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New Method Development for Nanoparticle Extraction of Water-Soluble β-(1→3)-D-Glucan from Edible Mushrooms, *Sparassis crispa* and *Phellinus linteus*

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Sparassis crispa and *Phellinus linteus* are edible/medicinal mushrooms that have remarkably high contents of β-(1→3)-D-glucan, which acts as a biological response modifier, but difficulty in cultivating the fruiting bodies and extraction of β-D-glucan have restricted detailed studies. Therefore, a novel process for nanoparticle extraction of Sparan, the β-D-glucan from *Sparassis crispa*, and Phellin, the β-D-glucan from *Phellinus linteus*, has been investigated using insoluble tungsten carbide as a model for nanoknife technology. This is the first report showing that the nanoknife method results in high yields of Sparan (70.2%) and Phellin (65.2%) with an average particle size of 150 and 390 nm, respectively. The extracted Sparan with β-(1→3) linkages showed a remarkably high water solubility of 90% even after 10 min of incubation at room temperature. Therefore, it is likely that this nanoknife method could be used to produce β-D-glucan for food, cosmetic, and pharmaceutical industries.

KEYWORDS: Nanometer-sized β-glucan; nanoknife; *Sparassis crispa*; *Phellinus linteus*; extraction; Sparan; Phellin

INTRODUCTION

Immunotherapy is widely applied to various diseases, such as infection, cancer, and autoimmune diseases (*1*). The immunomodulating substance (*2*), biological response modifier (BRM), or biotherapy is of particular interest for treatment of cancer and infectious diseases. β-glucan is a well-known BRM which is widely distributed in nature and is being added to medicine and food (*3, 4*). In particular, soluble glucans such as Lentinan from *Lentinus edodes* (*5*), Sonifilan from *Schizophillium commune* (*6*), and Krestin from *Grifola frondosa* (*7*) have been used for cancer immunotherapy.

At present, the content of β-glucan isolated from *Sparassis crispa* (SC, cauliflower mushroom), *Phellinus linteus* (PL), and *Ganoderam lucidum* (GL) is listed (*8*). SC has been found to be a good source material to prepare a high quantity of antitumor β-glucan (*9*). Following preliminary investigation, it was found that the β-glucan content of SC was 43.6% of the dry weight of the fruiting bodies as measured by the "enzyme method" of the Japan Food Research Laboratories (*10*) (Tokyo). According to chemical, enzymatic, and NMR analyses, the primary structure of the major polysaccharide recovered in the cold alkaline extract of the SC mushroom was a 6-branched β-(1→3)-D-glucan (*11*). These fractions showed antitumor activity to the solid form of Sarcoma 180 in ICR mice with strong vascular dilation and hemorrhage reaction. Ohno et al. (*12*) studied SC for its anticancer effects. Clinical trials showed that oral administration of SC powder to 14 last-stage cancer patients produced noticeable improvements in 9 patients. Hence, SC containing β-(1→3)-D-glucan has also been studied as a new natural material used in anticancer immunotherapy. The fruiting bodies of SC have been reported to exhibit an outstanding ability to cure gastric ulcers and esophageal cancers. These soluble β-glucans (i.e., mainly high molecular weight) are not digested in the human stomach and small intestine and survive mainly intact in the colon, at which point they are available for microbial fermentation (*13*).

PL is a well-known oriental medicinal fungus belonging to the *Hymenochaetaceae basidiomycetes*. Hot water extracts of this mushroom are reduced to powder form and commercially used as an anticancer agent. There is evidence that polysaccharide from PL is an active agent in stimulating humoral and cell-mediated immunity (*14*). Baker et al. (*15*) reported that the glycan fraction of a hot water extract from PL has been isolated

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following ammonium sulfate precipitation, dialysis/concentration, and anion exchange chromatographic steps. Analyses for monosaccharide composition showed glucose and mannose to be the major constituents. A core β-(1→3) linked glucan heavily substituted via (1→6) links with β-(1→3) linked mannose chains.

The nanometer-sized particles (nanoparticles, NPs) for sample extraction have garnered much attention due to their unique surface functionality and high surface-to-volume ratio. For example, magnetic NPs and gold NPs have been used for simultaneous enrichment and detection of small molecules combined with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (*16*, *17*). In addition, a higher surface-to-volume ratio of the micrometer-sized particles commonly used for solid-phase extraction is known to enhance the extraction capacity and efficiency (*18*). Another advantage of NPs is that there is surface functionality that can be easily modified to achieve selective sample extraction (*19*). The tungsten carbide (WC) hard metals are often employed in tools as they are wear-resistant materials and therefore can be used to produce NPs (*20*). Using the WC NPs can reduce the particle size, which is a central factor in the extraction of β-glucan, thereby enhancing biological activity. However, there is no literature reporting that the WC NPs have yet been used in nanoknife technology for β-glucan extraction. In this present paper, β-glucans from two edible mushrooms (SC and PL) were extracted using the NP-base extraction procedure, and their physicochemical properties were characterized.

MATERIALS AND METHODS SECTION

Materials. The strain of SC was kindly provided by Korea Forest Research Institute (Seoul, Korea). PL was purchased from Kyungdong retail market in Seoul, Korea. The antler and cap of GL (GLA and GLC) were collected from the mountain in the area of Yeoncheon-gun (Kyunggi-do, Korea) in October 2007. In order to extract β-glucan, the fruiting bodies of two mushrooms, SC, PL, GLA and GLC, were prepared according to the extraction method described by Choi (*21*). All chemical analyses of β-glucan from mushrooms were done in triplicate and reported on a dry matter basis. Phenylisothiocyanate (PITC) and acetate anhydrous were obtained from Aldrich (Milwaukee, WI, U.S.A.), and standard amino acids were purchased from Sigma (St. Louis, MO, U.S.A.). HPLC-grade triethylamine (TEA), acetonitrile (ACN), D_2O (deuteration degree min 99.96%), and Me_2SO-d_6 (99.96%) were from Merck (Darmstadt, Germany). All other reagents and solvents were of analytical reagent grade.

Preparation of NPs. The WC NPs (diameter of 10−500 nm) were obtained from Inframat Corporation, U.S.A. The particles were washed in acetone and then rinsed with water to remove any contaminants and were separated from the liquid by gravity centrifugation (IEC HN-SII Centrifuge) at a speed of 42000 g for 20 min. The supernatant was decanted, and the sediment was dried in a desiccator for 2 weeks (*20*).

Cultivation of *Sparassis crispa*. An isolated fraction of SC (150−200 mm × 150−200 mm) was prepared by following the procedure described in **Figure 1**. The culture was maintained on a potato dextrose agar medium (PDA, potato extract 4 g, dextrose 20 g, and agar 15 g/distilled water 1 L) slant at 4 °C. The inoculum of *S. crispa* was prepared using an agar plug of mycelia grown on PDA in 90 mm Petri dishes for 30 days at 25 °C in the dark. The fruiting bodies of SC were produced using a mixture of *Larix leptolepis* sawdust/actived carbon/wheat flour/yeast extract/calcium chloride (16:1:1:1:1, dry weight base) and adjusted to 65% moisture in a 850 mL polypropyrene bottle. The sawdust medium was sterilized by autoclaving for 90 min at 121 °C (15 psi pressure). Five hundred 20 mL bottles containing 10 g of sterilized sawdust were inoculated with a 5 mm diameter plug of SC from a 30 day old colony. After 60 days of incubation on a rotary shaker at 25 °C, the mycelia growth and density were observed. The measurement of mycelia growth was performed according to the method described by Choi (*21*). The humidity of the growing room was maintained at 90% during primordial induction, 70−80% during cap formation, and 30−40% during the final stage of fruiting body development. Light intensity at 50−450 lux was required during primordial formation and fruiting body development. After formation of the cap, the growing room was kept well ventilated.

Nanoknife Extraction of β-Glucan from Mushrooms. After air-drying, the water content of the powdered fruiting bodies was adjusted to 4% in SC, PL, GLA, and GLC. The fruiting bodies were then blended to pass 100 mesh using a Lab blender (FM 680T, Hanil, Co. Ltd., Seoul, Korea). One hundred grams of the blended material from each fungal sample (3.0−5.0 mm in diameter) was added to 2 L of distilled water adjusted to a pH of 3−12 with 1 N NaOH or HCl and was incubated for 2 h. Twenty percent (w/v) of insoluble WC NPs was added to each suspension in a tank (Il-Shin Co., Seoul, Korea) which contained propellers. The suspension was stabilized at 6000 g for 10 min, and the mixture was stirred at 20000 g for 40 min with a high-pressure emulsifier (Misung Co., Seoul, Korea) in a temperature-controlled water bath at 30 °C. The WC NPs acted as the nanoknife to improve the blending of the suspensions according to the method described by Roh and Choi (*22*). Twenty mL of hot water at 95 °C were added to the mixtures and stirred for 8, 15 and 30 h, and then, the mixtures were centrifuged for 20 min at 6500 g. The material was cleaned by rinsing the supernatants from Extract II (hot water) three times with three volumes of cold ethanol (95%, v/v) at 4 °C. After centrifuging for 20 min at 6500 g, the supernatants were kept at 4°C for 24 h. The water-soluble supernatant and the water-insoluble WC NP precipitate were separated by centrifuging for 20 min at 6500 g. The supernatant was freeze-dried, resulting in a creamy white powder named Sparan. The insoluble WC NPs were recovered from the pellet and reused.

Measurement of β-Glucan. β-Glucans in mushrooms were determined using a Mushroom β-glucan kit (K-YBGL, Megazyme Int., Wicklow, Ireland). One hundred mg of the milled mushrooms and 1.5 mL of 37% hydrochloric acid (v/v, 10 N) were added to each tube and stirred vigorously on a vortex mixer. The tubes were placed in a water bath at 30 °C for 45 min. The materials were mixed with 10 mL of distilled water, and then, the tubes were incubated in a boiling water bath at 100 °C for 2 h. The tubes were cooled at room temperature, and 10 mL of 2 N KOH was added. The samples were washed and adjusted to 100 mL volume with 200 mM sodium acetate buffer (pH 5.0). After centrifugation at 6500 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)-D-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 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 analyzed spectrophotometrically at 510 nm with the reagent blank using a Perkin-Elmer Lambda 25 Spectrometer (Perkin-Elmer, Shelton, CT). To measure the α-glucan content, 2 mL of 2 M KOH was added to each tube to resuspend pellets, and

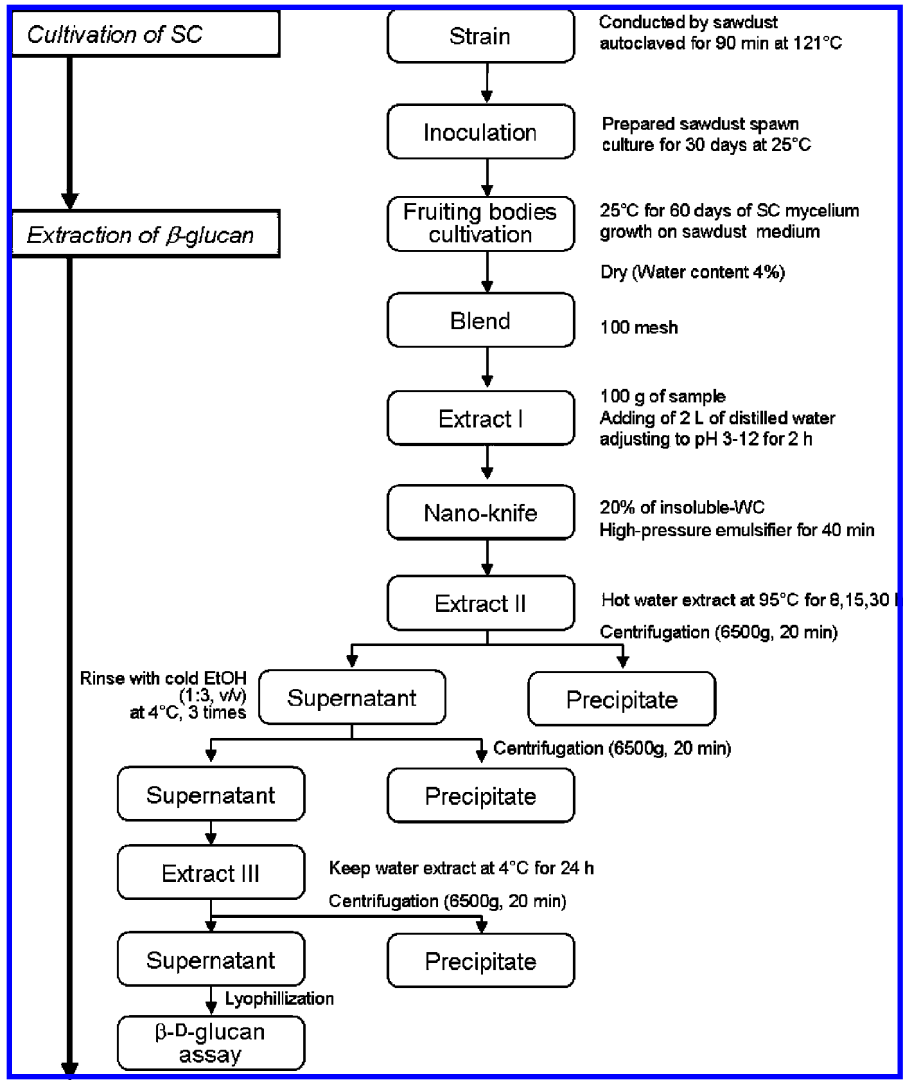

Figure 1. Schematic diagram for the extraction process of β-D-glucan from *Sparassis crispa.*

the phytoglycogen and starch 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 (3300 U/mL), and incubated in a water bath for 30 min at 40 °C. Tubes were centrifuged (10 min at 6500 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 glucose peroxidase reagent was conducted according to the total glucan content assay. The β-glucan content was determined by subtracting the α-glucan from the total glucan content.

Determination of the Carbohydrate Content. Total carbohydrates were determined on the powdered mushrooms using the phenol−sulfuric acid method. To analyze the neutral carbohydrate composition, 10 μL of 1% purified β-glucan was hydrolyzed with either 2 M trifluoroacetic acid or 6 N HCl for 4 h at 100 °C, followed by centrifugation for 30 min at 32600 g and a nylon syringe filtration (0.2 μm pore size, Chromatographic Specialties Inc., ON, Canada). The solutions were then evaporated to remove the remaining acid. Ten μL of hydrolyzed solution was injected into a high-pH anion exchange chromatograph (HPAEC) consisting of a Bio-LC DX-600 chromatograph equipped with a CarboPac PA1 column (4.5 × 250 mm and 2.0 × 250 mm; Dionex, U.S.A.) and an integrated amerometric ED50 detector. The neutral carbohydrate content was then determined.

Free Amino Acid Analysis. Quantitative estimation of amino acid in hydrolysates of Extract III was performed by HPLC after derivatization with PITC as described in Inglis et al. (*23*). Briefly, dried samples were hydrolyzed in 12 N HCl at 110 °C for 24 h using the Waters Pico-Tag work station and methanol. Our modified procedure incorporates a salt addition (8 μL of 2 M sodium methanesulfonate, pH 6.7) to the hydrolysate before vacuum drying (30 min). After reaction with the methanol/water/TEA/PITC (7:1:1:1) mixture at 25 °C for 20 min in the closed tube, sample diluent or water (5 μL) was added to dissolve the salt, and heptane (20 μL) was added to extract PITC. The solution was briefly vortexed, capped, and placed in a Wisp autosampler. The amino acid calibration mixture was treated at the same time and in exactly the same way as the hydrolysates. To the standard amino acid solution, a mixture solution of standard amino acids, except glutamine, cysteine, and cystine, was prepared at a concentration of 2.5/μmol/mL 0.01 M HC1. Standard solutions of glutamine and cysteine were prepared with water because of the conversion to pyroglutamic acid and cystine, respectively, on prolonged storage of these amino acids in HCl solution. Cystine was prepared at a concentration of 0.5/μmol/mL 0.01 M HCI. After drying, the samples were dissolved in 200 μL of solvent A (1.4 mM sodium acetate anhydrous, 0.1% TEA, 6.0% ACN, pH 6.1) and centrifuged at 30000 g for 10 min at 4 °C. The mobile phase consisted of 80% ACN at a flow rate of 1.0 mL/min. The column temperature was 40 °C.

The supernatants were processed on the Nova-Pak HR C_{18} reverse phase column (3.9 × 300 mm, I.D.; 4 μm dimethyloctadecylsilyl-bonded amorphous silica, Waters) with the support of a Hewlett-Packard 1100 series HPLC (PA, USA) and a fixed wavelength (254 nm) detector.

Determination of Molecular Weight. High-performance gel permeation chromatography (GPC) was performed on an LC-10 chromatograph equipped with a Shodex OHpak SB-804 column (8.0 × 300 mm; Showa, Japan) and an RI detector (Polymer Laboratory. Ltd., Japan). Thirty microliters of the 1% purified endopolysaccharide fraction was injected and eluted at a water flow rate of 1 mL/min at room temperature. Various molecular weights (MWs: 102000, 131400, 759400, and 136000 Da) of dextrans were used as standards and were purchased from American Polymer Standards (Mentor, OH, U.S.A.).

NMR Analysis. ^{1}H NMR spectroscopic analysis was performed using a Bruker Avance 500 spectrometer equipped with a TXI-xyz three gradient probe. Exchangeable protons were removed by suspending SC in D_2O (deuteration degree min 99.96%) and lyophilizing. This exchange process was repeated three times. All spectra were recorded in a mixed solvent Me_2SO-d_6/D_2O (6:1, 15 mg/mL) at 70 °C (*11*, *24*).

Particle Size Analysis. The particle size distribution of the produced precipitate was measured by a laser scattering particle size distribution analyzer (Granulometer 1064, CILAS, France). An amount of 0.1 g of sample powder was put in 100 mL of ethanol, and this underwent dispersion treatment by an ultrasonic dispersion unit for 60 s. The particle size was expressed as volume mean diameter (*25*).

Determination of Soluble and Insoluble β-Glucan. The content of soluble and insoluble β-glucan was determined according to a modification of the method of Lee and Kim (*26*). One hundred mg of β-gucan extract of SC was mixed with 2 mL of distilled water and then subjected to continuous shaking for 10, 30, 60, 120, and 180 min at 25, 40, 60, 80, and 100 °C, followed by centrifugation (20000 g, 10 min). The β-glucan content in the sediment was measured as insoluble β-glucan. Soluble β-glucan was determined by subtracting the insoluble from the total β-glucan content and calculated as a percentage of the total β-glucan content in the β-gucan extract of SC.

Statistical Analysis. The experiments were run in triplicate. Data were presented as mean values with standard deviations. Statistical differences were tested using the Student's *t* test, a one-way analysis of variance (ANOVA) with Duncan's multiple range tests. Statistical analyses were performed with the statistical program SPSS for windows (SPSS Inc., Chicago, IL, U.S.A.).

RESULTS

Effect of the Different Extraction Conditions on the Yield of β-Glucan from Edible Mushrooms. The WC NPs extraction of SC, PL, GLA, and GLC demonstrated that they all are glucan producers. The water-soluble extract of SC after Extraction III demonstrated that the yield and β-glucan content were found to be 54.2 and 70.2% at pH 10 for 30 h, respectively (**Figure 2A** and **B**). The yield and content of β-glucan of PL, GLA, and GLC were similar to those SC (data not shown). Ohno et al. (*10*) reported that the yield of β-glucan with a purity of 52.0% can be repeatedly extracted from SC by an extraction process using hot water, cold alkaline, followed by hot alkaline. They employed the chemical and enzymatic methodologies to analyze the structure of the polysaccharide fractions of these extracts. The yield content (54.2%) as well as the extractability of SC compares favorably to that reported by Ohno et al. (*10*). SC had the highest total carbohydrate productivity, producing up to 8.14 ± 0.7 g/mL ($p < 0.05$) (**Figure 3A**). The cold ethanol rinse step to obtain Extract III was more effective for isolation of β-glucan than no ethanol rinse (Extract II) (**Figure 3B**). Keeping Extract III at 4 °C for 24 h before centrifugation improves β-glucan content compared to the method developed by Roh and Choi (*22*). A higher β-glucan content was obtained from SC (70.2 ± 5.4%) and PL (65.2 ± 5.9%) than from GLA (48.8 ± 4.1%) and GLC (50.3 ± 4.8%). The content of the β-glucan isolated from the SC fruiting body by the alkaline (Extract I), hot water (Extract II), and cold water (Extract III) extraction methods were marginally higher than that given by Choi (*21*) and Roh and Choi (*22*), who found a purity of 46.3 and 51.1%, respectively. Following preliminary investigation, it was found that the β-glucan content of dried SC without WC NP extraction conducted by Hanabiotech Ltd. (Kyunggi-do, Korea) was 46.3% of the dry weight of the fruiting bodies (*21*). The β-glucan content of WC NP cold water extraction of SC by the Mushroom β-glucan kit (*22*) was 51.1%. However, the alkaline extraction and hot water extraction methods described in this manuscript gave a β-glucan content of 70.2%.

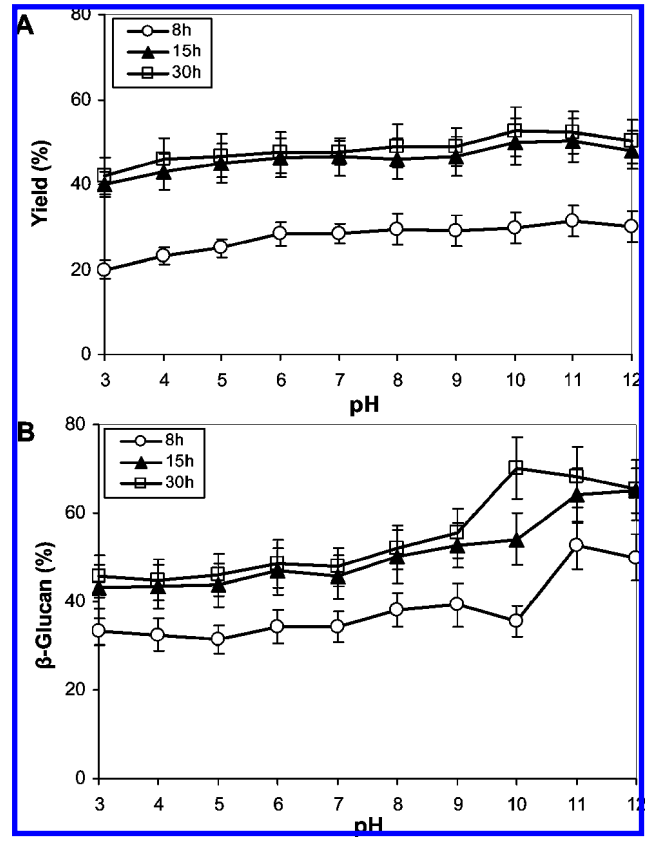

Figure 2. Water-soluble extracts from *Sparassis crispa* at different test conditions. (A) Yield of content from 100 g of air-dried fruiting body. (B) Contents of β-glucan extraction; β-glucan was determined using a β-glucan assay kit.

Other researchers reported that the β-glucan contents of 12 edible mushroom species were determined using a Mushroom β-glucan kit, *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*. There was a large variability in β-glucan content in the mushrooms which ranged from 4.71 to 46.20% on a dry weight basis (*26*). Manzi and Pizzoferrato (*27*) reported that β-glucan contents of edible mushrooms (*Pleurotus ostreatus*,

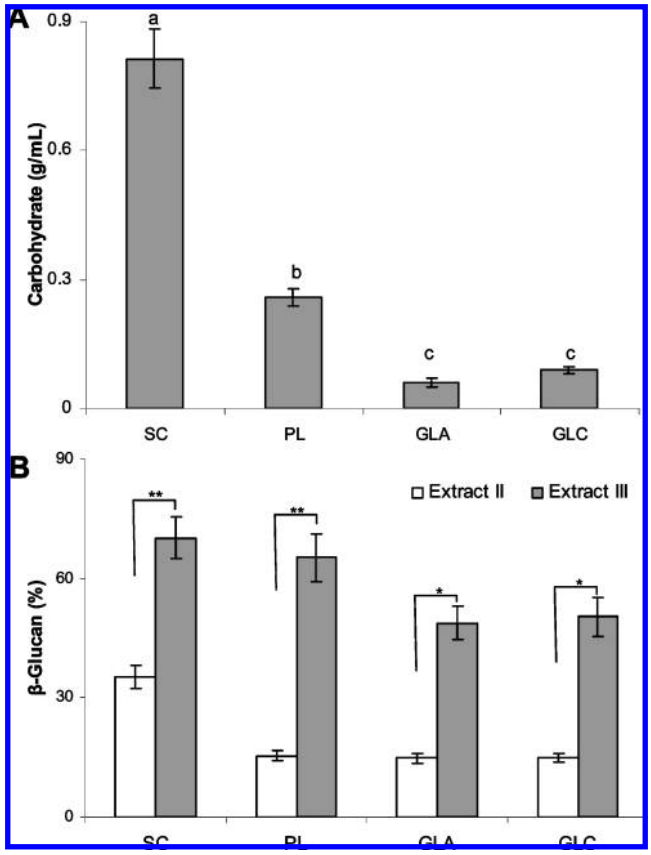

Figure 3. Comparison of β-glucans from mushrooms. (A) Total monosaccharide content by the phenol−sulfuric acid method. Values represent the mean ± standard deviation. Different letters on the bar indicate significant differences at $p < 0.05$. (B) β-glucan contents at the different steps in the extraction process. Statistically significant at $^{*}p < 0.05$ and $^{**}p < 0.005$ (Extraction II vs Extraction III).

Pleurotus pulmunarius, *Pleurotus eryng*ii, and *Lentinula edodes*) was very low, ranging from 0.22 to 0.53%. They used an enzymatic method involving lichenase and β-glucosidase hydrolyses. However, our β-glucan content (70.2%) was significantly higher using the WC NP extraction method described in this paper. Further analysis of the characteristics of the β-glucan was done on two highest yielding mushrooms, SC (Sparan) and PL (Phellin).

Contents of Carbohydrate and Amino Acid in Sparan and Phellin. Analysis of the monosaccharide compositions of the Sparan and Phellin showed that they contained 295.6 and 77.4 moles of glucose, respectively, which was about 3.8 times higher than that in Sparan and Phellin (**Table 1**). All of the other monosaccharides, that is, glucosamine, xylose, arabinose, and glucosamine, measured were present in lower levels than glucose, and all but galactose had a higher level in Phellin than that in Sparan. The ratio of the glucose content as a percent of other monosaccharides was 94.2% in Sparan.

The amino acid composition of the β-glucan was also different (**Table 2**). In the Sparan, the picomolar ratios of aspartic acid and glutamic acids having acidic side chains were 621.4 and 779.4 pmol, respectively. On the other side, histidine, arginine, and lysine, having positive charges, were 80.5, 113.3, and 267.7 pmol, respectively. Alanine and glycine were 113.3 and 511.5 pmol, respectively. The bulky side chains of alanine and glycine are considered important in promoting hydrophobic interactions with biomolecules (577.9 and 511.6 pmol, respectively).

Table 1. Neutral Carbohydrate Compositions of Sparan (*Sparassis crispa*) and Phellin (*Phellinus linteus*)

	concentration (M)	
composition[a]	Sparan	Phellin
glucose	295.6	77.4
galactose	11.3	7.5
mannose	4.1	22.6
glucosamine	2.5	5.0
arabinose	0.1	0.8
xylose	0.1	0.5
fucose	0	2.2
rhamnose	0	0.7
galactosamine	0	0.1

[a] The neutral carbohydrate composition was isolated by the nanoknife method, and the neutral and amino monosaccharide contents were analyzed by HPAEC equipped with a CarboPac PA1 column (2.0 or 4.5 × 250 mm).

Table 2. Amino Acid Compositions of Sparan (*Sparassis crispa*) and Phellin (*Phellinus linteus*)

	concentration (M)	
composition[a]	Sparan	Phellin
Cya[b]	80.7	298.5
Asx[c]	621.4	636.9
Glx[d]	779.4	472.6
Ser	439.1	505.3
Gly	511.5	473.5
His	80.5	32.7
Arg	113.3	29.1
Thr	299.1	409.6
Ala	577.9	455.5
Pro	239.7	343.0
Tyr	9.6	13.3
Val	273.1	301.8
Met	43.3	32.9
Ile	181.2	179.6
Leu	322.5	287.1
Phe	139.6	94.9
Trp	286.0	52.9
Lys	267.7	81.6

[a] Chromatogram of standard protein amino acid derivatives resolved on a Nova-Pak (300 × 3.9 mm) C_{18} column. [b] Cya is the sum of cysteine and cystine. [c] Asx is the sum of asparagine and aspartic acid. [d] Glx is the sum of glutamine and glutamic acid.

Determination of Molecular Weight and Particle Size Assay. Polysaccharides having MWs higher than 100−200 kDa are good immunogens and exhibit immunomodulating activity (*28*); Grifolan (from *G. frondosa*), lentinan (from *L. edodes*), and shizophyllan (from *S. commune*) had MWs of 5000, 400, and 450 kDa, respectively. The biological-response-modifying capabilities of polysaccharides are increased depending on their MWs (*29*). The MW of Sparan before the NP extraction was too high for an accurate determination. Tada et al. (*11*) reported that the MW of β-glucan from SC under physiological conditions might be quite high (MW > 2000 kDa) to be made to a viscous gel (*11*). However, the MW of Extract III from SC was estimated to be 510 kDa for Sparan, and 70.2% of Sparan showed a molecular distribution above 510 kDa (**Figure 4**A-i). On the other hand, The MW of Extract III was estimated to be 152 kDa for Phellin (**Figure 4**A-ii). Kim et al. (*30*) reported that the β-glucan was isolated from the mushroom PL by using ethanol precipitation methods followed by DEAE−cellulose and gel filtration chromatography, giving a MW of 150 kDa. While our WC NP extraction was similar with a MW of 152 kDa, the material had a small particle size (390 nm), which helps in digestion. These molecular characteristics suggest that Sparan could be used as a potential immunostimulant in livestock feed.

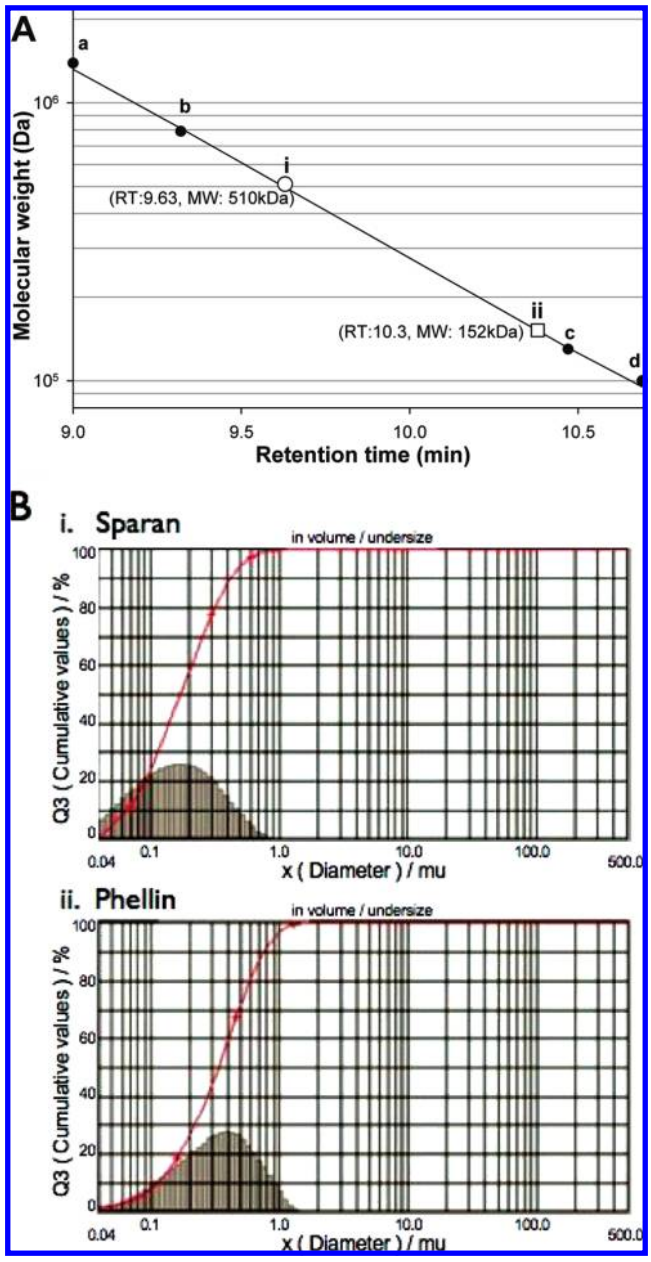

Figure 4. Molecular weights and particle sizes of Sparan and Phellin by nanoknife extraction isolated with insoluble WC. (A) Estimation of the MW of Sparan (○) and Phellin (□) by gel permeation HPLC with a GPC column. $R^2 = 0.994$. The standard compounds were dextrans with MWs of 1.4×10^6 (a), 7.6×10^5 (b), 1.3×10^5 (c), and 1.0×10^5 (d). (B) Particle size distribution. The flow through the module for particles in liquid was used with the 300 mm focal length lens, giving 56 channels of information between 0.04 and 500 μm diameter.

The WC NP is a superior material for using a nanoknife technology due to its hardness and other outstanding mechanical properties (*20*). The size distributions of Sparan and Phellin provided by this technology are monodispersed and narrow size distributions. The materials in Sparan and Phellin are finely ground substances with particle sizes of 150 and 390 nm, respectively (**Figure 4B-i** and **ii**). The average diameter of Sparan was 150 ± 14.1 nm (in volume diameter), and almost 90% of the particles were under 400 nm. This is in accordance with the results of β-glucan granule size distributions found by Roh and Choi (*22*). They reported the cold-water-extracted β-glucan had granules with an average particle size of 5.06 ± 0.9 μm, larger average particle sizes. This narrowly distributed

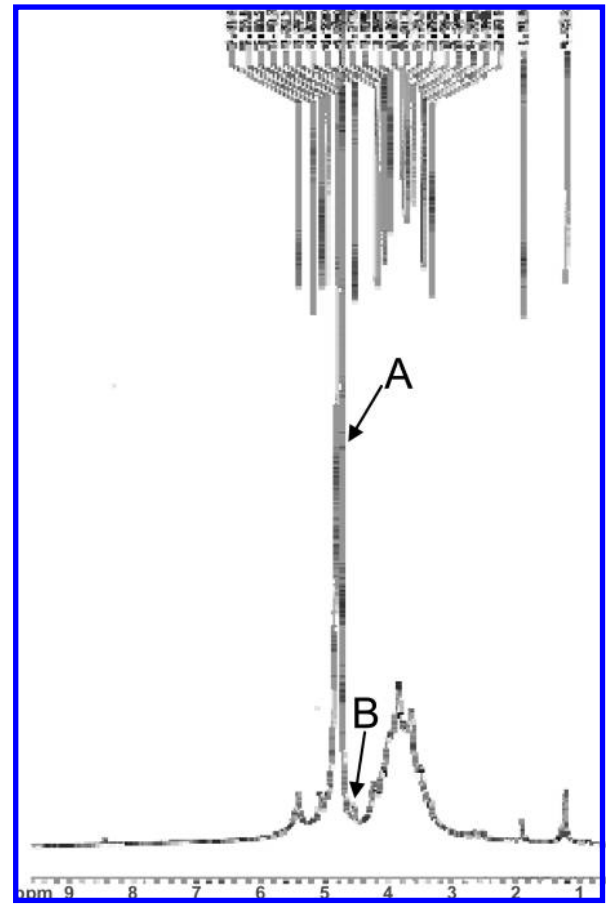

Figure 5. ^{1}H NMR spectra of Sparan. The samples were dissolved in Me_2SO-d_6 (6:1). The procedure was described in detail in the Materials and Methods Section and in the Results.

fine particle size produced by a WC nanoknife makes this process suitable for industrial application. It is potentially useful in the fields of drug delivery or nanotechnology as smaller particles are more easily absorbed by the human intestine (*31*).

Structural Characterization of Sparan. Sparan was repeatedly extracted with alkaline water (pH 10, Extract I), hot water (Extract II), and then cold water (Extract III), and the structures of the polysaccharide fractions of these extracts were analyzed by β-glucan structural characterization using a ^{1}H NMR spectrum. Besides the well-known antitumor β-(1→3)-D-glucans, a wide range of biologically active glucans with other structures have been described (*11*). The structure of the major polysaccharide fraction of Sparan dissolved in D_2O was primarily found to be 6-branched β-(1→3)-D-glucan at 4.78 ppm (**Figure 5A**). A β-(1→6)-D-glucan was also found at 4.55 ppm (**Figure 5B**). They were similar to other alkali-soluble fraction β-(1→3) and β-(1→6) D-glucans (*24*), and it was reported that the ^{1}H chemical shift of the anomeric region is observed at around 4−6 ppm (*32*) and that the anomeric signals for β-(1→3)-D-glucan and β-(1→6)-D-glucan appear at 4.7−4.8 and 4.5−4.6 ppm, respectively (*24, 33*). Additionally, the signals of branching points of β-(1→3)-branched β-(1→6)-D-glucan are observed at 4.55 ppm, and those of the nonreducing end are observed at 4.73 ppm. The molar ratio of β-(1→3)-D-glucan and β-(1→6)-D-glucan was about 99:1.

Water-Soluble and -Insoluble Fractions of Sparan. β-Glucan in mushrooms is distributed in the soluble and insoluble fractions of mushroom dietary fiber (*27*). **Figure 6A** shows the β-glucan contents in the water-soluble fraction from SC. The

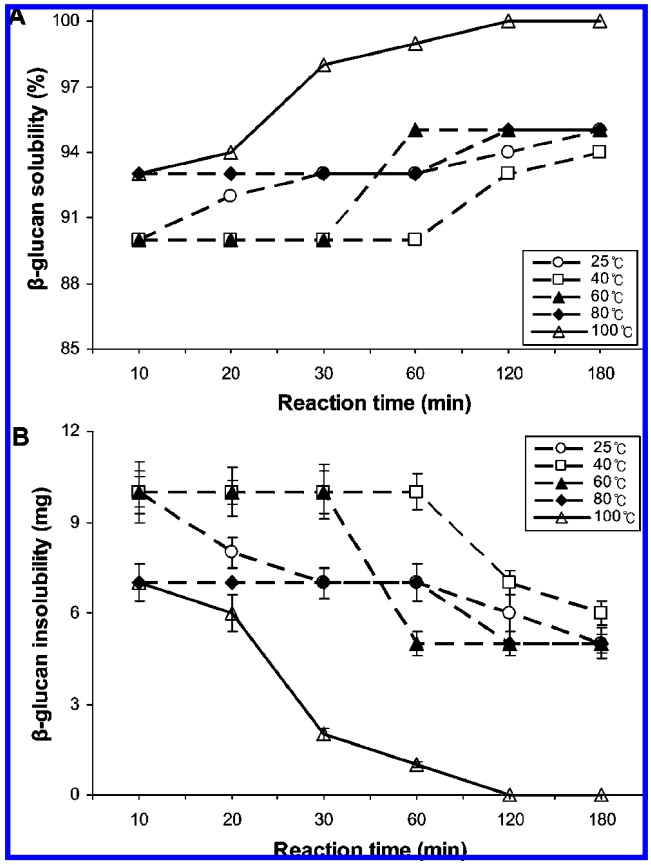

Figure 6. Solubility of Sparan at various incubation times. (A) Water-solubility of β-glucans; soluble compounds (% w/w, dry matter basis). Statistically significant at $p < 0.05$ between 25 and 100 °C by one-way ANOVA. (B) Insolubility of β-glucans; dry weight (mg). Values represent the mean ± standard deviation.

best conditions for recovery of soluble β-glucan occurred when the β-glucan extraction was incubated for more than 120 min at 100 °C. After incubation at 100 °C for 120 min, no insoluble β-glucan was recovered (**Figure 6B**). Even incubation at room temperature (25 °C) for only 10 min gave greater than 90% recovery of water-soluble β-glucan. These results were similar to those of Manzi and Pizzoferrato (*27*) who extracted β-glucan from the dietary fiber fractions of different mushroom species. Soluble components of Sparan using the nanoknife with simple procedures could provide us with an important supplement for the human diet.

DISCUSSION

This study reports the use of a nanoknife procedure for the β-glucan extraction from two mushroom species (SC and PL) known for their superior β-glucan content. Several mechanical techniques including crushing, grinding, and milling have been utilized as a final step in the multistage powder production process because conventional solvent-based crystallization and precipitation operations are unable to provide particles suitable for intended use. However, these mechanical procedures lead to a loss of material and a decrease of process yields. Other researchers have used spray-drying and freeze-drying but still have problems, such as thermal and chemical degradation of the product and broad particle size distribution. This paper demonstrates that the process can be improved by the use of a WC NP powder as a nanoknife in integrated mechanical and thermal activation. The resulting β-glucan particle size has a monodispersed, narrow particle size distribution (150 nm). This paper is the first report of NPs being used from SC and PL. Sparan extracted using this procedure has a narrow nanodispersed particle size and is a (1→6)-branched-type (1→3) glucan, with a MW of 510 kDa. Further studies may reveal that Sparan produced with the NP technology has superior immunomodulating potential as well as potential in signaling pathways for immune activation. Future work will focus on the search for pharmacologically active substances, anticancer agents, and the development of different health-related food products.

ABBREVIATIONS USED

BRM, Biological response modifier; SC, *Sarassis crispa*; PL, *Phellinus linteus*; NP, nanometer-sized particle; GLA, antler of *Ganoderma lucidum*; GLC, cap of *Ganoderma lucidum*; PDA, potato dextrose agar; WC, tungsten carbide; TEA, triethylamine; ACN, acetonitrile; TFA, trifluoroacetic acid; HPAEC-PAD, high-pH anion exchange chromatograph; PITC, phenylisothiocyanate; GPC, gel permeation chromatography; NMR, nuclear magnetic resonance.

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