Arch Pharm Res Vol 22, No 5, 515-519, 1999

Characterization of an Alkali-extracted Peptidoglycan from Korean *Ganoderma lucidum*

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(Received February 25, 1999)

The biologically active peptidoglycan was purified from the alkali fraction of the fruiting bodies of *Ganoderma lucidum* and the composition of the peptidoglycan was investigated by conventional analyses. The alkali-extracted peptidoglycan showed differences in chemical compositions from the water-extracted. The alkali-extracted peptidoglycan contained 6.9% protein and 75.9% carbohydrates composed mainly of β-glucose, mannose, and α-glucose. The molecular weight range of the peptidoglycan was determined as 2,000 kDa-17 kDa. The peptidoglycan is considered to be a hybrid molecule of polysaccharide chains covalently bound as a side chain to the polypeptide core.

Key words : *Ganoderma lucidum*, Fruiting body, Alkali extract, Peptidoglycan, Characterization

INTRODUCTION

The fruiting body of *Ganoderma lucidum* (Polyporaceae) distributed mainly in the oriental countries have long been used traditionally to cure chronic human diseases such as hepatitis, hypertension, hypercholesterolemia, bronchitis, insomnia, neurasthenia, asthma, and allergies (Sone *et al.*, 1985, Kobayashi, 1978, Lee *et al.*, 1990). Since the scientific investigation on antitumor activities of Basidiomycetes including *G. lucidum* have been reported (Ringler *et al.*, 1957), extensive studies on the antitumor components of Basidiomycetes, especially on polysaccharides have been carried out (Ikegawa *et al.*, 1968, 1969, Kim *et al.*, 1980, Miyazaki *et al.*, 1981, Mizuno *et al.*, 1984). The main components with this activity are thought to be polysaccharides. The mechanism to exert antitumor activity is assumed through potentiation of the host immunity rather than direct cytotoxicity on tumour cells (Mizuno 1993). It is widely accepted that activated macrophages, cytotoxic T cells and natural killer cells usually play important roles in tumour immunity (Cheong and Park 1996, Park *et al* 1996).

To characterize the peptidoglycan from Korean *G. lucidum* and to investigate its biological activity, the physiologically active peptidoglycan-protein bound polysaccharide was isolated from the fruiting body of Korean *G. lucidum*. The peptidoglycans in *G. lucidum* can be extracted using various solvents, and components can differ in variety according to the choice of solvents. The essential structure of water-soluble anti tumour polysaccharide isolated from *G. lucidum* is known to have a branched glucan core involving (1→3)-β-, (1→4)-β-, and (1→6)-β-linkages (Miyazaki and Nishijima, 1981, Usui *et al.*, 1983). The structure of an alkali-extracted polysaccharide of *G. lucidum* was highly branched and composed of 1,3,4-tri substituted D-mannopyranosyl and (1→4)-linked D-xylopyranosyl residues (Kim *et al.*, 1980, Miyazaki *et al.*, 1982). Mizuno *et al.* isolated only 0.007 % of water-soluble polysaccharides, but the amount of bioactive water-insoluble polysaccharide (10.2%) was much higher than that of water-soluble polysaccharide (Mizuno *et al.*, 1984). They isolated the water-soluble polysaccharide consisting of β-glucan and glucurono-β-glucan and the water-insoluble polysaccharide consisted of hetero β-glucan, xylo-β-glucan, xylomanno-β-glucan and manno-β-glucan. They also reported that the main water-insoluble polysaccharide, xylo-β-glucan (8.6%), has high molecular weight of 2,000 kDa which might affect the low solubility in the polysaccharide water of *G. lucidum*. Although plenty of investigations on the water-soluble fractions of *G. lucidum* have been reported, investigations of water-insoluble components are scarce. Since the biological activity of peptidoglycan was expected to be differ in variety according to

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extraction solvents, their essential chemical structures and fractionation method, the present study has been initiated with the extraction method and the investigation on physico-chemical properties of alkali-extracted petidoglycan from the fruiting bodies of Korean *G. lucidum*.

MATERIALS AND METHODS

Chemicals

Sigma (St. Louis, MO, USA) provided the chemicals Sepharose CL-4B, dextran standards (molecular weight marker; 2000, 124, 9.3 kDa), carbohydrates standard (CAR-11) and phenol. Amino acid standard, sulfuric acid, sodium hydroxide, metal standards (K, Ca, Mg, Na, Zn, Mn, Fe), sodium citrate, hydrochloric acid were obtained from Wako (Osaka, Japan). Trimethylchlorosilane (TMCS), hexamethyldisilazane (HMDS) and pyridine anhydrous (silylation grade) were purchased from Alltech (San Jose, CA, USA). All other chemicals were analytical grade and commercially available.

Instrumentation

The gas chromatographic system consisted of a Shimadzu GC-9A with a flame ionization detector (FID) and a borosilicate glass column (3 m × 3 mm id, Supelco) packed with 3% OV-17(80-100 mesh Shimalite. Both injector and detector were heated to 240°C. The oven temperature was raised by 2°C/min. from 120°C to 180°C. The gas flow rates for FID were 300 ml/min air, 40 ml/min. hydrogen and 40 ml/min. helium. A Beckman (Palo Alto, CA, USA) system 6300 amino acid analyzer was used with a sodium-high performance column (200 mm L. × 2.6 mm i.d., ion exchange resin no. 338076) along side a tungsten lamp detector and an auto sampler. The column temperature ranged from 50°C to 77°C in a timed gradient mode. The reaction bath was heated to 130°C. The reagent pressure was 7 kg/cm^2 and the buffer solution was 147 kg/cm^2, N_2 2.8 kg/cm^2. A Shimadzu UV spectrometer model 160A (Osaka, Japan) was used at a 490 nm wavelength. A Perkin-Elmer (Norwalk, NJ, USA) model 5100 atomic absorption spectroscope was used for analyzing the ionised forms of metals. Infrared spectroscopy by the KBr disc method (Bruker IFS-48 spectrometer, Germany) was used to identify a characteristic wavelength of absorption of the sample.

Sample preparation

Samples were prepared in quantities. Once cut into small pieces, the dried fruiting body of *Ganoderma lucidum* (1 kg, harvested at Kyungki prov., Korea, water content 11%) was stirred with distilled water for 2 h at 105°C. The water fraction was lyophilized *in vacuo* to produce 60 g of powder. The residue was extracted with 17 volumes of 8% sodium hydroxide for 5 h at 90°C. The supernatant substance was neutralized with acetic acid, ultrafilterated (molecular weight cut 10,000 amu) against running water for 2 days and then lyophilized again to produce 43 g of powder. The materials obtained through this process were analyzed.

Analytical method

To characterize the physico-chemical properties of the materials obtained, the total carbohydrate content was measured by the phenol sulfuric acid method (Chaplin and Kennedy, 1986). Neutral sugar components of the total carbohydrate were analyzed by hydrolysis and derivatized for gas chromatography. A sample of peptidoglycan (10 mg) was heated with 0.1 N-HCl (5 ml) at 100 °C for 5hrs in a vessel with a Teflon-lined screw cap. Fifteen ml of ethanol was added and the mixture was then left to stand for 24 h and filtered with a 0.2 μm micro filter. After evaporation at 50°C *in vacuo*, the residue was dissolved completely in 750 μl of pyridine anhydrous. In order to make trimethylsilyl derivatives of carbohydrates, 250 μl of trimethylchlorosilane (TMCS) :hexamethyldisilazane (HMDS) mixture (2:1) was added and heated at 105°C for 1 h. Then the reacted mixture (1 μl) was subjected to a gas chromatography. To determine the organic nitrogen content in the material, the Kjieldahl procedure was used with a semimicro-Kjieldahl apparatus. For identification of protein components, an amino acid analyzer was used. Four mg of the sample was dissolved in 1 ml of 6N-HCl, and air in the vessel was substituted with nitrogen gas and then sealed. Each mixture was hydrolyzed for 18 h at 110°C and then dried. The dried fraction was dissolved in 2 ml of a 0.2 N sodium citrate buffer (pH : 2.2) which was then injected into a amino acid analyzer.

Using Sepharose CL-4B, gel permeation chromatography was adopted to determine the molecular weight of peptidoglycan in the sample. Dextrans (Sigma Co., m.w. 2000, 124, 9.3 kDa) were used as a standard molecular weight marker. Pre-swollen Sepharose CL-4B gel was washed twice with two volumes of 0.3 M NaOH and degassed *in vacuo*. The gel was packed into a glass column (1.6 cm id. × 53 cm L) with 0.3 M-NaOH. The standard (10 mg) and sample were dissolved in the eluent and applied to the column. A peristaltic pump (Vision Scientific, Korea) was used to fill the column with 0.3N sodium hydroxide at a flow rate of 6 ml/hr and fractions of 2 ml were collected. The carbohydrate content of each fraction was determined by the phenol-sulfuric acid method by UV spectrometry.

RESULTS AND DISCUSSION

Total contents of carbohydrates and protein

Table I. Carbohydrate and protein contents in peptidoglycans of water- and alkali-extracts from *Ganoderma lucidum*

(unit: w/w dried %)

components	water extract	8%-NaOH extract
carbohydrate	83.50	75.98
protein	4.98	6.95

Table II. Monosaccharide components in peptidoglycans of Alkali-extracts from *Ganoderma lucidum*

(unit: w/w dried%)

saccharides	8%-NaOH extract	45%-NaOH extract
fructose	0.1918	0.1434
galactose	0.0152	N.D.*
α-glucose	0.2050	0.0826
β-glucose	0.4998	0.2303
ribose	0.0126	N.D.
xylose	0.1415	N.D.
mannose	0.3134	0.1012

*N.D.: not detected

Table III. Amino acid components in peptidoglycans of alkali -extracts from *Ganoderma lucidum*

(unit: w/w dried %)

amino acids	8 %-NaOH extract	45 %-NaOH extract
aspartic acid	0.3616	0.0085
threonine	0.0271	0.0019
serine	N.D.*	0.0083
glutamic acid	0.4963	0.0150
proline	0.1597	0.0042
glycine	0.0928	0.0176
alanine	0.2049	0.0103
valine	0.2035	0.0106
isoleucine	0.1739	0.0044
leucine	0.4102	0.0917
tyrosine	N.D.	0.0595
phenylalanine	0.2390	0.0106
histidine	0.0882	0.0169
lysine	0.2447	0.0019
arginine	0.0356	0.0057
methionine	0.1420	N.D.

*N.D.: not detected

The peptidoglycan purified from the alkali fraction contained 75.9% of carbohydrates and 6.9% of proteins while the compound from water fraction contained 83.5% of carbohydrates and 4.9% of proteins (Table I). It is considered that the peptidoglycan mostly contains a hybrid molecule of polysaccharide and proteins. It is also considered that this peptidoglycan is thought to be a polysaccharide chain covalently bound as a side chain to the polypeptide core.

Contents of monosaccharides and amino acids

The contents of monosaccharide in the petidoglycan from the alkali fraction are shown in Table II. The polysaccharide moiety of the peptidoglycan was found to be a hetero polymer consisting of several different monosaccharides such as β-glucose, mannose and α-glucose which was slightly different from other results. It was reported that the peptidoglycan from the water fraction contained glucose, xylose and arabinose (Kobayashi, 1978). It was also reported that the peptidoglycan from the water fraction contained glucose and mannose and the peptidoglycan from the alkali fraction contained mannose, xylose and fucose (Miyazaki and Nishijima, 1982). The sugar contents of peptidoglycan extracted with 45% NaOH solution were lower than the contents of peptidoglycan extracted with 8% NaOH.

Table III shows the amino acid contents of peptidoglycan. The amino acid contents of peptidoglycan extracted with 45% NaOH solution decreased. The peptidoglycan in the alkali fraction is thought to be a kind of acid mucopolysaccharide. It is assumed that immunological activities are attributed to 1, 3-β-D-glucan which is the main cell wall structure of mushrooms, chitin and their chemically modified structures (Sone *et al.*, 1985).

Molecular weight determination of peptidoglycan

The molecular weights of each peptidoglycan were determined by Sepharose CL-4B gel filtration using blue dextran as the molecular weight markers. The elution

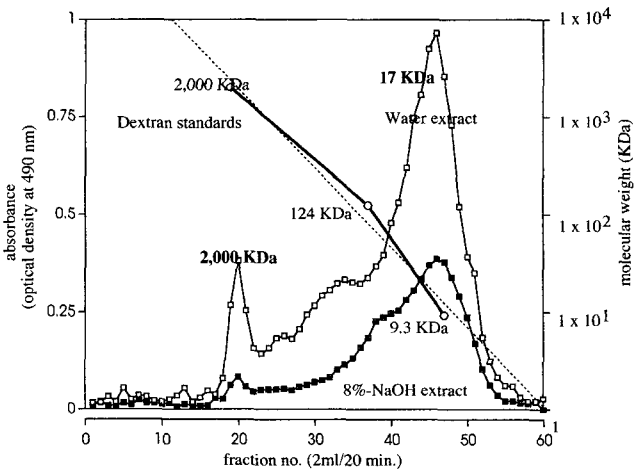

Fig. 1. Elution profiles of peptidoglycans extracted by water and alkali solution using gel permeation chromatography

Table IV. Elements in peptidoglycans of alkali-extracts from *Ganoderma lucidum*

(unit: w/w dried %)

element	8% - NaOH extract
Na	1.160
Mg	0.001
Fe	0.002
Ca	0.005
Zn	0.001
K	0.012
Mn	N.D.*

*N.D.: not detected

profiles of peptidoglycan on gel permeation chromatography are shown in Fig. 1. The molecular weights of the alkali extracted peptidoglycan are 2,000 kDa and 17 kDa which are similar to those of the water extract while the quantities of the alkali extract are lower.

Fourier transform infra-red spectroscopy

A KBr disc method was used and the spectra were almost identical showing a typical band absorbed at 3420 cm^{-1} for hydrogen bonded O-H stretching. 2924 cm^{-1} for aliphatic C-H stretching, 1635 cm^{-1} for C=O stretching, 1396 cm^{-1} for CH_3 bending, 1160 cm^{-1} and 1043 cm^{-1} for C-O stretching in polysaccharide moiety.

Elements of the alkali fraction

The alkali fraction was pretreated using acid digestion method and analyzed by atomic absorption spectroscopy. 1.16% (w/w) of sodium was considered to be attributed to the alkali extraction with NaOH solution. Traces of Mg, Fe, Ca, Zn and K were also found (Table IV).

CONCLUSION

The peptidoglycans of *G. lucidum* which exert biological activities can differ according to the choice of solvent. The peptidoglycan purified from the alkali extract of Korean *G. lucidum* contained 75.9% of carbohydrates and 6.9% of proteins. The molecular weights of the peptidoglycan were determined as 2,000 kDa and 17 kDa, and the peptidoglycan were considered to be a hetero polymer composed of several different monosaccharides-mainly in the order of β-glucose, mannose, and α-glucose. The authors concluded that the peptidoglycan obtained mostly contains a hybrid molecule of polysaccharide and proteins. This peptidoglycan is thought to be a polysaccharide chain covalently bound as a side chain to the polypeptide core. A FT-IR spectrum of this peptidoglycan shows the typical absorptional bands of polysaccharide. The physiological effects of this peptidoglycan of *G. lucidum* will be reported elsewhere.

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