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Structural and Biological Characterization of Sulfated-Derivatized Oat β-Glucan

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The structural, physicochemical, and biological properties of sulfated oat β-glucan were characterized. The degree of substitution of the sulfated oat-β glucan was obtained by elemental analysis, which was 0.68. Compared to native oat β-glucan, the FT-IR spectra of the derivative showed two new absorption bands at 1250 and 810 cm^{-1}, which would be attributed to (S═O) and (C−O−S) groups, respectively. The molecular weight of the sulfated β-glucan was determined to be 68 kDa and its viscosity decreased by almost 2 orders of magnitude while its solubility increased by more than 100% compared to that of the native β-glucan. In addition, the sulfation caused the reduction of in vitro bile acid binding capacity of oat β-glucan due to the new anionic character and decreased molecular weight. The sulfated derivative exhibited, however, anticoagulant activity which showed a concentration-dependent increase.

KEYWORDS: Oat β-glucan; sulfation; bile acid; anticoagulant activity

INTRODUCTION

As one of the naturally occurring polysaccharides, β-glucan, found mainly in the cell walls of barley and oats, is unbranched polymers composed of (1-3)- and (1-4)-β-D-glucose units with the (1-4) β-linkage predominating. It is well-recognized that it reduces the risk of heart-related disease by lowering cholesterol and reducing glycemic index (*1*, *2*). Due to the beneficial health effects, its importance as a dietary fiber has been growing rapidly with increasing popularity of nutraceuticals and functional foods. Consequently, it gives rise to a dramatic increase in the demand for β-glucan enriched products in the current market. It is also allowed by the FDA to make a heart-healthy claim on the label of the products that provide at least 0.75 g of β-glucan per serving (*3*, *4*).

Furthermore, there have been great efforts in the chemical modification of β-glucan because of commercial potentials as well as scientific interests. Specially, sulfation of β-glucan has been receiving continuous attention since the sulfate groups are shown to play an important role in a variety of biological activities. It is reported in the literature that sulfated polysaccharides have diverse physiological functions such as anticoagulant, antitumor, and anti-HIV infection activities (*5*, *6*). Likewise, various attempts have been previously made to improve or develop new biological activities of β-glucan by sulfation. (1-3)-β-Glucans isolated from the sclerotia of *Poria cocos* sclerotium and *Pleurotus tuber-regium* were sulfated and their antitumor and antiviral activities were investigated, respectively (*5*, *7*). Moreover, the sulfate derivatives of (1-3)-β-glucan from the soil bacterium *Alcaligenes faecalis* var. *myxogene* (*8*, *9*) and (1-6)-β-glucan from the lichenized fungus *Parmotrema mantiqueirense* Hale (*10*) exhibited anticoagulant and antithrombotic effects. However, the effect of sulfation on β-glucan from cereal sources has not been reported yet, to the best of our knowledge.

In this study, β-glucan was extracted from oats and its sulfated derivative was prepared. The changes in the solubility, molecular weight, and flow behavior of the sulfated oat β-glucan were then investigated. Moreover, its in vitro bile acid binding and anticoagulant activities were evaluated.

MATERIALS AND METHODS

Extraction of β-Glucans from Oats. β-Glucan from oats was extracted and purified as previously described by Shin et al. (*11*). Oats (*Avena sativa L.*) were provided by Bolak Co. Ltd. (Kyungki-do, Korea) and ground to pass through a 50-mesh sieve in a miller (Jinyoung, Korea). The oat powder was then suspended in distilled water (10%, pH 10.0) and kept at room temperature for 20 h, followed by the adjustment of its pH to 6.0. α-Amylase (0.5 mL, Termamyl 120 L, NOVO, Denmark) was added to the suspension, which was maintained at 95 °C for 2 h. After the pH was re-adjusted to 4.5, it was followed by the addition of amyloglucosidase (200 μL, AMG 300 L, NOVO, Denmark) over 4 h at 60 °C. The reaction mixture was heated to 100 °C for the enzyme inactivation and centrifuged at 1500*g* for 5 min. β-Glucan was precipitated by mixing the supernatant with three volumes of ethanol (95% v/v) followed by an overnight storage at room temperature. After centrifugation (1500*g*, 5 min), the precipitate was

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freeze-dried. This procedure was repeated again in order to increase the purity of β-glucan, which was determined to be 98%, based on the method of McCleary and Codd (*12*).

Sulfation of Oat β-Glucan. According to a previous study (*13*), β-glucan (15 g) was treated with formamide (150 mL) and chlorosulfonic acid (100 mL) at 80−90 °C for 4 h, followed by the addition of propylene oxide (500 mL). The precipitate was resuspended in distilled water and its pH was adjusted to 10.0−11.0. After the resulting samples were dialyzed against distilled water for 24 h, they were freeze-dried, producing sulfated β-glucan.

Structural Characterization. To investigate the structure of the sulfated β-glucan, powder samples were obtained by ball milling and mixing with KBr (1:20) to make pellets. Then, their FT-IR spectra were recorded with a FT-IR spectrometer (MAGNA-IR 760 E.S.P, Nicolet Instrument Corp., USA).

The elemental composition in each sample was investigated using an elemental analyzer (EA1110, CE Instrument, Italy).

Molecular Weight, Viscosity, and Solubility Measurements. The molecular weight of the β-glucan samples was determined by gel permeation chromatography (LC-900, Japan Analytical Industry Co., Ltd., Japan) connected with a refractive index detector (RI-50, Japan Analytical Instrument, Japan). Samples were injected into gel permeation columns in series (JAIGEL-W254, JAIGEL-W-253, JAIGEL-W252, Japan Analytical Instrument, Japan), which have exclusion limits of 2×10^6, 4×10^5, and 5×10^4 Da, respectively. The eluent was distilled water with a flow rate of 3.5 mL/min. Samples were filtered by a 0.45 μm membrane filter (MFS, Inc., Japan) and the injection volume was 3 mL.

For measuring the flow behaviors of native and sulfated β-glucans, the samples were suspended in distilled water at a concentration of 1%, heated at 90 °C for 15 min, and cooled at room temperature. The shear stress of each suspension was then investigated as a function of shear rate ($0-500\ s^{-1}$) using a controlled-strain rheometer (Rheostress RS1, Thermo Hakke, Germany). Parallel plates with 35 mm diameter were used and the gap was 1 mm. Measurements were made at 10 and 30 °C and the curves reported were mean values of two measurements.

The solubility of the β-glucan derivative was investigated by the method of Chang and Cho (*14*). The sample (3 g) was suspended in distilled water (5 mL) and the slurry was placed at room temperature for 24 h with stirring. After centrifugation at 1600*g* for 15 min, the supernatant (2 mL) was treated with 6 mL of ethanol and centrifuged at 3500*g* for 15 min. The solubility was then obtained from the weight of precipitate, which was dried in a vacuum oven (60 °C).

In Vitro Bile Acid Binding Capacity. Based on the method described previously (*11*, *15*), the effect of sulfation on the in vitro bile acid capacity of β-glucan was investigated. The sample (2.5 mg/mL) in sodium phosphate buffer (0.01 M, pH 7.0) containing bile acid (250 μM) was prepared and kept at 37 °C for 2 h with stirring, followed by filtration by a 0.2 μm syringe. The collected solution (0.2 mL) was mixed with 70% sulfuric acid (1 mL) for 5 min and 0.2 mL of 0.25% furfural was then added. After 1 h, the absorbance of the resulting solution was measured at 510 nm. The bile acid binding capacity of the samples was expressed relative to cholestyraminem which has been clinically used for cholesterol reduction in blood (*16*).

Anticoagulant Activity. The blood obtained from Sprague−Dawley rats (Samtako, Korea) was added to 3.13% sodium oxalate as a ratio of 10:1 and then centrifuged at 1600*g* for 25 min to produce platelet-poor plasma. The sample (50 μL) dissolved in saline at different concentrations was mixed with the plasma and then incubated at 37 °C for 3 min, followed by the addition of 5 mM $CaCl_2$ (100 μL). The clotting time when a fibrin clot appeared was then recorded and compared with that of heparin (Choongwae Pharma Co., Korea) as a reference. All assays were performed in triplicate.

RESULTS AND DISCUSSION

The sulfation of oat β-glucan was confirmed by FT-IR and elemental analysis. **Figure 1** presents the FT-IR spectra of native and sulfated β-glucans in the 400−4000 cm^{-1} region. The native β-glucan exhibited two strong absorption bands at 3447 and 1050 cm^{-1}, which would be associated with O−H and C−O bonding, respectively. It is interesting to note that several new bands in the region of 750−1500 cm^{-1} were observed in the sulfated β-glucan. The appearance of absorption bands at 1250 cm^{-1} would come from the vibrations of (S═O) groups. In addition, C−O−S bond would give rise to a FT-IR band at 810 cm^{-1} (*13*), indicating that the sulfation of oat β-glucan took place successfully.

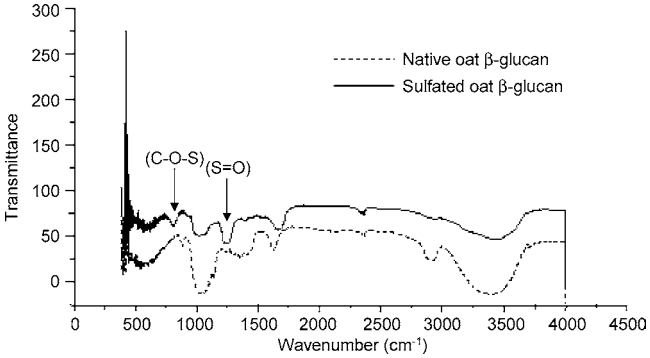

Figure 1. FT-IR spectra of native and sulfated oat β-glucans.

Table 1. Molecular Weight and Water Solubility of Native and Sulfated Oat β-Glucans

	Mw (kDa)	solubility (%)
native oat β-glucan	130	20.3
sulfated oat β-glucan	68	42.3

The degree of substitution (DS) of the sulfated derivative was estimated based on sulfur content by elemental analysis. The DS indicates the average number of sulfate groups attached to a glucose unit and was calculated by the following formula (*7*, *17*):

$$DS = \frac{162 \times \frac{S\%}{32}}{100 - \left(\frac{80}{32} \times S\%\right)}$$

The sulfate content obtained from elemental analysis was 10.02% and the DS was calculated to be 0.68. Therefore, the presence of sulfur groups in the β-glucan derivative was also confirmed by elemental analysis.

The molecular weight of the derivative was examined and compared with that of native β-glucan (**Table 1**). The sulfated oat derivative exhibited an average molecular weight of 68 kDa, which was much lower than that of native β-glucan. It indicates that a pronounced degradation took place during sulfation. Similar results have also been reported in several sulfated polysaccharides such as pullulan (*18*, *19*).

Table 1 shows that the sulfation caused a dramatic increase in the solubility of oat β-glucan. More incorporation of ionic groups and increased numbers of small fragments of β-glucan by sulfation would be responsible for the improved water solubility, which can have advantages in pharmaceutical and food applications. Solubility increase of sulfated polysaccharides has also been observed in the literature (*5*, *17*, *20*).

The shear stress vs shear rate plots of the β-glucan samples at 10 and 30 °C are shown in **Figure 2**. The decrease in the shear stress with increasing temperature was observed as expected. It is interesting to note that the sulfation led to a pronounced decrease in the shear stress of β-glucan at every shear rate tested. The shear stress of the derivative was almost 2 orders of magnitude lower than that of the underivatized

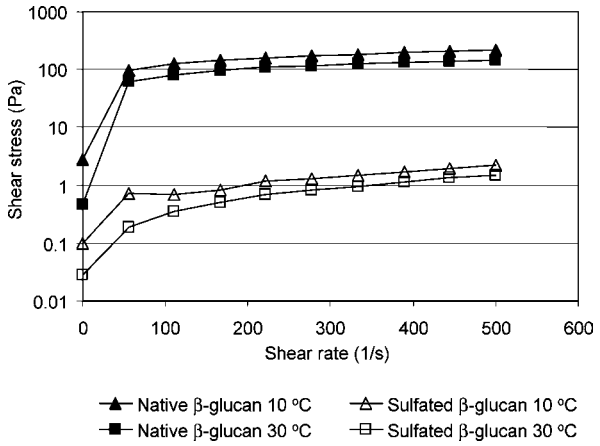

Figure 2. Flow behaviors of 1% native and sulfated oat β-glucan suspensions at 10 and 30 °C.

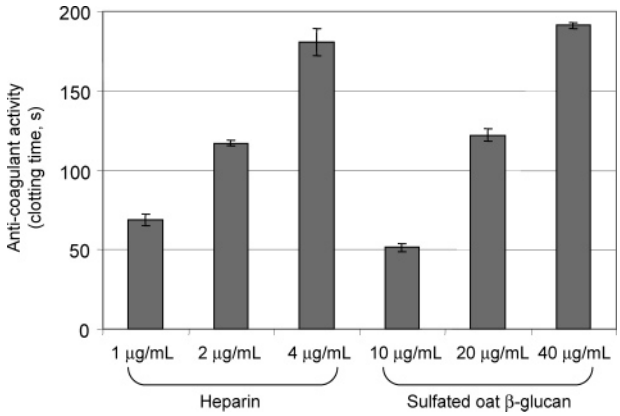

Figure 3. Comparison of the anticoagulant activity of heparin and sulfated oat β-glucan.

β-glucan, indicating substantial viscosity reduction by sulfation. Since viscosity is a function of molecular weight, its viscosity reduction could be expected from the decrease in its molecular weight (**Table 1**), which was consistent with several previous studies (*17*, *21*).

It is generally recognized that the positive effect of oat β-glucan on cholesterol level is due to its high viscosity (*22*). Ingested oat β-glucan increases the intestinal viscosity and traps bile acids, which suppresses reabsorption of the bile acids in a body and promotes their excretion. The body takes cholesterol out of the blood to produce more bile acids, consequently causing the reduction of blood cholesterol level. Hence, the sulfated β-glucan may be less efficient in reducing cholesterol due to its decreased viscosity.

In vitro bile acid binding capacity of the native and sulfated β-glucans was investigated. While the bile acid binding of the native β-glucan was 11.4%, that of the sulfated β-glucan reduced dramatically to be 1.1%. It is well-known that the bile acid binding activity is related to ionic interaction (*16*). Previous studies showed that cationic properties enhance the bile acid binding capacity (*11*, *23*). Therefore, the significant decrease in the bile acid binding capacity of the sulfated β-glucan could be caused by ionic repulsion due to its anionic characteristics. Since the bile acid binding activity is closely involved in cholesterol-lowering effects, the results indicate the negative effect of sulfation on cholesterol-lowering activity of β-glucan, which could also be involved in the viscosity results as mentioned in **Figure 2**.

Blood clotting is an essential mechanism to prevent further blood loss in a body. However, its overactivity can cause undesirable blood coagulation, which blocks a blood vessel. Consequently, it increases the risk of fatal complications such as stroke and heart attack. As an effective anticoagulant, heparin has been intravenously administered to help prevent clotting in a body. It is a linear polysaccharide consisting of sulfated repeating disaccharide units and 2.7 sulfate groups are included in the average heparin disaccharide, giving rise to high negative charge density (*24*). This anionic polymer is able to bind to and thereby activate antithrombin IIII which inhibits the enzyme playing a pivotal role in the blood clotting process (*6*). It was therefore used as a reference to compare the anticoagulant activity of the sulfated oat β-glucan in this study.

Figure 3 shows the comparative anticoagulant activities of heparin and sulfated β-glucan. They exhibited concentration-dependent increases in the anticoagulant activity. Since native β-glucan hardly showed the anticoagulant effect (data not shown), the anticoagulant activity of the β-glucan derivative could be attributed to sulfate groups in its structure, which give high levels of negative charge. Various studies have shown that sulfation gives polysaccharides the anticoagulant properties mainly due to interaction between the negative sulfate groups and positive peptide sequence of proteins involved in the coagulation process (*25*). In addition, the anticoagulant activity of sulfated polysaccharides parallels with increasing DS and molecular weight (*25*, *26*). Hence, the improvement of sulfation procedure to increase the DS and to minimize the molecular weight reduction will probably enhance the anticoagulant effect of sulfated oat β-glucan. **Figure 3** also shows that the sulfated β-glucan at around 10-fold higher concentrations showed similar anticoagulant effects to those of heparin. It is reported in the literature that sulfated glucan derivatives have anticoagulant activities ranging from less than 1% up to 135% of the heparin activity (*27*). Hence, it appears to have effective antithrombotic activity with less hemorrhagic risk due to less anticoagulant effect than heparin (*28*).

In an attempt to investigate new biological activities of β-glucans from cereals, oat β-glucan was subjected to sulfation and the biological activities of its resulting derivative were characterized with structural and physicochemical properties. Our results show that sulfation affected the molecular weight, water solubility, and viscosity of oat β-glucan. In addition, while the in vitro bile acid binding capacity of the sulfated β-glucan derivative decreased, it significantly exhibited anticoagulant activity in a concentration-dependent manner. It would be due to high negative charge density produced by the sulfated groups. Therefore, the sulfated oat β-glucan represents a promising candidate of an anticoagulant agent.

For better understanding of the anticoagulant mechanism of sulfated oat β-glucan, further research may be necessary to examine its structural patterns, which are important in its anticoagulant activity. Also, since the biological properties of sulfated polysaccharides are not limited to anticoagulant activity, it will be worthwhile to extend its study to other biological systems.

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