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Enhancement of Water-solubilities of Protein-bound Polysaccharides Contained in the Basidiocarps of *Ganoderma lucidum* by Hydrolyzing with Chymotrypsin

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Optimum conditions for hydrolysis were investigated to enhance water-solubilities of protein-bound polysaccharides in the basidiocarps of *Ganoderma lucidum* by treating chymotrypsin. We also attempted with *Ganoderma lucidum* residue remaining after extracting hot water-soluble components in *Ganoderma lucidum*. After hydrolyzing under optimum conditions (20 ppm chymotrypsin, 2% *Ganoderma lucidum* or 6% *Ganoderma lucidum* residue, at pH 10 and at 40°C), the amounts of total protein and carbohydrate of hydrolysate were measured. Michaelis constant, K_m and maximum rate, V_{max} calculated by Lineweaver-Burk plot for the hydrolysis of *Ganoderma lucidum* were 1.73% and 0.073%/min respectively and those for hydrolysis of *Ganoderma lucidum* residue were 2.40% and 0.033%/min respectively. The amount of polysaccharide isolated from *Ganoderma lucidum* (100 g) treated with chymotrypsin was only 3.07 g, but significantly increased amount (14.34 g) of polysaccharides was isolated from *Ganoderma lucidum* residue (100 g) treated with chymotrypsin. The protein-bound polysaccharide was isolated from the non-hydrolyzed and hydrolyzed samlpe and molecular weights of the polysaccharide were measured by Sepharose CL-4B gel filtration.

Key words : *Ganoderma lucidum*, Optimum conditions, Hydrolysis, Chymotrypsin, Michaelis constant, Polysaccharide, Molecular weight

INTRODUCTION

Much attention has recently been paid to the biological constituents of the fungus *Ganoderma lucidum* (Fr.) KARST (Polyporaceae), which is widely used as a traditional medicine for treatment of chronic diseases (Lee *et al.*, 1990, Nogami *et al.*, 1986, Kang *et al.*, 1980, Hyun *et al.*, 1990).

The protein-bound polysaccharides exerting antitumor activities have been isolated from natural sources such as yeasts (Bradner *et al.*, 1958), bacteria (Kato *et al.*, 1981) and fungi (Chihara *et al.*, 1970) including *Ganoderma lucidum*. The polysaccharides are considered to exert antitumor activities through an enhancement of the host-mediated immunity rather than a direct cytotoxicity to tumor cells. It is considered that activated macrophages, cytotoxic T cells and natural killer cells usually play an important roles in tumor immunity (Nakahara *et al*; Usui *et al.*, 1983; Sone *et al.*, 1985; Shin *et al.*, 1985).

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The essential structure of the water-soluble antitumor polysaccharide isolated from *Ganoderma lucidum* was a branched glucan core involving (1→3)-β-, (1→4)-β- and (1→6)-β-linkages (Miyazaki *et al.*, 1981). The structure of an alkali-extracted polysaccharide of *Ganoderma lucidum* was highly branched and involved 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.* (1985) 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.*, 1985). The water-soluble polysaccharide consisted 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. The main water-insoluble polysaccharide, xylo-β-glucan (8.6%) has the high molecular weight of 2,000 kD, which might affect the low solubility of polysaccharide of *Ganoderma lucidum* in water. There are some possibilities that water-insoluble polysaccharides might be surrounded by hydrophobic polypeptide chain which

might interfere the water-extraction of polysaccharide from *Ganoderma lucidum*. Therefore, if the polypeptide chains attached to the water-insoluble polysaccharide of *Ganoderma lucidum* are cleaved by hydrolyzing enzyme, the polysaccharide part might be more easily contacted with water and converted to be water-soluble. We reported that a significantly increased amount of polysaccharide was isolated from *Ganoderma lucidum* or *Ganoderma lucidum* residue after hydrolyzing with chitinase (5.46 folds from *Ganoderma lucidum* and 3.76 folds from *Ganoderma lucidum* residue) (Park *et al*, 1996). In this study, we attempted to convert water-insoluble polysaccharide contained in *Ganoderma lucidum* to water-soluble one by hydrolyzing with chymotrypsin. We also attempted to hydrolyze water-insoluble polysaccharide contained in *Ganoderma lucidum* residue remaining after extracting water-soluble materials in *Ganoderma lucidum*.

MATERIALS AND METHODS

Materials

The dried fruit bodies of *Ganoderma lucidum* harvested in Kang Hwa-Do and Ganoderma lucidum residue were kindly provided by the IL-Yang Pharma. Co.. α-Chymotrypsin (90 units/mg) from bovine pancreas, was purchased from Boehringer Mannheim. Sepharose CL-4B, molecular weight marker (dextran : MW 2.00×10^6~3.91×10^4) and bovine serum albumin were purchased from Sigma Chem. Co.. All other reagents were analytical grade and commercially available.

Treatment of *Ganoderma lucidum* with chymotrypsin

The fruit bodies of *Ganoderma lucidum* were disintegrated into powder. Chymotrypsin (5~60 ppm) was added to the 1.0~6.0% of *Ganoderma lucidum* in buffer solutions (pH 3.0~11.0) and incubated at 30~50°C for 2 hours in shaking incubater (200 rpm). The buffer solutions used were 0.05 M citrate-phosphate (pH 3.0~6.0), 0.01 M sodium-phosphate (pH 7.0), 0.05 M boric acid-borax (pH 8.0 and 9.0) and 0.05 M glycine-sodium hydroxide (pH 10.0 and 11.0) solutions. The 2 ml of hydrolyzed samples were taken at intervals and each sample was boiled at 100°C for 10 min and 2 ml of 0.4 M trichloroacetic acid (TCA) was added to stop the enzyme action.

Measurement of total contents of polysaccharide and protein

Hydrolysates were boiled at 121°C for 2 hours and centrifuged to extract water-soluble components. The content of polysaccharides in the supernatant was measured by anthrone test and the content of protein in the supernatant was measured by Lowry-Folin method.

Extraction of polysaccharide from fruit bodies

The extraction and separation of fractions soluble in water and in sodium hydroxide solution was conducted as follows. The basidiocarps of *Ganoderma lucidum* were disintegrated and boiled in distilled water for 30 min. and filtrated. The extraction process was repeated three times. The filtrates were collected and concentrated in a vacuum and three volumes of 95% ethanol were added to the concentrate. The mixture was allowed to stand at 4°C overnight to complete precipitation. The precipitate was dissolved in distilled water and dialyzed (molecular weight cut=12,000) against distilled water at 4°C for seven days. The filtrate was concentrated and lyophilized. The residues remaining after extracting with water were extracted with 0.1 M sodium hydroxide solution at 105°C for 30 min three times. The filtrates were collected and concentrated in a vacuum and three volumes of 95% ethanol were added to the concentrate and the mixture was allowed to stand at 4°C overnight. The precipitate was obtained by decantation and dissolved in 0.1 M sodium hydroxide solution and dialyzed against distilled water at 4°C for seven days. The filtrate was concentrated and lyophilized.

Molecular weight determination of the protein-bound polysaccharides

The molecular weight was determined by gel-filtration chromatography on a Sepharose CL-4B (Pharmacia-LKB) equilibrated with 100 mM sodium phosphate buffer(pH 6.8). The standards for the calibration of the column (2.5×85 cm) were Blue dextran (Sigma Chem. Co., USA, MW=2×10^6), T-480 (Sigma Chem. Co., USA, MW=4.8×10^5), T-60 (Nakarai Chem. Ltd., Japan, MW=6×10^4). The preswollen Sepharose CL-4B was washed with two volumes of 0.01 M sodium phosphate buffer (pH 6.8) twice and packed into a column (2.5×85 cm) with the same buffer. The blue dextran (10 mg) was dissolved in the eluent and applied to the column and eluted with 0.01 M sodium phosphate buffer (pH 6.8) at the flow rate of 1 ml/min (5 ml/fraction). The absorbance of each fraction was measured at 625 nm (anthrone test). The molecular weights of polysaccharides were determined by elution volumes and logarithm values of molecular weights of standard dextrans.

RESULTS AND DISCUSSION

Reaction time-activity profile of chymotrypsin

The activity of chymotrypsin was defined by the content (% w/w) of water-soluble protein in the total

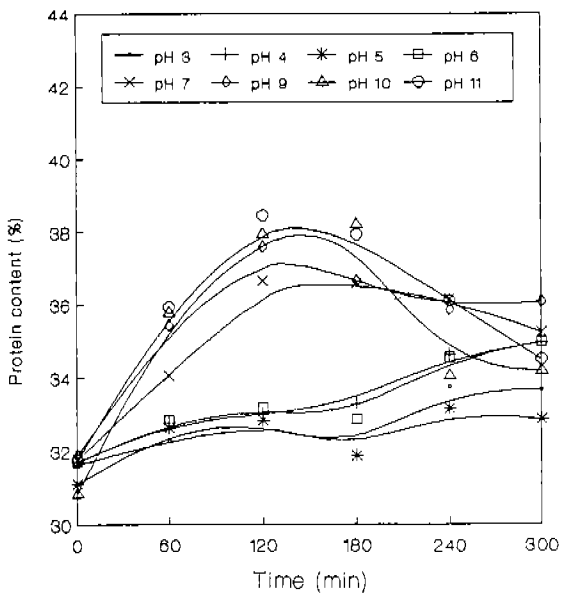

Fig. 1. Protein contents of *Ganoderma lucidum* as a function of time. *Ganoderma lucidum* was hydrolyzed with chymotrypsin (20 ppm) at 40°C at varied pH and protein content was measured.

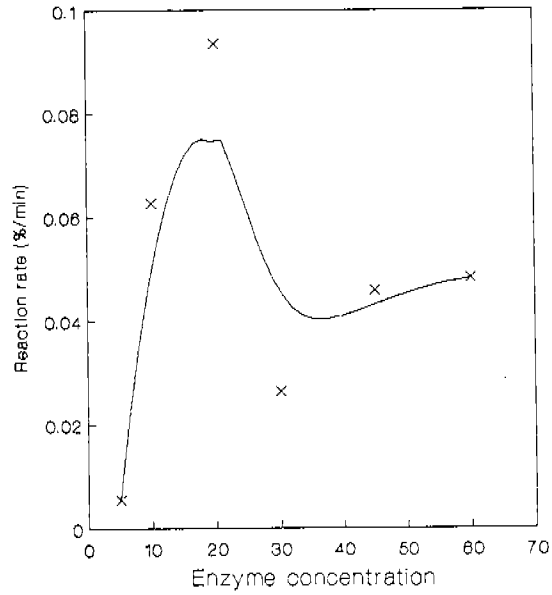

Fig. 2. Enzyme concentration-activity profile of chymotrypsin. *Ganoderma lucidum* was hydrolyzed with chymotrypsin at pH 10 at 40°C and protein content was measured.

protein of the sample. Fig. 1 shows the reaction time-activity profile of chymotrypsin for hydrolysis at different pH. At pH 3~6, the degree of hydrolysis was slightly increased as the hydrolyzing time was prolonged. But at pH 7~11, the degree of hydrolysis was significantly increased for 1~2 hours and afterwards decreased presumably due to inhibition of the hydrolyzing process by product.

Temperture and pH-activity profile of chymotrypsin

The effects of temperature and pH on the activity of chymotrypsin to hydrolyze polysaccharide were evaluated by relative values (V/V_{max} as a ratio of initial velocities (V) and maximum velocities of hydrolysis). The reaction temperature significantly influenced the hydrolysis, and the optimum temperature was 40°C. The hydrolyzing reactions significantly decreased below and over the optimum temperature presumably due to thermal unstability of chymotrypsin. The velocity of hydrolysis was highest at pH 10 and decreased gradually as the pH lowered to 3.

Enzyme concentration-activity profile of chymotrypsin

Fig.2 shows enzyme-activity profile of chymotrypsin at pH 10 at 40°C. There is no linear relationship between enzyme concentration and reaction rate and 20 ppm of chymotrypsin was chosen as optimum enzyme concentration to hydrolyze water-insoluble components contained in basidocarps of *Ganoderma lucidum* and *Ganoderma lucidum* residue.

Substrate concentration-activity profile of chymotrypsin

Fig.3 shows Lineweaver-Burk plot from the experimental data. The K_m value for the samples of *Ganoderma lucidum* was estimated to be 1.73%, while the value was 2.40% for the samples of *Ganoderma lucidum* residue. The similar K_m value of *Ganoderma lucidum* and *Ganoderma lucidum* residue implies that the process of extraction of the *Ganoderma lucidum* does not significantly alter the substrate binding affinity of the enzyme. The V_{max} value was estimated to be 0.073%/min for the samples of *Ganoderma lucidum* and 0.033%/min. for the samples of *Ganoderma lucidum* residue respectively.

Total contents of protein and carbohydrate

From the above results, hydrolysis was conducted under the following conditions; 20 ppm of chymotrypsin and 4% of *Ganoderma lucidum* or 6% of *Ganoderma luidum* residue at pH 10 at 40°C. The amounts of water-soluble protein extracted from *Ganoderma lucidum* was not increased by treating with chymotrypsin; 34.04% (without chymotrypsin treatment) and 34.40% (with chymotrypsin treatment). But the amount of water-soluble protein extracted from *Ganoderma lucidum* residue increased from 10.00% to 12.95% by treating with chymotrypsin. It is regarded that the hydrophobic domain of polypeptide chain of *Ganoderma luidum* residue may be changed to hydrolyze easily with chymotrypsin by extracting process of hot water-soluble components.

By treating with chymotrypsin, the amounts of water-soluble carbohydrate extracted from *Ganoderma lu-*

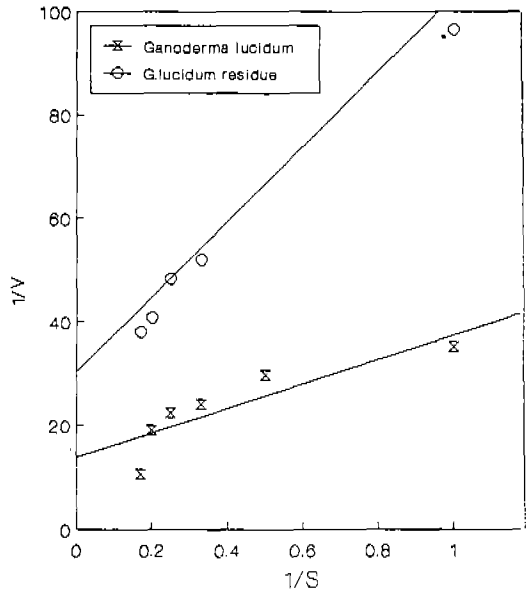

Fig. 3. Lineweaver-Burk plot of substrate concentration and initial velocity. *Ganoderma lucidum* or *Ganoderma lucidum* residue was hydrolyzed with 20 ppm of chymotrypsin at pH 10 at 40°C. The protein contents was measured as a function of hydrolyzing time and the initial velocities were calculated from the data respectively.

cidum increased from 17.00% to 29.60% (1.75 fold) and the amounts of water-soluble carbohydrate extracted from *Ganoderma lucidum* residue increased from 9.10% to 21.80% (2.40 fold). It is regarded that the domain of water-insoluble carbohydrate surrounded by hydrophobic polypeptide chain may be more exposed to water by cleaving hydrophobic polypeptide chain and converted to water-soluble one.

Contents of protein-bound polysaccharide

As shown in Table 1, 1.15 g of brown powder (water-soluble protein-bound polysaccharide) was extracted from the basidocarps (100 g) of *Ganoderma lucidum* and 3.07 g was extracted from *Ganoderma lucidum* (100 g) treated with chymotrypsin. But significantly increased amount (14.34g) of polysaccharides was extracted from *Ganoderma lucidum* residue after chymotrypsin treatment. It is assumed that water-insoluble polysaccharides surrounded by hydrophobic polypeptide chain may be more exposed to water by treating with chymotrypsin to cleave hydrophobic polypeptide chain. It is also assumed that *Ganoderma lucidum* residue contains more hydrophobic polysaccharides surrounded by hydrophobic polypeptides which can be hydrolyzed by chymotrypsin.

The amount of polysaccharide contained in *Ganoderma lucidum* and *Ganoderma lucidum* residue extracted with 0.1 M sodium hydroxide solution was not significantly changed by treating with chymotrypsin.

Table 1. Contents(g) of protein-bound polysaccharide in the basidocarps (100 g) of *Ganoderma lucidum* or *Ganoderma lucidum* residue

	Solvent	non-Hydrolyzed	Hydrolyzed[1]
G. lucidum	H_2O[2]	1.15	3.07
	NaOH[3]	1.43	1.99
G. lucidum residue	H_2O[2]	0	14.34
	$NaOH_3$	3.66	1.95

[1]Samples were hydrolyzed with chymotrypsin
[2]Samples were extracted with water
[3]Samples were extracted with 0.1M NaOH solution.

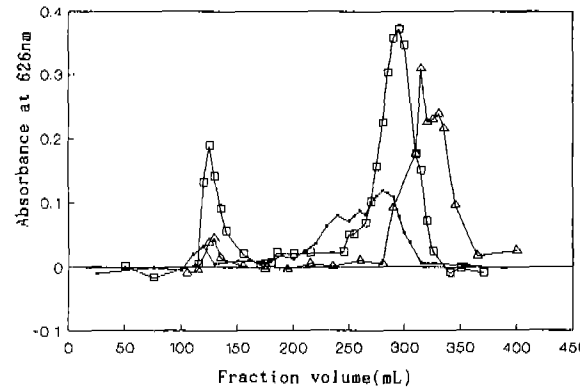

Fig. 4. Elution diagram of polysaccharide by Sepharose CL-4B gel filtration. Sample (5 mg) was dissolved in 0.01M sodium phosphate buffer solution (3 ml) and loaded.: □—□: *G. lucidum* extracted with distilled water, △—△: *G. lucidum* extracted with 0.1 M NaOH,●—● :*G. lucidum* residue extracted with 0.1 M NaOH.

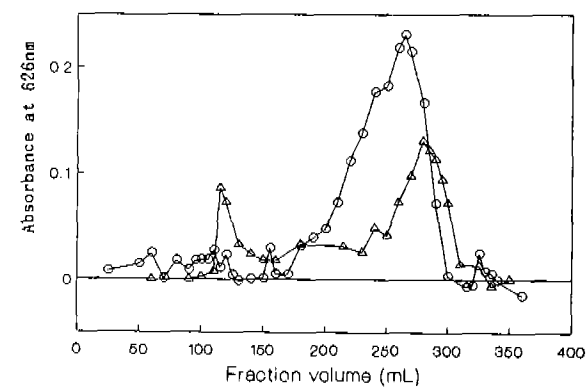

Fig. 5. Elution diagram of polysaccharide of *Ganoderma lucidum* hydrolyzed with chymotrypsin by Sepharose CL-4B gel filtration. Sample (4 mg) was dissolved in 0.01 M sodium phosphate buffer solution (3 ml) and loaded, △—△: extracted with distilled water, ○—○: extracted with 0.1 M NaOH.

Molecular weight determination of protein-bound polysaccharide

Molecular weights of protein-bound polysaccharides

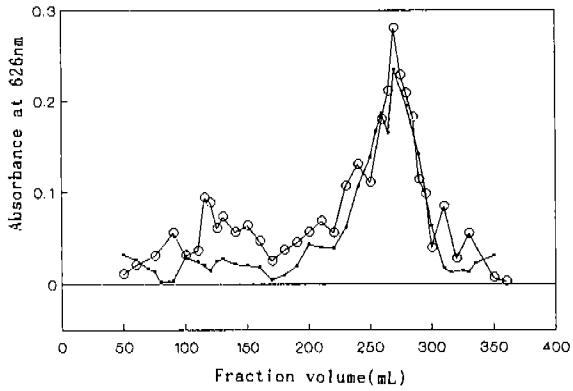

Fig. 6. Elution diagram of polysaccharide of *Ganoderma lucidum* residue hydrolyzed with chymotrypsin and extracted with distilled water by Sepharose CL-4B gel filtration. Sample (5 mg) was dissolved in 0.01 M NaOH (3 ml) and loaded, ○—○: extracted with distilled water, ●—●: extracted with 0.1 M NaOH.

Table 2. Molecular weights (kD) of protein-bound polysaccharide extracted from *Ganoderma lucidum* or *Ganoderma lucidum* residue

	Solvent	non-Hydrolyzed	Hydrolyzed[1]
G. lucidum	H_2O[2]	1,570, 25	2,290, 36
	NaOH[3]	1,570, 15, 10	740, 53, 10
G. lucidum residue	H_2O[2]	-	2,290, 46
	NaOH[3]	2,020, 60, 36	3,330, 60, 36

[1]Samples were hydrolyzed with chymotrypsin
[2]Samples were extracted with water
[3]Samples were extracted with 0.1 M NaOH solution.

were determined by Sepharose CL-4B gel filtration using blue dextran, T-480 and T-60 as standards. The elution patterns of each fraction from the basidiocarps of *Ganoderma lucidum* or *Ganoderma lucidum* residue extracted with water or NaOH solution are shown in Fig.4.

The elution patterns of polysaccharide of *Ganoderma lucidum* hydrolyzed with chymotrypsin and extracted with distilled water or with NaOH solution are shown in Fig.5. The elution patterns of polysaccharide of *Ganoderma lucidum* residue hydrolyzed with chymotrypsin and extracted with distilled water or with NaOH solution are also shown in Fig.6.

The molecular weights of each fraction are summarized in Table 2. The results are similar to the results (i.e. 2,000 kD and 30~70 kD) reported (Mizuno *et al.*, 1985). It was observed that higher molecular weigt (2,290 kD and 3,330 kD) of polysaccharide was isolated from the samples treated with chymotrypsin. It was also observed that similar polysaccharide (MW 46 kD) exerted antitumor activity was found in the sample of *Ganoderma lucidum* residue treated with chymotrypsin and extracted with water. It is regarded that the polysaccharide parts of high molecular weights and exerting antitumor activity were exposed to be easily dissolved in water by chymotrypsin treatment. It was also observed that the high molecular weight (1,570 kD) of polysaccharides contained in *Ganoderma lucidum* was disappeared after hydrolyzing with chymotrypsin and it is presumed that the polypeptide bond of protein-bound polysaccharide may be hydrolyzed by chymotrypsin and cleaved.

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