Preparation and Differential Scanning Calorimetric Studies of a New Water-absorbing Polysaccharide from a Bacterium Belonging to the Family *Oxalobacteraceae*

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Abstract: A Gram-negative bacterium, strain IM944, belonging to the family *Oxalobacteraceae* which produced a new water-absorbing polysaccharide (WAP) was isolated from soil. The water-absorbing capacity of the WAP purified by the cetyltrimethylammonium bromide treatment in a potassium hydroxide solution was 120 times as much as its own weight without stickiness. The differential scanning calorimetry (DSC) of WAP showed the reversible heat transition at a peak temperature of 60.5°C, suggesting that this polysaccharide sample is homogeneous and forms a highly ordered structure in aqueous solutions.

Key words: water-absorbing polysaccharide, new bacterium of *Oxalobacteraceae*, DSC analysis

In the course of our screening of water-absorbing polysaccharides, a new polysaccharide prepared from a culture of a soil bacterium has been found. In an investigation of chemical modification of carboxymethylcellulose (CMC), we found that CMC fibers having slightly been cross-linked by the treatment of dichloroacetic acid quickly absorbed water without growing sticky.¹⁾ We took an interest in this water-absorbing property and set out to search for polysaccharides of a bacterial origin with this property, because such polysaccharides may have a specific chemical structure and morphology in water, which can be used as a biodegradable bioabsorbent similar to that reported by Kurane and Nohata.²⁾ In this paper, the screening, purification, and differential scanning calorimetry (DSC) analysis of the new water-absorbing polysaccharide (WAP) are reported.

A small amount of soil sample collected in Niigata Prefecture, Japan, was suspended in water, and was spread on 1.5% (w/v) agar plates containing Czapek-Dox medium (composition (g/L): sucrose, 30; K₂HPO₄, 1; MgSO₄·7H₂O, 0.5; NaNO₃, 2; KCl, 0.5; FeSO₄·7H₂O, 0.01 at pH 7.0). After the cultivation at 30°C for 5 d, colonies swelling from water absorption were picked up. Each isolate was cultured by shaking at 120 rpm in a flask containing 80 mL of Czapek-Dox medium for 5 d at 30°C, and then autoclaved. Three times the volume of acetone was added to the culture broth, and the precipitate obtained was washed twice with 80% aqueous acetone (v/v) and dried by acetone substitution. Fifty-four isolates were tested for the production of extracellular materials with a water-absorbing capacity, and a bacterial strain IM 944 was selected. The 0.47 g precipitated by acetone (crude WAP) was yielded from 80 mL culture broth of

strain IM944, and the water absorption capacity was 200 times as much as its own weight without stickiness, and N content was 1.80% of the total weight (measured by the Institute of Science and Technology, Inc., Japan).

The taxonomic characteristics and the phylogenetic position of the strain IM944 are as follows. The organism was a Gram-negative, aerobic, non-motile rod. It was catalase positive but oxidase negative. Starch and gelatin were hydrolyzed. Reduction of nitrate, production of indole, hydrolysis of aesculine, arginine dihydrolase, β -galactosidase and urease were all negative. It assimilated L-arabinose, D-mannose, maltose, *n*-capric acid, DL-malic acid and phenyl acetate, but not D-mannitol, N-acetyl-D-glucosamine, gluconate, adipate or citrate. Acid was produced from D-glucose, D-fructose, D-mannose, maltose and sucrose, and weakly from L-arabinose, D-xylose, D-galactose, cellobiose, raffinose, starch, glycerol and D-mannitol, but not from lactose, L-sorbitol, inositol or α -methyl-D-glucoside. The 16S rRNA gene sequence analysis showed that the bacterium took a position in the phylogenetic radiation of the family *Oxalobacteraceae*. The sequence similarities of this gene between the organism and the neighboring species were 96.4% for *Duganella zoogloeoides*; 95.8% for *Duganella violaceinigra*, 95.6% for *Herbaspirillum lusitanum* and 95.4% for *Janthinobacterium lividum*. The sequence similarities among the genera *Duganella*, *Herbaspirillum*, *Janthinobacterium*, *Massilia*, *Telluria* and *Collimonas* are around 95%. The strain IM944 exhibited distinct phenotypic features from those of the species of the above mentioned genera. Based on phylogenetic and phenotypic characteristics, the strain IM 944 should constitute a new species but can not be assigned to the hitherto described genera, probably belonging to a new genus. In the present paper, the organism was tentatively identified as a new species, phylogenetically belonging to the family *Oxalobacteraceae*. Exact

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taxonomic position and nomenclature of the strain awaits further isolation of similar strains and further critical taxonomic studies of the family *Oxalobacteraceae* at a generic level. The taxonomic tests other than motility and acid formation from carbohydrates and the phylogenetic analysis for bacterial identification were done by NCIMB Japan Co., Ltd.

The water absorption capacity of a sample was measured by the "tea bag method." Tea bags made of organic polymers (polypropylene, polyester and polyethylene) were filled with a sample (40 mg). After the set of tea bags with and without a sample were immersed in water for 2 h, they were taken out and left for 4 min on tissue wipers, and then weighed. The water absorption capacity of a sample was calculated by the difference (g) between wet tea bag weight with and without a sample divided by the dried sample weight (g).

The acetone precipitate (crude WAP) being substantially insoluble in water was further purified by the treatment of cetyltrimethylammonium bromide (Cetavron, Wyeth-Ayerst) in potassium hydroxide solution.³⁾ Crude WAP (1.0 g) was added with sodium borohydride (50 mg) in 4% potassium hydroxide solution (w/w, 500 mL), and stirred for 24 h at ambient temperature, under a nitrogen gas stream, and then 0.2% Cetavron solution (w/w, 500 mL) was added to the mixture. The WPC-Cetavron complex was added with sodium chloride (20 g) and sodium borohydride (50 mg) in 4% potassium hydroxide solution (w/w, 250 mL), and was stirred for 24 h at ambient temperature, under a nitrogen gas stream, and then acetone (500 mL) was added. The precipitate obtained was neutralized and washed twice with 60% aqueous acetone solution (v/v), and then dried by acetone substitution to obtain the purified WAP (0.20 g from 80 mL culture broth) as white fibrous powder, $MP > 181^\circ\text{C}$ (gradually decomp.), N content 0.30%, IR (KBr) $1030\text{--}1150\text{ cm}^{-1}$ (characteristic saccharide band). Its absorbing capacity was 120 times as much as its own weight without stickiness.

The purified WAP did not include uronic acids mea-

sured by the method of Bitter and Muir,⁴⁾ nor amino sugars by the method of Rundle and Morgan.⁵⁾ The purified WAP (10 mg) was hydrolyzed with 0.75 mol/L sulfuric acid for 4 h at 100°C . The hydrolysate was neutralized with barium carbonate, deionized with Dowex 50W (H^+ form) resin, evaporated to a syrup, and was converted into the corresponding alditol acetate⁶⁾ for gas-liquid chromatography (GLC). Sugar analysis by GLC (column Shimadzu CBP5 $50\text{ m} \times 0.2\text{ mm}$, 220°C , carrier gas N_2) indicated the presence of mannose, glucose, and galactose with the molar ratio 1:3:3. To the best of our knowledge, a polysaccharide having this sugar composition and molar ratio is a new one, so the purified WAP seems to be a new type of polysaccharide.

The physicochemical properties of this polysaccharide WAP were investigated by means of differential scanning calorimetry (DSC). It is known that water-soluble polysaccharides such as schizophyllan or xanthan take stabilized and ordered structures in an aqueous solution undergoing conformational transitions during the process of temperature rising, and indicate characteristic DSC curves.^{7,8)} However, such an order-disorder transition has not been reported for substantially water-insoluble substances such as water-absorbing polysaccharides. We are particularly interested in the possibility whether this WAP has a specific morphology in water and gives a characteristic DSC curve. In addition, it is also expected that such a DSC curve may provide an evidence of homogeneity of substantially water-insoluble polysaccharides which has difficulty in determination. With such interest DSC was carried out for WAP. The WAP purified as mentioned above was suspended in water with the final concentration of 0.25% (w/v) and heated at 40°C for 3.5 h while being stirred, and allowed to stand at ambient temperature for a few days. The transparent sample solution obtained was put in the DSC sample cell and scanned at the heating rate of $0.5^\circ\text{K}/\text{min}$. The DSC curves were measured by using a differential scanning microcalorimeter model MCS DSC from Microcal Inc. (Northampton, MA, USA) attached with a 1.1644 mL sample vessel.

Fig. 1. DSC curves of a 0.25% WAP solution (w/v).

The sample was heated at the rate of $0.5^\circ\text{K}/\text{min}$. Heating and cooling were repeated: (1) 1st scan, (2) 2nd scan, (3) 3rd scan.

Figure 1 shows melting profiles of the polysaccharide obtained during the heating and cooling processes repeated three times. The endotherm in the heating trace corresponds to the transition between the ordered and disordered structures of the polysaccharide, suggesting that this polysaccharide forms a highly ordered structure in aqueous solutions similar to schizophyllan and xanthan, as was previously reported by Kitamura *et al.*^{7,8)} The three DSC curves (1st, 2nd and 3rd) with a peak top temperature of 60.5°C are very similar, indicating that the order-disorder transition of the polysaccharide is reversible, and that this sample is highly purified and homogeneous. A mean of DSC may give an evidence of the homogeneity of the substantially water-insoluble polysaccharide.

The polysaccharide (WAP) obtained in this study absorbs water quickly without growing sticky. These properties are different from those of the acidic polysaccharide reported by Kurane and Nohata,²⁾ which has a high water absorption capacity (more than 1000 times as much as its own weight) and a high viscosity. Structural study and detailed DSC analysis on WAP are under investigation now.

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Oxalobacteraceae 科細菌 IM944 株が生成する新規吸水性多糖の調製とその示差走査熱量測定

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カルボキシメチルセルロースの部分架橋により多量の水を速やかに吸収し、かつ粘稠性を生じない性質が発見した知見に基づき、同様の性質をもつ多糖を菌体外に生産する細菌の検索を行った。そのような菌体外多糖は、特異的な化学構造、あるいは、水中での高次構造をもつことが、また、生分解性の吸水性物質としての応用が期待できる。ツァベック・ドックス培地を用いて探索した結果、土壌より、自重の200倍の吸水能をもつ粗多糖(粗WAP)を生産する1菌株(IM944株)を分離した。IM944株は、形態学的性質、生理・生化学的性質、16S rRNA遺伝子の全塩基配列解析の諸結果より、*Oxalobacteraceae* 科に属する新種と推定された。また、粗WAPを水酸化カリウム水溶液中で、セチルトリメチルアンモニウムブロミドと処理することにより、マンノース、グルコース、ガラクトース(モル比1:3:3)を構成糖とする精製多糖(WAP)を得た。WAPは吸水倍率120倍、窒素含量0.3%の白色、繊維状の粉末で、ウロン酸、アミノ糖は含まれなかった。この多糖は構成糖およびそのモル比から、新規の多糖と推定された。WAPの示差走査熱量測定(DSC)を行った結果(Fig. 1)は、60.5°C付近に頂点のあるピークを示した。数回の昇降温をくり返しても、ピークの形状にほとんど変化がなかったことから、WAPは均質な試料であると判断された。また、WAPのDSC曲線が転移ピークを示したことは、実質的に水不溶性である吸水性多糖においても可溶性多糖と同様に、何らかの高次構造を形成していることを示唆した。DSC法は、均質性の証明が困難な水難溶性多糖の均質性の証明法として有用である可能性が示された。