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Physicochemical Properties and Biological Activities of DEAE-Derivatized *Sphingomonas* Gellan

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Physicochemical characteristics and biological activities of *Sphingomonas* gellan (S-gellan) were investigated. The S-gellan weight fractions of Glc and GlcUA were 0.45 and 0.25, respectively, and the molar ratio of Glc:Rha:GlcUA was approximately 4:2:3. The S-gellan was chemically derivatized with diethylaminoethyl chloride-HCl (DEAE-HCl), and the resulting modified S-gellan contained both positive and negative charges. The elemental and IR analyses were conducted to confirm the successful incorporation of DEAE groups into S-gellan. A large increase in nitrogen fraction was observed from the derivatized S-gellan by elemental analysis. The IR absorption bands induced by C−H, C−N, and C−O−C stretching were noticeable at 2950, 1310−1380, and 1000−1150 cm^{-1}, respectively, resulting from the DEAE substitution. The characteristic CH_3 and CH_2 peaks originated from the DEAE group were detected in the ^1H NMR spectrum of the derivatized S-gellan as well. The solubility of native S-gellan was improved almost twice from 40% to 75% after DEAE derivatization, while water holding capacity (WHC) drastically decreased from 10026% to 245%. Oil binding capacity (OBC) of S-gellan also significantly dropped from 1528% to 331% after the derivatization. The bile acid binding capacity of S-gellan was indirectly determined by measuring the holding capability of cholic acid inside the dialysis membrane (MWCO 12 000−14 000 Da). Once S-gellan was DEAE derivatized, there was substantial increase in the cholic acid retardation index (CRI). Up to 9 h of dialysis, the derivatized S-gellan released 29.3% less of cholic acid compared to the control group that did not contain S-gellan. From these results of the improved water solubility and stronger bile acid binding capacity, it would be suggested that the DEAE-derivatized S-gellan has more advantages than gellan itself for functional food applications.

KEYWORDS: Gellan; *Sphingomonas paucimobilis*; DEAE derivatization; cholic acid

INTRODUCTION

Microbial polysaccharides such as xanthan, dextran, curdlan, and pullulan have been commercialized and used for wide range of applications because they provided unique or excellent physical properties (*1*). These biopolymers have been mainly used as thickeners, gelling agents, stabilizers, lubricants, and flavor enhancers (*2*). Recently, natural and modified carbohydrate materials started to regain scientific interest as a target for developing new bioactive compounds. Various carbohydrate polymers have been recognized as bioactive compounds either by themselves or by structural modification (*3*). Because the structure of natural carbohydrate polymers is generally very complex, it is not easy to determine the exact chemical structure. Due to the lack of fine structural information, the structure−activity relationship of many polysaccharides has not been well established. Thus, studies focused on finding the exact chemical structures of bioactive materials and mimicking similar chemical structures to generate strong biological activity by enzymatic or chemical derivatization (*4*, *5*). At least, it has been generally accepted that chemical structure and physical properties were important factors affecting the biological activity of carbohydrate materials (*6*, *7*).

Gellan is an extracellular heteropolysaccharide produced by *Sphingomonas paucimobilis*, previously known as *Pseudomonas elodea* (*8*). It is composed of linear tetrasaccharide repeating units of β-D-glucose, β-D-glucuronic acid, β-D-glucose, and α-L-rhamnose. Occasionally it carries acetyl and glyceryl groups on glucose units (*8*, *9*). The degree of ester substitution directly influences the gellan properties in solution and gel (*8*, *10*, *11*). Aqueous solutions of gellan are highly viscous and show high

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thermal stability. The gellan gum exhibits good stability over a wide pH range of 3.5−8.0 (*12*). Acid stability of gellan gum is a distinct advantage in fruit-based products. According to its property to produce a thermoreversible gel, gellan gum can be substituted for agar-based cultivating medium (*13*, *14*). Due to the diversity of its structure and properties, gellan gum has a wide range of applications in the food, pharmaceutical, and other industries as texturizing, stabilizing, thickening, emulsifying, and gelling agents. The fine chemical structure of gellan was comparatively thoroughly investigated as mentioned above, and the relationship between chemical structure and physical properties was extensively studied as well. There were a number of methodological approaches to elucidate the structure−function relationship of polysaccharides (*15*, *16*). The structural modification by introducing new functional groups on the biopolymers may not only help understand the structure−function relationship but also enhance physical and biological properties of them.

High cholesterol level in blood is closely linked to the incidence of heart disease (*17*). It was reported that the coronary heart disease induced by high blood cholesterol level could be prevented through appropriate diet and medicine (*18*). Cholestyramin, which is one of the bile acid sequestrants, has been widely used for patients suffering from hypercholesterolemia. But a relatively large amount of cholestyramin was required to relieve the symptom, which leads to constipation, flatulence, and abdominal pain (*19*). Cholestyramin is possibly linked to colon cancer as well (*20*, *21*). These unpleasant side effects of cholestyramin prompted the discovery of new substitutes in nature and the creation of new structurally modified biomaterials. Removal of bile acids secreted into the digestive tract was shown to effectively decrease the blood cholesterol level (*22*). Highly viscous soluble dietary fibers were thought to be effective sequestrants of dietary cholesterol and bile acids by a physical entrapping mechanism (*23*). The ionic interactions between negatively charged bile acids and sequestrants were considered as another important factor, and the hydrophobic interactions could not be ignored as well.

In this study, gellan polysaccharide was chemically modified and evaluated as a biologically active material by means of improving its adaptability to the biological systems. For this purpose, the gellan produced from *Sphingomonas paucimobilis* was derivatized with diethylaminoethyl chloride-HCl, and various analytical techniques was adopted to investigate physical properties of the derivatized S-gellan. For the potential usage of gellan as a bioactive agent, the bile acid binding capacity of the derivatized S-gellan was determined.

MATERIALS AND METHODS

Production of Gellan Polysaccharide. *Sphingomonas paucimobilis* KTCC 31462 was cultivated to produce the exopolysacchairde, gellan, following the procedure of Dlamini et al. (*24*). A gellan-producing cell culture was inoculated with a seed culture and fermented at 30 °C for 3 days in a medium containing 0.5 g of K_2HPO_4, 0.1 g of $MgSO_4 \cdot 7H_2O$, 0.5 g of bactopeptone, 30 g of glucose, and 1 mL of salt solution in 1 L of culturing medium (pH 6.8). The pH of the final cultured broth was adjusted with 1 M KOH to 10.0, the broth was heated at 80 °C for 10 min, and then the broth was neutralized with 1 M trifluoroacetic acid to pH 7.0. The neutralized culture broth at 25 °C was spun-down at 20400*g* for 30 min, and the supernatant was recovered. The retrieved supernatant was treated with 2 vol of ethanol to precipitate the gellan polysaccharide at 4 °C for 24 h. The precipitates were recovered by centrifugation at 20400*g* for 15 min and then were dialyzed in deionized water for 4 days (MWCO 12 000−14 000 Da). The culture of *Sphingomonas paucimobilis* strain produced 1.5 g/L of the gellan polysaccharide, and the resulting gellan solution was lyophilized prior to further study.

Structural Analysis. The amounts of total sugars in S-gellan samples were analyzed by the phenol−sulfuric acid method (*25*), and the uronic acid content was determined by the *m*-hydroxydiphenyl method (*26*). The sugar composition of S-gellan was determined by anion-exchange chromatography using a Bio-LC system (Dionex). An analytical CarboPak PA-1 column (4 × 250 mm) was used to separate at 0.1 mL/min using 18 mM NaOH as an eluent. The gellan samples (∼3.0 mg) were hydrolyzed with 3 mL of 2 M trifluoroacetic acid (TFA) at 110 °C for 90 min in a refluxing condition. The TFA-hydrolyzed samples were neutralized with 2 M ammonium hydroxide solution to pH 7.0. Relative molecular weight (M_i) was determined by GPC analysis. The Sepharose CL-2B column (2.5 × 90 cm) was operated with deionized water as an eluent at 1.0 mL/min. Five milliliters of sample solution (0.2%) was loaded on the column, 4 mL fractions were collected, and the total carbohydrate content of each fraction was determined by the phenol−sulfuric acid method (*25*). The column was calibrated with blue dextran and pullulan standards.

DEAE Derivatization. The gellan samples were derivartized with DEAE following the procedure of Clifford et al. with some modification (*27*). The gellan (0.3 g) was reacted with 15 mL of diethylaminoethyl chloride-hydrochloride at 65 °C, and then 15 mL of prewarmed 3.0 M NaOH was carefully added to the reaction mixture. The reaction continued with stirring for 18 h, and then the reaction mixture was diluted twice with water. The solution was ultrafiltrated sequentially with 0.1 M HCl, 0.0001 M HCl, methanol, and diethyl ether and lyophilized.

The elemental analysis was performed to determine the level of substitution by using an elemental analyzer (EA1110, CE Instrument, Italy). The operating conditions of the elements followed the TCD CHNS PoraPak POS column 600s analysis time; 50−190 °C temperature range (*28*). The DEAE functional groups on gellan were also analyzed by IR analysis (Infrared spectroscope, MAGNA IR 760) (*22*). One part of the sample was mixed with 50 parts of KBr, and the mixture was ground and formed into a disk shape before the analysis.

The type of linkages formed during DEAE derivatization on gellan was investigated with 300 MHz ^{1}H NMR analysis (Unit INOVA, Varian Co.). The samples were dissolved in D_2O (*29*).

Physical Properties. The solubility of gellan and DEAE-gellan was determined as follows (*30*). The gellan dispersion (50 mg/mL) was agitated at 25 °C for 24 h. This supersaturated gellan dispersion was centrifuged at 5000*g* for 15 min, and the collected supernatant (0.2 mL) was precipitated with 3 vol of ethanol. The precipitates were recovered by centrifugation at 10000*g* for 5 min. The vacuum-dried sample at 50 °C was weighed, and the solubility was calculated. To determine the water holding capacity (WHC, %) of gellan, the sample (0.2 g) was dispersed in 10 mL of deionized water by being vortexed every 15 min for 1 h (*31*). The resulting dispersion was centrifuged at 16000*g* for 25 min, and the supernatant was discarded. Excessive water in the precipitate was removed by reclining the sample tube on a filter paper (Whatman No. 1) for 30 min, and then the precipitate was weighed. The WHC was represented by the following calculation: WHC (%) = [total sample weight after water absorption]/[total dry sample weight] × 100. The oil binding capacity (OBC, %) was determined by the method of Tadaya (1982) as well (*31*). Instead of using water as a dispersing medium, soybean oil was used to disperse gellan sample. The other steps were identical to the analysis procedure of WHC.

Biological Activity. The bile acid binding capacities of the gellan samples were indirectly determined by measuring the holding capability of cholic acid inside the dialysis membrane (MWCO >12 000 Da) (*32*). The dialysis membrane was soaked in 0.1% of sodium azide solution for 24 h. Gellan (0.2 g) was dissolved for 14 h in 50 mM phosphate buffer (6 mL, pH 7.0) containing 15 mM cholic acid plus 0.1% sodium azide. The dialysis was pursued in 50 mM phosphate buffer at 37 °C with constant stirring. At the different time intervals, 1 mL each of the dialyzate was collected to determine the released amount of cholic acid into dialysis buffer (*33*). An aliquot of 0.2 mL was taken from the collected dialysis buffer and was mixed with 1.0 mL of 70% sulfuric acid. After 5 min, 1.0 mL of furfural solution (0.25%) was added and

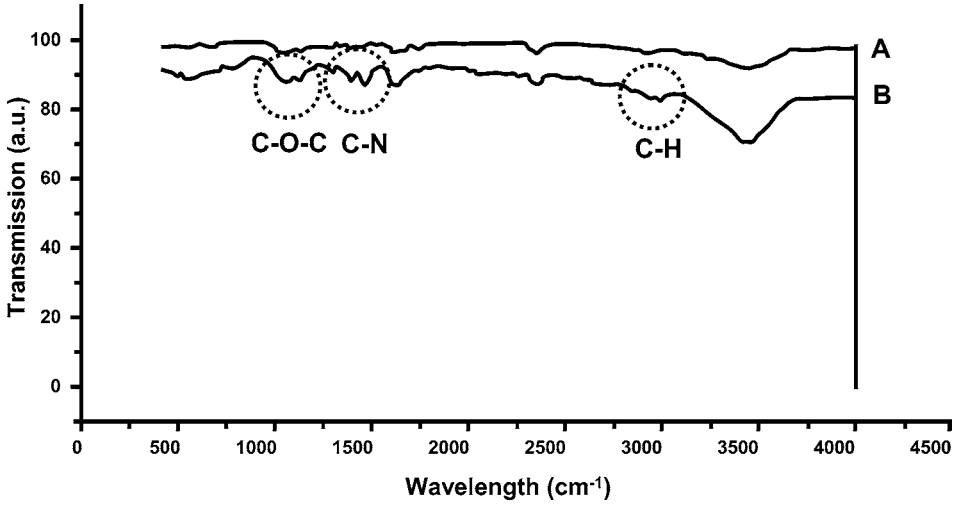

Figure 1. IR spectra of S-gellan (**A**) and DEAE-derivatized S-gellan (**B**).

Table 1. Elemental Analysis of Native and DEAE-Derivatized S-Gellans

sample	nitrogen (%)	carbon (%)	hydrogen (%)
S-gellan	1.4	36.4	6.2
S derivative	6.2	41.3	7.8

the pink color was developed for 60 min. The intensity of the color was measured at 510 nm by using a spectrophotometer. The binding capacity was represented by the equation of cholic acid retardation index (CRI, %) as follows: cholic acid retardation index (CRI, %) = (1 − [total amount of cholic acid diffused out from the sample group containing gellan]/[total amount of cholic acid diffused out from the control]) × 100.

Statistical Analysis. The CRI measurement for each sample was triplicated. Statistical analysis was performed with one-way ANOVA in SPSS program. Significance of difference at the 5% probability level was estimated by Duncan's multiple range test (*34*).

RESULTS AND DISCUSSION

Sugar Composition of S-Gellan. Neutral sugars were calculated by subtracting uronic acid content from total sugar content. The identity of the sugar components was confirmed by using the HPAEC method. The S-gellan consisted of three main components, such as glucose (Glc), rhamnose (Rha), and glucuronic acid (GlcUA). The weight fractions of Glc and GlcUA were 0.45 and 0.25, respectively, and the molar ratio of Glc:Rha:GlcUA was approximately 4:2:3. The general composition of *Sphingomonas paucimobilis* gellan (S-gellan) was known to be 60% of Glc, 20% of Rha, and 20% of GlcUA (*35*, *36*). The weight percent of individual sugar could be affected by the purity level of the gellan sample and the type of microbial strain, which resulted in the different final weight proportions from other studies (*13*, *37*). The molar ratio of individual sugars indicated that S-gellan isolated in this study has a relatively larger proportion of GlcUA in the polymeric structure.

Identification of DEAE Substitution on S-Gellan. In order to identify the covalently bonded DEAE groups on the modified S-gellan structure, elemental analysis was performed. A large increase in the weight fraction of nitrogen was detected from the DEAE-derivatized S-gellan, suggesting that the DEAE groups were successfully introduced into the S-gellan molecule (**Table 1**). The basal nitrogen content in native S-gellan could be explained by incomplete removal of cell protein residues (*13*). Relatively small increases in carbon and hydrogen were also rationalized by DEAE derivatization. We further investigated the structure of the DEAE derivatives by IR analysis. The IR absorption bands induced by C−H, C−N, and C−O−C stretching were noticeable at 2950, 1310−1380, and 1000−1150 cm^{-1}, respectively, resulting from the DEAE substitution (**Figure 1**; *22*). The result from ^1H NMR analysis supported that DEAE groups were incorporated into the gellan structure, which was judged by the occurrence of the characteristic peaks of CH_3 (δ 1.04; δ 1.29−1.41) and CH_2 (δ 2.61) (**Figure 2**). It was known that the primary C-6 hydroxyl groups had the highest reactivity for diethylaminoethylation (*38*).

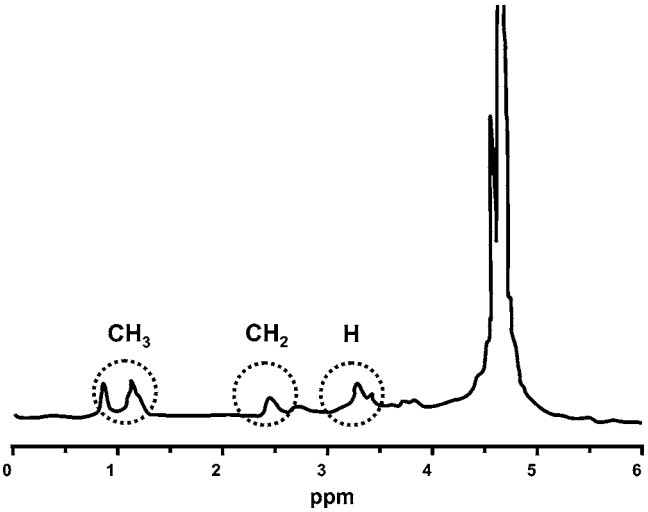

Figure 2. ^1H NMR spectrum of DEAE-derivatized S-gellan.

Table 2. Physical Property Changes Induced by DEAE Derivatization

sample	solubility (%)	water holding capacity (%)	oil binding capacity (%)
S-gellan	40.0 ± 0.6	10026.0 ± 0.6	1528.0 ± 0.6
S derivative	75.0 ± 1.2	245.0 ± 0.6	331.5 ± 0.6

Physical Properties of Native and Derivatized S-Gellans. The molecular weight (M_r) of S-gellan by GPC analysis was over 2.0×10^6, which was in good agreement with the result (1.64×10^6) reported by Whittaker et al. (*39*). The solubility of native S-gellan was improved almost twice from 40% to 75% after DEAE derivatization (*22*), whereas water holding capacity (WHC) drastically decreased from 10026% to 245% (**Table 2**).

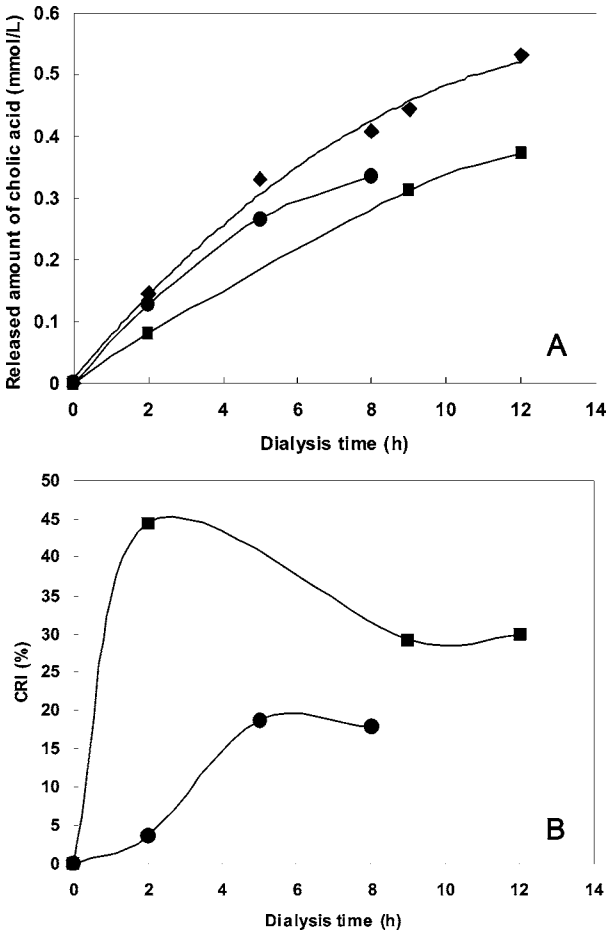

Figure 3. Released rate of cholic acid (**A**) and cholic acid retardation index (**B**). The symbols in the graph indicate control (◆), native S-gellan (●), and DEAE-derivatized S-gellan (■).

The decrease in gel forming ability was observed from the derivatized S-gellan, which resulted in the decrease in WHC. It was known that WHC is closely related to gel forming ability or gel rigidity (*40*). Oil binding capacity (OBC) of S-gellan also significantly dropped from 1528% to 331% after the derivatization (**Table 2**). The improved water solubility of DEAE-derivatized S-gellan could explain the substantial loss of oil binding capacity.

Effect of S-Gellan Derivatization on Bile Acid Binding Capacity. There was a report that positively charged methylan by introducing DEAE groups could be used as an alternative bile acid sequestrant (*41*). The bile acid binding capacity of gellan polysaccharides was represented by the holding amount of cholic acid inside the dialysis sack as described previously (*32*). In the control group not containing S-gellan, the cholic acid seemed to freely diffuse out from the dialysis sack up to 12 h dialysis (**Figure 3A**). In the presence of native S-gellan, the release of cholic acid was retarded (**Figure 3A**) and about 17.8% of cholic acid was either bound or physically trapped in gellan after 8 h dialysis (**Figure 3B**) (*42*). During the first 2 h dialysis, the CRI of native S-gellan was shown to be only 3.8%. This CRI value gradually increased up to 18.8% during 5 h dialysis, and then kept this level but small decrease by 1.0% during the subsequent 3 h dialysis. Once the S-gellan was DEAE derivatized, it was noticed that there were substantial increases in the CRI values. Up to 9 h of dialysis, the derivatized S-gellan released 29.3% less of cholic acid than did the control group. In turn, the derivatized S-gellan kept the same releasing rate. Therefore, these results indicated that the introduction of the positive charges on gellan structure by DEAE derivatization enhanced this particular biological activity.

ABBREVIATIONS USED

DEAE, diethylaminoethyl; WHC, water holding capacity; OBC, oil binding capacity; CRI, cholic acid retardation index.

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