 nm, has been extensively used by past researchers to evaluate the antioxidant capacity of complex mixtures and individual compounds (Miller and Rice-Evans, 1997). The scavenging ability of PPC-P11 on the ABTS free radical was observed in a dose-dependent manner, but with a lower activity than the reference standards of α -tocopherol, BHT and BHA (Figure 5B). Thus, the IC_{50} value for PPC-P11 in the ABTS radical scavenging assay was $48.1 \pm 0.74 \mu\text{g/mL}$, almost five-fold higher than that for the three standard compounds (IC_{50} values of 6.8 ± 0.05 , 7.3 ± 0.08 and $7.1 \pm 0.026 \mu\text{g/mL}$), and also with PPC-P11 only attaining a maximum ~75% scavenging level compared to ~95% for the three standard compounds. Nevertheless, PPC-P11 has a relatively strong scavenging power for ABTS radicals and merits further evaluation as a potential antioxidant. $ABTS^{+}$ reacts rapidly with antioxidants, and it can be used over a wide pH range to study the effects of pH on antioxidant mechanisms (Lemanska et al., 2001). Also, $ABTS^{+}$ is soluble in both aqueous and organic solvents and is not affected by the solvent ionic strength, and thus can be used in multiple media to determine both hydrophilic and lipophilic antioxidant capacities of extracts and body fluids (Awika et al., 2003). However, compounds of biological relevance are typically found to reduce DPPH less compared to the synthetic antioxidant compounds like BHT and BHA (Prior et al., 2005). One possible reason for reduced level of observed inhibition found in the DPPH assay when compared to ABTS antioxidant assay is simply the chemical nature (redox potential) of DPPH.

NO radical scavenging

NO is a free radical generated by various cell types including endothelial cells, macrophages and neurons, and it is involved in the regulation of various physiological processes including the inflammatory responses (Lata and Ahuja, 2003). Excess concentrations of NO and its reactions with superoxide radicals are associated with several diseases (Ialenti et al., 1993; Ross, 1993; Sainani et al., 1997). Sustained levels of production of this radical are directly toxic to tissues and contribute to vascular collapse associated with septic shock, whereas chronic expression of NO radical is associated with various carcinomas and inflammatory conditions including juvenile diabetes, multiple sclerosis, arthritis and ulcerative colitis (Tylor et al., 1997). The toxicity of NO increases greatly when it reacts with superoxide radicals, forming the highly reactive peroxynitrite anion ($ONOO^{-}$) (Huie and Padmaja, 1993). The NO generated from SNP reacts with oxygen to form nitrite. The extract inhibits nitrite formation by directly competing with oxygen in the reaction with NO. PPC-P11 also caused a moderate dose-dependent inhibition of NO with an IC_{50} of $23.9 \pm 0.3 \mu\text{g/mL}$, some 2.5-fold lower than that of Curcumin with an IC_{50} of $9.74 \pm 0.02 \mu\text{g/mL}$ (Figure 5C).

H_2O_2 scavenging

Hydroxyl radicals are the major active oxygen species in biological systems and cause lipid peroxidation and enormous biological damage (Aurand et al., 1977). They were produced in this study by incubating ferric-EDTA with ascorbic acid and H_2O_2 at pH 7.4, and reacted with 2-deoxy-2-ribose to generate a malondialdehyde-like product. This compound forms a pink chromogen upon heating with thiobarbituric acid at a low pH (Halliwell et al., 1987). When the PPC-P11 extract was added to the reaction mixture, it removed the hydroxyl radicals from the sugar and prevented the reaction in a dose-dependent manner (Figure 5D), but was a very poor scavenger of H_2O_2 ($IC_{50} = 18.4 \pm 0.5 \text{ mg/mL}$) compared to the standard sodium pyruvate ($IC_{50} = 4.37 \pm 0.05 \text{ mg/mL}$).

Polysaccharide extracts from fungi of different classes have previously been reported to show a high scavenger activity on free radicals (Wasser and Weis, 1999b), but the level of this activity was dependent on the protein portion linked to polysaccharide chain (Liu et al., 1997). Although the antioxidant mechanism of polysaccharide extracts is still not fully understood, factors related to polysaccharides, such as monosaccharide residues (mainly glucose), M_w and water solubility are very important.

Anti-proliferation/cytotoxicity assay for human malignant cell lines

The anti-proliferative or cytotoxic effect of the PPC-P11 extract was found to be dose-dependent against all five tested cell lines with the highest efficiency against the BT474 cell line with an IC_{50} value of $1.18 \pm 0.13 \mu\text{g/mL}$ down to the lowest for the HEP-G2 cell line with an IC_{50} of $5.18 \pm 0.23 \mu\text{g/mL}$ (Figure 6). However, the dose-dependent inhibition of proliferation and or cytotoxicity was different between the cell lines. For example, for the HEP-G2 cell line, although a larger IC_{50} was evident, a greater degree of inhibition over a narrower dose range was obtained than that seen with the BT474 cell line which displayed a 4.4-fold lower IC_{50} value but a lower maximal inhibition level spread over a larger PPC-P11 dose range. This could suggest different mechanisms, be that receptors with different K_a values, or differences in the number and duration of receptor crosslinking required or in internalization pathways, etc.

Extracts of multiple varieties of mushrooms have been shown to be protective in experimental cancer models; presumably because in part they boost anti-tumor immunity. These polysaccharides and PPCs are suggested to enhance cell-mediated immune responses *in vivo* and *in vitro* and act as biological response modifiers (Borchers et al., 1999). Potentiation of the host defense system may result in the activation of many kinds of immune cells that are vitally important for the maintenance of homeostasis. Polysaccharides or PPCs are considered as multi-cytokine inducers that are able to induce gene expression of

Figure 5c, d. (C) Nitric oxide radical scavenging activity, and (D) H₂O₂ scavenging activity, (○) PPC-P11 and the standard (●) curcumin or sodium pyruvate, respectively. Data are shown as the mean $\pm$ 1 SD, derived from 3 independent replications.

various immunomodulatory cytokines and cytokine receptors (Okamoto et al., 2004).

Conclusion

A potential PPC (PPC-P11) that displays antioxidant and anti-human malignant cell line proliferation was enriched from the mycelia of *P. portentosus* by a simple four-stage procedure (hot water extraction, precipitation with ethanol, DEAE-cellulose and Superdex G-200 column chromatography).

NMR and ATR-FT-IR spectroscopy indicated and confirmed that PPC-P11 contains polysaccharides from their functional groups and also contains a characteristic glucan-protein compound structure. PPC-P11 has a moderate dose-dependent antioxidant activity in four different assays. However, determination of the effective part (carbohydrate or peptide moiety) that play the important roles in malignant cell anti-proliferation process and the mechanism(s) of such activity of PPC, along with conformation of its multimeric state and role of such, await further research.

Figure 6. *In vitro* cytotoxic/anti-proliferation activity of PPC-P11 isolated from *P. portentosus* towards five cancer cell lines in tissue culture; (●) BT474, (○) CHAGO, (■) HEP-G2, (□) KATO-3, and (▲) SW620. Data are shown as the mean $\pm$ 1 SD, derived from three independent replications.

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