Fractionation and Characterization of Mushroom Dietary Fiber (Nonstarch Polysaccharides) as Potential Nutraceuticals from Sclerotia of *Pleurotus tuber-regium* (Fries) Singer

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The nonstarch polysaccharides (NSPs) in the total dietary fiber (TDF) from the sclerotia of *Pleurotus tuber-regium* (tiger milk mushroom) were fractionated by the sequential use of chemical solvents. About half of the TDF was solubilized and two major alkali-soluble fractions (1 and 4 N sodium hydroxide) that contained 126 and 293 g/kg TDF were obtained. Sugar analysis and infrared spectroscopy indicated that the NSPs in these alkali-soluble fractions were mainly β-glucans and chitin. These alkali-soluble NSPs were further purified by anion-exchange chromatography followed by gel permeation chromatographic separation. Methylation analysis revealed that these purified glucans were highly branched and contained a mixture of sugar linkages of β-1,3, β-1,6, and β-1,4. The potential use of these sclerotial β-glucans as nutraceuticals was discussed.

Keywords: *Mushroom sclerotium; nonstarch polysaccharides; Pleurotus tuber-regium; structure*

INTRODUCTION

Pleurotus tuber-regium (Fries) Singer is an edible mushroom from the *Basidiomycotina* capable of forming masses of hyphae compacted into dry structures known as sclerotia in order to survive periods of adverse conditions (Willetts, 1971). The sclerotia and fruiting bodies of *P. tuber-regium* are popularly consumed in Nigeria (Oso, 1977). In China, *P. tuber-regium* is commonly known as the tiger milk mushroom and has a growing economic importance (Huang et al., 1996). *P. tuber-regium* is consumed, not only for its flavor and nutritive value, but also for its beneficial medicinal effects (Zoberi, 1973). Notably, *P. tuber-regium* has a very high total dietary fiber (TDF) content (96.3% dry weight) (Cheung and Lee, 1998). Among the TDF in *P. tuber-regium*, the nonstarch polysaccharide (NSP) constituted more than 70% dry weight (Cheung and Lee, 1998). In the East, Mushroom NSPs are a popular nutraceutical product with biopharmacological effects such as antitumor activities and cholesterol-lowering properties (Cheung, 1996; Mizuno, 1995). Mushroom β-glucans with antitumor activity such as lentinan, schizophyllan and protein-bound polysaccharides (PSK, Krestin) are well-known products in the Asian nutraceutical market (Mizuno et al., 1995). In fact, potent antitumor polysaccharides have already been isolated from the fruiting bodies of two *Pleurotus* sp.: *P. sajor-caju* and *P. ostreatus* (Yoshioka et al., 1985; Zhuang et al., 1993).

As part of a continuing study of the biological activities of mushroom NSPs, we have now examined the isolation of the NSPs from the TDF of the sclerotia of *P. tuber-regium* by solvent extraction. The chemical composition and structural features of the purified mushroom β-glucans obtained are reported.

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MATERIALS AND METHODS

Sclerotia of *P. tuber-regium* were cultivated in the Sanming Mycological Institute in the Fujian Province of Mainland China. TDF were prepared from the mushroom sample by the AOAC total dietary fiber (TDF) method described previously (Cheung and Lee, 1998).

Solvent Extraction. The NSPs in the mushroom TDF were fractionated sequentially by solvent extraction using 0.01 M EDTA, 1 N sodium hydroxide, 50% acetic acid, and 4 N sodium hydroxide under the conditions shown in Figure 1. The mixture in each extraction was centrifuged (3000*g* for 20 min) to separate the extracting solvent from the insoluble residue. The supernatants in each step of the fractionation were pooled, dialyzed against distilled water, and freeze-dried. Precipitate (N4SF2) that formed during the dialysis of the 4 N sodium hydroxide extract was separated from the soluble fraction (N4SF1) by centrifugation. The final residue (INS) was also dialyzed and freeze-dried. A total of six fractions were obtained (Figure 1) and they were EDTA-soluble (ESF), 1 N NaOH-soluble (N1SF), acid-soluble (ASF), 4 N NaOH-soluble and water-soluble (N4SF1), 4N NaOH-soluble and water-insoluble (N4SF2), and final insoluble residue (INS).

Chromatographic Separation. The two alkali-soluble fractions (N1SF, N4SF1) found to be the major fractions (Table 1) were purified by an anion-exchange column (Pharmacia Biotech) packed with DEAE-Sepharose CL-6B (80 cm^3 in 26 mm i.d. x 20 cm) gels (Figure 1). The samples were dissolved in and eluted isocratically with a phosphate buffer (0.01 M, pH 7.8) at a sample concentration of 2.5 mg/mL. The flow rate was set at 1 mL min^{-1} for the first 100 mL followed by a continuous linear salt gradient (0−0.6 M NaCl in the next 200 mL of phosphate buffer). The fractions were collected at 2.5 mL each and the total carbohydrate content was monitored colorimetrically by the phenol/sulfuric acid assay (Dubois et al., 1956). All the fractions from the anion-exchange column were further purified by a gel permeation column (Pharmacia Biotech) packed with Sephacryl S400 (180 cm^3 in 16 mm i.d. × 100 cm) (Figure 1). Each sample was dissolved in and eluted with 0.1 M ammonium acetate buffer at a sample concentration of 5 mg/mL and a flow rate of 0.2 mL min^{-1}. Fractions were collected at 5 mL each and were tested for their total carbohydrate content by the same method mentioned above.

10.1021/jf000382s CCC: $19.00
Published on Web 07/15/2000

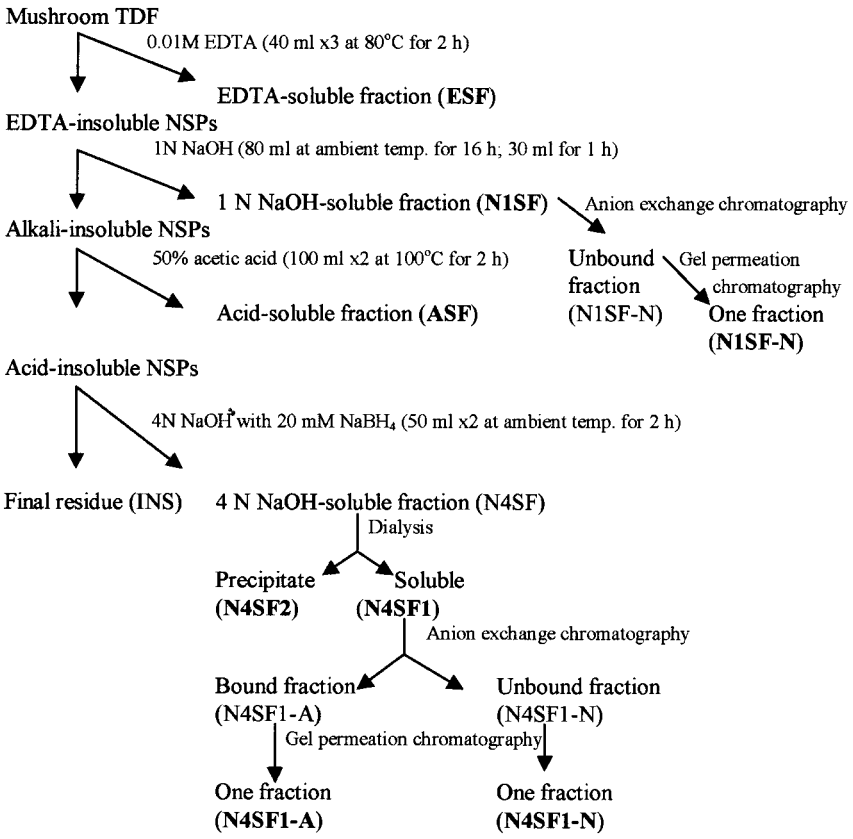

Figure 1. Fractionation scheme of the nonstarch polysaccharides in the total dietary fiber of the sclerotium of *P. tuber-regium*.

Table 1. Monosaccharide Composition and Yield of Nonstarch Polysaccharide Fractions from Total Dietary Fiber of the Sclerotium of *P. tuber-regium* (g/kg TDF)[a]

	ESF	N1SF	ASF	N4SF1	N4SF2	INS	total [b]	TDF
fucose	0.10	1.10	0.10	0.20	2.10	4.10	7.70	2.70
arabinose	0.50	nd[e]	nd	nd	nd	nd	0.50	1.00
xylose	0.30	nd	nd	0.40	2.50	1.30	4.50	nd
mannose	0.60	0.60	0.01	0.10	0.30	0.70	2.20	4.80
galactose	nd	nd	nd	0.10	nd	nd	0.10	1.10
glucose	1.50	78.1	0.10	6.30	198	280	563	633
glucosamine	1.40	5.20	0.40	1.70	8.00	57.7	74.4	52.0
uronic acids	nd	nd	nd	0.30	4.50	7.40	12.2	14.8
NSP[c]	4.40	84.9	0.50	9.10	215	351	665	710
yield [d]	55.1	126	11.2	28.9	254	464	939	1000

[a] Mean values of three replicates. [b] Sum of all fractions. [c] Sum of all sugars. [d] Gravimetric yield. [e] nd, not detected.

Sugar and Linkage Analysis. The neutral sugar composition and uronic acid content of all the isolated mushroom NSP fractions were determined by gas chromatography (GC) and colorimetry as described previously (Cheung and Lee, 1998). The glycosidic linkages of some of the purified mushroom NSPs were determined by methylation analysis according to the procedures of Harris et al. (1984) as modified by Mukerjea et al. (1996). In brief, methylsulfinyl carbanion (dimsyl) was prepared from potassium hydride and dimethyl sulfoxide (DMSO). Mushroom NSP fractions that were insoluble in DMSO were premethylated by dimsyl and methyl iodide prior to the normal methylation procedures. Partially methylated alditol acetates (PMAA) samples were prepared from the methylated mushroom NSPs by acid hydrolysis (2 M trifluoroacetic acid at 121 °C for 60 min), reduction of the hydrolyzed sugars by sodium borohydride, and acetylation by acetic anhydride. The PMAA samples were analyzed by a GC−MS (GCMS-QP5050, Shimadzu, Japan). The GC conditions were as follows: HP-5MS capillary column (5% phenyl methyl siloxane, 30 m × 250 μm i.d., Hewlett-Packard); helium as carrier gas at a flow rate of 1.2 mL/min; initial oven temperature at 130 °C, followed by 4 °C/min rise to 280 °C; injector temperature at 280 °C; interface temperature at 250 °C; and split ratio at 30:1. The MS conditions were as follows: ion source temperature at 250 °C, ionization energy at 70 eV, detector volts at 1.5 kV, and mass range from 50 to 350. Each PMAA was identified by matching its mass spectrum with known standards and quantified after corrections for PMAA molar response factors, according to the effective carbon-response theory (Sweet et al., 1975).

Infrared Spectroscopy. Infrared spectrum was measured by preparing potassium bromide disks containing the isolated mushroom NSPs using a Fourier transform Infrared spectrometer (Magna-IR 560, Nicolet, Madison WI).

RESULTS AND DISCUSSION

Fractionation of Mushroom NSPs. The fractionation scheme (Figure 1) of mushroom TDF followed the conventional alkali−acid−alkali sequence used for isolated fungal cell wall preparations (Bartnicki-Garcia and Nickerson, 1962). Since fungal cell walls are not lignified, the hypochlorite delignification step was omitted in the fractionation procedure. The total NSP content recovered from all the fractions (665 g/kg TDF) was in agreement with that of the mushroom TDF (710 g/kg TDF) that was the starting material (Table 1),

Table 2. Monosaccharide Composition in the Fractions Separated by Anion Exchange and Gel Permeation Chromatography from the Alkali-Soluble NSPs of the Sclerotium of *P. tuber-regium* (Normalized Percentage)[a]

	N1SF-N	N4SF1-N	N4SF1-A
fucose	0.35	0.86	2.58
arabinose	nd[b]	nd	nd
xylose	nd	0.61	4.90
mannose	nd	0.55	1.85
galactose	nd	nd	2.57
glucose	97.8	93.8	61.4
glucosamine	1.84	3.56	20.9
uronic acids	nd	0.62	5.77

[a] Mean values of three replicates. [b] nd, not detected.

indicating that the loss of fiber material was less than 10%. More than half of the mushroom TDF was solubilized during the solvent extraction. Among the various soluble fractions, the 1 N and 4 N alkali fractions (N1SF and N4SF, respectively) contained 126 and 293 g/kg TDF. Almost all the solubilized NSP was only found in the N1SF and N4SF fractions (27 and 71% of the total extractable NSP from TDF, respectively) (Table 1). In terms of NSP content in the fractions, N4SF2 had the highest level (846 g/kg fraction) followed by N1SF (674 g/kg fraction). ESF and ASF were the minor soluble fractions which contained less than 100 g/kg of NSP but with more than 900 g/kg non-NSP components. In terms of sugar composition, all the alkali-soluble fractions (N1SF, N4SF1, and N4SF2) and the final alkali-insoluble residue (INS) had glucose as the major polysaccharidic sugar (as glucan), followed by glucosamine (as *N*-acetylglucosamine from chitin). The presence of IR absorption bands at 2920−2925, 1375−1380, and 895−898 cm^{-1} (data not shown) suggested the prevalence of the β-glycosidic linkage in these mushroom NSP fractions (Michell and Surfield, 1967). Moreover, the IR spectra also had absorption bands near 1640 and 1550 cm^{-1} (data not shown), which are the characteristics of chitin (Michell and Surfield, 1970). Such information suggested that chitin and possibly β-glucans were both present in these fractions (Table 1). The fact that about half of the NSP remained insoluble in the final residue (INS) suggested that they might be covalently linked to other polymers such as chitin in the INS. In fact, chitin−β-glucan complexes are present in the alkali-insoluble fractions from a number of fungi (Holan et al., 1981; Sietsma and Wessels, 1977) via an amino acid linkage (Sietsma and Wessels, 1979).

Purification of Fractionated Mushroom NSPs. The two major soluble fractions, N1SF and N4SF1, were subjected to further chromatographic separation (Figure 1). Anion exchange chromatography had revealed that N1SF contained only a single unbound fraction (N1SF-N) while N4SF1 contained an unbound neutral fraction (N4SF1-N) and a bound acidic fraction (N4SF1-A) of approximately equal proportion (data not shown). All these fractions from the anion exchange chromatography only gave a single peak in gel permeation chromatography (data not shown). The sugar composition of the neutral fractions (N1SF-N and N4SF1-N) indicated that glucose was the predominant sugar with only a trace amount of glucosamine (Table 2), implying the presence of homoglycan (most probably glucans). The acidic fractions (N4SF1-A) contained glucose as the major sugar followed by glucosamine and uronic acids (Table 2), implying that heteroglycans (possibly glucans, chitin, and polyuronides) were present.

Table 3. Major Sugar Linkages (mol %)[a] of the Purified β-Glucan Fractions from the Sclerotium of *P. tuber-regium*

type of linkage	N1SF-N	N4SF1-N
terminal groups	26.1	26.2
1,3	21.7	30.8
1,6	9.50	10.4
1,4	20.3	14.9
1,3,6	22.4	17.7

[a] Mean values of three replicates.

Linkage Analysis of the Purified Mushroom Glucans. Methylation analysis indicated that the purified glucans (N1SF-N and N4SF1-N) appeared to be a highly branched polysaccharide containing a mixed β-1,3, β-1,6, and β-1,4 linkages (Table 3). It also showed that hardly any cross-linkage occurred between glucan chains, for the amount of terminal groups was almost equal to the amount of branching points (Table 3). The ratio of the 1,3:1,6:1,4 linkages for N1SF−N and N4SF1−N was approximately 2:1:2 and 3:1:1.5, respectively. Although such linkage types were different from the other *Pleurotus* mushroom polysaccharides (Saito et al., 1976; Yoshioka et al., 1985), which contained mainly β-1,3 and β-1,6 linkages only, they were consistent with the polysaccharides isolated from sclerotia of *Sclerotinia libertiana*, which had a ratio of β-1,3:β-1,6:β-1,4 of 3:1:2 (Oi et al., 1966). The findings of the existence of β-1,4 linkages in the mushroom NSP would imply the presence of cellulose in the sclerotia of *P. tuber-regium*. It is unusual to have two structural cell wall polysaccharides, cellulose and chitin, coexisting in the basidiomycetes (Bartnicki-Garcia, 1970). However, it is possible that cellulose from the compost may be associated with the fungal hyphae during the formation of sclerotia, leading to its inclusion in the mushroom TDF. How β-1,4 cellulose remained associated with the β-1,3 and β-1,6 glucans despite vigorous chromatographic separations remains unclear.

Conclusions. From the above studies, it can be concluded that the NSPs in the sclerotia of *P. tuber-regium* contained β-1,3 and β-1,6 glucans, chitin, and cellulose as the major polysaccharides with some polyuronides. The fact that β-glucans are the major reserve polysaccharides (more than 60% dry weight) in the sclerotia of *P. tuber-regium* is consistent with the observations in other sclerotial glucans such as sclerotan in *Sclerotium libertiana* (Oi et al., 1966) and pachyman in *Poria cocos* (Hoffman et al., 1971). Structurally, the β-1,3 and β-1,6 glucans in *P. tuber-regium* are similar to the other β-glucans such as pachyman and lentinan that have biological activity including immunopotentiating, antitumor, and hypocholesterolemic activity (Mizuno, 1995). Therefore, the above sclerotial glucans from *P. tuber-regium* will be further tested for their biological activities as an important step leading to the development of novel mushroom nutraceuticals.

ABBREVIATIONS USED

NSP, nonstarch polysaccharides; TDF, total dietary fiber; ESF, EDTA-soluble fraction; N1SF, 1 N NaOH-soluble fraction; ASF, acid-soluble fraction; N4SF1, 4 N NaOH-soluble and water-soluble fraction; N4SF2, 4 N NaOH-soluble and water-insoluble fraction; INS, final insoluble residue.

LITERATURE CITED

Bartnicki-Garcia, S. Cell wall composition and other biochemical markers in fungal phylogeny. In *Phytochemical Phylogeny* (Harborne, J. B., Ed.), 1970; pp 81−103. Academic Press: London and New York.

Bartnicki-Garcia, S.; Nickerson, W. J. Isolation, composition, and structure of cell walls of filamentous and yeast-like forms of *Mucor rouxii*. *Biochim. Biophys. Acta* **1962**, *58*, 102−119.

Cheung, P. C. K. The hypocholesterolemic effect of extracellular polysaccharide from the submerged fermentation of mushroom. *Nutr. Res.* **1996**, *16*, 1953−1957.

Cheung, P. C. K.; Lee, M. Y. Comparative chemical analysis of fiber material prepared by enzymatic and chemical methods from two mushrooms (*Pleurotus sajor-caju* and *Pleurotus tuber-regium*), *J. Agric. Food Chem*. **1998**, *46*, 4854−4857.

Dubois, M.; Gilles, K. A.; Hamilton, J. K.; Rebers, P. A.; Smith, F. Colorimetric method for determination of sugars and related substances. *Anal. Chem*. **1956**, *28*, 350−356.

Harris, P. J.; Henry, R. J.; Blakeney, R. A.; Stone, B. A. An improved procedure for the methylation analyses of oligosaccharides and polysaccharides. *Carbohydr. Res.* **1984**, *127*, 59−73.

Hoffmann, G. C.; Simson, B. W.; Timell, T. E. Structure and molecular size of pachyman. *Carbohydr. Res.* **1971**, *20*, 185−188.

Holan, Z.; Pokorny, V.; Beran, K.; Gemperle, A.; Tuzar, Z.; Baldrain, J. The glucan−chitin complex in *Saccharomyces cerevisiae*. V. Precise location of chitin and glucan in bud scar and their physicochemical characterization. *Arch. Microbiol*. **1981**, *130*, 312−318.

Huang, N.; Guo, M.; Huang, L. Tiger milk mushroom (*Pleurotus tuber-regium* (Fr.) Sing) and its cultivation. *Edible Fungi China* **1996**, *16*, 13−14.

Michell, A. J.; Surfield, G. Composition of extracted fungal cell walls as indicated by infrared spectroscopy. *Arch. Biochem. Biophys.* **1967**, *120*, 628−637.

Michell, A. J.; Surfield, G. An assessment of infrared spectra as indicators of fungal cell wall composition. *Aust. J. Biol. Sci.* **1970**, *23*, 345−360.

Mizuno, T. Bioactive molecules of mushrooms: Food function and medicinal effect of mushroom fungi. *Food Rev. Intern*. **1995**, *11*, 7−22.

Mizuno, T.; Saito, H.; Nishitoba, T.; Kawagishi, H. Antitumor-active substances from mushrooms. *Food Rev. Intern*. **1995**, *11*, 23−61.

Mukerjea, R.; Kim, D.; Robyt, J. F. Simplified and improved methylation analysis of saccharides, using a modified procedure and thin-layer chromatography. *Carbohydr. Res*. **1996**, *292*, 11−20.

Oi, S., Konishi, I.; Satomura, Y. Structural studies on 'Isosclerotan', a new glucan isolated from *Sclerotinia* fungus, by physical, chemical and enzymatic methods. *Agric. Biol. Chem*. **1966**, *30*, 266−273.

Oso, B. A. *Pleurotus tuber-regium* from Nigeria. *Mycologia* **1977**, *69*, 271−279.

Saito, H.; Ohki, T.; Yoshioka, Y.; Fukuoka, F. A ^{13}C nuclear resonance study of a gel-forming branched (1→3)-β-glucan, A_3, from *Pleurotus ostreatus* (Fr.) Quel: Determination of the side-chains and conformation of the polymer-chain in relation to gel-structure. *FEBS Lett.* **1976**, *68*, 15−18.

Sietsma, J. H.; Wessels, J. G. H. Chemical analysis of the hyphal wall of *Schizophyllum commune*. *Biochim. Biophys. Acta* **1977**, 4*96*, 225−239.

Sietsma, J. H.; Wessels, J. G. H. Evidence for the covalent linkages between chitin and β-glucan in a fungal wall. *J. Gen. Micobiol*. **1979**, *114*, 99−108.

Sweet, D. P.; Shapiro, R. H.; Albersheim, P. Quantitative analysis by various GLC response factors theories for partially methylated and partially ethylated alditol acetates. *Carbohydr. Res.* **1975**, *40*, 217−225.

Willetts, H. J. The survival of fungal sclerotia under adverse environmental conditions. *Biol. Rev. Cambridge Philos. Soc.* **1971**, *46*, 387−407.

Yoshioka, Y.; Tabeta, R.; Saito, H.; Uehara, N.; Fukuoka, F. Antitumor polysaccharide from *P. ostreatus* (Fr) Quel.: isolation and structure of a beta-glucan. *Carbohydr. Res.* **1985**, *140*, 93−100.

Zhuang, C.; Mizuno, T.; Shimada, A.; Ito, H.; Suzuki, C.; Mayuzumi, Y.; Okamoto, H.; Ma, Y.; Li, J. Antitumor protein-containing polysaccharides from a Chinese mushroom Fengweiguor Houbitake, *Pleurotus sajor-caju* (Fr.) Sings. *Biosci. Biotechnol. Biochem*. **1993**, *57*, 901−906.

Zoberi, M. H. Some edible mushrooms from Nigeria. *Nigerian Field* **1973**, *38*, 81−90.

Received for review March 23, 2000. Revised manuscript received June 6, 2000. Accepted June 6, 2000. This work is financially supported by the Research Grant Council of the Hong Kong SAR Government.

JF000382S