INTRODUCTION

Polysaccharides from natural sources raise remarkable interest among several biotechnological products and have a large range of industrial and commercial applications. Some of them, for example, showing strong antigenic and pathogenic activities, are employed successfully by the pharmaceutical industry for the formulation of vaccines, and others are used as industrial food additives because of their chemical-physical properties (Tombs et al., 2006). Polysaccharides, because of their unusual multiplicity and structural complexity, 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 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 such as man (Sharon and Lis, 1993; Chang, 2002; Geremia and Rinaudo, 2005).

A numerous polysaccharides and polysaccharide-protein complexes have been isolated from mushrooms and used as a source of therapeutic agents. The most promising biopharmacological activities of these biopolymers are their immunomodulation and anti-cancer effects. They are mainly present as glucans with different types of glycosidic linkages such as (1→3), (1→6)-β-glucans and (1→3)-α-glucans, and as true herteroglycans, while others mostly bind to protein residues as polysaccharide-protein complexes (Kidd, 2000; Wasser, 2002; Geremia and Rinaudo, 2005). Unique anticancer preparations were developed from mushrooms such as polysaccharide lentinan from *Lentinus edodes* (Sasaki and Takasuka, 1976; Chihara et al. 1989), 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 United States and Canada. The most significant medicinal effect of mushrooms that attracted the attention in the recent years is their antitumor property. The scientific and clinical research on medicinal mushrooms is among the most exciting. Medicinal mushroom extracts are ideally suited for improving general health and they are considered to be fast growing commodity in the field of neutraceuticals. Although the mechanism of their antitumor action is still not completely clear, these polysaccharides and polysaccharide-

protein complexes are suggested to enhance cell-mediated immune responses *in vivo* and in vitro and act as biological response modifiers (Mizuno, 1999).

Edible mushroom are considerate as a rich food because they contain protein sugar, lipid, vitamins, amino acids and crude fiber (Chang, 1996). They also contain important minerals required for normal functioning of the body (Gbolagade et al., 2006; Kalac, 2009). Recently, fruit bodies of mushrooms have become attractive not only for food, flavor and texture but also as functional food and as a source of physiologically benefical medicines, while being devoid of undesirable side effects (Sadler, 2003). *Phaeogyroporus portentosus* (Berk. & Broome) McNabb, a mychorrhizal mushroom in the Order *Boletales* and the Family *Gyrodontaceae*, is a popular edible mushroom in Thailand. In recent years, mushroom polysaccharides have been demonstrated to play an important role as free radical scavengers and antioxidants for the prevention of oxidative damage in living organisms. Recently, because of demand as a health food, the average mushroom production in Thailand has increased dramatically. *P. portentosus* have been used traditionally as food in Thailand for long time, however its medicinal value has hardly been studied, and there few data in the literature on its β-glucan content. Therefore, the aims of this study were to purify and characterize the polysaccharide-protein complex from the dried mycelia and to assay them for bioactivity including antioxidation and cytotoxicity assay for human malignant cell lines activities.

MATERIALS AND METHODS

Chemical and Biological Materials

Ammonium ferrous sulfate, 2, 2-azinobis (3-ethylbenzothiazoline-6-sulfonate) (ABTS), buthylated hydroxyanisol (BHA), buthylated hydroxytoluene (BHT), curcumin from *Curcuma longa* (Turmeric),1,1-Diphenly-2-picrylhydrazyl (DPPH), MTT (3-[5,5-dimethylthyazol-2-yl-2,5-diphenyltetrazolium bromide), napthylethylenediamine dihydrochloride (NED), potassium persulphate, sodium nitroprusside (SNP), α-tocopherol, and xylenol orange were purchased from Sigma. All other unlabeled chemicals and reagents were 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.

Microrganism and maintenance

P. portentosus was obtained from Mushroom Research and Development Group, Biotechnology Research and Development, Department of Agriculture, Bangkok, Thailand and maintained on potato dextrose agar (PDA) slant at 4 °C and subcultured every 2 months. The mycelia of *P. portentosus* were grown in potato dextrose broth (PDB) 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 washed 3 times with the same volume of distilled water. Collected mycelia were freeze-dried.

Extraction of crude polysaccharide-protein complex (PPC)

Dried mycelia of *P. portentosus* were homogenized in liquid nitrogen with a pestle. The 500 g of dried mycelium powder of *P. portentosus* were dissolved in distilled water at 1 g per 20 mL. The suspension was stirred on the water bath at 95 °C for 3 h. Then allowed it to be cooled before kept overnight at 4 °C. After that, it was centrifuged for 30 min, 15,000 × g at 4 °C. The supernatant was concentrated in a rotary evaporator under pressure at 50 °C and filtrated. The filtrate was precipitated with 4

volumes of 95% ethanol and the suspension was kept overnight at 4 °C. The precipitate was assembled by centrifugation for 45 min, 15,000 × g at 4 °C. Washed with ethanol, air dried and then dissolved in distilled water. The supernatant was dialyzed against distilled water for 72 h and precipitated by 4 volumes of 95% ethanol and kept overnight at 4 °C. After centrifugation for 45 min, 15,000 × g at 20 °C, crude PPC was washed by 95% ethanol and freeze dried for 24 h, giving the dried crude PPC.

Further purification 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 injected into a DEAE-cellulose anion exchange column. The size of the column was 1.6 cm of diameter and 20 cm of height. 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, determined by phenol sulfuric acid method, were then purified by Superdex G-200 gel filtration column chromatography, eluted with 1,000 mL of distilled water at a flow rate of 0.5 mL/ min and collecting 10 mL fractions. The size of the column was 1.6 cm of diameter and 60 cm of height. 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 dilutions of a known concentration of bovine serum albumin 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 (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 five 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 coresolved 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

Fourier Transform Infrared Spectrophotometer (FT-IR)

The 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 (KBr) to form a pellet. Spectra of liquid samples were recorded as thin film on a sodium chloride (NaCl) cell.

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

The ^{1}H-NMR, and ^{13}C-NMR spectra were recorded on a Varian Spectrometer operated at 400 MHz for ^{1}H nuclei and at 100 MHz for ^{13}C nuclei, with the sample dissolved in D_2O.

Gel Permeation Chromatography (GPC)

The molecular weight of purified PPC was determined on a Waters model 600E composed of a Waters Ultra-hydroseal linear 1 column and a refractive index detector.

The purified polysaccharide solution 2 mg/mL (20 μL) was injected with 0.05 M sodium bicarbonate buffer (pH 11) as a mobile phase at 0.6 mL/min. Pullulan was used as a standard (MW of 5,900-788,000 Da).

In vitro antioxidant activity

DPPH radical scavenging assay

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

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

where A_{sample}, A_{blank} and $A_{control}$ were defined as absorbance of sample and DPPH, sample without DPPH, and DPPH without sample, respectively.

ABTS radical scavenging assay

ABTS assay was carried out according to the method of Cai et al. (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. ABTS cation radical solution (3 mL) was added to the test samples (0.1 mL) of various concentrations (0-200 μg/mL) and mixed vigorously. The absorbance was measured at 734 nm after standing for 6 min. α-tocopherol, BHT, and BHA were used as the positive control. The ABTS scavenging effect was calculated as follows:

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

where A_{sample}, A_{blank} and $A_{control}$ were defined as absorbance of sample and ABTS, sample without ABTS, and ABTS without sample, respectively.

Nitric oxide radical scavenging

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

Hydrogen peroxide scavenging

This activity was determined according to a previously described method (Soares et al., 2009) with minor changes. An aliquot of 50 mM H_2O_2 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 H_2O_2-sample solution was mixed with 10 μL methanol and 0.9 mL FOX reagent was added (prepared in advance by mixing 9 volumes of 4.4 mM BHT in methanol with 1 volume of 1 mM xylenol orange and 2.56 mM ammonium ferrous sulfate in 0.25 M H_2SO_4). The reaction mixture was then vortexed and incubated at room temperature for 30 min. The absorbance of the ferric-xylenol 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 *in vitro* antiproliferative activity (including cytotoxicity without discrimination of the two activities) towards five human malignant cell lines, BT474 (breast), CHAGO (lung), HEP-G2 (hepatoma), KATO-3 (gastric) and SW620 (colon), was performed in routine tissue culture. Cells were maintained in complete media, comprised of RPMI-1640 supplemented with 10% (v/v) FCS and 2.0 mM L-glutamine at 37 ^{o}C under a 5% (v/v) CO_2 atmosphere. Cells were trypsinized, aspirated and washed before being seeding at a final density of 5×10^3 cells/μL in 200 μL of complete media 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 complete media) into each well, mixed and the cultures incubated for 72 h. Next, 10 μL of MTT (3-[5, 5-dimethylthyazol-2-yl-2,5-diphenyltetrazolium bromide) 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 (w/o FCS and other supplements) 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 CH-Liver cell line w/o P11 as the positive and negative controls, respectively.

RESULTS AND DISCUSSION

Higher Basidiomycete mushrooms have been used in folk medicine throughout the world since ancient times. In the last decade, several medicinally active basidiomycetes were 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). A water soluble polysaccharide-protein complex, named PPC, was extracted, purified, and biological activities test from the mycelium of *P. portentosus* as described below.

Enrichment of the purified PPC from *P. portentosus*

Crude PPC from *P. portentosus* was obtained from mycelium by drying, grinding and extracting from the dry powder by hot water and subsequent precipitation by ethanol, as detailed in the methods section, and is summarized in Table 1. The subsequent extraction and purification steps of crude PPC, detailed in the 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, called P1, and a bound fraction, called P2, respectively (Figure 2A). Since P1 was eluted by water it is likely to be a neutral PPC, whereas since P2 was eluted by 0.5 M NaCl it is likely to be an acidic PPC. The various protein contents in each polysaccharide fraction as determined by direct reading of the absorbance at 280 nm. Fraction number 15s of the latter showed a slighter absorbance at 280 nm, however, which obviously might be with much smaller amounts. The direct photometric reading of the absorbance at 280 nm has long been used for determination of proteins; yet, the chromophores that responsd to such a spectrometric measurement include only the minor amino acids tryptophan, tyrosine, and phenylalanine.

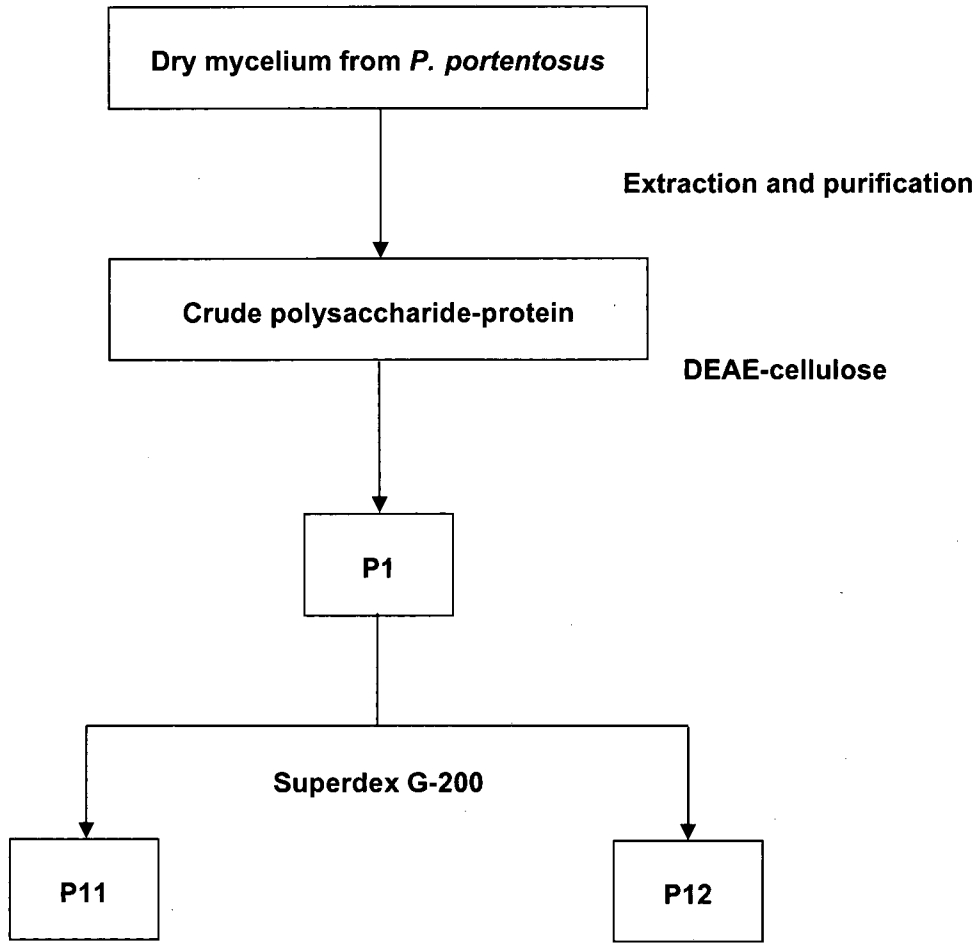
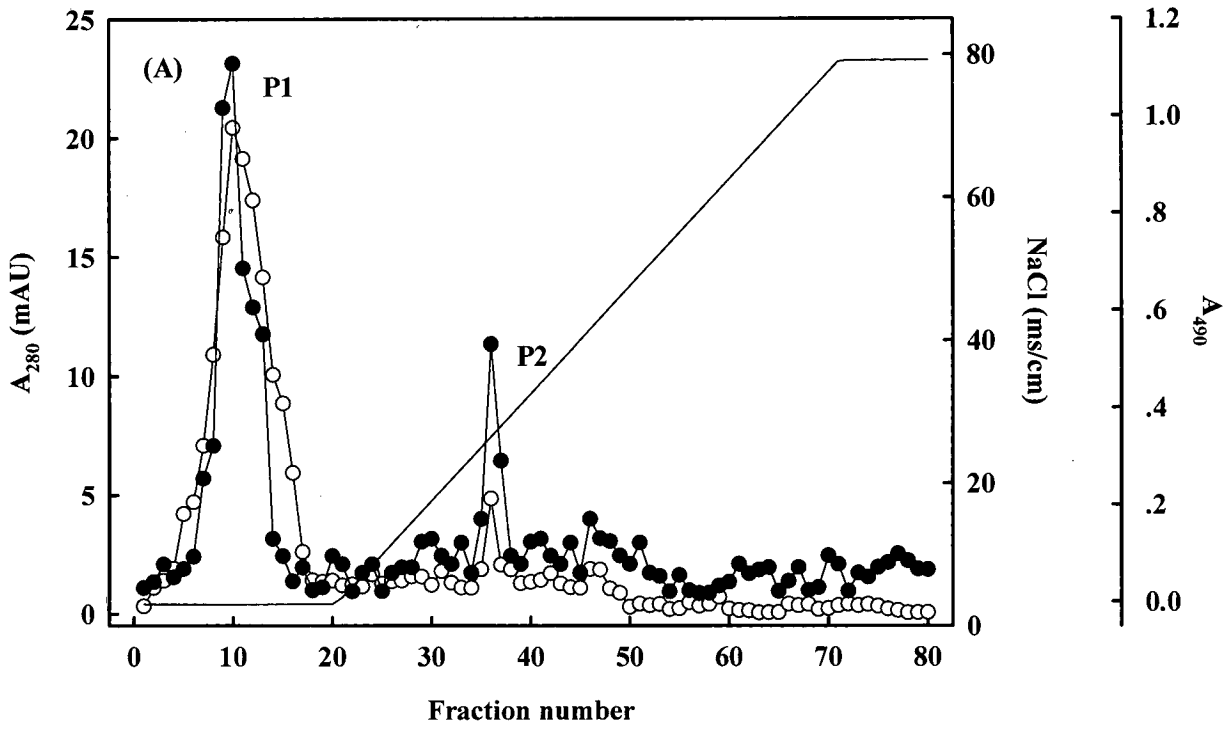
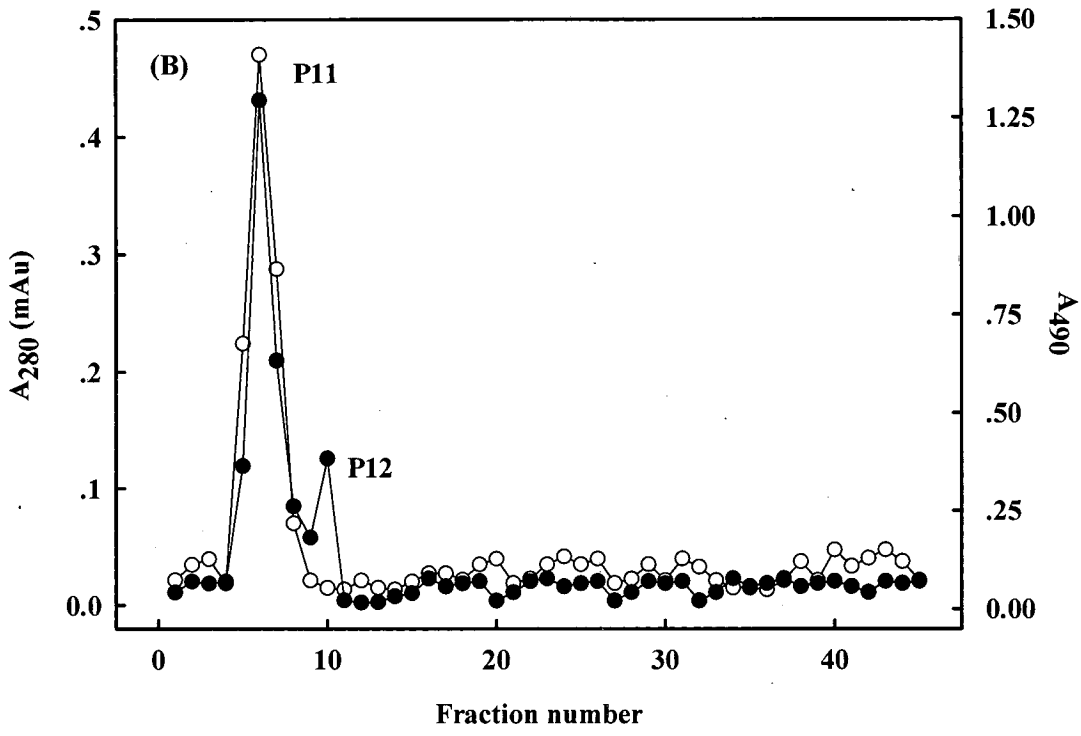


Figure 1. Schematic diagram summarizing the main stages in the extraction and purification of polysaccharides-protein complex (PPC) from dry mycelium of *P. portentosus*.


(A)
P1
P2
A_{280} (mAU)
NaCl (ms/cm)
A_{490}
Fraction number
0
5
10
15
20
25
0
20
40
60
80
0.0
.2
.4
.6
.8
1.0
1.2
0
10
20
30
40
50
60
70
80


(B)
P11
P12
A_{280} (mAu)
A_{490}
.5
.4
.3
.2
.1
0.0
1.50
1.25
1.00
.75
.50
.25
0.00
0
10
20
30
40
Fraction number

(C)

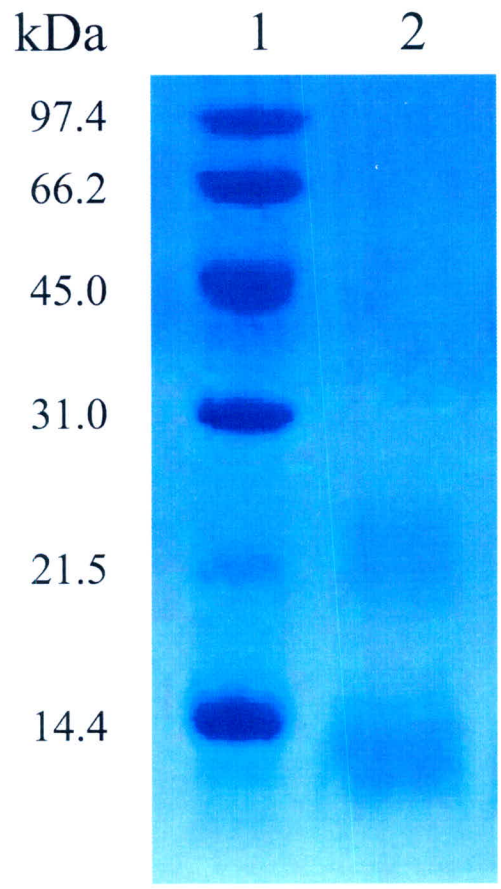


Figure 2. (A) DEAE-cellulose ion exchange column chromatogram showing the separation profile of crude PPC, giving P1 and P2 from distilled water and 0.5 M NaCl elution, respectively. (B) Superdex G-200 gel filtration column chromatogram for fraction P1 from the distilled water elute. (C) SDS-PAGE (12.5%) of purified PPC (P11) from *P. portentosus*. Lane 1, molecular weight markers; 2, P11.

In essence, the absorbance at 280 nm determines the true amount of these three amino acid contents, rather than the whole protein. Naturally, one would expect a more intense absorbance in 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. Further analysis of the protein content is obviously alternative interesting research work that remains ahead. The P1 was then subjected to further purification using Superdex G-200 gel filtration column chromatography, resulting in only a single peak from fraction P1, two peaks were separated, called P11 and P12, at fraction numbers 5-7 and 10, respectively (Figure 2B). Due to the low yield attained for P12, 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 had two major protein bands, which had molecular weights lower than 21.5 kDa and around 12-15 kDa (Figure 2C).

Various methods have been used for the isolation of fungal polysaccharides and polysaccharide-peptide complexes. Repeated extraction with hot water and cold NaOH has been used for isolating polysaccharides from *Agaricus blazei* and *Sparassis crispa* (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 has been used by Mizuno et al. (Mizuno et al., 1995) to isolate polysaccharides from *Tricholoma giganteum*, and by Zhang et al. (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 purified PPC

Major purified PPC (P11) from purification by using DEAE-cellulose and Superdex G-200 column was characterization which composed of finding their functional group by using FT-IR spectroscopy, and NMR spectroscopy, and determination molecular weight by using gel permeable chromatography (GPC). The results showed as below.

The functional group of purified PPC by FT-IR spectroscopy

The results from FT-IR spectroscopy was used to confirm that purified PPC (P11) was polysaccharides (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 ~2,992.72 cm^{-1} represent C-H stretching in the sugar ring. PPC have residual water bands around ~1,634.81 cm^{-1}, whilst bands around 1,363.84 cm^{-1} represent the C-H and O-H deformation vibrations, and those around 888.05 cm^{-1} the C-H deformation vibrations. The band at ~1,052.03 cm^{-1} indicates the C-O-C and O-H residues in a pyran structure.

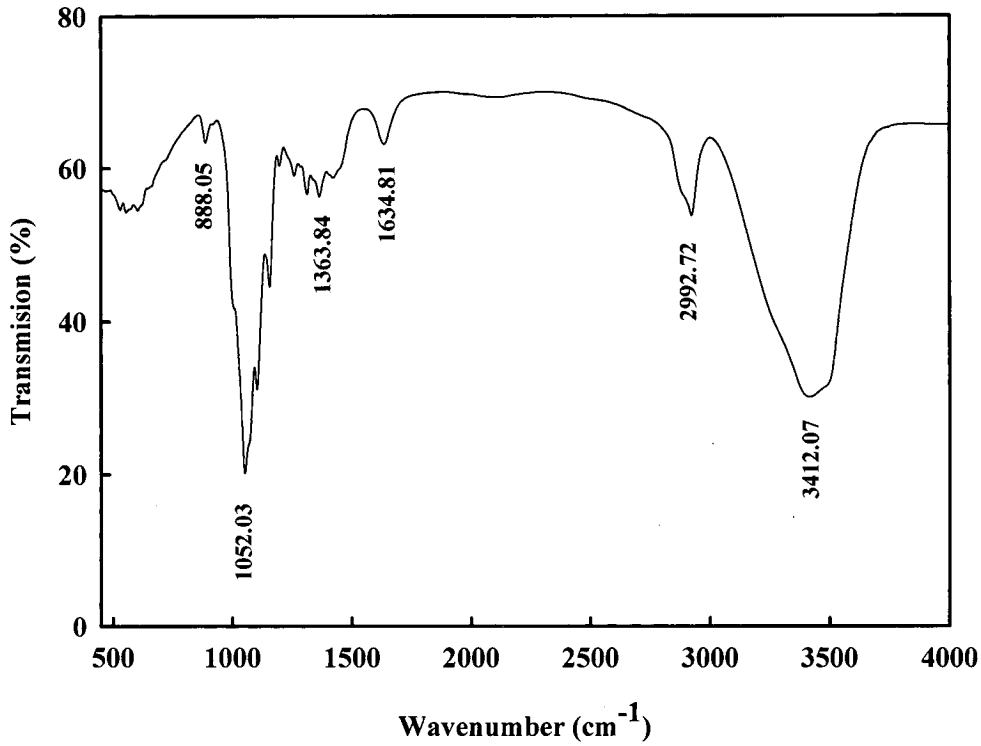


Figure 3. FT-IR spectroscopy of P11 from *P. portentosus*.

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

The ^{13}C NMR spectroscopy analysis of purified PPC (P11) revealed a relative simplicity. This is due to the homopolysaccharide type present in these organisms. Figure 4A shows that the ^{13}C NMR spectrum of the P11 from *P. portentosus*, the absence of signals at 180-120 ppm in this figure shows that this spectrum was not contaminated by phenolic compounds. The presence of glucose was observed through a signal at 103.5 ppm, characteristic of the β-configuration. Other important signals in the present spectra are those in the area of 60-80 ppm, where they are related to C2, C3, C4, C5 and C6 of that carbohydrate. This spectrum displays signals in the area between 28.5 and 33.0 ppm. According to Gonzaga et al. (Gonzaga et al., 2005), the presence of signals between 20 and 40 ppm suggests characteristic glucan-protein compound structure. The ^{1}H spectra (Figure 4B) 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 (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.

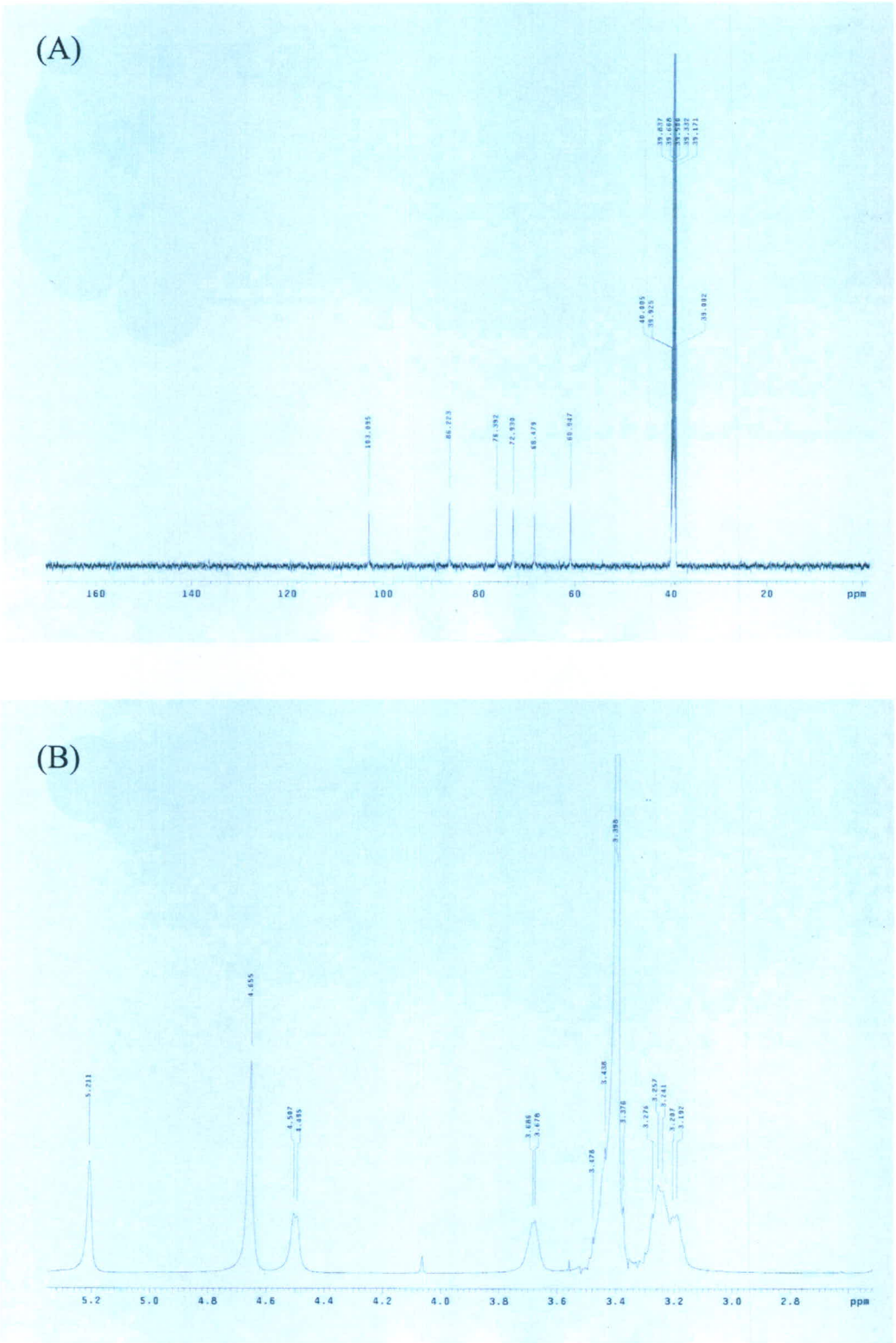


Figure 4. (A) ^{13}C NMR spectroscopy of P11 from *P. portentosus*. (B) ^{1}H NMR spectroscopy of P11 from *P. portentosus*.

The molecular weight of purified PPC by GPC

The results from gel permeable chromatography indicated that P11 had average molecular weight of 294,725 Da (Figure 5). The fact that P11 was eluted by 1.0 M NaCl in DEAE-cellulose anion exchange chromatography and PPC had prominently negative charges on its molecule indicated the P11 is acidic polysaccharide. Due to PPC is acidic polysaccharide so it can be eluted by using sodium bicarbonate buffer (pH = 11) from GPC.

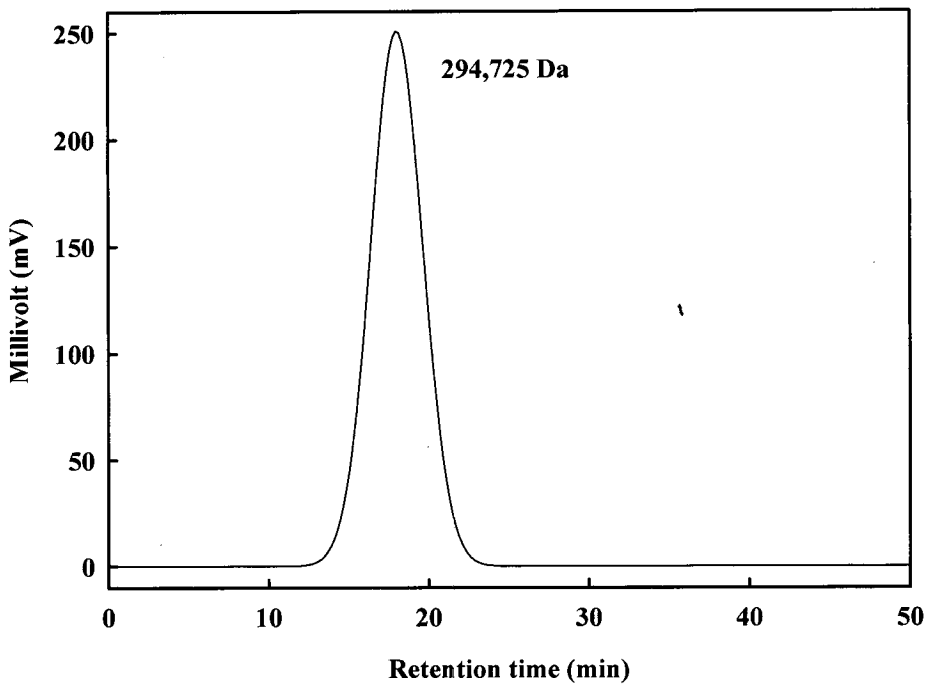


Figure 5. The molegular weight of P11 from gel permeable chromatography.

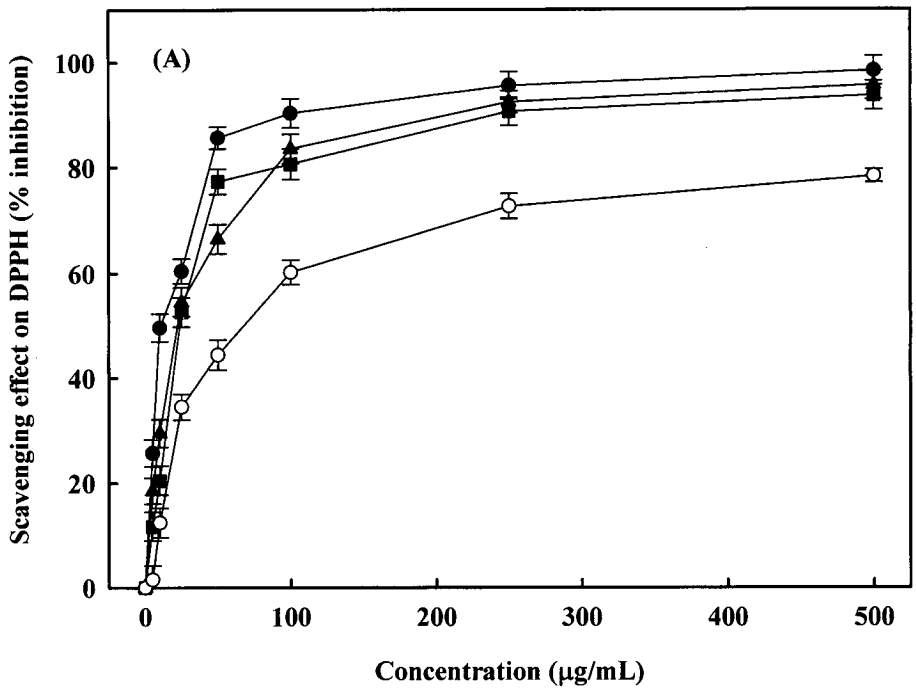
In vitro antioxidant activity

Free radicals are chemical entities that can exist separately with one or more unpaired electrons. The generation of free radicals can thus cause extensive tissue damage. Lipids, proteins and DNA are all susceptible to attack by free radicals (Cheeseman et al., 1995). Antioxidants may offer resistance against oxidative stress by scavenging the free radicals. It is that when the balance between ROS production and oxidant defenses is lost, oxidative stress which 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, Alzhimer's disease, retinopathy and rheumatism (Tiwari et al., 2004).

DPPH radical scavenging assay

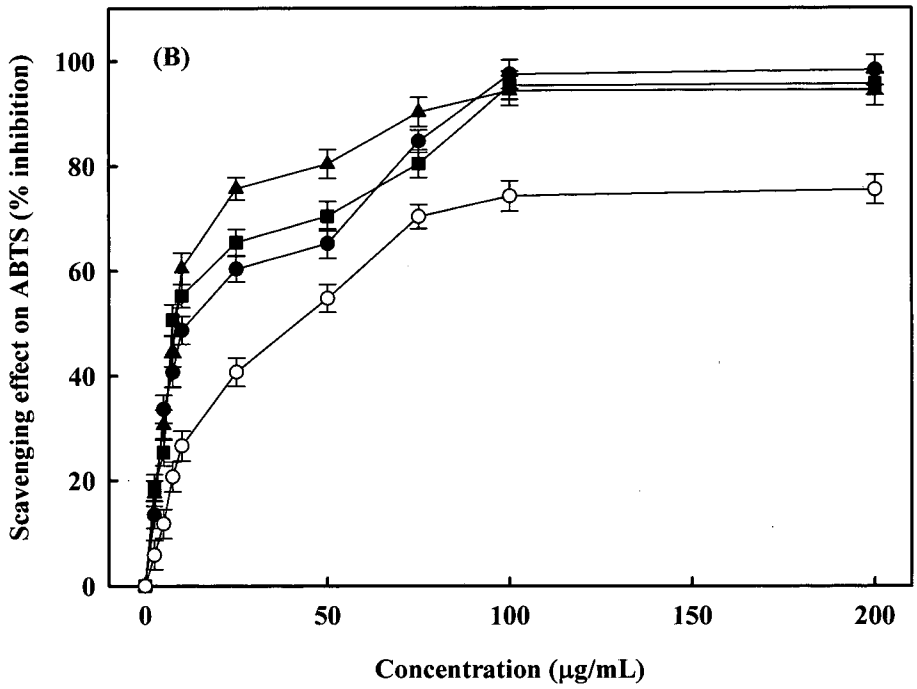
Unlike other free radicals such as the hydroxyl radical and superoxide anion, DPPH has the advantage of being unaffact by certain side reactions, such as metal ion chelation and enzyme inhibition (Amarowicz et al., 2007). A freshly prepared DPPH solution exhibits a deep purple colour with absorption maximum at 517 nm. The purple colour generally fades or disappears when an antioxidant is present in the medium. Thus, antioxidant molecules can quench DPPH free radicals (i.e., by providing hydrogen atoms or by electron donation, conceivably via a free-radical attack on the DPPH molecule) and convert them to a colourless (i.e., 2, 2-diphenyl-l-hydrazine, or a substituted analogous hydrazine), resulting, in decreases in 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 o DPPH is that its reduction can be measured directly in the reaction medium by a continuous spectrophotometric assay. DPPH assay is known to give reliable in formation concerning the antioxidant ability of the tested compounds. Free radical scavenging capacities of P11, measured by DPPH assay, are shown in Figure 6A. All concentration studied showed free radical scavenging activity. The 50% of inhibition value for P11 seems to be fairly significant when compared to commonly used synthetic

antioxidant α-tocopherol, BHT, and BHA (IC_{50} = 92.473 ± 0.129 μg/mL was necessary to obtain 50% of DPPH degradation).


(A)
Scavenging effect on DPPH (% inhibition)
100
80
60
40
20
0
0
100
200
300
400
500
Concentration (μg/mL)

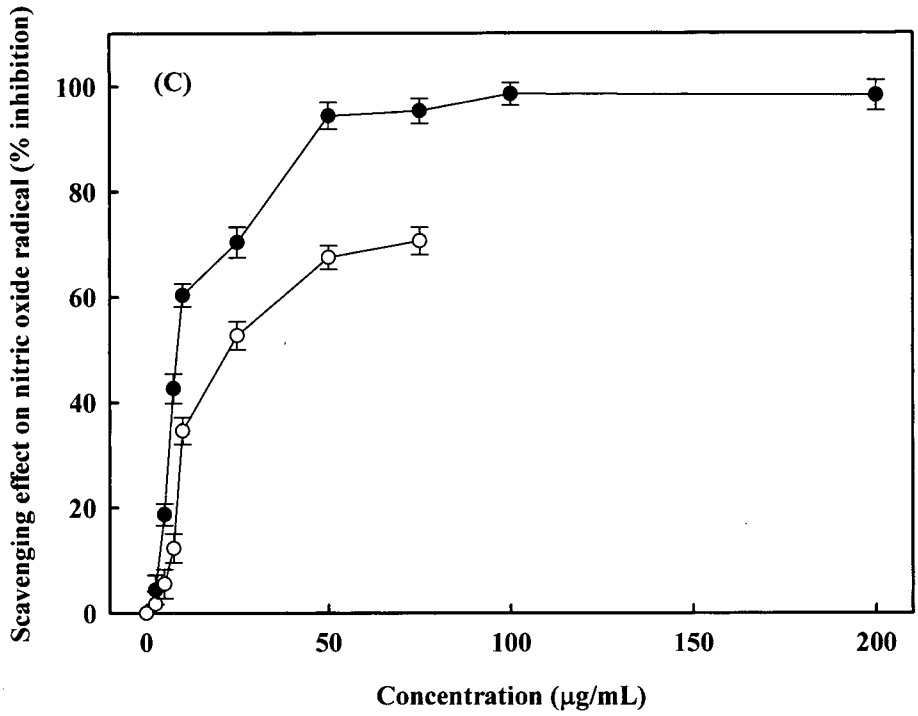
ABTS radical scavenging assay

The reactions with $ABTS^{+\bullet}$ radicals involve a single-electron transfer process. Bleaching of a preformed solution of the blue-green radical cation $ABTS^{+\bullet}$, which has an absorption at 734 nm, has been extensively used by past researchers to evaluate the antioxidant capacity of complex mixtures and individual compounds (Miller et al., 1997). The scavenging ability of P11 on ABTS free radical was shown in Figure 6B. The scavenging powers of P11, α-tocopherol, BHT, and BHA correlated well with increasing concentrations. With regards to scavenging ability on ABTS radicals, IC_{50} values of the P11 from the studied mushroom was 48.113 ± 0.743 μg/mL. Therefore, the results indicated that P11 had strong scavenging power for ABTS radicals and should be explored as novel potential antioxidants. $ABTS^{+\bullet}$ 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, $ABTS^{+\bullet}$ is soluble in both aqueous and organic solvents and is not affected by 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). But the compounds involved in biological relevance were found to reduce DPPH less when compared to the synthetic antioxidant compounds like BHT and BHA (Prior et al., 2005). One possible reason for lesser amount of inhibition found in DPPH assay when compared to ABTS antioxidant assay would be because of the chemical nature of DPPH.


(B)
Scavenging effect on ABTS (% inhibition)
100
80
60
40
20
0
0
50
100
150
200
Concentration (μg/mL)

Nitric oxide radical scavenging

Nitric oxide is a free radical generated by endothelial cells, macrophages, neurons, etc. and involved in the regulation of various physiological processes (Lata and Ahuja 2003). Excess concentration of nitric oxide is associated with several diseases (Ialenti et al., 1993). Oxygen reacts with several diseases (Sainani et al., 1997). It is well known that nitric oxide has an important role in various inflammatory processes. Sustained levels of production of this radical are directly toxic to tissues and contribute to the vascular collapse associated with septic shock, whereas chronic expression of nitric oxide 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 radical, forming the highly reactive peroxynitrite anion ($ONOO^-$) (Huie and Padmaja, 1993). The nitric oxide generated from sodium nitroprusside reacts with oxygen to form nitrite. The extract inhibits nitrite formation by directly competing with oxygen in the reaction with nitric oxide. P11 also caused a moderate dose-dependent inhibition of nitric oxide with an IC_{50} of 23.921 $\pm$ 0.312 μg/mL (Figure 6C).


(C)
Scavenging effect on nitric oxide radical (% inhibition)
100
80
60
40
20
0
0
50
100
150
200
Concentration (μg/mL)

Hydrogen peroxide scavenging

Hydroxyl radicals are the major active oxygen species causing 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 (MDA)-like product. This compound forms a pink chromogen upon heating with TBA at low pH (Halliwell et al., 1987). When PPC extract was added to the reaction mixture, it removed the hydroxyl radicals from the sugar and prevented the reaction. Figure 6D shows that the plant extract is a very poor scavenger of H_2O_2 (IC_{50} = 18.442 ± 0.521 mg/mL) compared to standard sodium pyruvate (Figure 5D).

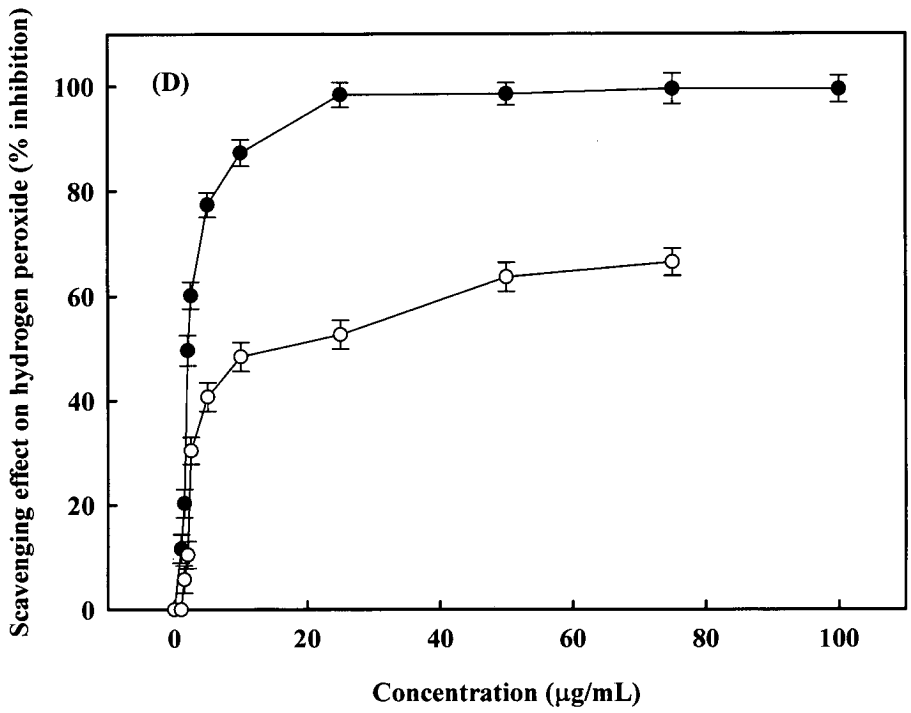


Figure 6. (A) Dose response curves of DPPH radical and (B) ABTS cation scavenging activity of P11 isolated from *P. portentosus*. Different concentration of P11 and standard as: (○) P11, (●) α-tocopherol, (■) BHT, and (▲) BHA. (C) Dose response curves of nitric oxide radical scavenging activity of P11 isolated from *P. portentosus*. Different concentration of P11 and standard as: (○) P11, (●) curcumin. (D) Dose response curves of hydrogen peroxide scavenging activity of P11 isolated from *P. portentosus*. Different concentration of P11 and standard as: (○) P11, (●) sodium pyruvate. The data are shown as the mean ± 1 SD and are derived from three replicate experiments. Means with a different lowercase letter above them are significantly different ($p<0.05$; Duncan's multiple means test).

Studies of antioxidant activity with P11 showed that the polysaccharide-protein complex extract of this mushroom has an inhibitory action on the formation of hydroxyl radicals in a dose-dependent way. These radicals are highly deleterious. Polysaccharide extracts from fungi of different classes have showed high scavenger activity on free radicals (Wasser and Weis, 1999b). This activity is dependent on protein portion linked to polysaccharide chain (Liu et al., 1997). Although the antioxidant mechanism of polysaccharides extracts is still not fully understood, factors related to polysaccharide such as monosaccharide residues (mainly glucose), molecular weight and water solubility are very important.

Anti-proliferation /cytotoxicity assay for human malignant cell lines

The anti-proliferative or cytotoxic effect of P11 highest efficiency against BT474 (breast) with an IC_{50} value of 1.178 ± 0.129 μg/mL down to the lowest for HEP-G2 (hepatoma) with an IC_{50} of 5.183 ± 0.229 μg/mL. However, the dose-dependent effect (inhibition of proliferation and or cytotoxicity) was different between the two cell lines. 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 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 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 polysaccharide-protein complexes 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 polysaccharide-protein complexes are considered as multi-cytokine inducers that are able to induce gene expression of various immunomodulatory cytokines and cytokine receptors (Okamoto et al., 2004).

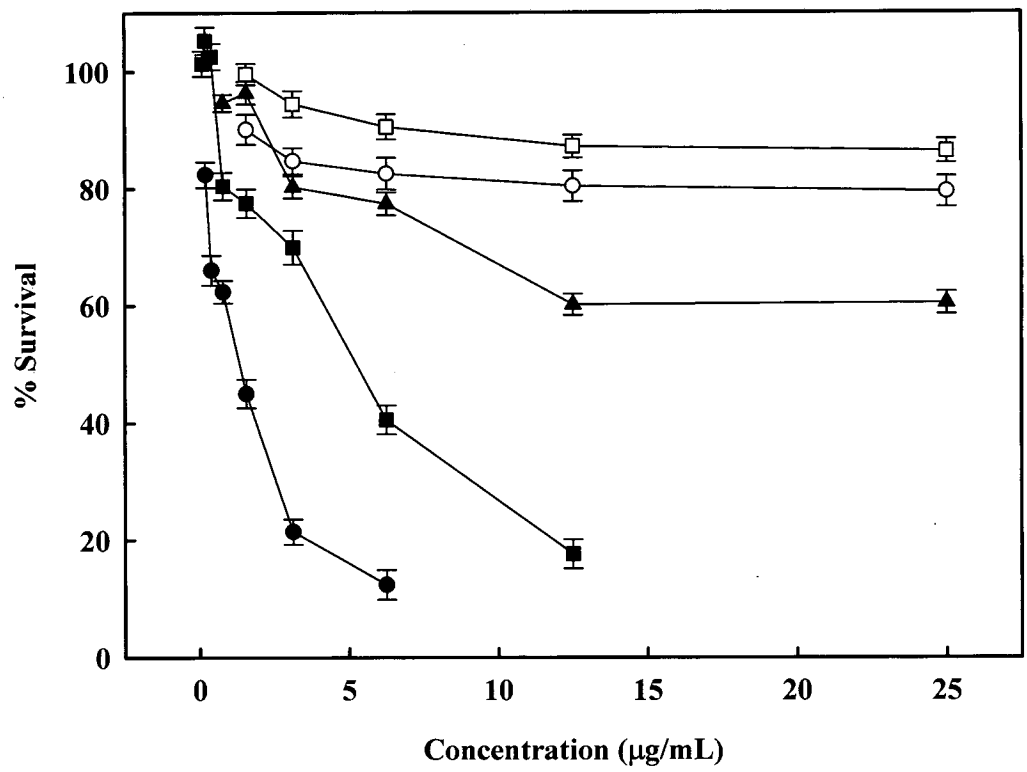


Figure 7. Inhibitory activity of P11 isolated from *P. portentosus* towards cancer cell line. Different concentration of P11: (○) KATO-3 (gastric), (●) BT474 (breast), (■) HEP-G2 (hepatoma), (□) SW620 (colon), and (▲) CHAGO (lung). Each data point represents the mean ± 1 SD of triplicate determinations. Means with a different lowercase letter above them are significantly different ($p<0.05$; Duncan's multiple means test).

CONCLUSION

A potential polysaccharide-protein complex (PPC) that displays antioxidant and anti-humanmalignant cell line proliferation was enriched from the mycelia of *P. portentosus* by a simple four-stage (extracted by hot water, precipitate with ethanol, DEAE-cellulose and Superdex G-200 column chromatography). The results from NMR, and FT-IR spectroscopy indicated and confirmed that purified PPC (P11) is polysaccharides from their functional groups and also contain characteristic glucan-protein compound structure. Findings from this research showed that P11 have moderate antioxidant activity which is significantly correlated with protein and polysaccharide content. However, determination of the effective part (carbohydrate or peptide moiety) that play the important roles in malignant cell anti-proliferation process and themechanism(s) of such activity of P11, along with conformation of its multimeric state and role of such, await further research.

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PROJECT OUTPUT

Karnchanatat A, Sihanonth P, Piapukiew J Sangvanich P (2012). An antioxidation and antiproliferation of polysaccharide-protein complex extracted from *Phaeogyroporus portentosus* (Berk. & Broome) McNabb. Afri. J. Micro. Res. (Under review; Appendix A).