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Improved Purity and Immunostimulatory Activity of β-(1→3)(1→6)-Glucan from *Pleurotus sajor-caju* Using Cell Wall-Degrading Enzymes

Saranya Satitmanwiwat,[†] Khanok Ratanakhanokchai,*[,†] Natta Laohakunjit,[†] Louis Kuoping Chao,[‡] Shui-Tein Chen,[§] Patthra Pason,[∥] Chakrit Tachaapaikoon,[∥] and Khin Lay Kyu[†]

[†]Division of Biochemical Technology, School of Bioresources and Technology, King Mongkut's University of Technology Thonburi, Bangkok 10150, Thailand

[‡]Department of Cosmeceutics, China Medical University, Taichung 404, Taiwan

[§]Institute of Biological Chemistry and Genomics Research Center, Academia Sinica, Nankang, Taipei 115, Taiwan

[∥]Pilot Plant Development and Training Institute, King Mongkut's University of Technology Thonburi, Bangkuntien, Bangkok 10150, Thailand

ABSTRACT: The objective of this work was to improve the purity of β-(1→3)(1→6)-glucan in the native triple helical structure from the fruiting bodies of *Pleurotus sajor-caju* for effective biological function using cell wall-degrading enzymes. A crude carbohydrate was extracted with hot water, then treated with crude xylanase and cellulase from *Paenibacillus curdlanolyticus* B-6. β-Glucan in the extract was purified to homogeneity with a single and symmetrical peak using 650M DEAE Toyopearl and Sepharose CL-6B column chromatography. The purity of β-glucan was confirmed by high-performance size-exclusion chromatography. Purified β-glucan was obtained at a purity of up to 90.2%. The Congo red reaction and atomic force microscopy indicated that the purified β-glucan exhibited a triple helix conformation. Purified β-glucan was able to effectively up-regulate the functions of macrophages such as nitric oxide (NO) and tumor necrosis factor (TNF-α) production.

KEYWORDS: *cellulase, cell wall-degrading enzymes, immunostimulatory activity, Pleurotus sajor-caju, xylanase, β-(1→3)(1→6)-glucan*

1. INTRODUCTION

For millennia, edible mushrooms have been of much interest to humans. This interest mainly concerns the use of mushrooms as both a food and a health food due to their multipurpose medicinal attributes.[1] Moreover, there are various applications for mushrooms, due to their hypoglycemic, antithrombotic, antibiotic, antitumor, antiviral, antihypertensive, anti-inflammatory, and blood lipid level lowering activities.[2] *Pleurotus sajor-caju* (Fr.) sing. is a tasty edible mushroom belonging to the family *Pleurotaceae* within the genus *Pleurotus*. This mushroom originated in India, growing naturally on a succulent plant (*Euphorbia royleans*) in the foothills of the Himalayas. Now, this mushroom is widely used in Asian countries. It has unique nutritional and medicinal values, and a characteristic aroma and taste.[3]

Generally, mushroom structure can be divided into three layers: the outer layer is composed of a water-soluble glucan called mucilage, the second is a tissue composed of alkaline-soluble glucan (α-(1→3)-glucan), and the innermost layer is composed of alkaline-insoluble glucan (β-(1→3)-glucan) and more rigid chitin.[3] The cell walls of mushrooms contain 50−90% polysaccharides. Among the components of polysaccharides, β-glucan is the most abundant in the cell walls of mushrooms. Other polysaccharides such as glycogen, chitin, hemicellulose (mainly xylan), and cellulose are trapped between the fibrils of β-glucan in the cell walls.[4,5] β-Glucans of mushrooms have β-(1→3)-linkages in the main chain and additional β-(1→6) side branches, but lack β-(1→4) linkages.[2] β-(1→3)(1→6)-Glucan is an interesting functional part of mushroom cell walls, and its triple helical structure shows biological effects.[1,6] Previous studies revealed that the triple helical β-glucan structure has increased the potential to contribute to immunological and antitumor activities because of their interaction with the immunological cell surface receptors.[7]

Effective production of β-glucans in terms of yield and structural conformation, which play an important role in their antitumor activities, has been a main concern.[8] Many extraction methods have been developed by researchers for producing mushroom β-glucans.[3] Alkaline and hot water extractions are usually used to extract β-glucan from the cell walls of mushrooms.[4] Hot water extraction is a simple method, and the conformation of the extracted β-glucan is thought to be a triple helix. However, β-glucan extracted by alkaline extraction methods has a single helical structure because the alkaline pH effectively breaks apart the hydrogen bonds of the β-glucan triple helix. Alkaline extraction is performed in harsh conditions, leading to β-glucan structural changes during processing. Moreover, the cross-linked structure of β-glucan with other polysaccharides is a problem that affects the purification of mushroom β-glucan, as other components are also extracted along with β-glucan during the extraction process, thereby reducing the purity and recovery yield.[9]

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Hence, a process is needed that does not destroy the native structure of β-glucan and removes the other polysaccharides. This has led to a modification of the process for the extraction of β-glucan, which is a combination of hot water and enzymatic treatments under gentle conditions. The enzymatic treatment has advantages for the preparation of β-glucan because the gentle conditions allow for the recovery of β-glucan in its native form with minimal degradation.[9] To date, there are four reports on the use of enzymes in the extraction method with different enzymes. Rhee et al.[10] reported the preparation of the alkaline-soluble crude β-glucan content using α-amylase and amyloglucosidase. Ookushi et al.[11] reported that β-D-glucan was extracted from the water-insoluble residue of *Hericium erinaceum* by proteolytic and chitin degrading enzyme treatments, followed by microwave irradiation. Wong et al.[12] reported the use of enzymes, such as α-amylase, amyloglucosidase, and protease, in the preparation of dietary fiber, including β-glucan from three mushrooms sclerotia, namely, *Pleurotus tuber-regium*, *Polyporus rhinoceros*, and *Wolfiporia cocos*. Yin et al.[13] reported the alkaline-soluble polysaccharide production from the fruiting body of *Tricholoma matsutake* using a papain, pectinase, and cellulase assisted extraction method. The β-glucan content ranged from 8.3−42.3% and was obtained from the above reports. These processes were used for pilot-scale production.[14] Concerning the composition of *P. sajor-caju*, although cellulose and hemicellulose are present (∼15%), no report was found using xylanase and cellulase to remove those impurities. Nevertheless, chemical and enzymatic treatments of mushroom materials may result in a different degree of degradation to the fiber materials, especially cell wall polysaccharides.[15] Hence, using xylanase and cellulase in the extraction and purification steps may lead to different chemical and physical characteristics of β-glucan. This may affect the properties of this material when used in food applications, specifically in terms of its impact on human health. Following these ideas, this study presents a process for the extraction of β-glucan from mushroom *P. sajor-caju*. This method includes extraction by hot water, along with the use of cell wall-degrading enzymes, xylanase, and cellulase from *P. curdlanolyticus* B-6; this would allow for the retention of the native structure of β-glucan and the removal of undesirable polysaccharides from mushroom cell walls, followed by purification by column chromatographies. Finally, functional parameters were investigated, such as the release of NO and the production of cytokines by macrophages activated by the purified β-glucan as part of the innate immune response.

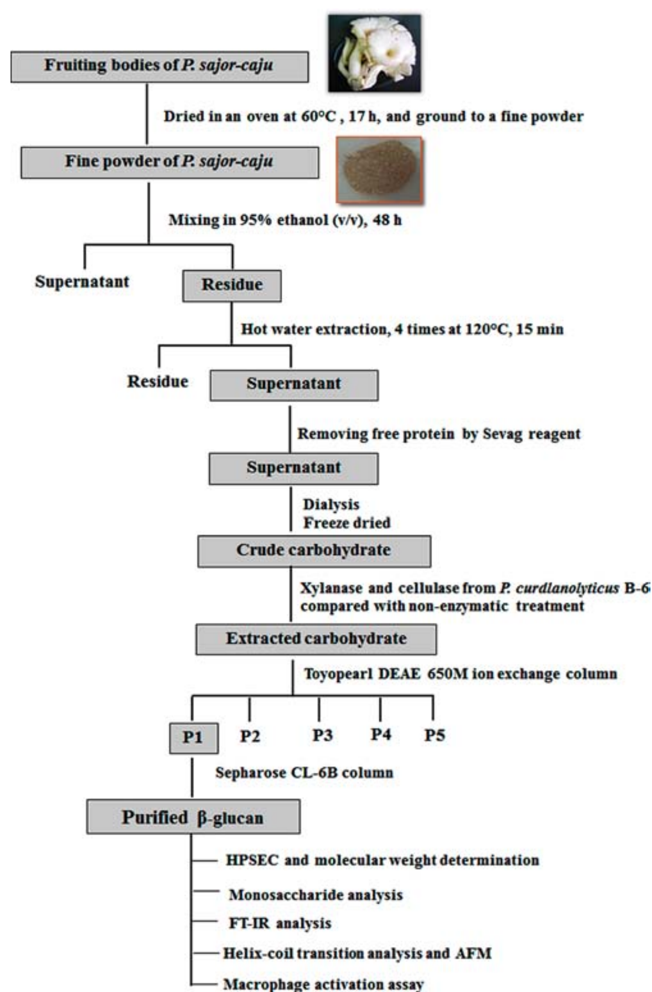

Figure 1. Detailed procedures of extraction and purification of β-(1→3)(1→6)-glucan from *P. sajor-caju*..

2. MATERIALS AND METHODS

2.1. Materials. Fresh fruiting bodies of the mushroom *P. sajor-caju* were purchased from a farm in Ratchaburi Province, Thailand. The mushrooms were washed with water, crushed, dried in an oven at 60 °C until the weight was constant (17 h), and ground using a mill to obtain a fine powder.

2.2. General Methods. UV−visible absorption spectra were recorded with a UV-1601 Shimadzu spectrophotometer. Total carbohydrate content was determined by the phenol−sulfuric acid method using D-glucose as the standard.[16] Protein was analyzed by the Lowry method.[17] The detailed procedures of extraction and purification of β-(1→3)(1→6)-glucan from *P. sajor-caju* are described in Figure 1.

2.3. Enzyme Production. Alkaliphilic *Bacillus firmus* K-1 (formerly known as *Bacillus* sp. strain K-1),[18] a cellulase-free xylanase-producing strain, was used as a source of xylanase. *P. curdlanolyticus* B-6 was used as a xylanase- and cellulase-producing strain. The crude enzymes were prepared according to the method of Sornyotha et al.[19]

2.4. Enzyme Assays. Xylanase, cellulase, amylase, and chitinase activities were determined as previously described.[20] β-Glucanase activity (β-(1→3)(1→6)-glucanase) was assayed under the same conditions as those described above using laminarin (Sigma-Aldrich, St. Louis, MO, USA) as the substrate.

The concentration of reducing sugars was determined by the Somogyi−Nelson method.[21] One unit of enzyme activity was defined as the amount of enzyme that liberated 1 μmol of product per minute under the assay conditions.

2.5. Extraction of β-Glucan from Mushrooms Using Cell Wall-Degrading Enzymes. *2.5.1. Preparation of Crude Carbohydrate from Mushroom.* The powder from fruiting bodies of *P. sajor-caju* was first defatted with 95% ethanol at room temperature for 48 h with stirring to remove any low molecular weight compounds; the supernatant was then removed. Hot water extraction was applied to the residue by autoclaving in distilled water (1:2 w/v) at 120 °C for 15 min, four times, according to the modified method of Izydorczyk et al.[22] Next, the aqueous filtrates were combined and concentrated to a small volume using a rotary evaporator at 40 °C. The Sevag reagent (chloroform/butanol 4:1, v/v) was applied to remove free proteins, followed by exhaustive dialysis through regenerated cellulose tubes (pore size about 10,000 Da) against water for 48 h. Then, the deproteinized sample was lyophilized to give a crude carbohydrate.

2.5.2. Hydrolysis and Extraction of Crude Carbohydrate by Xylanase and Cellulase. The crude carbohydrate was hydrolyzed with the crude enzyme containing 2.0 U/mg protein of cellulase-free xylanase from *B. firmus* K-1 or the crude enzyme from *P.*

curdlanolyticus B-6 containing 2.0 U/mg protein of xylanase and 0.08 U/mg protein of cellulase at 50 °C and pH 6.0 (50 mM sodium phosphate buffer). Samples were withdrawn at different intervals, and the reducing sugar content was determined by the Somogyi−Nelson method.[21] After that, the sample from the optimization condition (2.0 U/mg protein of xylanase and 0.08 U/mg protein of cellulase from the strain B-6 at pH 6.0, 50 °C for 90 min), was centrifuged, and the supernatant was dialyzed and lyophilized. The no-enzymatic treatment was also produced under the same conditions as those describe above but with no enzymes added.

2.6. Purification of β-Glucan. The extracted carbohydrate was dissolved in distilled water, centrifuged, and applied to a Toyopearl DEAE (Tosoh, Japan) column (2.0 × 45.0 cm) that was previously equilibrated with distilled water following the method of Sun et al.[23] After loading the sample, the column was eluted with distilled water, then with stepwise solutions of aqueous NaCl (0.2, 0.5, and 1.0 M) at a flow rate of 0.6 mL/min. Fractions of 4 mL were collected, and the total carbohydrate content of each tube was determined by the phenol−sulfuric acid method. Absorption at 280 nm was determined for the protein in each fraction. After that, each fraction containing only carbohydrate was dialyzed with distilled water and lyophilized for further purification. The polysaccharide sample obtained above was dissolved in distilled water and applied to a Sepharose CL-6B column (1.5 × 60.0 cm). The column was eluted with distilled water at a flow rate of 0.4 mL/min. Fractions of 2 mL were collected and assayed for total carbohydrate by the phenol−sulfuric acid method. The fractions of each peak containing carbohydrate were collected and lyophilized for further study.

2.7. Determination of β-(1→3)(1→6)-Glucan. β-Glucan of mushroom samples was determined using a mushroom β-glucan kit (K-YBGL 10/2005, Megazyme Int., Wicklow, Ireland). One hundred milligrams of the milled mushroom powder and 1.5 mL of 37% hydrochloric acid (v/v, 10 N) were added to each tube and stirred vigorously using a vortex mixer. The tubes were placed in a water bath at 30 °C for 45 min. Next, the materials were mixed with 10 mL of distilled water, and the tubes were incubated in a boiling water bath at 100 °C for 2 h. The tubes were cooled to room temperature, and 10 mL of 2 N KOH was added. The samples were washed and adjusted to a 100 mL volume with 200 mM sodium acetate buffer (pH 5.0). After centrifugation at 6,500*g* for 10 min, 0.1 mL aliquots of the supernatants were combined with 0.1 mL of a mixture of exo-β-(1→3)-glucanase (20 U/mL) plus β-glucosidase (4 U/mL) in 200 mM sodium acetate buffer (pH 5.0) and incubated at 40 °C for 60 min. To measure the total glucan content, 3.0 mL of glucose peroxidase reagent was added and incubated at 40 °C for 30 min. The absorbance of all solutions was measured at 510 nm against the reagent blank. To measure the α-glucan content (the phytoglycogen), 2 mL of 2 M KOH was added to each tube to resuspend the pellets, which were dissolved by stirring for 20 min in an ice water bath. The suspension was added to 8 mL of 1.2 M sodium acetate buffer (pH 3.8), mixed with 0.1 mL of amyloglucosidase (3,300 U/mL), and incubated in a water bath for 30 min at 40 °C. Tubes were centrifuged (10 min at 6,500*g*), and 0.1 mL aliquots (0.1 mL) of the supernatants were combined with 0.1 mL of sodium acetate buffer (200 mM, pH 5.0). Chromophore development employing a glucose peroxidase reagent was conducted according to the total glucan content assay. The β-glucan content was determined by subtracting the α-glucan content from the total glucan content.

2.8. Homogeneity of β-Glucan. The homogeneity for purity of β-glucan was determined by high-performance size exclusion chromatography (HPSEC) using a Shimadzu LC-10Avp HPLC system equipped with a ZORBAX PSM 1,000S column (7.8 mm i.d. × 300.0 mm) (Agilent, USA), an LC-20AD pump, and a RID-10A refractive index detector. The sample solution (20 μL, 3 g/L) was injected and eluted with distilled water at a flow rate of 0.8 mL/min at room temperature. The fractions containing carbohydrate were collected for further β-glucan determination.

2.9. Molecular Weight Determination. The average molecular weight of the purified β-glucan was determined by a gel-chromatographic technique on a Sepharose CL-6B column (1.5 × 60.0 cm), eluted with distilled water at a flow rate of 0.4 mL/min. The elution volume of the purified β-glucan was plotted on a standard calibration curve prepared by plotting the elution volume of standard dextran (M_w ∼25 kDa, M_w ∼80 kDa, M_w ∼150 kDa, and M_w ∼270 kDa from *Leuconostoc mesenteroides*, Sigma-Aldrich, St. Louis, MO, USA) against the logarithm of their respective molecular weights.

2.10. Monosaccharide Analysis. For monosaccharide analysis, 0.5 mg of polysaccharide was methanolyzed with 0.2 mL of 0.5 N methanolic HCl (Supelco, Bellefonte, PA, USA) at 80 °C for 16 h, re-N-acetylated with 500 mL of absolute methanol, 10 mL of pyridine, and 50 mL of acetic anhydride, then treated with Sylon HTP trimethylsilylation reagent (Supelco, Bellefonte, PA, USA) for 20 min at room temperature, after which it was dried under nitrogen stream and redissolved in hexane. GC-MS analysis of the trimethylsilylated derivatives was carried out using an Agilent Technology Gas Chromatograph 7683B connected to an Agilent Technology 5973 mass selective detector. Samples were dissolved in hexane prior to splitless injection into an HP-5MS fused silica capillary column (0.25 mm i.d. × 30 m). The column head pressure was maintained at around 8.2 psi to give a constant flow rate of 1 mL/min using helium as the carrier gas. The initial oven temperature was held at 60 °C for 1 min, increased to 140 at 25 °C/min, then to 250 at 5 °C/min, and then to 300 at 10 °C/min. Sugar identification was performed by comparison with reference sugars.

2.11. Amino Acid Analysis. Amino acid analysis of the carbohydrate−protein complex was performed in a sealed ampule for 22 h at 110 °C using 6 M HCl. The amino acid constituents were separated on a reversed phase Hypersil gold column (4.6 mm i.d. × 150 mm, 3 μm) and analyzed on high-performance liquid chromatography (HPLC) (Waters Alliance 2695, USA) with the mobile phase composed of sodium acetate buffer at pH 4.9 and 60% acetonitrile. Preliminary calibration of the column was conducted using amino acid standards.

2.12. Fourier Transformed Infra-Red (FT-IR) Analysis. Samples were ground with KBr powder and pressed into 1 mm pellets for FT-IR measurement. FT-IR spectra were obtained on an FT-IR spectrometer (Spectrum one, Perkin-Elmer Co., USA) in the range of 400−4000 cm^{-1}.

2.13. Helix−Coil Transition Analysis. *2.13.1. Congo Red Test.* The analysis was performed following the method of Ogawa et al.[24] The conformation of β-glucan was determined by the Congo red binding assay that consists of the formation of a complex between the biopolymer and the Congo red dye, which shows a shift in the λ_{max} in the visible spectrum. This method to determine the conformation of β-glucan is based on the ability of Congo red to interact with β-glucan, possibly due to hydrogen bond formation with the innate free hydroxyl linkages in the triple helix form. The conformation of the triple helix can be affected by increasing the pH. Aqueous alkali reagents (NaOH or KOH) disrupt hydrogen bonding, causing a transition from the triple helix. The β-glucan solution (1 g/L) containing 91 mM Congo red was subjected to different concentrations of NaOH and, after 3 h of interaction, the visible absorption spectra were recorded from 400−700 nm at 25 °C with a UV/visible spectrophotometer at various alkali concentrations.

2.13.2. Atomic Force Microscopy (AFM). The purified β-glucan was dissolved in distilled water to make a 1 mg/mL stock solution after stirring for a few days. The samples for the AFM test were made by diluting the stock solution to 5−10 μg/mL with distilled water and prepared by the drop deposition method. AFM images were obtained with a Seiko instrument (SPI400, Japan) and collected in tapping mode. All measurements were performed in air at ambient pressure and humidity. The resulting imaging force was estimated to be 0.05−1.60 nN, and the resonant frequency was about 180 kHz.

2.14. Macrophage Activation Assay. *2.14.1. Cell Culture.* Murine macrophage J774A.1 cells were obtained from ATCC (Rockville, MD), propagated in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Hyclone Co., Logan, UT) and 2 mM L-glutamine (Life Technologies, Inc., MD), and cultured at 37 °C in a 5% CO_2 incubator.[25]

2.14.2. Determination of NO Production. After preincubating murine macrophage J774A.1 cells (2×10^6 cells/mL) for 24 h, purified β-glucan (100 μg/mL), crude carbohydrate (100 μg/mL), or lipopolysaccharide (1 μg/mL; LPS), and control (nothing added) in the mixture were incubated for an additional 24 h. Nitrite was measured in the culture supernatants by adding 100 μL of the Griess reagent (1% sulfanilamide and 0.1% *N*-[1-naphthyl]-ethylenediamine dihydrochloride in 5% phosphoric acid) to 100 μL of the samples. The nitrite concentration was determined at 540 nm using $NaNO_2$ as the standard.

2.14.3. Determination of TNF-α Production. Murine macrophage J774A.1 cells (2×10^6 cells/mL) were incubated for 24 h with purified β-glucan (100 μg/mL), crude carbohydrate (100 μg/mL) or LPS (1 μg/mL), and control (nothing added). Supernatants were harvested, and the concentration of TNF-α was determined using a mouse TNF-α enzyme linked immunosorbent assay (ELISA) kit (BD Biosciences Pharmingen) according to the manufacturer's instructions.

2.14.4. Monitoring of LPS Contamination. In order to rule out possible LPS contamination of purified β-glucan, J774A.1 cells were preincubated with or without 10 μg/mL of polymyxin B (PMB) for 30 min, followed by treatment for the determination of NO production and TNF-α production with purified β-glucan (100 μg/mL), crude carbohydrate (100 μg/mL) or LPS (1 μg/mL), and control (nothing added).

2.15. Statistical Analysis. Each result is expressed as the mean $\pm$ SD of data obtained from triplicate experiments. Statistical analysis was performed by a paired Student *t*-test. Differences at $p < 0.01$ are considered statistically significant.

3. RESULTS AND DISCUSSION

3.1. Extraction of Crude Carbohydrate. In this study, crude carbohydrate was obtained from the outer layer cell wall of fruiting bodies of *P. sajor-caju* via hot water extraction. In order to remove the remaining undesirable components, such as xylan and cellulose, which might also be extracted along with β-glucan in the crude carbohydrate of *P. sajor-caju* by hot water treatment, a comparison was conducted between the ability of a crude enzyme containing xylanase alone from strain K-1 and a crude enzyme containing xylanase and cellulase from strain B-6. Figure 2 shows the experimental values of the released reducing sugars. The crude enzyme containing xylanase alone (2.0 U/mg protein) from strain K-1 had the ability to hydrolyze xylan to form reducing sugars and reached a maximum at 7.5 mg/g mushroom powder at 90 min. However, the crude enzyme containing xylanase (2.0 U/mg protein) and cellulase (0.08 U/mg protein) from strain B-6 could hydrolyze xylan and cellulose in the crude carbohydrate and achieved a maximum reducing sugar content of 14.0 mg/g powder at 90 min. These results indicate that both xylanase and cellulase play an important role in the hydrolysis of xylan and cellulose. Wong et al.[12] reported that the purity of analytical or industrial enzymes affects the yield of sclerotial β-glucan in the enzymatic preparation of mushroom dietary fiber. Contamination of β-glucanases during enzymatic preparation of the crude enzyme might cause endo/exodepolymerization of β-glucan. Thus, the crude enzymes from strains K-1 and B-6 were investigated for β-glucanase activity. The results show that the crude enzymes from both strains were free of β-(1→3)(1→6)-glucanase. Therefore, the crude enzymes produced from both strains did not depolymerize the β-glucan of the mushroom. The liberated reducing sugars were the hydrolytic products of xylan and/or cellulose, which were associated with β-glucan in the crude carbohydrate of *P. sajor-caju*. From these results, enzymatic treatment with xylanase and cellulase of strain B-6 provided more hydrolytic products following hydrolysis of the crude carbohydrate than xylanase alone from strain K-1. Moreover, Pason et al.[20] reported that strain B-6 produced an extracellular xylanolytic−cellulolytic enzyme system which existed as a multienzyme complex. The multienzyme complex is dedicated to hydrolyzing crystalline cellulose and insoluble xylan because of the presence of their binding ability to insoluble substrates via cellulose- and xylan-binding modules. In addition, the different xylanases and cellulases of the strain B-6 which differ in their activities against various substrate structures and modes of action were observed.[20] Thus, the cellulolytic−xylanolytic multienzyme complex of the strain B-6 are increasingly attracting attention because of the degradation of cellulose and xylan in the crude carbohydrate of *P. sajor-caju* to reducing sugars that can eliminate these impurities from crude carbohydrate efficiently. Therefore, the crude enzyme from strain B-6 was selected for further study.

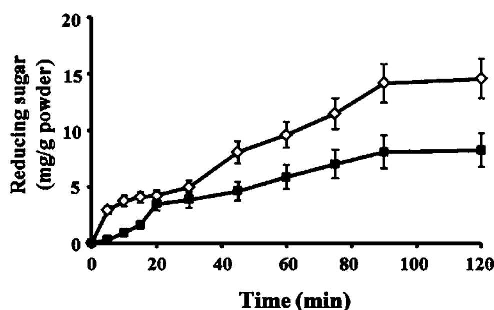

Figure 2. Time course of hydrolysis of *P. sajor-caju* cell walls by cell wall-degrading enzymes, from strain K-1 (■) and strain B-6 (◊). The data represent as the mean ± SD ($n = 5$).

Different steps and enzymes were used in the extraction and purification processes of β-(1→3)(1→6)-glucan compared to previous studies.[10−13] In the first step, the crude carbohydrate was extracted by hot water extraction as this is the most common and effective means, then free proteins were removed by the Sevag method because chemical methods seem to be more effective in removing protein from mushrooms compared to enzymatic (protease) methods.[15] Next, the crude carbohydrate was mixed with xylanase and cellulase from *P. curdlanolyticus* B-6 for the hydrolysis of xylan and cellulose. The β-(1→3)(1→6)-glucan contents of 12 edible mushroom species such as *Ganoderma lucidum*, *Lentinus edodes*, *Coriolus versicolor*, *Agaricus bisporus*, *Flammulina velutipes*, *Agaricus blazei* Murill, *Pleurotus ostreatus*, *Phellinus linteus*, *Gyrophora esculenta*, *Auricularia auricular*, *Inonotus obliquus*, and *Pleurotus eryngii* have been determined using a β-glucan kit. There is a large variability in β-glucan content in these mushrooms, ranging from 4.7 to 46.2% on a dry weight basis.[26] The β-glucan content of *P. sajor-caju* powder obtained in our study was 30.5% (dry weight basis). The extraction and purification steps for obtaining β-glucan from *P. sajor-caju* are shown in Table 1. The crude carbohydrate extracted by hot water contained 44.9% β-glucan (dry weight basis). After the enzymatic treatment of the crude carbohydrate by xylanase and cellulase from strain B-6, the β-glucan content increased to 54.6% (dry weight basis) and was higher when compared with that in the no-enzymatic treatment (45.1% dry weight basis), indicating that the β-glucan content increased by 9.5% (dry weight basis) compared to that in the no-enzymatic treatment. The higher percentage of β-glucan after the enzymatic extraction process may be because xylanase and cellulase degrade xylan and cellulose, which buried

Table 1. Extraction and Purification of β-(1→3)(1→6)-Glucan from *P. sajor-caju*

steps	no-enzyme treatment[a] (control)		enzyme treatment[b]	
	β-glucan[c]	%yield	β-glucan[c]	%yield
mushroom powder	30.5 ± 0.41	100.0 ± 0.27	30.5 ± 0.29	100.0 ± 0.31
hot water treatment	44.9 ± 0.06	27.1 ± 0.12	44.9 ± 0.34	27.1 ± 0.21
treatment for extraction	45.1 ± 0.38	15.3 ± 0.16	54.6 ± 0.15	11.7 ± 0.22
Toyopearl DEAE 650M column (from peak 1)	56.7 ± 0.24	4.7 ± 0.10	88.1 ± 0.30	3.9 ± 0.20
Sepharose CL-6B column	59.7 ± 0.37	4.1 ± 0.08	90.2 ± 0.13	3.2 ± 0.12

[a]Control: 0.5 mM phosphate buffer, pH 6.0, no enzyme added. [b]Xylanase (2.0 U/mg protein) and cellulase (0.08 U/mg protein) from strain B-6. [c]Data expressed as % dry weight.

β-glucan in the crude carbohydrate and resulted in the high extractability of β-glucan.

3.2. Purification of β-Glucan. As the carbohydrate of mushrooms contained both neutral (β-glucan) and acidic carbohydrate, anion-exchange chromatography can be used to remove acidic carbohydrate.[3] The extracted crude carbohydrate was separated on a Toyopearl 650M DEAE column; this afforded two independent peaks of carbohydrate (peaks 1 and 2) detected by the phenol−sulfuric acid method, while the other peaks (3, 4, and 5), which were eluted with 0.5 M NaCl, contained only protein that was more negatively charged than in peak 2 (Figure 3a). However, peak 2, which was eluted with

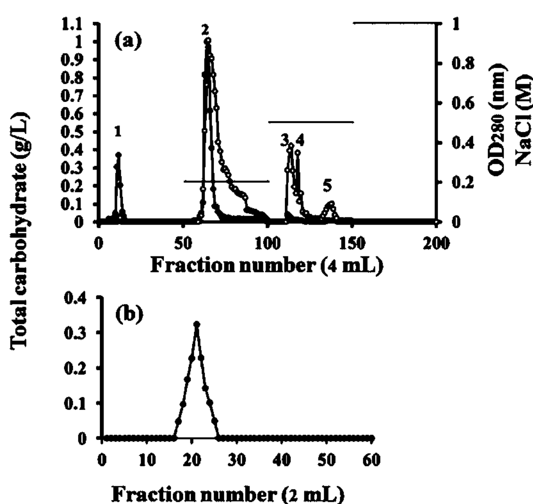

Figure 3. Stepwise elution curves of extracted carbohydrates and OD_{280} from the Toyopearl DEAE 650M column (a) and the elution curve of carbohydrate fractions (peak 1) on the Sepharose CL-6B column (b). Total carbohydrate (●), protein (○), and NaCl (——).

0.2 M NaCl, contained not only carbohydrate but also protein and was not studied further because it contained a negatively charged carbohydrate−protein complex. Amino acid analysis revealed that the protein of the complex contained high amounts of glutamic acid (10.75% w/v) and aspartic acid (10.69% w/v). These amino acids may cause negative charge in carbohydrate−protein complex. Thus, when peak 1 was pooled and sequentially passed through a Sepharose CL-6B column, a single peak occurred (Figure 3b). The fractions of this peak were collected for subsequent analyses. This extract did not respond to the Lowry test, and there was no absorption at 280 or 260 nm in the UV spectrum, indicating the absence of protein and nucleic acids. The following step gave 90.2% β-glucan (dry weight) (Table 1), whereas the nonenzymatic treatment gave 59.7% β-glucan (dry weight). The β-glucan content following enzymatic treatment was 30.5% higher than that from the nonenzymatic treatment. Interestingly, this result revealed that xylanase and cellulase have the potential to improve the purity of β-(1→3)(1→6)-glucan by releasing not only xylan and cellulose but also the carbohydrate−protein complex and proteins bound to the crude carbohydrate from the cross-linked structure of β-glucan from *P. sajor-caju*. When both xylan and cellulose were eliminated after being treated with xylanase and cellulase, the carbohydrate−protein complex and proteins may be easily removed from the cross-linked structure; they were separated in the step of anion exchange chromatography.

3.3. Monosaccharide Composition. The major sugar constituents of the purified β-glucan from *P. sajor-caju* were glucose (87.12% of total monosaccharides), fructose (8.29% of total monosaccharides), and galactose (4.60% of total monosaccharides). Considering the purity, we showed that there was no influence from the glucose in glycogen on the isolation procedure as determined by amyloglucosidase treatment; glycogen was not found in purified β-glucan. However, other components (∼13.00%) might be fructose and galactose, present as monosaccharides in the β-glucan extract, or they might be products of the degradation of an unidentified polysaccharide during methanolyzation in preparation of GCMS analysis. Fructose and galactose are monosaccharides found in some mushrooms, such as *Pleurotus ostreatus*, *P. eryngii*, and *Russula virescens*.[5,23] Thus, glucose was the most predominant sugar as reflected by β-glucans.

3.4. Homogeneity and Molecular Mass. In Figure 4, the HPSEC profile showed a single symmetrical peak, indicating that the single peak from the Sepharose CL-6B column was a homogeneous carbohydrate reflecting both purity and homogeneity of the molecular weight. The molecular weight of the

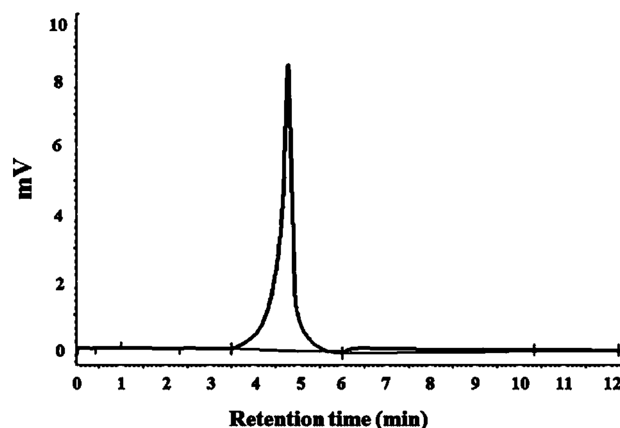

Figure 4. Profile of the purified β-glucan on HPSEC.

purified β-glucan was estimated at ~45 kDa, while the polysaccharide of *P. sajor-caju* obtained using other methods gave molecular weights of 28, 35, and 240 kDa.[2,27,28] This result indicates that different extraction and purification methods give dissimilar molecular weights of β-glucan. Moreover, this peak could also be hydrolyzed by exo-β-(1→3)-glucanase and β-glucosidase in the β-glucan kit. In many studies, mushroom polysaccharides are present mostly as glucans with different types of glycosidic linkages, but they often lack β-(1→4) glycosidic linkages.[4] Therefore, these results indicate that this peak was composed of β-(1→3)(1→6)-glucan.

3.5. Identification of Anomeric Configuration. FT-IR spectroscopy is extensively applied in polysaccharide analysis. In the case of laminarin, the characteristic peaks for (1→3)- and/or (1→6)-linked β-D-glucans are 1160, 1078, 1041, and 889 cm^{-1}.[29] Figure 5 shows the FT-IR spectrum of the purified

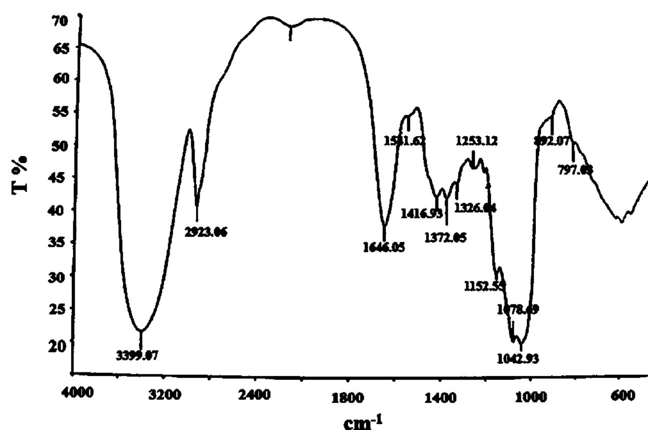

Figure 5. FT-IR spectrum of the purified β-glucan.

β-(1→3)(1→6)-glucan from *P. sajor-caju* at 1152, 1078, 1042, and 892 cm^{-1}, which was similar to laminarin. The peak at 892 cm^{-1} is specific for the β-glycosidic bond and, therefore, indicates the presence of β-glucans. The other absorption peaks at 3399, 2923, and 1646 cm^{-1} are hydroxyl stretching vibration, C–H stretching vibration, and bound water, respectively. The peaks at 1152, 1078, and 1042 cm^{-1} (pyranose ring) are from the corresponding sugar residues, fructose, glucose, and galactose.[30]

3.6. Helix–Coil Transition Analysis. This assay is based on the fact that β-glucan can bind with Congo red dye to form a Congo red–glucan complex and the λ_{max} of Congo red dye in the complex, and is, therefore, affected by the structure of the β-glucan, which can be a triple helix, single helix, or random coil. The conversion of the triple helices to single helices and single helices to random coil can be effected by increasing pH. As shown in Figure 6, the complex of Congo red and the purified β-glucan exhibited the native conformational state of a triple helix. At low NaOH concentrations (0.05–0.20 M), the λ_{max} shifted to a longer wavelength between 508–510 nm. The behavior of the λ_{max} shift was observed in a complex of Congo red and purified β-glucan. When the NaOH concentration was increased by more than 0.25 M, the λ_{max} dropped due to the disruption of triple helix. Similarly, a β-glucan from *Pleurotus tuber-regium* presented a maximum absorption at λ_{530}, which declined after the addition of more than 0.16 M NaOH.[31,32] However, no shift in λ_{max} was observed for dextran α-(1→6)-glucan, which does not exist in a triple helix conformation. In contrast, laminarins (*Laminaria digitata*) are lesser-branched β-

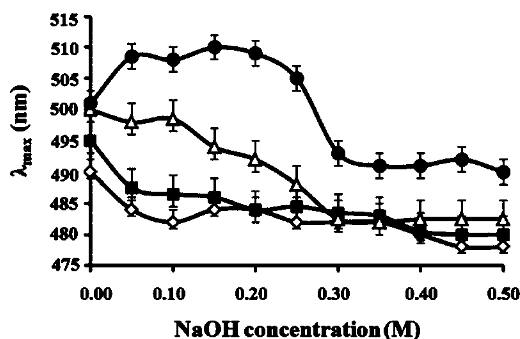

Figure 6. Helix–coil transition analysis of the purified β-glucan according to the change in the maximum absorption of the Congo red polysaccharide complex at various concentrations of NaOH. Congo red, dextran, and laminarin were used as controls. Purified β-glucan (●), laminarin (Δ), Congo red (■), and dextran (◊). The data represent the mean ± SD (n = 5).

(1→3)(1→6)-glucans with 5–10% ramification.[28,29] This lower ramification is responsible for the changes in its triple helix conformation at lower NaOH concentrations. Wang et al.[33] reported that temperatures lower than 130 °C do not destroy the triple helix conformation of lentinan or β-(1→3)(1→6)-glucan, which was isolated from *Lentinus edodes*. Thus, the hot water treatment, enzymatic extraction, and purification methods used in this study did not affect the structure of β-glucan during the extraction process. The crude enzyme from strain B-6 is a good alternative for selective improvement by directly removing xylan and cellulose from crude carbohydrate and retaining its native structure as a triple helix.

3.7. Morphology of the Purified β-Glucan. AFM has become a valuable metrological tool to characterize surface topology, on the nanometer scale, of particles, macromolecules absorbed to surfaces, biopolymers, and the linear and circular triple helix structures of β-glucan.[34] Figure 7 shows the AFM

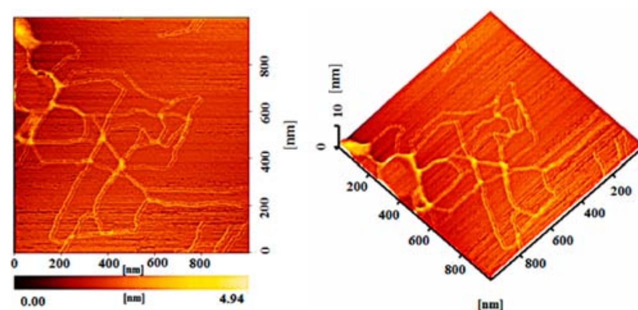

Figure 7. Atomic force microscopy of the purified β-glucan from the fruiting bodies of *P. sajor-caju*.

images; the purified β-glucan reveals almost rod-like structures. The measured mean thickness of the purified β-glucan structures was 1.0 ± 0.3 nm. The chain thickness of the purified β-glucan was consistent with that of the native triple helix chains of lentinan, scleroglucan, and schizophyllan (~1.0 nm).[35,36] These results provide further evidence of the purified β-glucan existing as triple helical chains in dilute solution.

3.8. Macrophage Activation by β-Glucan. Macrophages, which are part of the innate immune system, play an essential and pivotal role in protecting the body from microbial infection and cancer through direct action. However, they also play an indirect role in antitumor activity by the production of

cytokines. It is well-documented that polysaccharides extracted from edible mushrooms have immunostimulatory and antitumor activities.[37,38] Stimulated macrophages release a broad spectrum of cytokines, including TNF-α and inflammatory molecules, such as NO, which inhibit cancer cell growth.

To verify that purified β-glucan obtained from the fruiting bodies of *P. sajor-caju* was able to stimulate macrophages, macrophage-like J774A.1 cells were used. Figure 8a shows that

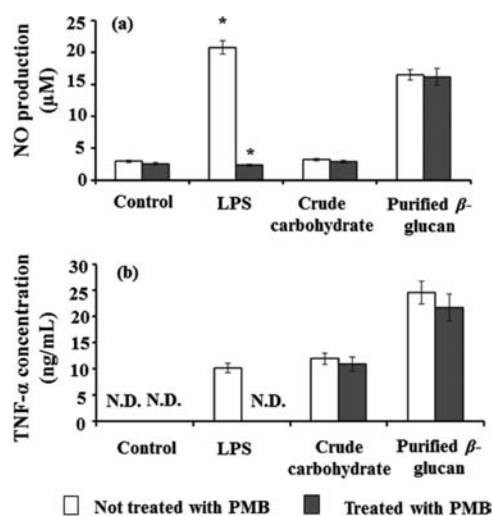

Figure 8. Immunostimulatory effects of the purified β-glucan in murine macrophage J774A.1 cells; effect of the purified β-glucan on NO production (a) and effect of the purified β-glucan on TNF-α production (b). N.D. indicates "not detectable." The data are represented as the mean $\pm$ SD (n = 3). Statistically significant differences between non-treated samples and samples treated with PMB are indicated (*$p < 0.01$).

cells treated with purified β-glucan produced greater amounts of NO (16.50 μM) than crude carbohydrate-treated cells (3.21 μM), which increased about 5-fold. It was found that the difference in NO production between crude carbohydrate-treated cells and control cells was only 0.27 μM. However, NO production was not as great after stimulation with purified β-glucan compared to LPS, the major cell wall component of Gram-negative bacteria, which stimulates macrophages to secrete various inflammatory cytokines. In addition, ELISA assays were used to examine the concentration of pro-inflammatory cytokines to verify that purified β-glucan also stimulated innate immune responses in macrophages. The results show that purified β-glucan triggers the expression of the pro-inflammatory cytokine TNF-α (Figure 8b). We found that the TNF-α concentration increased ~2-fold in the purified β-glucan-treated cells (24.56 ng/mL) compared to crude carbohydrate-treated cells (11.97 ng/mL). However, the TNF-α concentration was higher after stimulation with purified β-glucan and crude carbohydrate than after LPS stimulation. To ensure that the effects of the purified polysaccharide were not due to endotoxin contamination, cells were treated with PMB, an inhibitor of LPS activity. The results showed that cells incubated with PMB, followed by stimulation with purified β-glucan, are able to produce NO and TNF-α with no statistical difference between samples treated with PMB and nontreated samples (Figures 8a and 8b). These results indicate that the NO and TNF-α production of macrophages of crude carbohydrate and purified β-glucan was not due to endotoxin contamination. PMB bound to LPS almost completely and did not affect samples. Thus, the purified β-glucan exhibited potent macrophage-activating properties, resulting in the modulation of NO and TNF-α production. However, compared at equal β-glucan content (90.2%), the crude carbohydrate extract produced NO (4.84 μM) and TNF-α (18.08 ng/mL) less than purified β-glucan (NO; 16.53 μM and TNF-α; 24.55 ng/mL). Thus, the purified β-glucan could stimulate macrophages to a greater extent than the crude carbohydrate. This might be due to differences in the process of extraction since the crude carbohydrate was not treated with xylanase and cellulase from strain B-6, so other components (xylan, cellulose, carbohydrate–protein complex, and proteins) buried β-glucan in the network and reduced the effectiveness of β-glucan on macrophage stimulation. On the basis of these results, the extraction process affected the amount of impurities in the crude carbohydrate extract obtained from *P. sajor-caju* fruiting bodies. Similar to other β-glucans obtained from medicinal mushrooms, the purified β-glucan in this study had a triple helix conformation, which has proven to be important for immunostimulatory activity.[4,37]

3.9. Conclusions. This study described modified methods for the extraction and purification of β-(1→3)(1→6)-glucan from the mushroom *P. sajor-caju*. These methods involve harmless treatments with xylanase and cellulase from *P. curdlanolyticus* B-6, improving the purity of β-glucan, avoiding chain disruption, and providing β-glucan in its native conformation (triple helix coil conformation), which has been shown to be important for immunostimulatory activity.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: +66 2470 7755. Fax: +66 2470 7760. E-mail: khanok.rat@kmutt.ac.th.

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Notes

The authors declare no competing financial interest.

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