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Pressurized Water Extraction of Polysaccharides as Secondary Metabolites from *Lentinula edodes*

TIFFANY CHIEN TING LO,† HSIN HSIN TSAO,§ AI YIH WANG,§ AND C. ALLEN CHANG*,†

Department of Biological Science and Technology, National Chiao Tung University, Hsinchu, 75 Po-Ai Street, Hsinchu 300, Taiwan, Republic of China, and Department of Radiological Technology, Yuanpei University of Science and Technology, Hsinchu, No. 306 Yuanpei Street, Hsinchu 300, Taiwan, Republic of China

The suitability of pressurized water extraction (PWE) of crude polysaccharides as secondary metabolites from *Lentinula edodes* was investigated. A series of experiments were carried out to examine the effects of extraction times and pressures. The results indicated that the maximum recovery of polysaccharides was about 90% of the crude polysaccharides from mycelia pellets when the pressure was at 10.1 MPa for 70 min (28 °C). This was a drastic improvement over that of boiling water extraction (BWE) at 0.1 MPa for 40 min, which gave only 27.9% recovery. A nitroblue tetrazolium (NBT) reduction assay was used to examine the macrophage stimulating activities (MSA), and it was found that the PWE polysaccharides retained the MSA. The morphology of the macrophage cells treated by PWE polysaccharides was also examined and found to be similar to that of the positive control lipopolysaccharides treated. Finally, gel chromatographic and NMR experiments revealed that both PWE and BWE polysaccharides showed the presence of four similar molecular mass components and the α-(1→4)-D-Glc*p* and *β*-(1→6)-D-Glc*p* linkage residues. The improved PWE efficiency is probably due to the possibility that under high pressure, the solid polysaccharide's hydrogen bonding is partially destroyed to increase structure elasticity and water solubility.

KEYWORDS: ***Lentinula edodes*; pressurized water extraction; polysaccharides; macrophage stimulating activities; macrophage morphology; structures; molecular masses; NMR**

INTRODUCTION

Mushroom polysaccharides have been used as a medical resource to fight cancer for a long time in the Far East, the United States, and Canada. Polysaccharides from mushroom do not attack cancer cells directly, but produce their antitumor effects by activating different immune responses in the host (*1*, *2*). The polysaccharides are known to stimulate natural killer cells, T cells, B cells, and macrophage-dependent immune system responses. A variety of polysaccharides with different structures and biological activities have been isolated from various sources using different extraction processes (*3*−*5*).

The basidiomycetous mushroom, *Lentinula edodes* (shiitake), is the second most popular mushroom in the global market (*6*). Three polysaccharide products have been isolated from *L. edodes*, including lentinan (extracted from the cell wall of a fruiting body), KS-2 (an α-mannan peptide extracted from culture mycelia), and LEM (a *β*-glucan−protein complex extracted from solid medium), which are shown to be immunopotentiators and exhibit anticancer activities (*7*−*10*). However, few attempts have been made to produce the polysaccharides from the submerged cultures of *L. edodes*.

Recently we have made major efforts to isolate polysaccharides from the submerged cultures of 10 different strains of *L. edodes* and have determined their monosaccharide compositions, molecular masses, structural linkage types, and immunomodulating activities (*11*, *12*). The isolates had a molecular mass distribution between 1×10^2 and 3×10^3 kDa. The monosaccharide composition analysis revealed that they were heterogeneous and contained glucose, mannose, xylose, galactose, fucose, rhamnose, and arabinose in different ratios. A major form of polysaccharide linkage with a backbone of (1→4)-glucan and side chains of (1→6)-glucan has been identified (**Figure 1**).

The isolation of solid polysaccharides from various sources remains a major problem because they are heterogeneous with low water solubility, and the water extraction yield was normally quite low. In our experience, the boiling water extraction (BWE) of the polysaccharides was usually lower than 30% of the obtained crude products (*4*, *13*−*15*). Because the submerged cultivating process of *L. edodes* is rather time-consuming and normally takes several weeks, it is always necessary to optimize

* Corresponding author (e-mail changca@cc.nctu.edu.tw; telephone +886-35712121, ext. 56903; fax +886-35729288).
† National Chiao Tung University.
§ Yuanpei University of Science and Technology.

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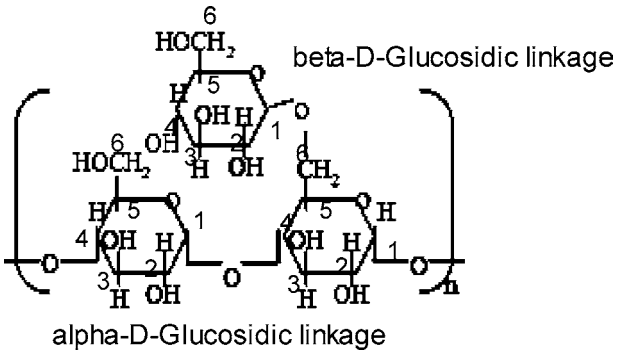

Figure 1. Structural unit of polysaccharides from *Lentinula edodes* showing the α-D-glucosidic linkages within the backbone and the β-D-glucosidic linkages of the side chains.

the polysaccharide extraction yield for further research and application purposes.

Extractions of polysaccharide from mushrooms with boiling water, sodium hydroxide, ammonium oxalate, and pulsed electric field were also reported for improved efficiency (*13*, *16*). However, the structures and bioactivities of a number of polysaccharides extracted under these conditions were not compared before and after the extractions. On the other hand, extractions with supercritical fluids at high pressures have been demonstrated to be quite useful for many food and biological samples (*17*−*22*). To the best of our knowledge, few papers have been published on the use of pressurized water extraction (PWE) for polysaccharides from *L. edodes*. In this paper, we report the results of using a PWE method to improve the extraction efficiency and to retain the general structures and bioactivity of the polysaccharides.

MATERIALS AND METHODS

Strains, Media, and Growth Conditions. The culture growth conditions were optimized by first incubating the *L. edodes* (Japanese 271) for 3, 5, 7, 12, 14, 17, 19, 21, 24, 26, 28, and 31 days at 26 °C. The culture broth of *L. edodes* was obtained from a medium (pH 4.5) containing 2% oat, 0.5% yeast extract, 0.1% KH_2PO_4, 0.05% $MgSO_4$, and 0.15% $CaCO_3$, with reciprocating shaking (150 rpm) and fermentation for 14 days at 26 °C. The fermentation culture broth was filtered (Millipore, 0.22 μm) to collect the culture broth filtrate (CBF) for immunoassay and further experiments. The mycelia pellets were collected and washed with distilled water. The mycelia pellet dry weight was determined by drying in an oven for 7 days at 60 °C.

Crude Polysaccharide Isolation and PWE. The CBF was added to 3 volumes of 95% ethanol and stored at 4 °C overnight. The precipitate was collected by centrifugation, followed by washing with 4-fold volumes of 75% ethanol, and then freeze-dried to obtain the culture precipitate. The crude polysaccharides were then treated with pressurized water (ISCO, 260D) at different pressures and time periods to obtain the PWE products. Normally, 35 mg of crude polysaccharides was added to each extraction cell (10 mL). The effects of the two factors, that is, pressure and time, were investigated in the ranges of 2.5−25.3 MPa and 10−80 min at 28 °C. The PWE polysaccharides were filtered (Millipore, 0.22 μm) for immunoassay, and the residues were dried to determine recovery yields. The BWE polysaccharides obtained at 0.1 MPa for 40 min were used for control experiments (*23*). The data of the above experiments were presented as a mean and relative standard deviation (RSD, %) of three replicate measurements.

Macrophage Stimulating Activity (MSA). The O_2^- production of the macrophage cell line (mouse BALB/C macrophage, RAW 264.7) stimulated by the polysaccharides was measured by a modified nitroblue tetrazolium (NBT; Sigma) reduction assay (*24*, *25*). The macrophage cells were cultured in 18 mL of Dulbecco's modified Eagle's medium (DMEM, Gibco) containing 2 mL of fetal bovine serum (FBS, Hyclone). The cells were then placed into the wells of a 96-well microtiter plate (2.5×10^5 cells/mL) and treated with 20 μL of CBF or BWE (3.5 mg/mL) or PWE polysaccharides (3.5 mg/mL) obtained as extracts of different pressures for 48 h at 37 °C. After removal of the supernatant, the macrophage cell line monolayer was added to 100 μL of 2 mg/mL of NBT. The plates with stimulated cells were incubated for 4 h at 37 °C. The reduced formazan within the macrophage cell line was solubilized in 100 μL of DMSO (Merck). Optical density was measured using an ELISA reader at 570 nm. The BWE polysaccharides were used for control experiments. Phosphate-buffered saline (PBS) was used as a blank solution for optical density experiments. The percent NBT reduced is estimated using the following equation: {[(sample average) − (blank average)]/[(control average) − (blank average)]} × 100.

Macrophage Morphology. Macrophage cell lines were placed into wells of a microtiter plate (2.5×10^5 cells/mL) and were treated separately with 100 μL of PBS (10 μg/mL) as a blank, lipopolysaccharide (LPS; 10 μg/mL) as a positive control, and BWE and PWE polysaccharides (10.1 MPa, 70 min). They were then incubated for 24 h at 37 °C. After the supernatant had been decanted, a cell culture medium (DMEM) was added at the end of incubation. The macrophage morphologies were observed using differential interference contrast (DIC) microscopy (Leica DMRXA, photo CCD-Leica DC500).

Molecular Mass Determinations. The molecular mass fractions of BWE (3.5 mg/mL) and PWE (2.5, 10.1, and 25.3 MPa) polysaccharides were determined using a gel chromatographic technique with H_2O as the mobile phase (mix D column, PL Aqua-gel-OH, Waters 1515 isocratic pump, 717 plus injector, and 2410 RI detector, flow rate = 1 mL/min, 25 °C). A calibration curve was constructed with standard dextrans (Polymer Standards Service) within the molecular mass range of 180−2 100 000 Da.

NMR Studies. The ^1H and ^{13}C NMR and distortionless enhancement by polarization transfer (DEPT) experiments of PWE polysaccharides (10.1 MPa, 70 min) were recorded in D_2O (99.96% atom ^2H, Aldrich) solutions at 600 and 150 MHz, respectively, on a Bruker DMX-600 spectrometer at 25 °C (*26*−*28*). Methanol was used as an internal standard (49 ppm) for the ^{13}C spectrum. The ^1H NMR spectrum was recorded by adjusting the HOD signal at 4.70 ppm.

RESULTS AND DISCUSSION

Optimization of *L. edodes* Culture Growth and PWE of Polysaccharides. Figure 2 shows the time course of mycelia growth curve expressed as plots of dry weights versus time. The results indicated that the mycelia dry weight reached 461.2 mg/50 mL at the 12th day and that the growth rate of mycelia could be roughly divided into three phases. The first one appeared from the 3rd to the 7th day and had a slow growth rate. The second one appeared from the 7th to the 12th day with the best mycelia growth rate. After the 14th day, the mycelia growth moved to a stationary phase. The MSA measured by using the NBT reduction assay on the collected culture broth filtrates (CBF) from the mycelia culture at various days are also plotted in **Figure 2**. Although the MSA was slightly greater for the 17th day polysaccharides, because of the considerations of (1) the quantity of polysaccharides, (2) polysaccharide bioactivity, and (3) time, the operational time to harvest the mycelia growth and crude polysaccharides production were set at the 14th day (Supporting Information, Table S1).

BWE (40 min at 0.1 MPa, as control experiments) and PWE (between 10 and 80 min at 25.3 MPa) of the crude polysaccharides as secondary metabolites from *L. edode* culture broth filtrate were performed to obtain the optimum extraction time; 25.3 MPa was the highest pressure the instrument could achieve. Under this pressure the optimum extraction time was obtained, and at this optimum extraction time, the pressure was then reduced to find the optimum extraction efficiency (*17*, *18*).

It was found that BWE recovered only 27.9% of the crude polysaccharides, which was much less than those extracted with

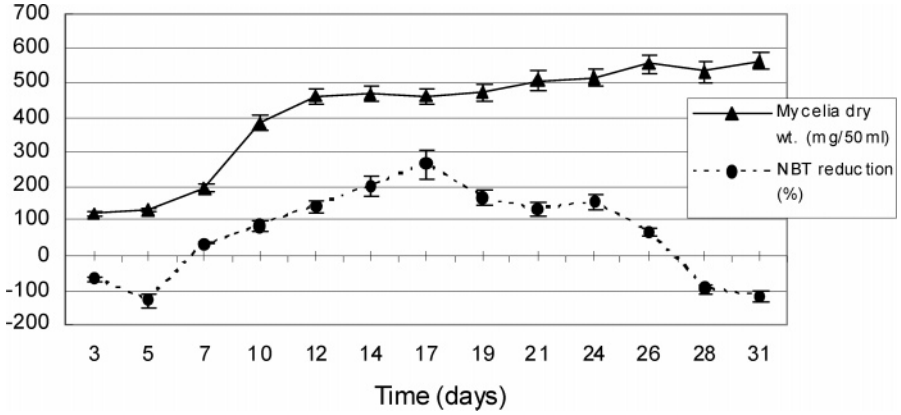

Figure 2. Plots of isolated mycelia dry weights (▲, mg/50 mL) and the macrophage stimulating activities (●, % NBT reduction) of the culture broth filtrates (CBF) versus time (days) during the mycelia growth of *L. edodes*.

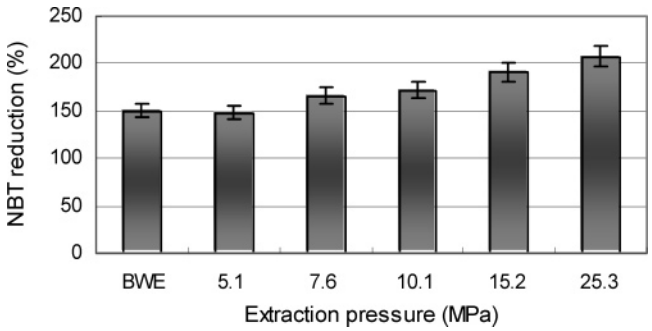

Figure 3. Macrophage stimulatory activities of PWE polysaccharides extracted at different pressures.

PWE at all time conditions (**Table 1**). When the PWE time was increased from 10 to 70 min, the polysaccharide recovery increased from 80.6 to 88.6%. The greatest recovery of polysaccharides was 88.6% at a pressure of 25.3 MPa for 70 min. As shown in **Table 1**, increasing the extraction time to 80 min did not increase polysaccharide recovery. Thus, extraction time was set at 70 min for subsequent experiments.

Table 1 also shows the polysaccharide extraction data at various pressures at 70 min. The recoveries of polysaccharides slightly increased from 85.1% at an extraction pressure of 2.5 MPa to 90.0−90.3% at 10.1−15.2 MPa and then diminished to 88.6% at 25.3 MPa. A maximum recovery (∼90%) at 10.1 MPa was obtained. Thus, an optimum condition for PWE was therefore set at 10.1 MPa and 70 min. At 2.5−25.3 MPa, the yield (i.e., solubility) of polysaccharides was about 29.8−31.6 mg/10 mL (calculated from the initial 35 mg/10 mL of crude polysaccharides). The extraction reproducibility was expressed by RSD (%). It was found that for BWE, the RSD was 21.5%, which was much greater (and hence less reproducible) than those

Table 1. Recoveries of Crude Polysaccharides by Pressurized Water Extraction (PWE) at Different Time Periods and Pressures[a]

pressure (MPa)	time (min)	recovery ± SD (%)
0.1 (BWE)	40	27.9 ± 21.0
25.3	10	80.6 ± 1.5
25.3	20	81.4 ± 1.3
25.3	30	82.0 ± 0.9
25.3	40	85.8 ± 1.4
25.3	50	85.8 ± 1.0
25.3	60	87.5 ± 0.9
25.3	70	88.6 ± 1.3
25.3	80	88.6 ± 0.5
2.5	70	85.1 ± 2.7
5.1	70	85.7 ± 1.0
7.6	70	85.8 ± 0.4
10.1	70	90.0 ± 1.5
15.2	70	90.3 ±1 .2

[a] Boiling water extractions (BWE) were performed at 0.1 MPa for 40 min. Data are the average of triplicate experiments.

of PWE, which were normally <2.7%. This was due, probably in part, to the lack of precise control of pressure and temperature under the traditional extraction processes.

Generally, the extracted amount of polysaccharides depends on temperature, pressure, pH of the solvent, ionic strength, and fineness of solid particle grinding. The polysaccharide Lentinan was first isolated and studied by Chihara (*23*). The extractions were performed using 80−100 °C boiling water for 8−16 h. Mizuno et al. later modified the extraction method, and the extractions of high molecular mass polysaccharides were performed with three successive steps, that is, with water (100 °C, 3 h), 2% ammonium oxalate (100 °C, 6 h), and 5% sodium hydroxide (80 °C, 6 h) (*4*, *13*). A strong base such as NaOH alone in the extraction medium resulted in a higher extraction

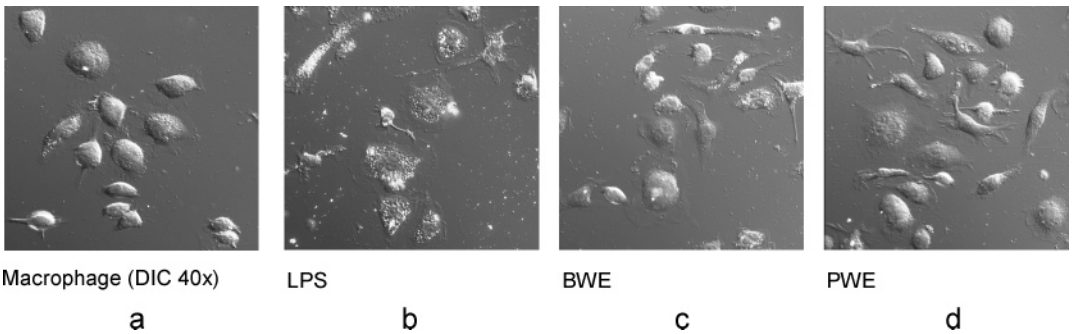

Figure 4. Differential interference contrast images of macrophage morphological changes induced by (**a**) PBS, (**b**) 10 μg/mL LPS, (**c**) BWE, and (**d**) PWE polysaccharides.

Table 2. Selected Gel Chromatographic Retention Data of the BWE and PWE Polysaccharides

sample	fraction[a]	retention time (min)	% area
BWE (0.1 MPa)	A	5.7	12.0
	B	8.2	23.1
	C	9.2	18.8
	D	10.1	46.2
PWE (2.5 MPa)	A	5.6	12.2
	B	8.3	25.7
	C	9.2	15.8
	D	10.6	46.3
PWE (10.1 MPa)	A	5.7	12.4
	B	8.3	22.3
	C	9.2	16.1
	D	10.6	49.3
PWE (25.3 MPa)	A	5.7	12.6
	B	8.4	25.3
	C	9.2	16.8
	D	10.6	45.3

[a] A, mol mass > 2750 kDa; B, mol mass ~ 2700 kDa; C, mol mass ~ 500 kDa; D, mol mass ~ 10 kDa.

yield than pure water at all temperatures tested (*14*, *29*, *30*). This modified method was more efficient and relatively less costly when compared to the original Chihara method (*15*, *23*, *31*). However, the use of boiling water was not convenient, and dilute alkaline solution may alter the structures and bioactivities of the polysaccharides (*14*). Note that the −OH groups in the anhydrous glucose units are ionized at high pH (i.e., pH 12.5), which might increase the polysaccharides' water solubility and structure elasticity in addition to the reduction of polymer chain agglomeration and molecular degradation (*32*, *33*).

MSA and Macrophage Morphology. It has been reported that polysaccharides possess an immunomodulating effect by the activation of a variety of macrophage functions, for example, superoxide anion production and cytokine production (*1*, *2*). The MSA of the PWE polysaccharides were examined by using the NBT assay, and the results are shown in **Figure 3**. The NBT reduction of macrophage activities at 5.1, 7.6, 10.1 15.2, and 25.3 MPa extractions were 148, 166, 172, 192, and 207%, respectively. These data were close to that of BWE (151%), indicating that both the PWE and BWE methods were able to retain the bioactivities of the polysaccharides. It is noted that CBF has higher macrophage activities, probably due to the presence of other metabolites in addition to polysaccharides.

When macrophage cells were cultivated for 24 h with LPS, BWE, or PWE polysaccharides, dramatic morphological changes were observed (**Figure 4**). The LPS is a cell wall component of a Gram-negative bacteria and can activate monocytes and mocrophages (*34*, *35*). **Figure 4b** shows that the macrophage cells appeared to have distinct dendritic-like morphology after 24 h of culturing in the presence of the LPS positive control as compared with those of unstimulated macrophage cells (**Figure 4a**). Macrophage cells cultured with both BWE and PWE polysaccharides show morphology similar to those of the LPS-treated (**Figure 4c**,**d**). The macrophage cells in **Figure 4b**−**d** were characterized by a dramatic increase in size, larger nuclei, prominent nucleoli, extended processes, and cytoplasm with increased granularity as compared with untreated macrophage cells.

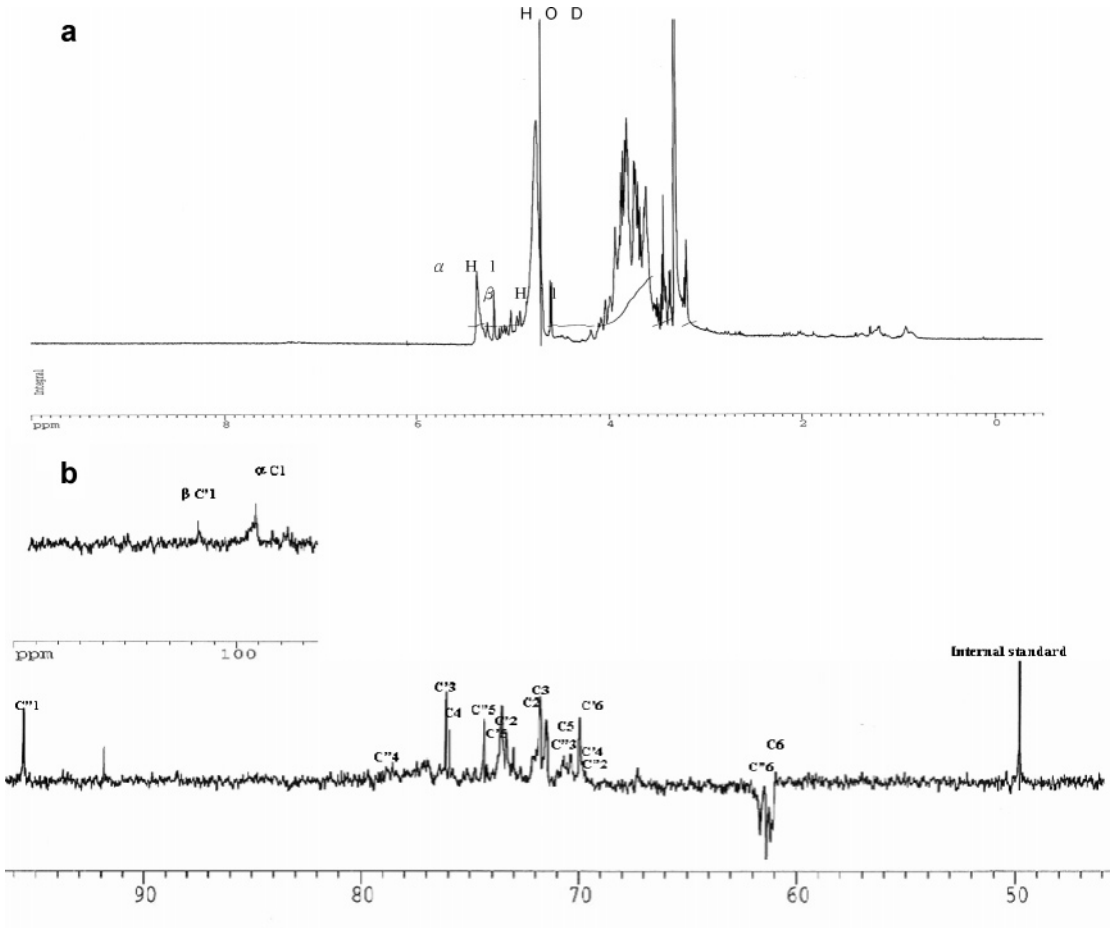

Figure 5. NMR spectra of PWE polysaccharides (100 atm, 70 min) in D_2O: (**a**) ^1H NMR; (**b**) ^{13}C NMR.

Table 3. ^{13}C NMR Chemical Shifts of BWE and PWE Polysaccharides in D_2O

α-(1→4)-D-Glc*p*	C1	C2	C3	C4	C5	C6
BWE	99.68	71.55	71.28	76.06	70.09	60.86
PWE	99.67	71.56	71.31	76.06	70.07	60.87
β-(1→6)-D-Glc*p*	C′1	C′2	C′3	C′4	C′5	C′6
BWE	102.43	73.16	76.79	69.75	73.45	66.96
PWE	102.27	73.2	76.8	69.72	73.42	66.97

Molecular Mass Determinations. Gel permeation chromatographic studies show that the BWE and PWE polysaccharides have four similar molecular mass fractions (designated A, B, C, and D, **Table 2**; Supporting Information, Figure S1). The polysaccharides show a broad molecular mass range from 10 to >2750 kDa. Careful examinations revealed that these molecular mass fractions A (>2750 kDa), B (~2700 kDa), C (~500 kDa), and D (~10 kDa) were present in all extracts with minor variations in fractions ratios (area percent).

The PWE was utilized to improve the extraction yields of polysaccharides from solid or semisolid samples. High pressure could modify considerably the physical properties of the extraction solvents, with the effect of increasing selectivity in the extraction (*21*). The PWE could minimize strong hydrogen bonding of solid crude polysaccharides and increase structure elasticity to increase water solubility without changing most polysaccharide structures and, therefore, similar molecular mass distributions and bioactivities were found after the extraction (25−250 atm, 10−80 min).

NMR Studies. The proton and ^{13}C NMR spectra of the PWE polysaccharides (100 atm, 70 min) are shown in **Figure 5**. The two groups of anomeric proton signals centered at 5.36 and 5.01 ppm (**Figure 5a**) were assigned to the α-(1→4)-D-Glcp and *β*-(1→6)-D-Glcp protons, respectively (*27*, *28*). These assignments of α-(1→4)-D-Glcp and *β*-(1→6)-D-Glcp protons were similar to those of BWE at 5.33 and 4.97 ppm. The assignments of the C1 and C′1 chemical shifts of the glucose residues were reported in the range of 98−104 ppm with the α form being downfield and the *β* form upfield (*28*, *36*, *37*). The two carbon peaks at 99.67 and 102.27 ppm were assigned to α-(1→4)-D-Glcp and *β*-(1→6)-D-Glcp carbons, respectively (**Figure 5b**; **Table 3**). It should be noted that although the chemical shifts of the two C1 peaks of the backbone glucose units may theoretically be different, we and others have found them to be similar.

The anomeric carbon peaks C1 and C′1 of the BWE polysaccharides were similar to those of PWE, and the corresponding chemical shifts were at 99.68 and 102.43 ppm. The peaks at 71.56, 71.31, 76.06, 70.07, and 60.87 ppm were assigned, respectively, to C2, C3, C4, C5, and C6 of the α-(1→4)-D-Glcp carbons, due to their relatively higher peak intensities. The other signals at 73.20, 76.80, 69.72, 73.42, and 66.97 ppm were assigned, respectively, to C′2, C′3, C′4, C′5, and C′6 of the *β*-(1→6)-D-Glcp carbons. The tentative ^{13}C NMR peak assignments for the mannopyranosyl carbons are C″1 (96.02 ppm), C″2 (69.65 ppm), C″3 (70.35 ppm), C″4 (78.5 ppm), C″5 (74.25 ppm), and C″6 (61.2 ppm), based on those assignments of related studies (*38*, *39*). The assignment of the peak at 92.0 ppm was not certain but could be attributed to a monosaccharide carbon residue (*40*).

In conclusion, although some previous studies indicated that increased extraction yields of polysaccharides were obtained by adjusting the pH or other extraction conditions, few reported if the structures and bioactivities were the same or similar before and after the improvement of extraction yield. The data presented in this paper indicated that the yield of the polysaccharides extracted by the PWE method could be increased up to 90% of the total crude polysaccharides from CBF, which was ~3 times that by the BWE method. Moreover, the extracted polysaccharides by both the PWE and BWE methods showed similar macrophage stimulating activities, macrophage morphologies, molecular mass distributions, and NMR spectral properties. The PWE method for polysaccharides is easy to practice and is recommended for future applications in agricultural and food research and industrial product developments.

Supporting Information Available: Polysaccharide dry weight, gel permeation chromatograms, and molecular mass calibration curve. This material is available free of charge via the Internet at http://pubs.acs.org.

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