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Determination of Uronic Acid in the Polysaccharides from the Slime Producing Coryneform Bacteria and Their Immunological Study

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Introduction

Acidic polysaccharides, PS C-1 to C-8, are produced from glucose in more than 50% yields by coryneform bacteria, Strains C-1 to C-8, respectively, which have been isolated from soil and waste of starch factories[11]. These polysaccharides were estimated to be composed of mannose, galactose, glucose, glucuronic acid, pyruvic acid, in the molar ratio of 1: 1: 1: 2.1–3.7: 1[11]. However, the values for uronic acid were not so precise, because estimation of them was made directly in the polymer by the carbazole method[6]. Concerning PS C-8, the whole structure of its repeating unit has been clarified by partial methanolysis, methylation and enzymic analysis[12,13]. It has two characteristic moieties: Mannose is accompanied with pyruvic acid linked to *O*-4 and *O*-6, and galactose is surrounded by three uronic acids (see Fig. 5). The physical properties were also reported by Tako *et al.*[19-21]

Difficulties in constitution analysis of polysaccharides containing uronic acids may probably lie not only in the resistance of glycosiduronic moiety to acid hydrolysis but also in the hardness of the uronic acid to quantitative reduction[2].

In this study, the compositions of these coryneform polysaccharides were confirmed as follows: The polysaccharides were first degraded by methanolysis to give the methyl-glycosides of the sugar constituents. The methyl ester of glycosiduronic acid in the methanolysate was then reduced quantitatively with an aqueous solution of $NaBH_4$ under the pH-controlled conditions. After hydrolysis, the neutral monosaccharides were determined by liquid chromatography. This paper dealed also with the serological identification of PS C-1 to C-8 and estimated a moiety common to these polysaccharides, which was recognized as an antigenic determinant by rabbit anti-PS C-8 serum.

Materials and Methods

Preparation of Coryneform Polysaccharides — Coryneform bacteria, Strain C-8, was cultivated in 3.2 liter of medium containing 3% glucose, 0.25% yeast extract, 0.1% KH_2PO_4 and 0.1% $MgSO_4 \cdot 7H_2O$ in a jar fermenter equipped with a pH controller (pH 6.7), with stirring and aeration at 33°C. Extracellular polysaccharide was harvested from the 68-h broth and precipitated with ethanol to obtain "crude PS C-8" according to the previous method[11]. Glucose in medium was converted to the crude PS in good yield of more than 60%. For further purification, the crude PS solution

was acidified to pH 3 to reduce its viscosity, and was centrifuged (40,000×g, 30 min). After the supernatant was neutralized, the polysaccharide was precipitated with two volumes of ethanol. The resultant precipitate was collected by centrifugation and dissolved in water, and the solution was passed through a column of Amberlite IR 120 (H^+). After dialyzed and neutralized, the purified PS C-8 was obtained as Na^+ form through lyophilization. Other polysaccharides, PS C-1, 2, 4 and 7, were also harvested from the broth and purified by the same method. The yields of crude PS to glucose were 50–80% and those of purified PS to crude PS 37–50%.

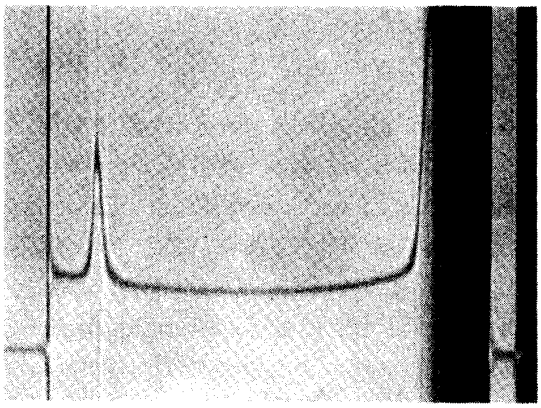

Fig. 1. Ultracentrifugal analysis of coryneform polysaccharide PS C-8.
Ultracentrifugation was performed at 60,000 rpm by a Hitachi UCA-1A at 10.7°C. A concentration of the polysaccharide was 3.3 mg per ml of 0.83% citrate solution. The sedimentation coefficient was obtained to be 1.42×10^{-13} s.

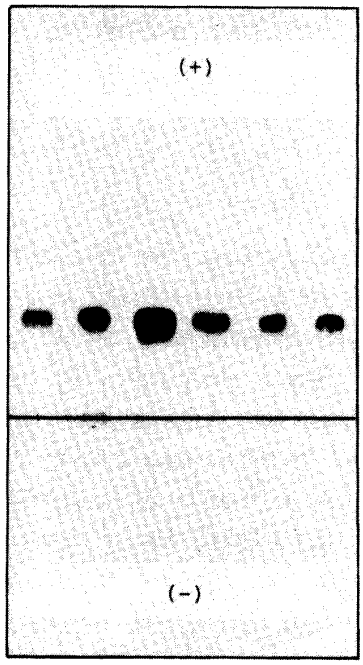

Fig. 2. Cellulose acetate electrophoresis of coryneform polysaccharides.
Electrophoresis was carried out with 1M acetate-pyridine buffer (pH 3.5) in a current of 0.5 mA per cm. After electrophoresis for 20 min, the polysaccharides were stained with 0.5% toluidine blue.

The purified PS C-8 was shown to consist of a single component by ultracentrifugal analysis (Fig. 1). In cellulose acetate electrophoresis with a solvent system of pyridine-acetic acid, each of the purified polysaccharides, as a single band, migrated to the same position of 1.5 cm towards the cathod (Fig. 2). The same result was obtained with another solvent system of calcium acetate[17]. This indicated that all of the coryneform polysaccharides were electrophoretically identical to one another.

Analysis for Sugar Composition — Phosphorus pentaoxide-dried sample (25 mg) was subjected to methanolysis with 5 ml of 1 N HCl in methanol in a sealed tube at 80°C for 24 h and the solution was neutralized with Ag_2CO_3. After removal of methanol by evaporation, the residue was dissolved in water and then an aqueous solution of 2 M $NaBH_4$ (6 ml) was added to the solution dropwise for 1 h at room temperature, retaining the pH to 7.0–7.4 with a pH controlling appearatus. After evaporation to dryness, the methylglycosides were extracted with hot ethanol from the residues, desalted with ion exchange resin, and hydrolyzed in 2 N H_2SO_4 in a sealed tube at a reflux

temperature for 6 h. The hydrolysate was neutralized with $BaCO_3$. The remaining borate was removed by further addition of methanol, followed by repeated evaporations[24].

Quantitative analyses of the neutral sugars in the hydrolysates obtained before and after the reduction were performed by a liquid chromatograph (JEOL JLC-6AS) equipped with a column of JLC R-3, using an elution system of borate buffer.

Glucuronic acid was determined by a modified carbazole method[6].

Pyruvic acid in the polysaccharides was determined spectrophotometrically in acid hydrolysate, using dinitrophenylhydrazine reagent[4].

Preparation of Modified Polysaccharides — Depyruvated PS C-8 was obtained by partial acid hydrolysis in 0.1 N H_2SO_4 at 80°C for 1 h (70% depyruvated) or 5 h (95% depyruvated).

Methyl ester of PS C-8 was prepared with diazomethan[7]: The freeze-dried polysaccharide (1 g) was dissolved in dimethy lsulfoxide (20 ml) with stirring overnight, and methanol (10 ml) was added to the solution. A solution of diazomethan in dioxan was added dropwise until there was no further effervescence. The modified polysaccharide was collected by precipitation with ethanol.

Preparation of Rabbit Anti-PS C-8 Serum — Three rabbits were immunized by means of two times intramuscular injections of the purified PS C-8 (20 mg) in Freund's complete adjuvant, and the succeeding intravascular injection of the polysaccharide (15 mg) in phosphate-buffered saline (PBS) at 2-week intervals. The antisera from two rabbits showed enough titers to carry out the following serological experiments.

Immunological Diffusion Test — Microscale immunodiffusion tests were carried out in a 1% agarose layer by the technique of Ouchterlony[15,23]; the center well was filled with 0.25 ml of undiluted antiserum, and the sample wells were filled with 0.1 ml of polysaccharides (1%, in PBS). The precipitin lines were observed after standing for 2 days at room temperature.

Quantitative Precipitin Test — The quantitative precipitin test of anti-PS C-8 serum (4-fold diluted, 0.4 ml) with various amounts of antigen PS (0.4–10 μg) in PBS was performed in the final volume of 0.8 ml. The reaction mixture was incubated at 37°C for 90 min, and was allowed to stand for 2 days at 4°C. The precipitate was washed twice with ice-cold PBS by centrifugation at 3,500 rpm for 30 min[16,23]. The protein precipitate was determined by the method of Lowry *et al.*[10].

Results and Discussion

Constitution Analysis — Generally, polysaccharides containing uronic acids resist acid hydrolysis, and uronic acids of monomers are very difficult to be quantitatively reduced to the corresponding neutral sugars, even with repeated treatments with $NaBH_4$. However, in the polymer, the uronic acid can be readily reduced, provided that the methyl ester is formed first[1,18]. Therefore, in most of the recent works for determining uronic acids the polysaccharides were methylated prior to the reduction.

In the present study, methanolysis of the polysaccharide was performed first to split the glycosidic bonds linked to the uronic acid residues. Then, the methyl ester of the methyl glycosiduronic acid was quantitatively reduced with an aqueous solution of $NaBH_4$ under the pH-controlled conditions. In our preliminary experiment with the authentic specimens, more than 95% of methyl ester of methyl glucosiduronic acid was reduced under the condition as shown in Table I. Molar ratio of uronic acid can be estimated on the basis of the increment of the corresponding neutral sugar after the reduction (Table II).

Table I. Reduction of methyl ester of methyl glucosiduronic acid with an aqueous solution of $NaBH_4$ under pH-controlled conditions

$NaBH_4$	Time	pH Adjust	pH	Reduction
0.5 g	15 min	No	9.8–10	84%
0.5 g	18 h	No	10.8	90%
0.5 g	15 min	Yes	7.0–7.4	97%
0.7 g	1 h	Yes	7.0–7.4	98%

The amount of the sugar analyzed is 4.3 mg in all the experiments. "Reduction" was calculated from the remaining glucuronic acid determined by the modified carbazole method[6].

Table II. Chemical composition of the coryneform polysaccharides

PS	Man	Gal	Glc[a]	Glc[b]	GlcUA[c]	PyrA[d]
C–1	1.09	1.00	0.87	4.01	3.14	1.39
C–2	1.16	1.00	0.93	4.08	3.15	1.40
C–4	1.15	1.00	0.91	3.95	3.04	1.43
C–7	1.14	1.00	0.95	4.10	3.15	1.45
C–8	1.17	1.00	0.95	4.01	3.06	1.29

Glucose was determined in a) methanolysate and b) the reduced methanolysate by liquid chromatography. c): The values were calculated by subtracting a) from b). d): This was determined in acid hydrolysates of the polysaccharides by the dinitrophenylhydrazine method[4].

Dutton *et al.* reported a similar method for determining uronic acid content, but did not describe the extent of the reduction[5]. By the present method for maintaining the pH neutral during the reaction period, the quantitative reduction of uronic acid was achieved, even without an additive reagent of carbodiimide[22].

Pyruvic acid is usually to be released from the saccharide at the step of hydrolysis. The pyruvate content determined spectrophotometrically was also represented in Table II. The polysaccharides from the coryneform bacterial strains were confirmed to be all equal in chemical compositions, Man: Gal: Glc: GlcUA: PyrA=1: 1: 1: 3: 1.

Production of Anti-PS C–8 Serum — The production of antiserum to pure polysaccharide, especially in rabbits, is not an easy matter even with the aid of an adjuvant, and the titers obtained, if any, are usually quite low[16,23]. In this experiment, the two rabbits among three, immunized with electrophoretically homogeneous PS C–8, were found to yield anti-PS C–8 serum with about 500 μg of precipitable protein per milliliter.

Immunological Identification of Coryneform Polysaccharides — As described above, the coryneform polysaccharides, C–1 to C–8, were proved to have the same compositions of sugars and pyruvic acid. Then, the authors examined whether these polysaccharides could be serologically descriminated from one another. In a double diffusion test of anti-PS C–8 serum with the antigen PS C–8, two lines were observed: and outside line towards the sample well (polysaccharide) and a broader inside line towards the center well (antiserum), as shown on (A) of Fig. 3. Schiffman described that immunodiffusion against antiserum made to crude polysaccharide would often give multiple bands[16]. However, PS C–8 used as antigen appeared homogeneous by the criteria of

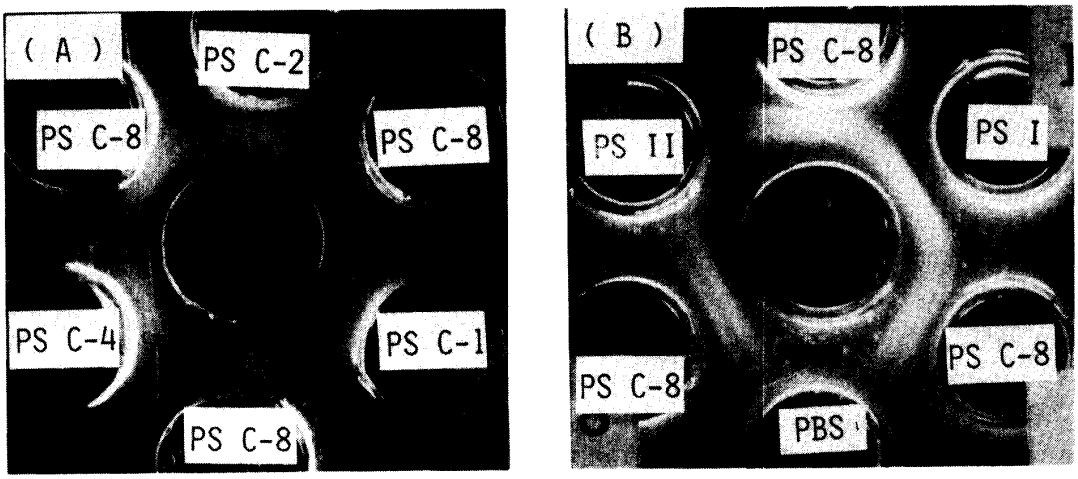

Fig. 3. Double diffusion test of anti-PS C-8 serum (center well) with (A) coryneform polysaccharides (C-1, 2 and 4), and (B) *E. coli* polysaccharides (PS I and PS II) besides the original antigen PS C-8. A concentration of each polysaccharide was 10 mg per ml of PBS.

ultracentrifugation and electrophoresis (Figs. 1-2), as has already been mentioned. The same result was obtained when the polysaccharide was treated with "pronase" digestion[14].

The coryneform polysaccharides other than PS C-8 also formed two diffusional lines which fused themselves to the respective lines of PS C-8 (Fig. 3), although the outer lines of them were

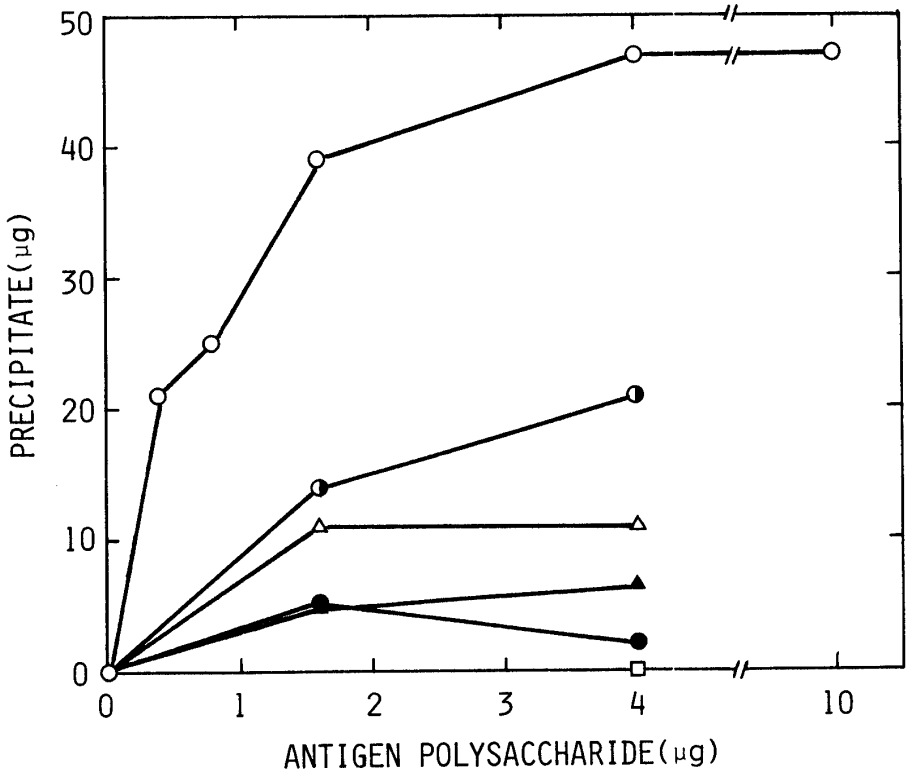

Fig. 4. Quantitative precipitin test of anti-PS C-8 serum with the intact and the modified PS C-8, and *E. coli* polysaccharides, PS I and PS II.

The experimental conditions are described in "Materials and Methods". ○, intact PS C-8; ◑, 70% depyruvated PS C-8; ●, 95% depyruvated PS C-8; △, PS C-7; ▲, PS I; □, PS II and methyl ester of PS C-8

very weak. In the quantitative precipitin tests, 10 μg of precipitate was determined in the reaction of the serum with PS C-7, while about 50 μg of precipitate was yielded in the reaction with PS C-8 (Fig. 4).

The alternate arrangement of the reaction was also examined with the anti-PS C-7 serum obtained from a rabbit sensitized by purified PS C-7. Besides PS C-7, PS C-8 (4 μg) was reacted with the antiserum to form the specific precipitate, the amount (60 μg) of which was even larger than that (25 μg) for the original antigen PS C-7. The authors have no explanation for this intriguing results. Anyhow, the above serological experiments revealed that all these coryneform polysacchrides can be taken as serologically close to one another as well as chemically.

Estimation of Immunological Determinant in PS C-8 — A moiety of polymer that contributes most to the antigenic specificity is termed antigenic determinant. In polysaccharide antigen, the determinant usually repeats along the polysaccharide chain. The antigenic specificity may also be due to noncarbohydrate substituents such as pyruvic acid[3]. Then, the authors tried to examine the anitgenic determinant for the anti-PS C-8 serum, using modified PS C-8 and other polysaccharides, the sugar components of which are similar to those of PS C-8.

In quantitative precipitin test of anti-PS C-8 serum with depyruvated PS C-8, 70% depyruvated PS C-8 yielded the precipitate of 24 μg, which is about half for the intact PS C-8, and 95% depyruvated PS C-8 gave a small amount of precipitate (Fig. 4). This indicates that pyruvic acid in PS C-8 plays an important role in antigenic specificity. No precipitate was observed in the reaction of the serum with the methyl ester of PS C-8, in which both the carboxyl groups of uronic and pyruvic acids were masked (Fig. 4). Uronic acid would also participate in the serological reactions to some extent.

The following commercially obtainable polysaccharides did not react with anti-PS C-8 serum in both the tests of immunodiffusion and quantitative precipitin: chondroitin sulfate, hyaluronic acid, arabic gum, tragacanth gum, pectic acid, alginic acid, dextran, yeast mannan and konjakmannan.

The polysaccharides, PS I and II, produced by *E. coli* 36 M and 72 M, respectively, have the same components of sugars and pyruvic acid as the coryneform polysaccharides, though the compositions and the sequence are different[8,9] (Fig. 5). Both saccharides are composed of mannose,

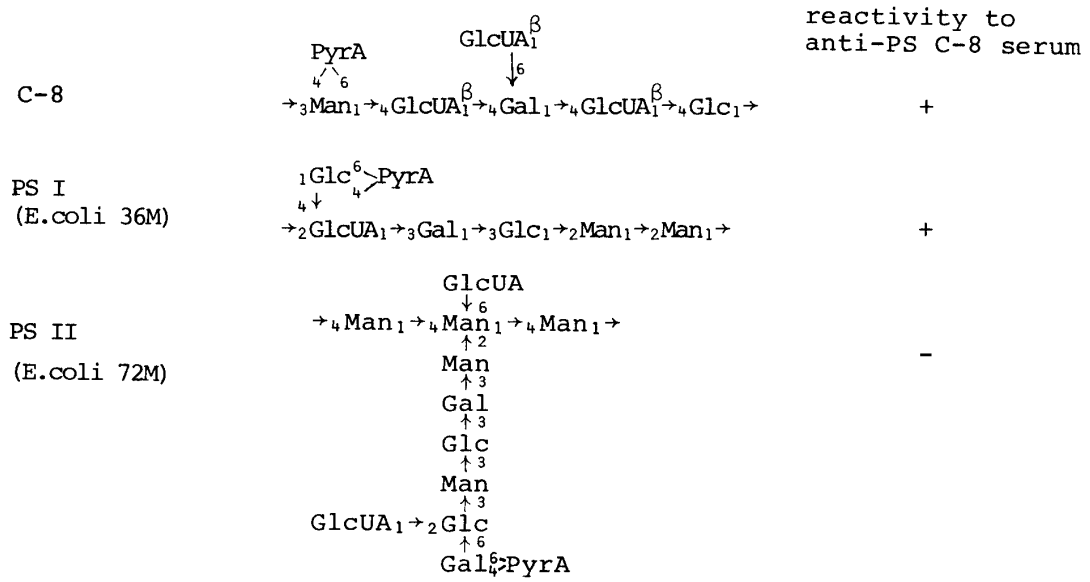

Fig. 5. Structures of PS C-8 and *E. coli* polysaccharides, PS I and PS II.

galactose, glucose, glucuronic acid and pyruvic acid. The pyruvic acid is linked to the terminal sugar of a branch chain in a ketal form. Nevertheless, PS I was reactive to anti-PS C-8 serum, whereas PS II was not, in the immunological tests (Figs. 3 and 4).

The repeating structure of PS C-8 has been clarified by methylation analyses of the intact polysaccharide and of its fragments obtained by a partial methanolysis[13], as shown in Fig. 5. PS C-8 has a structure comprised of pyruvic acid ketal-linked to mannose *on the main chain*, which differs from the *E. coli* polysaccharides. The immunological experiments with the modified PS C-8 suggested the important role of acidic moieties of PS C-8, especially pyruvic acid, in the antigen-antibody reaction. In both reactive polysaccharides, PS C-8 and PS I, we can see a common moiety: an uronic acid neighboring a sugar (X) having a pyruvic acid ($\overset{\text{PyrA}}{\overset{\|}{\text{X}}}$–GlcUA). This moiety seems to be a likely immunological determinant against the anti-PS C-8 serum. On the other hand, PS II, non-reactive to the antiserum, does not have the common structure, though it contains pyruvic and uronic acids.

Summary

The acidic polysaccharides, PS C-1, 2, 4, 7 and 8, produced by coryneform bacteria Strains C-1, 2, 4, 7 and 8, respectively, were studied chemically and serologically. For accurate determination of the sugar compositions of them involving uronic acid, the respective polysaccharides were degraded into monomers by methanolysis, then were treated with $NaBH_4$ and hydrolyzed. The quantitative (98%) reduction of the methyl ester of glucosiduronic acid in the methanolysate was achieved with an aqueous solution of $NaBH_4$ maintaining the pH neutral. Thereby, all sugar constituents of the polysaccharides could be determined as neutral, reducing sugars by liquid chromatogarphy. Thus, the chemical compositions of all the polysaccharides were confirmed to be in the same molar ratio of Man: Gal: Glc: GlcUA: PyrA=1: 1: 1: 3: 1.

On the other hand, a rabbit antiserum to purified PS C-8 was prepared with Freund's adjuvant. On double diffusion tests of the serum, the coryneform polysaccharides formed two precipitin lines and the respective lines fused with one another, in other words, the polysaccharides could not be serologically discriminated from one another. Then, the quantitative precipitin reactions with the intact and the modified polysaccharides of PS C-8 indicated that acidic moieties, pyruvic acid and uronic acid of the polysaccharide play some important role in antigenic determinant. Basing on the results of experiments with similar polysaccharides produced from *E. coli* 36 M and 72 M, the determinant was estimated to be a moiety of uronic acid neighboring mannose linked to pyruvic acid (–$\overset{\text{PyrA}}{\overset{\|}{\text{Man}}}$–GlcUA–) in the coryneform polysaccharides.

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