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Solubilization of water-insoluble β-glucan isolated from *Ganoderma lucidum*

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Abstract: *The fungal β-D-glucan is a biological response modifier (BRM), but a major obstacle to the clinical utilization of β-glucan BRMs is their relative lack of solubility in aqueous media. Water insoluble fungal glucans extracted by alkali from the mycelia of Ganoderma lucidum were sulfated to yield their corresponding water-soluble derivatives. Insoluble glucan is dissolved in methyl sulfoxide and urea, and is partially sulfated with sulfuric acid. The sulfated glucan (SGL) yield prepared from insoluble glucan (IGL) was 85%, the sulfation degree of SGL was about 14.9%, and the solubility of SGL was above 95% in water. The monosugar SGL content was 34.9% α-glucose and 35.9% β-glucose. The mean molecular weight (MW) of SGL was shown as a single peak on Sepharose CL-4B column chromatography, and their MW was approximately 9.3 kDa. The ^{13}C NMR spectrum analysis shows that SGL has a high similarity with the β-(1→3)-linked triple-helical control.*

Key words: *β-Glucan, Sulfated glucan, Biological response modifier, Ganoderma lucidum*
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Introduction

Ganoderma lucidum (Fr.) Karst, a popular medicinal mushroom, has been used in traditional medicines in many Asian countries. The chemical constituents of *G. lucidum* include polysaccharides, proteins, nucleosides, fatty acids, sterols, cerebrosides and triterpens (Yeung *et al*., 2004). *Ganoderma* polysaccharides belong to the class of drugs known as biological response modifiers (BRMs). Special attention has been paid to this fungal polysaccharide as a functional food and a source for the development of biomedical drugs. Polysaccharides represent a structurally diverse class of biological macromolecules with a wide range of physicochemical properties. The major bioactive *Ganoderma* polysaccharides are β-(1→3) and β-(1→6)-D glucan (Paterson, 2006), which have been shown to slow the growth of sarcoma cells growing in mice (Cao and Lin, 2004). A major obstacle to the clinical utilization of β-glucan BRMs is their relative lack of solubility in aqueous media. Specifically, (1→3)-β-D-glucan exists as an insoluble microparticulate upon initial isolation from *G. lucidum*. While topical administration of insoluble microparticulate (1→3)-β-D-glucan induces no toxicity, intravenous administration of the microparticulate form is associated with hepatosplenomegaly, granuloma formation, microembolization, and enhanced endotoxin sensitivity. Williams *et al*. (1991, 1992) have demonstrated that insoluble (1→3)-β-glucan from yeast, *Saccharomyces cerevisiae* is water-soluble and immunologically active. Other studies have shown that several partially synthesized sulfated polysaccharides, including dextran sulfate, are highly inhibitory to the *in vitro* replication of human immunodeficiency virus (HIV) (Yoshida *et al*., 1990).

Although several investigations have been reported on optimal culture conditions and medicinal properties of *G. lucidum*, preparation of soluble β-glucan from this mushroom have not been studied in detail to utilize as BRM. One goal of this study is to develop a water-soluble polysaccharide based on the introduction of a sulfated group in enhancing the immuno-modulating activity of mushroom polysaccharide. Thus far, the chemical structure of the sulfated β-glucan derivatives from *G. lucidum* have been never published. Therefore, the mass production of insolubleβ-glucan was isolated from *G. lucidum* mycelia by means of fermentation. To increase the solubility, water-insoluble β-glucan was sulfated, and physicochemical characterization was then discussed.

Materials and Methods

Organism and growth: *G. licudum* was grown on potato dextrose broth (Han *et al*., 1995; Demir, 2004) in shake cultures in 500 ml flasks containing 100 ml medium (pH 4.5, 27°C, 120 rpm, 6 days). Mycelia were harvested by centrifugation, washed in deionized water, and freeze-dried by a lyophilizer. The strain of *G. lucidum* was preserved on the potato dextrose agar. It was inoculated in a liquid medium containing 15% glucose, 1% peptone, 1% yeast extract, 0.05% KH_2PO_4, 0.05% K_2HPO_4 and 0.05% $MgSO_4 \cdot 7H_2O$.

Polysaccharide production in a bioreactor: Cultivation was carried out in a 5 l air-lift and stirred-tank fermenter (Kobiotech, KL-5 l, Korea) with a 3 l working volume. The medium contained (gl^{-1}) glucose 20, yeast extract 2, K_2HPO_4 5, and $MgSO_4 \cdot 7H_2O$ 0.01, at pH 4.5. The experiment was conducted at an air flow rate of approximately 1 vvm and 300 rpm at 27°C (Han *et al*., 1999).

Table - 1: Solubility properties of sulfated glucan (SGL) prepared from insoluble glucan (IGL)

Characteristics \ Glucan	Insoluble β-glucan	SGL
Color	Dark brown	Yellowish brown
Yield (%)	-	85
Solubility (%)	Below 5	Above 95

Table - 2: Total carbohydrate contents of insoluble β-glucan and water soluble sulfated β-glucan

Samples	Insoluble β-glucan	Sulfated β-glucan
Total carbohydrate	72.4 ± 2.9	84.2 ± 0.05

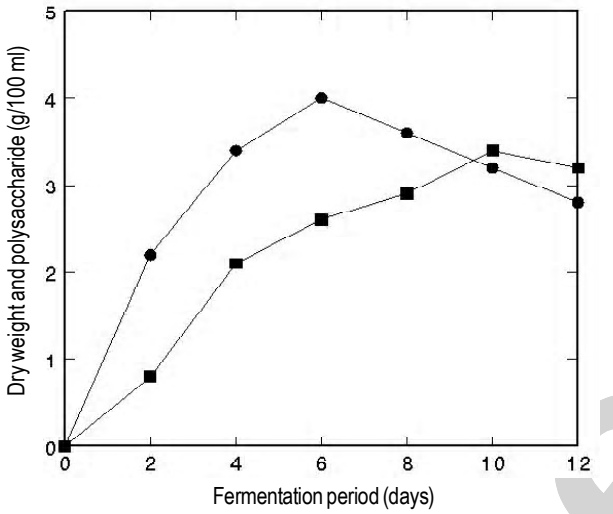

Fig. 1: Mycelium dry weight and production of polysaccharide in the fermenter. Mycelium dry weight (●), polysaccharide content (■)

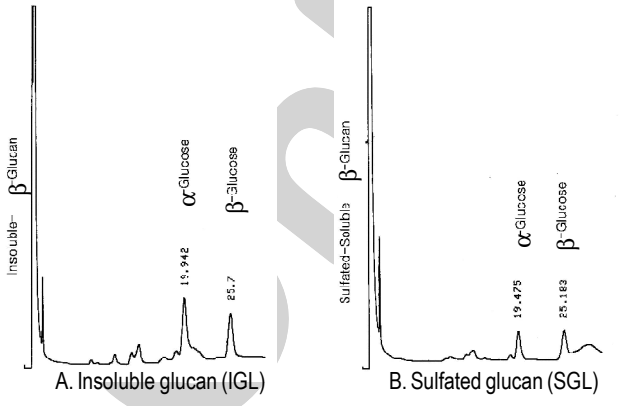

Fig. 2: GLC pattern and monosaccharide composition of the water-insoluble glucan and sulfated soluble glucan (SGL) obtained from *G. lucidum*

Insoluble β-glucan preparation: Particulate β-glucan was prepared from the cultured mycelia of *G. lucidum* by a modification of the method of Han *et al.* (1999). The dried β-glucan powder was prepared sequentially by using the alkali extraction and ethyl alcohol precipitation method. The cultured mycelia were immersed in 2 N NaOH solution at room temperature. The mixture was stirred overnight and was then neutralized with acetic acid and centrifuged at 6,000 x g for 20 min in order to obtain a supernatant. The resulting polysaccharide was precipitated by the addition of 3 volumes of absolute ethyl alcohol, and recovered by centrifugation at 6,000 x g for 20 min to remove the supernatant. It was dissolved in a minimum volume of distilled water and then dialyzed with a regenerated cellulose tube (MW cut-off 10,000, Sigma, USA) against distilled water for 5 days. The polysaccharide was finally dried with a lyophilizer (Il-Shin Inc, Korea) to yield a purified insoluble β-glucan sample.

Preparation of sulfated β-glucan: Soluble sulfated β-glucan was prepared by a modification of the method of Williams *et al.* (1992). Water-insoluble glucan (4 g) was dissolved in 100 ml of Me_2SO containing 72 g of urea (8 M). In a separate flask, Me_2SO (100 ml) and concentrated H_2SO_4 (10 ml) were thoroughly mixed, and then added drop-wise to the glucan-Me_2SO-urea solution with stirring. The solution was heated to 100°C in a water bath with stirring, and the reaction was carried out for 4 hr. The solution was cooled to ambient temperature and diluted in 4 l of ultrapure deionized water obtained from a water purification system (Millipore, Bedford, MA). The glucan sulfate solution was passed through a Millipore prefilter (1.2 µl) to remove unreacted microparticulate glucan. The glucan sulfate solution was purified with an ultrafiltration system (Satorius. Co. SM 17521), using a 10,000 MW cut-off filter. The solution was dissolved with 100 l of ultra-pure deionized water, concentrated to 500 ml, frozen in the bottle and lyophilized to dryness (Il-Shin Inc, Korea). The sulfated polysaccharide was coded as SGL. The carbohydrate content of SGL was determined by the phenol-sulfuric method (Chaplin and Kennedy, 1994).

Gas chromatography for sugar content of SGL: Sugar composition was analyzed by gas chromatography (Chaplin and Kennedy, 1994). Briefly, sugars were separated on a stainless steel column packed with 3% OV-17 [(80-100 mesh Shimalite, 3 mm (D) x 3 m (L) boronsilicate glass column)] in Shimadzu GL 9A fitted with an FID (flame-ionized detector). The carrier gas was He and the column temperatures ranged within a 150-180°C gradient. The injector and detector temperatures were 240°C. SGL was hydrolyzed by 0.1 N HCl at 100°C for 5 hr. Monosaccharide residues were precipitated with ethyl alcohol and removed solvents with a vacuum evaporator, and then into trimethylsilation reagent [(pyridine (10 vol.) with hexamethydisilazane (2 vol.) and trimethychlorosilane (1 vol.)] and examined by GC. Sugar contents of each sample were analyzed and represented by the mean and standard deviation resulting from three independent experiments.

FT-IR of sample: Infrared spectra (IR) of the insoluble β-glucan (IGL) and sulfated-β-D-glucan (SGL) samples were recorded with

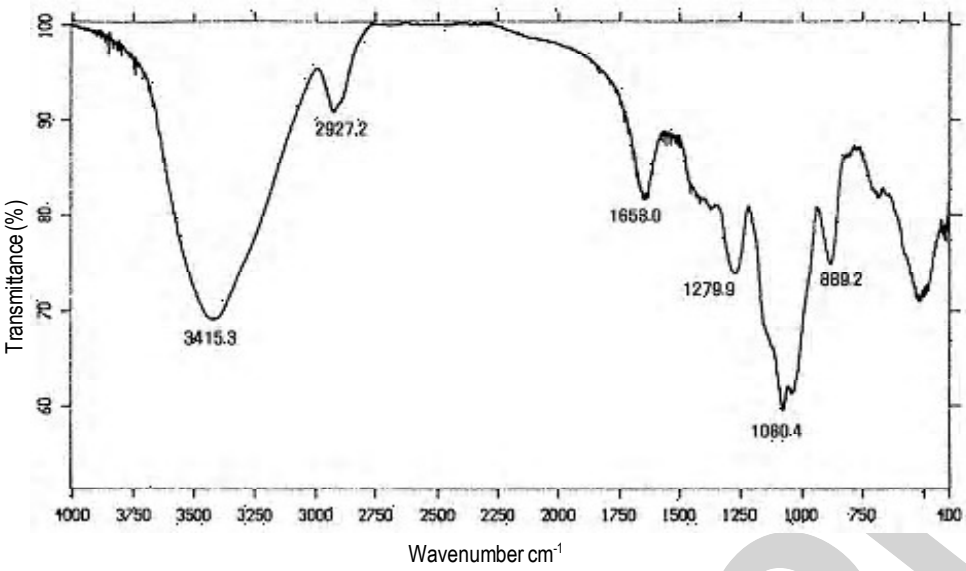

Fig. 3: IR spectrum of the sulfated soluble glucan (SGL) obtained from the mycelia of *G. lucidum*

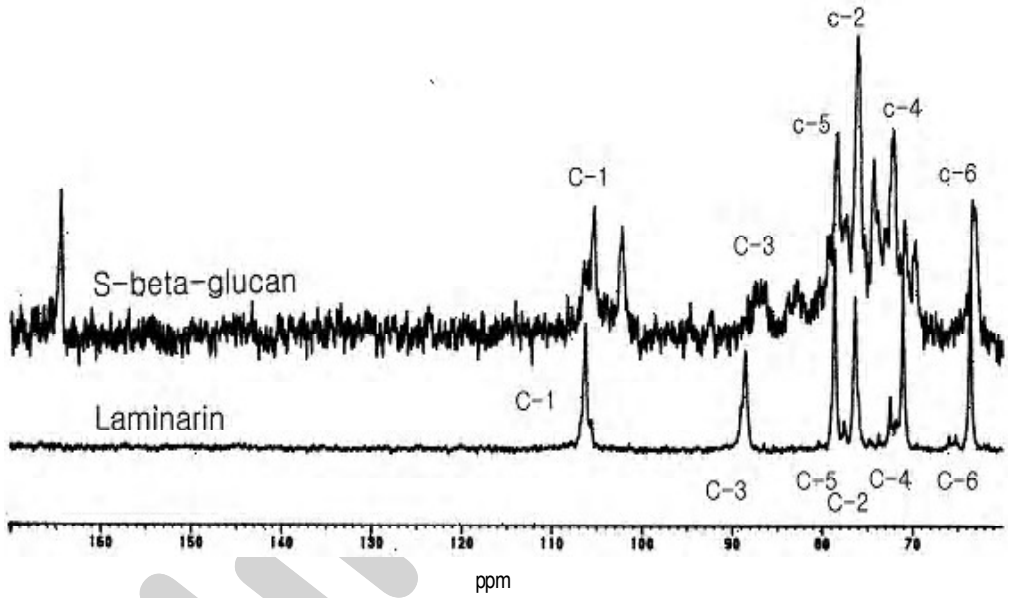

Fig. 4: ^{13}C NMR pattern of the sulfated soluble glucan (SGL) obtained from the *G. lucidum* Control β-glucan; laminarin (from *Laminaria digitata,* Sigma, USA)

an FT-IR spectrometer (Bruker, IFS-48, Billerica, MA) in the range of 4000~400 cm^{-1} using the KBr-disk method.

^{13}C NMR of sample: To investigate the type of interchain linkage and to elucidate the polymer backbone, IGL and SGL samples were dissolved in D_2O and analyzed by ^{13}C NMR spectroscopy (Zhang *et al*., 2001; Tao *et al*., 2006). Analyses were performed on a Bruker 200-MHz spectrometer (Bruker, AM-200, Billerica, MA) operating in the pulsed Fourier transform mode. Broadband, proton-decoupled spectra were obtained for using D_2O as a solvent. The spectral width was 200 ppm. The samples were dissolved in D_2O to obtain a concentration of 30 mg ml^{-1}.

Molecular weight of samples: The molecular weights of IGL and SGL samples were estimated using Sepharose CL-4B column gel chromatography. The gel-filtration profile of the insoluble glucan (IGL) and sulfated glucan (SGL) was analyzed in 0.3N NaOH using the following system: Sepharose CL-4B column 16 mm (D) × 530 mm (L). The column was calibrated using standard dextran (2000, 124, 70 and 9.3 kDa) under the same conditions. The sample IGL and SGL (10 mg) were dissolved in 0.3 N NaOH, and were then injected onto a column of Sepharose CL-4B equilibrated with 0.3 N NaOH solution. After loading with sample, the column was eluted with 0.3 N NaOH at 6 ml hr^{-1}. The volume of each fraction was 2 ml per tube.

Table - 3: The sugar compositions of SGL and IGL from *G. lucidum* mycelium. Mannan and Laminarin; control polysacchride purchased from Sigma

Polysaccharide	Sugar component (%)				
	Fructose	Mannose	Galactose	α-glucose	β-glucose
SGL	6.4 ± 1.2	17.9 ± 3.4	4.8 ± 0.3	34.9 ± 1.8	35.9 ± 2.1
IGL	4.3 ± 0.2	4.24 ± 0.3	9.4 ± 0.4	44.8 ± 1.3	23.8 ± 1.9
Mannan	9.7 ± 0.9	88.7 ± 3.9	-	1.6 ± 0.2	-
Laminarin	2.2 ± 0.4	-	8.1 ± 0.3	42.7 ± 1.4	47.0 ± 2.0

* Mannan and Laminarin; control polysacchride purchased from Sigma company

Table - 4: ^{13}C-N.M.R chemical shifts of the SGL and laminarin. Control β-glucan; laminarin (from *Laminaria digitata*, Sigma, USA)

Compound	C-1	C-2	C-3	C-4	C-5	C-6
SGL	105.2	76.0	87.2	72.2	78.0	63.5
Laminarin	106.2	76.5	88.5	71.5	78.8	63.3

* Control β-glucan; laminarin (from *Laminaria digitata*, Sigma, USA)

Table 5: Sulfate contents of insoluble β-glucan and sulfated soluble β-glucan (SGL)

Glucan / Sulfate group	Insoluble β-glucan	Sulfated β-glucan
Sulfate (%)	0.2	14.9
Molar ratio : SO_3^- / sugar	SO_3^- (0.2%) / Sugar (72.4%): One SO_3^- per 815 sugar residues	SO_3^- (14.9%) / Sugar (84.2%): One SO_3^- per 12.8 sugar residues
Molar ratio : SO_4 / sugar	SO_4 (0.2%) / Sugar (72.4%): One SO_4 per 680 sugar residues	SO_4 (14.9%) / Sugar (84.2%): One SO_4 per 10.8 sugar residues

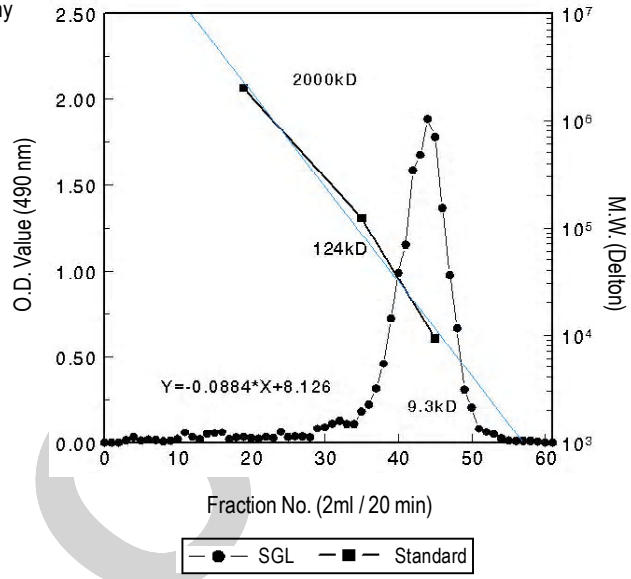

Fig. 5: Gel-filtration profile of the SGL on a Sepharose CL-4B column. The volume of each fraction was 2 ml. The elutes were checked by measuring absorbance at 490 nm after a phenol-sulfuric acid reaction for carbohydrates. Closed circle (●), Sulfated β-glucan (SGL); Closed square (■), standard dextran (MW)

The elutes were checked by measuring absorbance at 490 nm wavelength after the phenol-sulfuric acid reaction for carbohydrates (Dubois *et al*., 1956).

Determination of the content of the introduced sulfate group: The content of the sulfate group was evaluated as the amount of sulfur (%) in the sample as outlined by Chaplin and Kennedy (1994). Ten mg of sulfated β-glucan was dissolved in distilled water, and the solution was adjusted to 60% (w/v) by the addition of 90% (w/v) formic acid. The sample solution was hydrolyzed for 8 hr at 100°C in tightly sealed tubes, and dried over KOH pellets in a desiccator maintained under vacuum conditions. The dried hydrolysate was resuspended in distilled water (0.5 ml). Standard solution was made of 1.813 g K_2SO_4 in 100 ml distilled water (final volume 100 ml); this solution was used as a standard and contains 10 mg SO_4^{2-} per ml. The hydrolysate or standard solution (0.2 ml) was mixed with 3.8 ml of reagent A (4% TCA in distilled water) and 1 ml of reagent B (gelatin/$BaCl_2$ reagent; 2.0 g of gelatin in 400 ml D.W. and 20 g of added $BaCl_2$). The mixture was allowed to stand for 15 min, and the opacity of the solution was then read at 500 nm. The value obtained was compared with a standard curve using K_2SO_4 (standard solution). The assay was linear between 20 and 200 µg of SO_4^{2-}.

Results and Discussion

Mycelial growth and production of polysaccharide: The maximum yields of polysaccharides and mycelial dry biomass were obtained on the day 10 (3.3 gl^{-1}) and day 6 (4.0 gl^{-1}), respectively, in the 5 l jar fermenter (Fig. 1). Mycelial polysaccharide accumulated on the cell surface during logarithmic growth phase, and was subsequently increased in the stationary phase.

Sulfation of insoluble glucan and its solubility: In terms of the commercial importance of polysaccharides, water-insoluble polysaccharides show little bioactivity, whereas glucan derivatives such as dextran sulfate, lentinan sulfate, and pullulan sulfate exhibit high anti-HIV activities and low anticoagulant activities (Wang *et al*., 2005). However, the aqueous solubility of the glucan was as poor as that of glucan prepared by other processes, meaning that it was less suitable for pharmaceutical applications. The abundance of -OH groups in glucan facilitates the formation of hydrogen bonds, causing the natural glucan to exist as a compact triple-stranded helix, which in turn contributes to poor aqueous solubility of glucan. In order to

estimate the water solubility after the sulfation, the solubility of the sulfated glucan in water solution was measured. In distilled water, the solubility of 1% insoluble glucan was below 5%, but that of its sulfated β-glucan (1%) was above 95%. Table 1 summarizes the solubility and yields of the insoluble and sulfated soluble glucan. Furthermore, the sulfated glucan samples were readily dissolved without heating, and were yellow-brown in color. It is worth noting that introduction of only a small proportion of sulfate groups changes the water solubility drastically.

Sugar contents: Total carbohydrates of SGL and IGL are listed in Table 2. SGL contents were 84.2% carbohydrates. The sugar composition of the sulfated-α-glucan SGL included 34.9% β-glucose and 35.5% β-glucose, together with mannose, fructose, and galactose. For IGL, α-glucose was the major sugar, followed by β-glucose. Control β-glucan polysaccharide, Laminarin (Sigma), was composed of 42.7% α-glucose and 47% β-glucose (Fig. 2 and Table 3).

IR spectrum: The infrared (IR) spectra of the sulfated β-D-glucan (SGL) samples were recorded with an FT-IR spectrometer (Bruker, IFS-48, Billerica, MA) in the range 4000~400 cm^{-1} using the KBr-disk method. The IR spectrum of SGL is shown in Fig. 3. The typical signal pattern expected for a carbohydrate moiety and several bands in the anomeric region were present. SGL exhibited the typical absorption peaks at 889.2 cm^{-1}, characteristic for β-D-glucan. Obvious characteristic peaks were observed at both 850 and 920 cm^{-1} for α-D-glucan and at 890 cm^{-1} for β-D-glucan. SGL exhibited the main absorption peak at 890 cm^{-1} for the β-configuration of β-D-glucan (Mathlouthi and Koenig, 1986).

^{13}C-NMR analysis: To investigate the type of interchain linkage and to elucidate the polymer backbone, SGL samples were dissolved in D_2O and analyzed by ^{13}C NMR spectroscopy. Fig. 4 shows the ^{13}C-NMR spectra of SGL and laminarin (*Laminaria digitata*, Sigma, USA) in 30 mg/ml of D_2O solvent at 60°C. The six strong signals at 105.2, 76.0, 87.2, 72.2, 78.0, and 63.5 ppm in the SGL spectrum were assigned to the C-1, C-3, C-5, C-2, C-4, and C-6 of SGL (sulfated β-(1→3)-D-glucan). Comparison of the chemical shifts of insoluble glucan and SGL with laminarin confirmed the β-(1→3)-assignment (Table 4). The ^{13}C NMR spectra of SGL in D_2O at 60°C are shown to be similar to the results of other studies on sulfated β-glucan derivatives obtained from *Ganoderma tsugae* (Chen *et al.,* 1998) and *Poria cocos* (Huang and Zhang, 2005). Interestingly, the other signals with lower intensity were also observed in the spectrum of SGL, which may be assigned to sulfate residues. Therefore, the SGL polysaccharides have different structures from that of the corresponding laminarin, a water-insoluble linear (1→3)-β-D-glucan from *Laminaria digitata* (Bao *et al*., 2001).

Molecular weight of SGL: To determine the molecular weights, SGL (10 mg) was dissolved in 0.3 N NaOH, and was then injected to a column of Sepharose CL-4B equilibrated with 0.3N NaOH (Dubois *et al*., 1956). After the sample was loaded, the column was eluted with 0.3 N NaOH at 6 ml/hr. The volume of each fraction was 2 ml per tube. The total carbohydrate content of elutes were determined by measuring absorbance at 490 nm after reacting with phenol sulfuric acid. As shown in Fig. 5, the mean values of molecular weight of SGL samples was 9.3 kDa.

Determination of the content of the introduced sulfate group: The solubility of polymers always depends on their degree of polymerization, degree of branching, and chemical derivation (Wang *et al*., 2005). Glucan depolymerization by acidic or alkali hydrolysis, enzymatic degradation, and supersonic treatment, along with sulfation and phosphation (Williams *et al*., 1992), has been developed to enhance glucan solubility in aqueous solution. Among them, sulfation is a preferred method due to its positive impacts on their biological functions. Sulfate contents of SGL prepared from IGL are shown in Table 5. The sulfate content of nonsulfated-insoluble β-glucan (IGL) was 0.2%, but after sulfation (sulfated β-glucan: SGL) it was increased to14.9%. The molar ratio (SO_3^- per sugar(%)) of insoluble β-glucan has one SO_3^- per 815 sugar residues and SGL has one SO_3^- per 12.8 sugar residue. In terms of the molar ratio of SO_4 per sugar, insoluble β-glucan has one SO_4 per 680 sugar residues, and SGL has one SO_4 per 10.8 sugar residues.

In conclusion, a water-insoluble β-glucan was isolated from mushroom *G. lucidum* mycelia cultivated using a pilot scale fermenter by means of submerged fermentation. The water-insoluble β-glucans were reacted with Me_2SO and sulfuric acid to obtain water-soluble sulfated β-glucan (SGL). The results from physiochemical properties indicated that SGL increases to 98% solubility compared to water-insoluble β-glucan. In our study, the water-soluble sulfated β-glucan derivatives showed conformational similarity with native β-glucan, except for sulfate groups. This suggests that the mushroom immunostimulators, in the form of water-soluble β-glucan derivatives, will be obtained from mass production via fermentation and the solubility was increased through the sulfation process without the loss of its chemical backbone.

Acknowledgments

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