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Chemically Cross-Linked Gels Formed by Novel Supergiant Polysaccharide, Sacran

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We extracted the novel sulfated polysaccharide (Sacran) from cyanobacteria, *Aphanothece sacrum* (*A. sacrum*). Sacran contained sulfate, carboxyl, and amide groups as functional ones. The constituent monosaccharides of Sacran were mainly neutral sugar such as glucose, galactose, and mannose, and we additionally confirmed uronic acids and slight amount of amino sugars such as GalN, and Mur. Then sacran can be regarded as glyco-ampholytes with imbalanced charge ratio. Furthermore it was found that Sacran was supergiant biopolymers with a high molecular weight, ca. 1.6 x 10^7 Da, and radius gyration was ca. 400 nm. Sacran solution with a concentration of 1 wt.% showed a very high zero-share viscosity value (83 000 cps). Sacran was successfully cross-linked with cross-linking agents such as L-lysine and adipoyl dihydrazide to form tough gels with a high swelling degree, ca. 400 times to the weight of the dried gel.

Key words: polysaccharides, cyanobacteria, polyampholytes, chemical cross-linking, gels

1. INTRODUCTION

Cyanobacteria are photoautotrophic prokaryotes and have been regarded as good candidates for various biotechnological applications for food, feed, pharmaceutical and other industrial materials [1]. Cyanobacteria are also known to synthesize polysaccharide outside the cell walls to overcome external various stressors such as desiccation [2]. Cyanobacterial polysaccharides are reported that they have high viscosity in their aqueous solutions and capability of forming gels with good tensile strength [3].

Especially *Aphanothece sacrum*, which is a freshwater unicellular cyanobacterium indigenous to Japan and mass-aquacultured in rivers, produces much jelly-like extracellular matrix (ECM) mainly composed of polysaccharides (PS). The aqueous solution of ECM of *A. sacrum* was very viscous. From the result, we can imagine that ECM contains a giant biopolymer. We have previously reported that the extraction method of PS using alkaline solution (1 N), and one of the constituent monosaccharides was sulfated fucose, just like as fucoidan [4]. One important point of the structure of the extracted PS is the presence of carboxylic acid, differently from fucoidan. Hyaluronan which is a biopolymer with high molecular weight and high solution viscosity can be cross-linked by cross-linking agents such as bis-epoxide, dihydrazide and polyisocyanate and so on, to form hydrogels owing to have carboxyl groups [5,6]. Since the extracted PS also contains carboxyl groups, we expected that they could give gels by chemical cross-linking.

In this paper, we attempt to make gels of the PS by chemical cross-linking, and found that the PS was successfully cross-linked by diamines such as L-lysine and adipoyl dihydrazide (ADH) to form hydrogels whose swelling degree was about 400 times. Furthermore, organogel preparation was also achieved in DMSO. Solvent replacement from DMSO to water gave the opaque hydrogels.

2. EXPERIMENTAL PROCEDURES

2.1 Extraction of Sacran from A. sacrum

Extraction of polysaccharide from *A. sacrum*, as shown follows. The *A. sacrum* samples were freeze-thawed and washed in pure water, followed by lyophilization. The samples were washed three times using a large amount of isopropanol with agitation, and then collected by filtration using gauze. The isopropanol-washed samples were put into 0.1 M NaOH aq. at 100 °C, and agitated at constant temperature for 4 h to yield the transparent solution. The solution was dialyzed with pure water for more than 72 h using the regenerated cellulose membrane (MWCO: 14000) until the pH value decreased to 8.0–9.0, and then filtrated. Then the filtrate was concentrated by rotary evaporator to create a highly viscous solution. The viscous solution was slowly poured into 100 % isopropanol (1000 ml) to precipitate white fibrous material. The fibers were dissolved in hot water again, concentrated, and reprecipitated, and these operations were repeated three times in total. The fibrous precipitates in isopropanol were collected and dried using vacuum oven.

2.2 Spectroscopic analysis

Fourier transform infrared spectroscopy (FT-IR) spectra of PS were recorded at 25 °C on a Perkin Elmer Spectrum One spectrometer between 4000-600 cm^{-1} using a diamond-attenuated total reflection (ATR) accessory. Ultraviolet-visible absorption spectra of SC aqueous solution were measured at 25°C on a Perkin Elmer Lambda 25 UV/VS spectrometer. Elemental analysis was made by Yanako CHN coder MT-6 (at Center for Organic Elemental Microanalysis at Kyoto University).

2.3 Monosaccharide analysis

Monosaccharides constituting the jelly matrix were analyzed by the following procedure. For gas chromatography (GC) analyses, the dried PS was acid-hydrolyzed with 4M TFA at 110°C for 78h. After

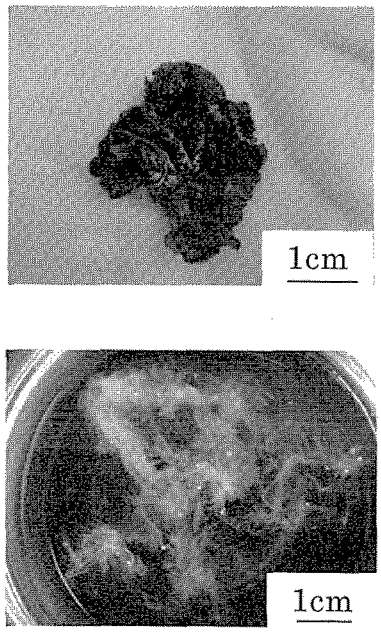

Fig.1b Image of extracted PS (Sacran)

removal of TFA, the hydrolyzed sample (1mg) was methylated in a mixture of dry-MeOH (50 ml)/ CH_3COCl (0.6 ml) at 60 °C for 16 h and was trimethylsilylated with TMSI-C. The trimethylsilylated samples were analyzed by GC (GC-18A, Simadzu, Kyoto, Japan).

2.4 Measurement of an absolute molecular weight

Multi-angle laser light scattering (MALLS) was performed in static mode at 30°C with a fully computerized DLS-7000 system including a compact goniometer system (Super Dynamic Light scattering spectrometer, Otsuka Electronics). The angles ranged between 15, 17, 19, 22, 25, 30, and 40°. A He-Ne laser (λ_0 =632.8 nm) was used as the light source, and the scattering of toluene was used as the primary standard. The refractive index increment, dn/dc, was chosen at 0.1435 $mL \cdot g^{-1}$. PS in 0.1 M $NaNO_3$ solutions with concentrations of 0.27, 0.18, 0.135, and 0.09 $mg \cdot ml^{-1}$ were used.

2.5 Zero-shear viscosity of sacran solution

Zero-shear viscosity of the PS solution was measured as follows. The PS (100 mg) were dissolved in hot water (10 ml) and cooled to give a homogeneous solution. A cone plate (25 mmϕ) was used as a probe for the static rotation viscosity, which was measured by a rotation viscometer (physica MCR301, Anton-Paar). The solution thickness was 1 mm (thickness at center position: 0.053 mm). The probe was pre-rotated at an angular velocity of 0.01 rpm for 60 seconds, and then the measurement was started. The rotation viscosity was recorded with changes in angular velocity from 0.01 to 1 rpm. Zero-shear viscosity was estimated by extrapolation of the plots to zero velocity.

2.6 Formation of chemically cross-linked gels

PS were cross-linked to yield hydrogels and organogels by the following procedure. In the case of hydro-gel formation, L-lysine was added into a PS solution (1 wt.%, 100 ml) and then 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl salt (2 g) was added as an condensation reagent. The solution was stirred strong, centrifuged to degas, and kept at 4°C for 72 hrs in the refrigerator, to yield the hydrogels. Organogels were prepared in DMSO by the same procedure. The hydrogels and organogels were purified by immersing in water and DMSO for 3 days at room temperature to remove unreacted compounds, respectively. The solvent exchange from DMSO to water was performed by immersion of organogels in distilled water for 1 week with replacing the water.

3. RESULTS AND DISCUSSIONS

Aphanothece sacrum has an abundance of a jelly-like ECM (**Fig. 1a**) as related in introduction. When PS was extracted from ECM using 1N NaOH aq, functional groups may be damaged. Then in this study, the PS were extracted by alkaline solution (0.1 N NaOH). The PS was successfully extracted as fibers (**Fig.1b**), and the yield kept high (50-80 wt.%). The PS were soluble in hot water to give a homogeneous solution regardless of pH. The IR absorption (**Fig.2**) and XPS spectra data of

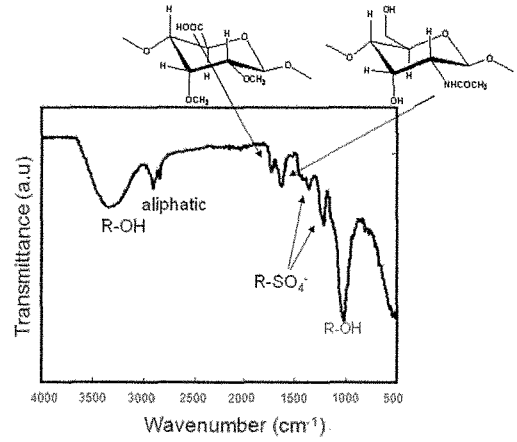

Fig.2 IR spectrum of Sacran and chemical structure of two main monosaccharide constituents containing carboxylic acid and amide groups.

the PS extracted here demonstrated the presence of sulfate, carboxyl, and amide groups in the PS, as reported previously [4]. The uronic acid content was estimated at 22 % by the carbazole-sulfuric acid method (525 nm). CHN S elemental analyses of the PS showed the following composition: C: 36.04 %, H: 5.91 %, N: 0.30 %, and S: 2.07 %. GC analyses of trimethylsilylated samples of methanolyzed PS indicated that the main monosaccharides were Glc, Gal, Man, Xyl, Rha, Fuc, GalA, and GlcA, with a composition of 25.9: 11.0: 10.0: 16.2: 10.2: 6.9: 4.0: and 4.2. We also confirmed the presence of trace amounts of Ara, GalN, and Mur. One can presume that the N element detected by elemental analysis was derived from GalN and Mur. As a result of PS search by data base, SciFinder Scholar ver.2006 (American chemical society), the PS containing such monosaccharide constituents were not reported so far and can be regarded as a new substance and we named the PS Sacran. It was demonstrated that Sacran was an ampholytic one with an imbalanced charge ratio resulting in a high content of anionic sugars

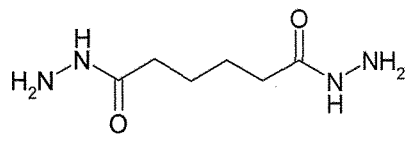

Fig. 3a. Chemical structure of adipoyl dihydrazide (ADH).

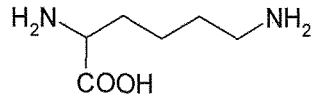

Fig. 3b. Chemical structure of L-lysine.

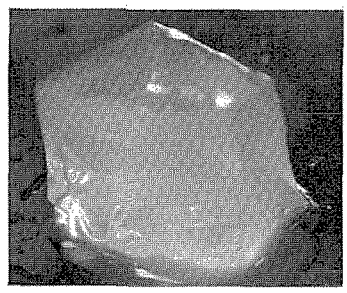

Fig.4a Image of a representative gel cross-linked by ADH

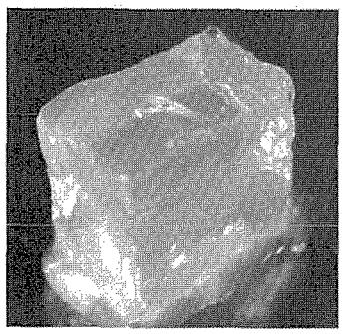

Fig.4b Image of a representative gel cross-linked by L-lysine

with sulfate and carboxylic acid groups, and a low content of cationic amino sugars (anions/cations; ca. 30/1). An absolute molecular weight and radius of gyration, $<s^2>^{1/2}$ of Sacran were estimated at 1.6 x 10^7 Da and 402 nm, respectively. We successfully obtained a typical Zimm-Berry plot with a very small error of 1.4%. To the best of our knowledge, this is the first confirmation of an absolute Mw over 10^7 Da for a water-soluble bio-derived polymer. The rotation viscosity measurement of the extracted Sacran solution (1 wt.%) showed a very high zero-share viscosity value (83 000 cps) whereas hyaluronic acid (Mw~150-180 kDa) showed a value of 8 900 cps. Such a large difference in the viscosity can be attributed to the difference in the *Mw*.

In general, it is reported that the macromolecules with high molecular weight form gels efficiently [7]. Therefore we tried to prepare the gels of Sacran by chemical cross-linking. The chemical cross-linking of polysaccharide is most commonly made using the less efficient reaction of hydroxyl groups with cross-linkers [7]. On the other hand, Sacran contained carboxyl groups and then we decided to use a high efficient reaction such as dehydration reaction of calboxylic acids with diamines. It has been reported that hyaruronic acid successfully reacted with ADH (structure; Fig.3a) by using the abovementioned dehydration [7]. Then, we examined the gelation of Sacran using ADH by the procedure similar with the formation of hyaruronic acid gel and obtained the aimed gels (Fig.4a). However ADH is not a natural product and then it has some possibilities for militating against environments and bad for ecology. We also investigated whether amino acid containing two amino groups L- lysine (structure; Fig.3b) could be used as cross-linker, by following procedures. Sacran solution in water and DMSO were prepared, and L-lysine was added, and then condensation agent 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl salt was added. Since the reaction did not occur immediately, we had enough time to deaerate the solution by centrifugation to gather all air bubbles on the top of solution. Thereby, homogeneous solution without bubbles was obtained. The gelation after three days was confirmed by turning a sample bottle containing reactants upside down. Then, the gels were took out from a sample bottle and immersed in water for several days to remove impurities. The swelling degree of hydrogels (amount of cross-linking agent added was 10 mol% to mol of carboxylic acid in Sacran) was about 400 times. Although the value is very high comparing with other hydrogels reported so far, the hydrogels were tough to process into the rectangular shape by a normal cutter (Fig.4b). When the quantity of L-lysine was decreased to 5 mol%, the gel was formed but it was more fragile than that prepared at 10 mol%. Further, the gelation was not confirmed when [3 mol%. The swelling degree of hydrogel when use of ADH as a cross-linker was about 240 times. Therefore it seemed that reaction efficiency rate of L-lysine was somewhat lower than that of ADH, presumably due to a lower nucleophilicity of α-amine of L-lysine than that of hydrazide amine of ADH. However we discuss that L-lysine which is produced by fermentation method has an advantage in the green-chemical aspect and can propose it a cross-linking agent for PS containing carboxylic acids. Sacran chains may associate through electrostatic and/or hydrogen interactions in water, which possibly affects the gel structures. Moreover organogel of Sacran was formed using L-lysine as a cross-linker in DMSO to create transparent gels. Next, the organogel was immersed into pure water to replace DMSO into the water, and then the gel shrunk and turned harder and opaque. One can consider that some sorts of self-assemblies were induced by solvent replacement, but the studies in detail were remained.

4. CONCLUSION

We extracted novel sulfated polysaccharide (Sacran) from *Aphanothece sacrum* using dilute alkaline solution (0.1 N NaOH). Results from various spectroscopic analyses, it was found that Sacran has sulfate, carboxyl, and amide groups. Gas chromatography (GC) analyses of methanolyzed SC indicated that the main monosaccharides were Glc, Gal, Man, Xyl, Rha, Fuc, GalA, and GlcA, and that trace amounts of Ara, GalN, and Mur. An absolute molecular weight and radius of

gyration, $<s^2>^{1/2}$ were estimated at 1.6 x 10^7 Da and 402 nm, respectively from multi angle laser light scattering (MALLS). The rotation viscosity measurement of the extracted Sacran solution (1 wt.%) showed a very high zero-share viscosity value (83 000 cps). Chemically cross-linked gels of Sacran using cross-linkers such as ADH and L-lysine were successfully formed. Furthermore, the organogels of Sacran was also formed using L-lysine in DMSO. After the organogel was put into pure water to replace DMSO into water, the gels shrink and turn harder and opaque. Since Sacran hydrogels have a high swelling degree and toughness enough high to process, it is possible to apply in the field of environmentally-benign super-absorbers such as planting materials and diapers.

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