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Description	

Macroscopic birefringence in liquid crystals from novel cyanobacterial polysaccharide with an extremely high molecular weight

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Abstract: We report an efficient method for extraction of anionic polysaccharides (PS) from cyanobacteria, *Aphanothece sacrum*; we used a hot alkaline solution (0.01 N NaOH) as an elution solvent in the first step of the extraction and isopropanol as a precipitation solvent in the last step. Thin fibers of PS were obtained at a high yield (50-80 % to the weight of the raw cyanobacterial sample). The spectroscopy and elemental analyses indicated the PS contains fucose, uronic acids (14.2 % by a carbazole-sulfuric acid method), a sugar unit containing amides. The solution of PS with a concentration of 1 wt% showed a very high viscosity (80 000cps) implying a high molecular weight, and a strong macroscopic birefringence with a texture typical of nematic liquid crystals was confirmed by crossed-polarizing microscopy (more than 0.5 wt%). The PS from *A. sacrum* may form a special structure rigid-rod enough to show LC phase and macroscopic birefringence.

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INTRODUCTION

The study of structure-property relationships in liquid crystalline (LC) polysaccharides (PS) is very important in terms of not only the development of PS materials but also mechanical and photonic roles of the PS in the organism. In a previous study, we extracted novel PS from cyanobacteria with jelly matrix, *Aphanothece sacrum* (Sur.) Okada. *A. sacrum*, which was biologically classified in the 19th century by Suringar [1], is a freshwater unicellular cyanobacterium indigenous to Japan. *A. sacrum* lives only in the shallow river filled with limpid, fresh water where the sunlight transmits with the low scattering. Otherwise, *A. sacrum* is sensitive to the light strength and sometimes is damaged when floating in the upper side of the river for a long time. These phenomena imply that the photonic environment may be very significant for *A. sacrum* life activity. From this information, we can analogize that extracellular PS of *A. sacrum* assumes a vital role in a photonic function to keep the life. For example, PS may control the waveform of sunlight to perform efficiently in the photosynthesis.

In this study, we improve the extraction efficiency of PS by adjusting the elution solvent and precipitation condition, and investigate the LC properties of PS aqueous solution to find that the macroscopic birefringence domain of LC is automatically formed.

METHODS

Microorganism samples

A. sacrum cultivated in a river farm was kindly provided by Kisendo corporation (Amagi, Japan). Raw samples used for the extraction were wavy jelly sheets (thickness: 1-2 mm, area: 100-1600 mm²) colored brown-green. Prior to the extraction operation, the raw samples were thoroughly washed using a large amount of tap water, and rinsed in pure water. Microscopic observation confirmed that no other microorganisms contaminated the inside of the jelly matrix for *A. sacrum*.

Extraction and purification of PS

PS was extracted from *A. sacrum* by the following procedure. The *A. sacrum* samples were freeze-thawed and washed in pure water, and were washed three times using a large amount of ethanol with shaking (120 rpm) overnight, and then collected by filtration using gauze. The ethanol-washed samples were put into 0.01 N NaOH aq (1000 ml) 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: 14 000) 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 (10 ml) was slowly poured into pure isopropanol (1000 ml) to precipitate white microfibrinous material containing a large amount of solvent. The microfibrers were dissolved in hot water again, concentrated, and reprecipitated, and

these operations were repeated three times in total. The aqueous solutions of the fibers showed a pH value of about 6.0-7.0. Further purification was achieved by gel filtration chromatography (Sephadex LH-20, GE Healthcare) to produce a transparent solution containing PS solute. The aqueous solution showed no specific absorption in the wavelength range of 220-600 nm in ultraviolet-visible (UV-vis) spectroscopy, which confirmed there was no contamination by proteins, nucleic acid, chromophores, and/or other chemicals with aromatic rings.

Structure 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 PS aqueous solution were measured at 25 °C on a Perkin Elmer Lambda 25 UV/VS spectrometer. ^1H nuclear magnetic resonance (NMR) of partially-hydrolyzed PS was measured at 25 °C in DMSO solution on a Varian FT-NMR spectrometer (UNITY 500 plus, 500 MHz), using the residual proton resonance of DMSO as an internal standard (2.55 ppm). Elemental analysis was made by Yanako CHN coder MT-6 (at Center for Organic Elemental Microanalysis at Kyoto University).

Measurement of solution properties

Rotation viscosity of PS aqueous solution was measured by Anton Paar Physica MCR 301. The sample (gap: 1 mm) was statically sheared by rotation of a cone plate at 30 °C and zero-shear viscosities were estimated by extrapolation to the zero-shear condition. The liquid crystallinity was evaluated by observing the white light transmitting through the PS solution under crossed-polarizer; the solution was not in a LC state when the transmitting light was invisible while the solution was in a LC state when the light was bright. The relative brightness was calculated by measuring the whiteness of the PS solution using the image analysis application (WinROOF, Mitani-corp.) The texture within the bright region was carefully observed in order to identify the LC phase. The orientation behavior of PS solution was estimated by a crossed-polarizing microscope (Olympus BX51) equipped with a high-resolution digital camera (Olympus DP70, 12 500 000 PIXELs). The samples were sandwiched between two glass plates set on the rotary stage.

RESULTS AND DISCUSSION

A. sacrum is a cyanobacterium with a large quantity of jelly matrix. One can see that many cell bodies with a diameter of 3-4 μm are dispersed over the jelly matrix, forming a group with a size range less than 80 μm . In addition, about half of the area of the matrix contains no cells. From the results of the microscopic observation, we could roughly estimate the volume percent of the

extracellular jelly matrix as 70-85 vol%. The swollen weight of *A. sacrum* compared to its dry weight was 62-64.

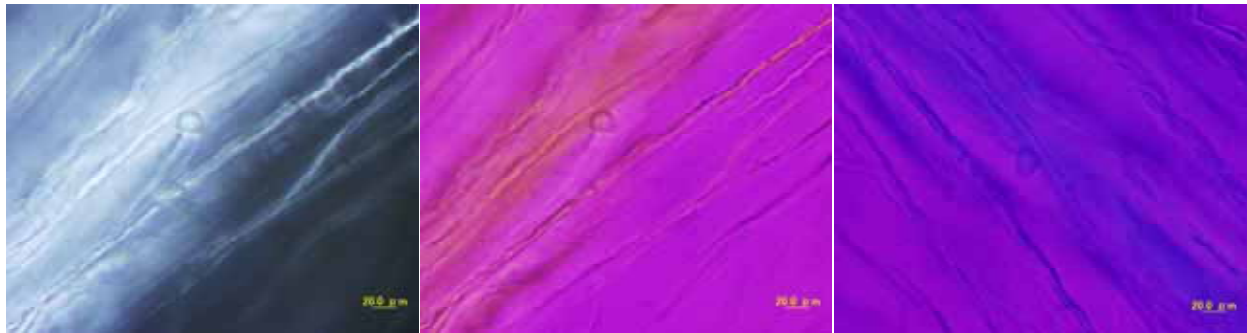


Fig.1 Crossed-polarizing microscopic photos of PS fibers (a) no plate (b) (c) 530nm plate

PS extraction

The raw sample of *A. sacrum* is brown-green, but the freeze-thaw treatment made it easy to remove the purple pigments such as phycoerithrin and phycocyanin by simple water washing. The following ethanol washing removed the green lipid pigment, such as chlorophylls. The sample surface was not slimy, which made us imagine no spontaneous PS elution from the jelly matrix. Actually, the hot water treatment of the *A. sacrum* sample gave difficulty. We used alkaline solution (NaOHaq, 0.01 N) for PS extraction, since cyanobacterial PS generally have uronic acid units, according to the literature [2-5]. And the solution was agitated for 4 hours in order to degrade other biopolymers such as proteins and ribonucleotides. After some insoluble matter was filtrated, the solution was dialyzed with pure water to remove low molecular weight impurities. We confirmed impurity removal by recording the sufficient electric resistance (more than $10^5 \Omega$) of the solution outside of the dialysis membrane. When the solution inside the dialysis membrane was concentrated, the solution became highly viscous, which implies the existence of PS. Slow pouring of the solution into isopropanol created fibrous precipitates. Reprecipitation was performed twice more to give colorless fibers, which were confirmed as PS by the spectroscopic study shown in the next section. The aqueous solution of PS (1 g/dL) showed a zero-shear viscosity of 80 000 cps at 30°C, which is such a high value that the molecular weight may exceed 1 MDa. A trace of low molecular weight impurity (< 10 kDa) was removed from PS by gel filtration. When the concentrated PS solution was poured into isopropanol, the gel-like matters composed of thin fibers appeared. The polarized microscopic observation showed that the gels were composed of oriented fibers with a thickness of less than 10 μm . (Fig. 1a). Fig.1a shows a microscopic photo of the microfibers taken under the cross-nicol and Figure 1b and 1c show a corresponding photo taken using a first-order retardation

plate ($\lambda=530$ nm) inserted in the light path. The fiber birefringence was negative, as evidenced by both subtractive birefringence (blue color, Fig.1c) in the fiber lying from upper left to lower right and additive birefringence (orange color, Fig.1b) in the fiber lying from upper right to lower left. The negative birefringence strongly suggests the PS main chains along the fiber axis.

Spectroscopic study

The UV spectra did not show any absorption peaks of protein, nucleotides, or pigments in the wavelength range of 600 to 230 nm, but rather the typical absorption of PS which is clearly visible at less than 230 nm, indicating PS isolation. The elemental analysis of desalinated PS by a cation exchange resin (DOWEX 50W-X8, 50-100 mesh, H form) showed results as follows; C; 36.04 %, H: 5.91 %, N:0.30 %, S:2.07 %. If average molecular weight for a sugar unit is assumed to be ranged 162-180, the molar composition of S to the total PS can be estimated as 10 mol%.

FT-IR spectra of PS showed several distinct peaks at wavenumbers of 3354 (hydroxyls), 2924 (aliphatic chains), 1736 (carbonyls of carboxylic acid), 1613 (carbonyls of amide), 1415 (aliphatic chains), 1363 (sulfates), 1223 (sulfates), and 1022 cm^{-1} (hydroxyls) (Fig.2). The spectra indicated that PS has carboxylic acid, amide, and sulfate groups, as well as the typical groups of sugars. As a result of the carbazole-sulfuric acid assay (525 nm) using a glucuronic acid standard, the presence of uronic acids was confirmed and their content was estimated as 14.2 %. The IR peak of the carboxylic acid can be assigned to 6-carbon of uronic acids as shown in Fig.2.

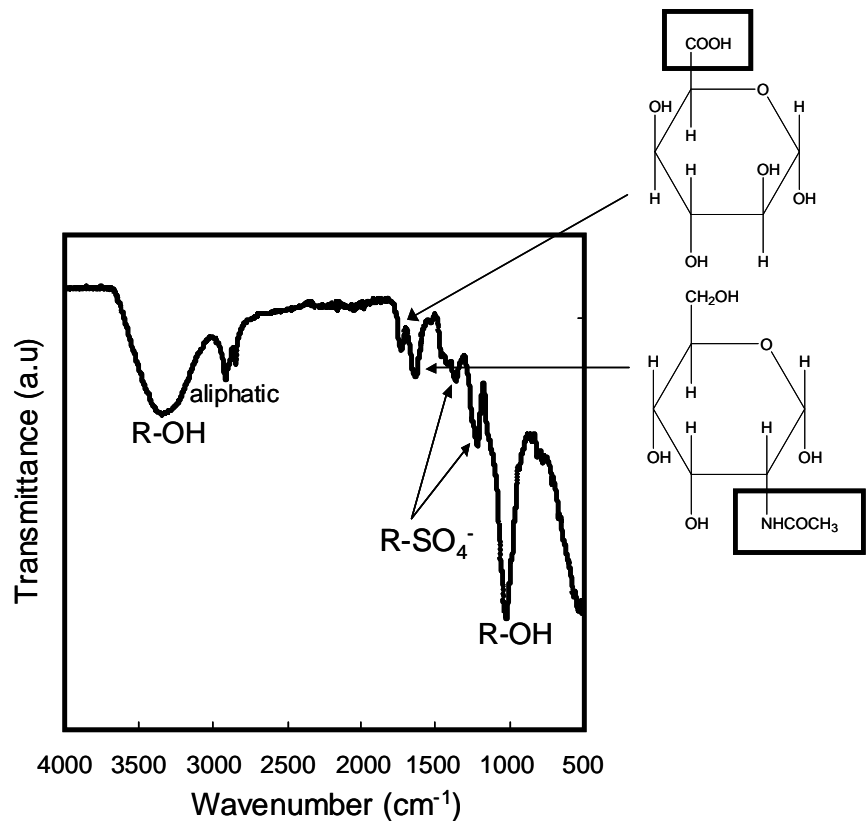


Fig.2 IR spectrum of extracted polysaccharides. Inset structures showed the representative sugar unit having a carboxylic acid and amide groups.

We tried to measure ^1H NMR spectra of PS in $\text{DMSO-}d_6$, but the solution was too viscous to put into the NMR tube (5mm ϕ) without bubbles remaining. In order to reduce the solution viscosity, the PS was partially hydrolyzed in methanol/HCl solution for 8 hours.

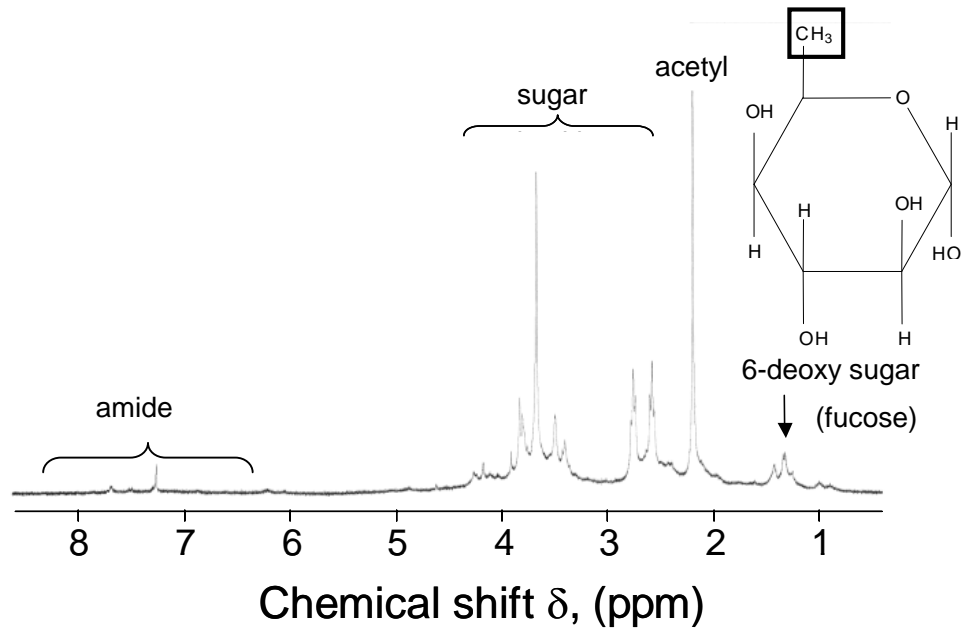


Fig.3 ^1H NMR spectrum of extracted polysaccharides. Inset structure shows the fucose unit.

Fig. 3 shows the ^1H

NMR spectra of the degraded PS in $\text{DMSO-}d_6$, showing broad but distinct signals, whose maximum lie at chemical shifts of δ 1.32 ppm and δ 6-8 ppm, and multiple signals with several local maximum at δ 2.2, 2.6, 2.8, 3.5, 3.7, and 3.8 ppm. While a signal at δ 1.32 ppm indicates the presence of fucose, and signals at δ 2.2 ppm and δ 6-8 ppm may be assigned to acetyl and amide groups of N-acetylosamines, respectively, whose representative structure is shown in Fig.2. Then, one can say that the PS extracted from *A. sacrum* may have not only sulfate among its groups but also the fucose derivative and amide group of N-acetylosamine as one of its sugar units. Studies of other monosaccharide constituents are under investigation.

LC properties

Fig. 4 shows the image of the PS aqueous solution (3 ml) put on a glass dish with a round bottom. The solution was irradiated by the white back-light under the crossed-polarizers. The solution was observed very bright to show a birefringence. The birefringence in the solution is generally caused by the crystalline particle dispersions or the molecular orientation of the solutes. In the present case, the microscopic observation did not confirm the crystalline particles, and the brushes (black lines) were confirmed in Fig. 4. The observation indicates that PS chains were not in the crystalline state but in the LC state. Since the blush lines were accompanied by point defects (circles in Fig.4), the LC was identified as the nematic phase. It was noticeable that the domain surrounded by the black lines was very big (millimeter to several centimeter scale) even without any treatment by external

forces. The PS chains automatically aligned in the macroscopic range. Cellulose, which is a very rigid β 1,4-glucan, is not soluble in water but the modification makes it soluble to create the LC phase where the large orientation domain [6]. On the other hand, other PS chains such as starch do not have so high rigidity that they show a high solubility but no LC properties. The present PS showed a high solubility and might not be so rigid. Then we can speculate that the LC phase may be attributed to a special form of PS by some interchain interactions such as hydrogen bonds of carboxyls and/or amides, ionic interaction of sulfates, and hydrophobic interaction of the 6-deoxy carbon group, as a microbial polysaccharide, Schizophyllan, forms a triple helix form which is very rigid to show a LC phase [7].

Thus the aqueous solution of PS extracted from *A. sacrum* showed nematic LC phase where the PS chains automatically formed a macroscopic orientation domain. The macroscopic orientation can polarize the light transmitting through the solution to reduce the quantity of light. If PS chains were in the liquid crystalline state on the matrix of living *A. sacrum*, the microorganism may utilize PS to control the sunlight transmitting into the cell body.

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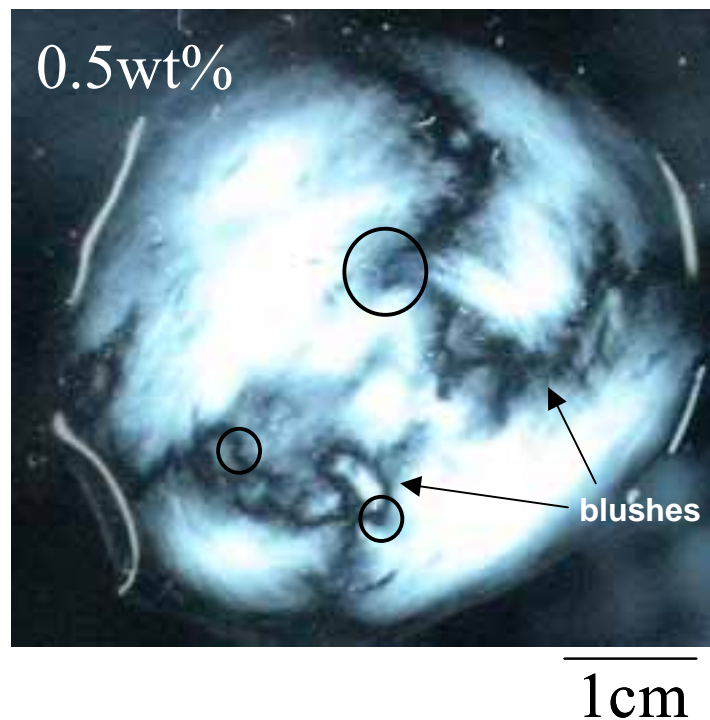


Fig. 4 The image of the PS solution taken under the crossed-polarizer

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