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Structure of Extracellular Polysaccharides of *Escherichia coli* Strains 36M,[1] 72M and 29M isolated from Coligranuloma of Chick Intestine. II. Polysaccharide from *E. coli* 29M

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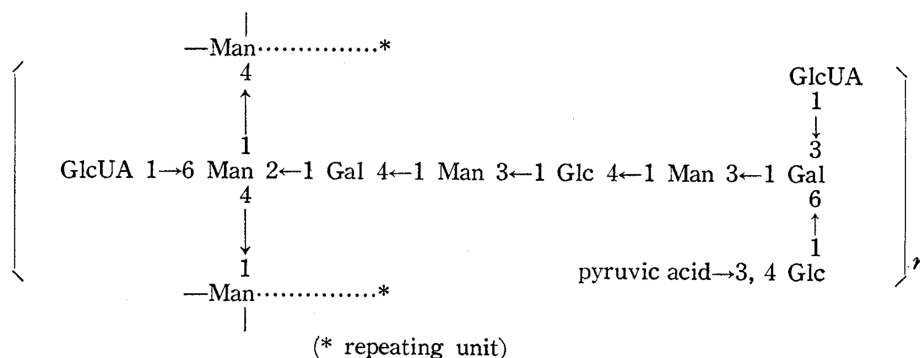
An extracellular polysaccharide (referred to as Ps-III), which showed a positive immune activity to anti-Ps-III-sera obtained from a rabbit immunized with *Escherichia coli* 29M organisms, was isolated from the culture broth of *E. coli* 29M.

By methylation, Smith degradation, partial acid hydrolysis and methanolysis of Ps-III, its repeating structure was postulated as given below:



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Keywords——structure of extracellular polysaccharide; *Escherichia coli*, isolated from chick intestine; polysaccharide, with immune activity; highly branched polysaccharide; *Escherichia coli*, produced type-specific polysaccharide

Ogawa *et al.*[3] isolated from a coligranuloma of the chick intestine three types of *Escherichia coli* (termed 36M, 72M and 29M) which produced antigenic, type-specific extracellular polysaccharides (referred to as Ps-I, Ps-II and Ps-III, respectively) with immune activity to each antisera from rabbits immunized with each of *E. coli* organisms.

The purpose of this work is to elucidate the structural difference among these three kinds of extracellular polysaccharides. In the previous paper,[4] we reported the postulated structure of Ps-I from *E. coli* 36M. This paper is a study on the structure of extracellular polysaccharide of *E. coli* 29M.

Experimental

Growth of *Escherichia coli* 29M——The organisms were grown in liquid culture at 37° in the growth medium described previously.[4a)]

1) A. Kamei, N. Takeuchi, S. Akashi, and K. Kagabe, *J. Biochem.*, **83**, 1009 (1978).
2) Location: *Yagoto, Tenpaku-ku, Nagoya, Aichi, 468, Japan.*
3) *a*) T. Ogawa and N. Onaka, *Nagoya Med. J.*, **4**, 4 (1944); *b*) T. Ogawa, N. Onaka, and T. Suzuki, *Nagoya Med. J.*, **5**, 1 (1944); *c*) T. Ogawa, *Nagoya Med. J.*, **12**, 1284 (1952).
4) *a*) A. Kamei, K. Nakazawa, N. Takeuchi, S. Akashi, and K. Kagabe, *J. Biochem.*, **82**, 599 (1977); *b*) A. Kamei, N. Takeuchi, S. Akashi, and K. Kagabe, *J. Biochem.*, **83**, 1009 (1978).

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Analytical Procedures——Total sugar was determined by tryptophan-H_2SO_4 method,[5] hexose by anthrone-H_2SO_4 method,[6] and glucuronic acid by carbazole-H_2SO_4 method.[7] Pyruvic acid was detected by the method of Nogare *et al.*[8]

A reducing-end hexose and the molar ratios of component sugars in oligosaccharides were determined by gas chromatography and/or gas chromatography–mass spectroscopy as described previously.[9]

Isolation of Extracellular Polysaccharide——The procedure for extraction and purification of the extracellular polysaccharide was essentially the same as described previously.[4a] It is briefly outlined: cells were removed from the culture broth of *E. coli* 29M by centrifugation followed by filtration, and the resulting filtrate was dialyzed against running water. The dialyzed solution was concentrated to about 0.02 volume. To this solution $(NH_4)_2SO_4$ was added to give 100% saturation. The precipitate formed was removed by centrifugation and the supernatant was dialyzed against running water. After concentration, the solution was treated with pronase[10] and deproteinized according to the Sevag's procedure.[11] The resulting solution, after dialysis, was concentrated and the polysaccharides were precipitated from the syrup with ethanol. The polysaccharide was purified by chromatography on a Sephadex G-150 column (7×90 cm) with 0.5 M NaCl as eluant. Fractions of 20 ml were collected and tubes 21—33 were combined. Then, the fraction was submitted to a DEAE-cellulose column (1.5×20 cm) (OH^-) and the column was eluted with a linear gradient of NaCl (0.1—2.0 M in 0.001 M HCl). The final yield of antigenic extracellular polysaccharide was approximately 100—150 mg from 10 liters of culture broth. This substance with immune activity is referred to as Ps-III.

Paper Chromatography, Thin-Layer Chromatography and Paper Electrophoresis——Ascending paper chromatography and paper electrophoresis were carried out on Whatman 3MM or Toyo No. 51A filter paper in the same solvent systems and the buffer solutions as those described previously.[4a] Sugars were detected on chromatograms and electrophoretograms with alkaline silver nitrate[12] or aniline hydrogen phthalate.[13]

Gas Chromatography——Sugars and products of Smith degradation and methylation were analyzed with a Shimadzu 5A gas chromatograph equipped with digital integrator, ITG-4A, and flame ionization detector with glass columns as described previously.[4a]

Acid Hydrolysis——For complete acid hydrolysis, the material was treated with 0.5 M H_2SO_4 at 100° for 5—42 hr, and partial acid hydrolysis to prepare oligosaccharides from Ps-III was conducted in 0.5 M H_2SO_4 for 45 min. The sugars liberated by the complete or partial acid hydrolysis were analyzed by paper chromatography, gas chromatography and/or mass fragmentography after removing the acid in the hydrolyzates by precipitation with $BaCO_3$.

Methylation of Ps-III——Methylation was performed on Ps-III (50—100 mg) by Hakomori's method,[14] carboxy-reduction of the permethylated Ps-III with $LiAlH_4$ by the method of Sandford *et al.*,[15] and hydrolysis of the permethylated Ps-III with H_2SO_4 by the method of Garegg *et al.*[16] De-*O*-methylation of methylated monosaccharides was carried out by the method of Bonner *et al.*[17]

Gel Filtration Chromatography——Chromatography of Ps-III was performed on a Sephadex G-150 column (7×90 cm) using 0.5 M NaCl. Products of acid hydrolysis and Smith degradation were separated by chromatography on a Sephadex G-25 column (2.6×133 cm) and on a Sephadex G-15 column (2.6×130 cm) using 1 M NaCl as eluant.

Periodate Oxidation and Smith Degradation of Ps-III——About 50 mg of Ps-III was dissolved in 50 ml of 0.04 M $NaIO_4$ and periodate consumption at 4° in the dark was measured until no further consumption occurred (about 120 hr),[18] then a few drops of ethylene glycol were added to the reaction mixture and it was dialyzed against running water to remove sodium iodate. The products were reduced with $NaBH_4$ by the method of Smith *et al.*[19] The reaction was terminated by the addition of ice-cold 4 M acetic acid to the reaction mixture and the solution was dialyzed and lyophilized. Yield, 42.4 mg. Portions of 3 mg of lyophilized sample were hydrolyzed with 0.1 M HCl for the controlled Smith degradation or 0.5 M H_2SO_4 for complete

5) J. Badim, C. Jackson, and M. Schubert, *Proc. Soc. Exp. Bio. Med.*, **84**, 288 (1953).
6) W.E. Trevelyan and J.S. Harrison, *Biochem. J.*, **50**, 298 (1952).
7) T. Bitter and H.M. Muir, *Anal. Biochem.*, **4**, 330 (1962).
8) S.D. Nogare, T.O. Norris, and J. Nitchell, *Anal. Chem.*, **23**, 1473 (1951).
9) A. Kamei, H. Yoshizumi, S. Akashi, and K. Kagabe, *Chem. Pharm. Bull.* (Tokyo), **24**, 1108 (1976).
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11) M.G. Sevag, *Biochem. Z.*, **273**, 419 (1934).
12) W.E. Trevelyan, D.P. Procter, and J.S. Harrison, *Nature* (London), **166**, 443 (1950).
13) S. Partrige, *Nature* (London), **164**, 443 (1949).
14) S. Hakomori, *J. Biochem.*, **55**, 205 (1964).
15) A. Sandford and H.E. Conrad, *Biochemistry*, **5**, 1508 (1966).
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17) T.G. Bonner, E.K. Bourne, and S. McNally, *J. Chem. Soc.*, **1960**, 2929.
18) P.F. Fkury and J. Lange, *J. Pharm. Chem.*, **17**, 107 (1933).
19) F. Smith and J.W. Van Cleve, *J. Am. Chem. Soc.*, **77**, 3091 (1955).

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hydrolysis to analyze sugars which were not oxidized by periodate. a) The controlled Smith degradation: hydrolysis was carried out by placing the sample solution in 0.1 M HCl at room temperature overnight, and then the solution was treated with NH_2OH–HCl according to the method of Yamaguchi *et al.*[20] The solution was evaporated to dryness under reduced pressure. The hydrolyzate was converted into trimethylsilylate by the general method,[21] and then its derivative was submitted to gas chromatography using 5% SE-30 column (80—100 mesh Chromosorb W, 0.3 × 200 cm) for analysis of the controlled Smith degradation products. b) Complete hydrolysis: hydrolysis was performed with 0.5 M H_2SO_4 at 100° for 24 hr. A portion of the hydrolyzate was reduced with $NaBH_4$ and then the boric acid was removed by treating with methanol. The resulting solution was dried by evaporation under reduced pressure. The residue was dissolved in 1 ml of pyridine and 1 ml of acetic anhydride for acetylation, and mixed solution was heated at 100° for 5 hr. Alditol acetates obtained were analyzed by gas chromatography using column of 3% ECNSS-M (100—120 mesh Gas Chrom Q, 0.3 × 300 cm). The other portion of the hydrolyzate was treated with NH_2OH–HCl according to the method of Yamaguchi *et al.*,[20] and then converted into trimethylsilylate.[21] The resulting derivatives were analyzed by gas chromatography using the column described above.

Preparation of Pyruvic Acid-carrying Sugar and Ketal Exchange Reaction of Permethylated Ps-III with Acetone——The experimental procedures for the isolation and characterization of pyruvic acid from Ps-III, pyruvic acid-carrying sugar and the conditions for ketal exchange reaction were described in detail in the previous paper.[4a)]

Biological Assay——Immune activity of Ps-III to anti-Ps-III-sera obtained from rabbits was measured according to the method of Williams *et al.*[22] The precipitate formed by antigen–antibody reaction was dissolved in 1% Na_2CO_3 solution and the protein content was measured at 280 nm.

Results and Discussion

Isolation and Composition of Ps-III

The extracellular polysaccharide obtained from the culture broth was separated into two major fractions on Sephadex G-150 column as shown in Fig. 1. The component, which had high molecular weights and was partially excluded from the gel (Fraction 1), comprised

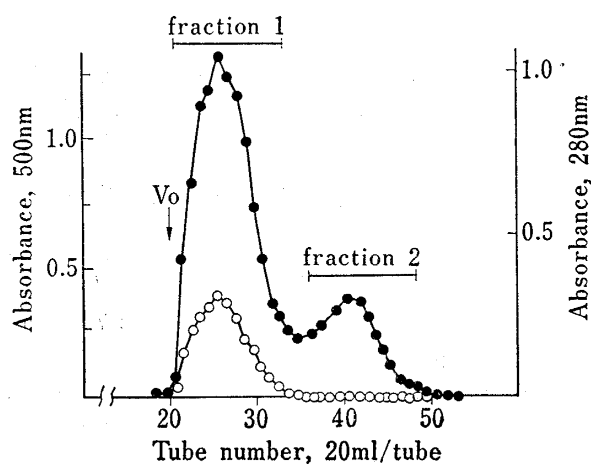

Fig. 1. Fractionation of Extracellular Polysaccharide on a Sephadex G-150

A 500 mg of extracellular polysaccharide was chromatographed on a column (7 × 90 cm) of Sephadex G-150 with 0.5 M NaCl as eluant. Fractions of 20 ml were collected. Tubes 21—33 were pooled. Carbohydrates in each fraction were analyzed by tryptophan-H_2SO_4 method. —●—: Absorbance at 500 nm in tryptophan-H_2SO_4 reaction; —○—: Absorbance at 280 nm of protein precipitated by specific precipitin test (see text in details).

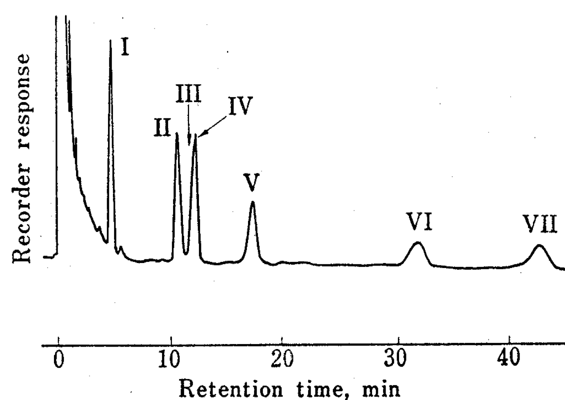

Fig. 2. Gas Chromatographic Analysis of partially Methylated Alditol Acetates prepared from Carboxy-reduced, Re-methylated Ps-III

Gas chromatography was performed with column 1 (see text for conditions). Table II represents the numbered peaks identified.

20) H. Yamaguchi, T. Ikenaka, and Y. Matsushima, *J. Biochem.*, **63**, 553 (1968).
21) C.C. Sweeley, R. Bentley, M. Makita, and W.W. Wells, *J. Am. Chem. Soc.*, **85**, 2479 (1963).
22) C.A. Williams and M.W. Chase, "Method in Immunology and Immunochemistry," Vol. II, Academic Press, New York–London, 1968, p. 349.

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Table I. Chemical and Physical Properties of Ps-III

Molar ratio of constituents[a)]	D-GlcUA	D-Man	D-Glc	D-Gal	Pyruvic acid
	1.00	1.50	1.01	0.99	0.47
$[\alpha]_D$	+1.52° (0.46% in water)				
ORD	(+) Simple curve (0.5% in water)				
pK_a	3.7 (measured by titration with 0.02 M NaOH)				

a) Molar ratios to glucuronic acid.

Table II. Gas Chromatographic Analysis of partially Methylated Alditol Acetates derived from Permethylated Ps-III (A) and Its Carboxy-reduced, Remethylated Product (B)

Peaks	Relative retention time[a)]		Mol % of toal methylated product		Tentative assignment[b)]
	(A)[c)]	(B)[d)]	(A)[c)]	(B)[d)]	
I	1.00	1.00	1.5	23.1	1,5-Di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-glucitol
II	2.04	2.04	28.9	22.3	1,3,5-Tri-*O*-acetyl-2,4,6-tri-*O*-methyl-mannitol
III	2.38	2.38	13.9	11.2	1,4,5-Tri-*O*-acetyl-2,3,6-tri-*O*-methyl-galactitol
IV	2.45	2.45	13.9	11.2	1,4,5-Tri-*O*-acetyl-2,3,6-tri-*O*-methyl-glucitol
V	3.65	3.65	12.8	9.9	1,3,4,5-Tetra-*O*-acetyl-2,6-di-*O*-metyl-glucitol
VI	6.45	6.45	14.7	11.4	1,3,5,6-Tetra-*O*-acetyl-2,4-di-*O*-methyl-galactitol
VII	8.39	8.39	14.3	10.9	1,2,4,5,6-Penta-*O*-acetyl-3-*O*-methyl-mannitol

a) Relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol.
b) Based on the results of gas-chromatographic analyses with columns 1 and 2, for details see text.
c) (A), permethylated Ps-III.
d) (B), carboxy-reduced and then remethylated Ps-III.

about 70% of the total extracellular polysaccharide, and had a positive immune activity to the rabbit antisera against Ps-III. Since the substance with lower molecular weight consisted of hexose only (mainly mannose) and had no immune activity, this fraction was not studied. Fraction 1 was further purified by chromatography on the same column followed by DEAE-cellulose chromatography with a linear gradient of NaCl. A single peak, tryptophan-H_2SO_4-reactive, was eluted at the position of 0.35 M NaCl concentration. Purified fraction 1 (referred to as Ps-III) showed a single band with a mobility of 4.5 cm/40 min (1 mA/cm) on cellulose acetate paper electrophoresis (pyridine–acetic acid–water, 8: 71: 921, v/v. pH 3.5). Staining was carried out with 3% alciane blue.

Table I summarized the chemical and physical properties of Ps-III. Ps-III was an acidic polysaccharide containing pyruvic acid and glucuronic acid. Its optical rotatory dispersion showed a positive simple curve. Acid hydrolysis of the material resulted in a progressive decrease in $[\alpha]_D$ value with the time of hydrolysis. Therefore, Ps-III is a polysaccharide containing an α-D-glycosidic linkage.

Methylation Analysis of Ps-III

Methylated monosaccharides derived from permethylated Ps-III and its carboxy-reduced, remethylated product were analyzed by gas chromatography (column 1: 3% ECNSS–M on 100—120 mesh Gas Chrom Q, 0.3×300 cm; column 2: 15% butane-1,4-diol-succinate polyester on 80—100 mesh Celite, 0.3×200 cm). Table II and Fig. 2 show the results of methylation analysis of Ps-III with column 1. By gas chromatographic analysis of permethylated Ps-III and its carboxy-reduced product using column 2, the following peaks were identified from each material: permethylated Ps-III, 2,3,6-tri-*O*-methyl-D-galactose (t_R*, 3.26); 2,4,6-tri-*O*-methyl-D-mannose (t_R*, 4.18); 2,3,6-tri-*O*-methyl-D-glucose (t_R*, 4.46); 2,3,4-tri-*O*-methyl-

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D-glucuronic acid methyl ester (t_R*, 2.46 and 3.26); unidentified peak (t_R*, 8.77); carboxy-reduced, remethylated permethylated Ps-III, 2,3,4,6-tetra-*O*-methyl-D-glucose (t_R*, 1.00 and 1.43); 2,3,6-tri-*O*-methyl-D-galactose (t_R*, 3.26); 2,4,6-tri-*O*-methyl-D-mannose (t_R*, 4.18); 2,3,6-tri-*O*-methyl-D-glucose (t_R*, 4.46); unidentified peak (t_R*, 8.77). t_R* was relative to methyl-2,3,4,6-tetra-*O*-methyl-β-D-glucopyranoside. Each peak was identified by co-chromatography with authentic compounds and by a comparison with the retention times reported by Aspinall[23] and Björndal *et al.*[24] Monomethylated sugar, corresponding to peak VII on gas chromatogram was obtained from hydrolyzate of permethylated Ps-III by paper chromatography (R_G, 0.42, relative to 2,3,4,6-tetra-*O*-methyl-α-D-glucose; solvent, *n*-butanol–ethanol–water–28% ammonia, 40: 10: 49: 1, v/v),[25] and identified by de-*O*-methylation and periodate oxidation reaction. The compositions were calculated from individual peak areas of the gas chromatographic tracing with an integrator coupled to the gas chromatograph apparatus. As seen in Table II, peak I obtained from carboxy-reduced, remethylated Ps-III was characterized as alditol derived from 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucopyranose by comparing its retention time with that of the authentic sample. The 2,3,4,6-tetra-*O*-methyl-D-glucose originated in glucuronic acid residues in the polysaccharide. The glucuronic acid should be non-reducing terminals. These results show that the polysaccharide is composed of 1,3- and 1,2,4,6-linked mannose, 1,4- and 1,3,4-linked glucose, 1,4- and 1,3,6-linked galactose and 1-linked glucuronic acid.

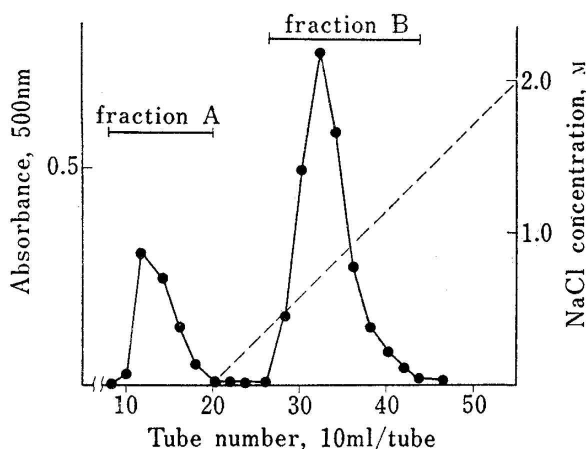

Fig. 3. Fractionation of Oligosaccharides obtained from Ps-III by Partial Acid Hydrolysis

A 500 mg of Ps-III was hydrolyzed as described under "Experimental Procedures" and the hydrolyzate was applied to a column (2×20 cm) of DEAE-cellulose (OH^-) in water. The column was first eluted with 200 ml of water and then with a linear gradient of NaCl (0.1—2.0 M) in 1/1000 M HCl. Tubes 10—20 (fraction A) and 29—43 (fraction B) were separately pooled for further analysis.

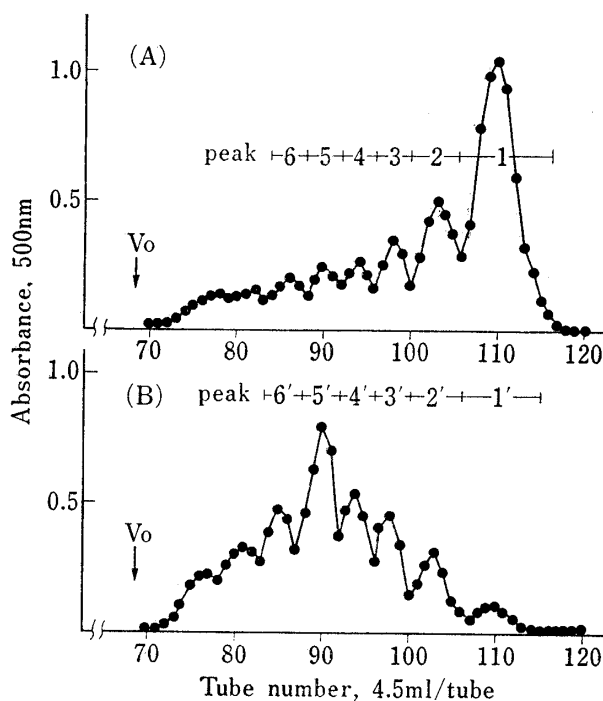

Fig. 4. Sephadex G-25 Chromatography of Fractions A and B obtained by DEAE-cellulose Chromatography (*cf.* Fig. 3)

Each fraction was chromatographed on a column (2.6×133 cm) of Sephadex G-25 with 1 M NaCl as eluant. Carbohydrate in each fraction was detected by tryptophan-H_2SO_4 method. Panel A: elution pattern of fraction A; Panel B: elution pattern of fraction B.

23) G.O. Aspinall, *J. Chem. Soc.*, **1963**, 1676.
24) H. Björndal, B. Lindenberg, and S. Svensson, *Acta Chem. Scand.*, 21, 1801 (1967).
25) E.L. Hirst, L. Hough, and J.K.N. Jones, *J. Chem. Soc.*, **1949**, 928.

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Preparation of Oligosaccharides from Ps-III by Partial Acid Hydrolysis and Their Compositions

The partial acid hydrolyzate of Ps-III yielded two fractions when applied to DEAE-cellulose column and the column was eluted with a linear gradient of NaCl (Fig. 3). Fractions A and B were separately desalted on a Sephadex G-10 column (1.5×100 cm), and submitted to gel filtration on a Sephadex G-25 column. Fig. 4 shows the elution profiles of fractions A and B. Six main peaks were obtained from both fractions. The peak fractions were collected, desalted, concentrated and purified by rechromatography on the same column, then by paper chromatography (pyridine–isoamylalcohol–benzene–water ,5: 5: 3: 2, v/v, for the oligosaccharides from fraction A; pyridine–isoamylalcohol–water, 7: 7: 6, v/v, for those from fraction B), and by high-voltage paper electrophoresis (0.05 M sodium borate, pH 9.2,

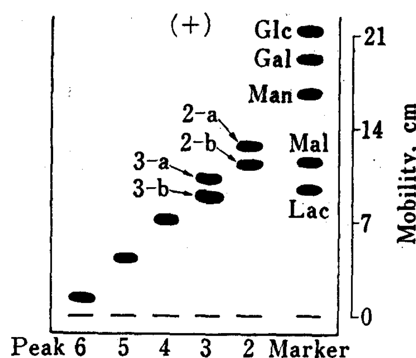

Fig. 5-A. Purification of Each Peak Fraction of Gel Filtration (*cf.* Fig. 4-A) by High-Voltage Paper Electrophoresis

Electrophoresis was performed in 0.05 M sodium borate, pH 9.2. Guide strips were cut out and stained with $AgNO_3$-reagent. $AgNO_3$-reacting zones were eluted from the remainder of the strips with water for further analysis.

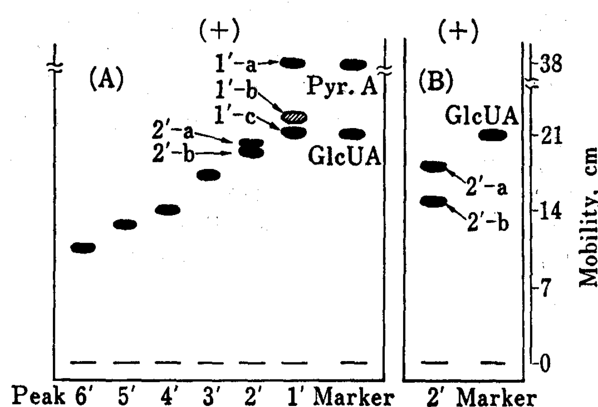

Fig. 5-B. Purification of Each Peak Fraction of Gel Filtration (*cf.* Fig. 4-B) by High-Voltage Paper Electrophoresis

Panel A: electrophoretogram of each fraction in pyridine-acetic acid–water, 5: 0.2: 95, v/v, pH 6.0; Panel B: electrophoretogram of peak 2′ in 0.05 M sodium borate, pH 9.2.

Table III. Quantitative Analyses of Products from Fractions A and B by Partial Acid Hydrolysis

Samples	mol % of of constituents				
	Glc	Gal	Man	GlcUA	Pyruvic acid
Fraction A					
Peak 2–a	50.8	0	49.2[a]	0	0
Peak 2–b	49.7[a]	0	50.3	0	0
Peak 3–a	32.6	0	67.4[a]	0	0
Peak 3–b	33.1	32.9	34.0[a]	0	0
Peak 4	24.6	25.1	50.3[a]	0	0
Peak 5	39.6	19.8	40.6[a]	0	0
Peak 6	33.5	32.9[a]	33.6	0	0
Fraction B					
Peak 1′–b	53.1[a]	0	0	0	46.9
Peak 2′–a	0	49.1[a]	0	50.9	0
Peak 2′–b	0	0	49.8[a]	50.2	0
Peak 3′	34.1	33.2[a]	0	32.7	0
Peak 4′	25.3	24.9	25.3[a]	24.5	0
Peak 5′	19.3	20.8	40.8[a]	20.1	0
Peak 6′	33.3	16.3	33.1[a]	17.3	0

a) Underlined sugars are reducing-end hexoses in each oligosaccharide.

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45—50 volts/cm, for the oligosaccharides from fraction A; pyridine–acetic acid–water, 5: 0.2: 95, v/v, pH 6.0, 50—65 volts/cm, for those from fraction B). Figure 5 shows electrophoretograms by high-voltage paper electrophoresis in which peaks-2, -3, -1′ and -2′ obtained by gel filtration (*cf.* Fig. 4) were separated into two (peaks 2-a and 2-b), two (peaks 3-a and 3-b), three (peaks 1′-a, 1′-b and 1′-c) and two (peaks 2′-a and 2′-b) fractions, respectively. Peaks 1′-a and 1′-c were identified to be pyruvic acid (R_G, 1.21) and glucuronic acid (R_G, 0.31 and 1.52 as lactone form) (R_G represents mobility relative to glucose), respectively, by thin layer chromatography (on Avicel SF, solvent: ethylacetate–pyridine–acetic acid–water, 5: 5: 1: 3, v/v). Table III shows the analytical data on the sugar composition and reducing terminal sugar of the resulting oligosaccharides. The molecular sizes of each oligosaccharide were estimated from its *K*av value by comparing with that of mono- (glucose), di-(maltose) and trisaccharide (melezitose) of authentic standards. The higher oligosaccharides over peaks 6 and 6′ were not analyzed.

Pyruvic acid-carrying sugar was obtained from peak 1′-b. The substance obtained from peak 1′-b consisted of equimolar amounts of pyruvic acid and glucose. These results suggest that the pyruvic acid-carrying sugar is glucose.

Periodate Oxidation and Smith Degradation of Ps-III

Ps-III consumed 0.72 mol of periodate per mol of hexose (as glucose, measured by tryptophan-H_2SO_4 method) and produced 0.23 mol of formic acid per mol of hexose. The uptake was completed within 120 hr.

The products obtained by the controlled Smith degradation of Ps-III was submitted to gel filtration on a Sephadex G-15 column, where three fractions positive in tryptophan-H_2SO_4 reaction were eluted as shown in Fig. 6. Fraction I was polymer consisting of mannose only. Fraction II was eluted in the position expected for trisaccharide (melzitose) and was composed of glucose, galactose and mannose as sugar in a molar ratio of 0.98: 1.02: 1.00. Fraction III was eluted in the position expected for monosaccharide (glucose) and mannose only as sugar was detected from the fraction. Analysis of sugar in each fraction was carried out by gas chromatography using a column of 3% ECNSS–M (100—120 mesh Gas Chrom Q, 0.3×300 cm).

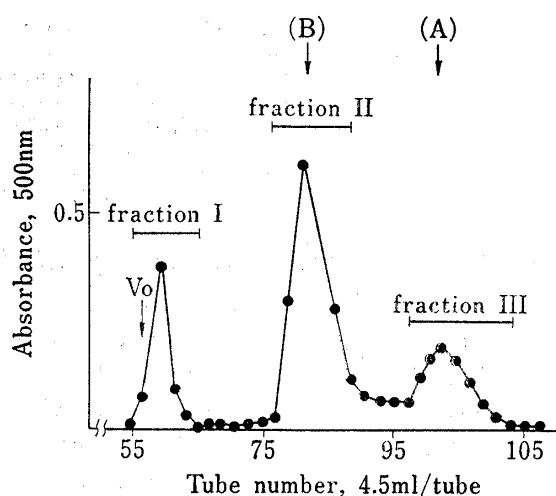

Fig. 6. Fractionation of the Controlled Smith Degradation Product of Ps-III.

The total sugar in the fraction were assayed by tryptophan-H_2SO_4 method. Fractions I, II and III were separately pooled. Arrows indicate the elution position of glucose (A) and melezitose (B).

Table IV. Products obtained from Ps-III by Smith Degradation

Conditions for acid hydrolysis	Products	mol %
0.1 M HCl at room temp. for 24 hr	Glycolaldehyde (oxime)	98.4
	Glycerol	1.6
0.5 M H_2SO_4 at 100° for 24 hr	Glycolaldehyde (oxime)	66.1
	Glycerol	1.6
	Erythritol	32.3
(unoxidized sugars with IO_4^-)	Mannose	61.2
	Glucose	18.3
	Galactose	20.5

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These results indicate that the core sugar-chain consisting of mannose polymer exists in Ps-III and side sugar-chains extend from each mannose. The fact that the trisaccharide consisting three kinds of sugar in fraction II was detected, shows that these three sugars were linked with 1,3-bonds each other. Further, the fact that mannose was detected in fraction III, shows that the mannose was linked to the sugars which have the linkage type oxidizable with periodate.

The type of sugar linkage can be assigned on the basis of the Smith-degradation products (Table IV), and by methylation analysis of materials of peaks 2′-a and 2′-b, fraction I as shown in Table V. These assignments are compatible with the results of methylation and partial acid hydrolysis.

Table V. Gas-Chromatographic Analysis of Methylated Monosaccharides derived from Materials of Peaks 2′–a and 2′–b, and Fraction I

Samples	Tentative assignment	Retention time
Peak 2′–a[a]	2,4,6-Tri-*O*-methyl-galactose	2.28[b]
Peak 2′–b[a]	2,3,4-Tri-*O*-methyl-mannose	2.45[b]
Fraction I[c]	2,3,6-Tri-*O*-methyl-mannose	4.85[d]

a) The disaccharides were obtained by partial acid hydrolysis of Ps-III. Methylated monosaccharide was analyzed as alditol acetate with column 1.
b) Relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol.
c) The oligosaccharide was obtained by Smith degradation of Ps-III. Methylated monosaccharide was analyzed as methyl glycoside.
d) Relative to methyl-2,3,4,6-tetra-*O*-methyl-*α*-D-glucopyranoside.

Identification of Pyruvic Acid and Pyruvic Acid-carrying Sugar

The general methods and the main results were reported in the previous paper,[4a] *e.g.*, Ps-III contained 3.2% of pyruvic acid. Pyruvic acid methylester was obtained from permethylated Ps-III by ketal exchange reaction with acetone. On partial acid hydrolysis and methanolysis of Ps-III, acid-carrying sugar was released. Methanolysis of Ps-III was carried out according to the method of Hirase.[26] Analysis of the pyruvic acid-carrying sugar obtained by the methanolysis, by gas chromatography using column 1 (*cf.* Methylation Analysis) and by IR-spectra indicated that the pyruvic acid-carriying glucose in Ps-III existed as 3,4-*O*-(1-carboxy ethylidene)-D-glucopyranoside and this glucose is linked to the position 6 of galactose: pyruvic acid→3,4 Glc 1→6 Gal.

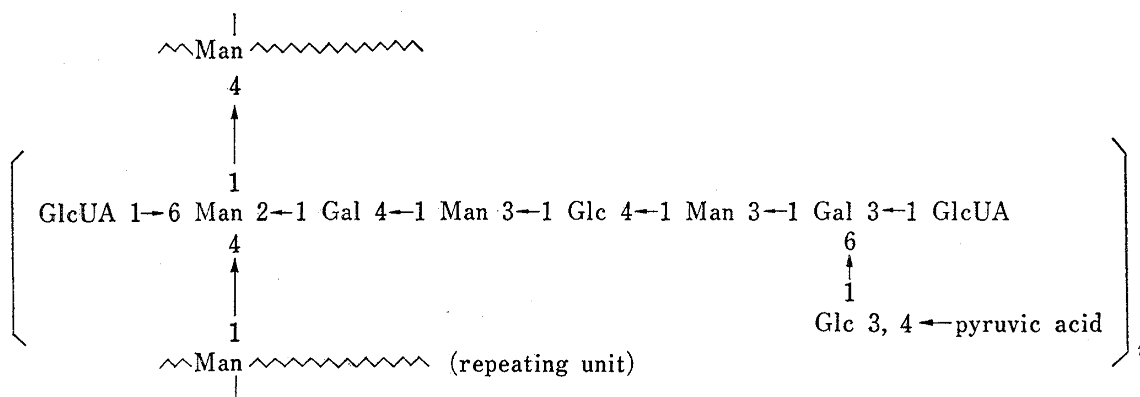

Fig. 7. Postulated Structure of Ps-III

26) S. Hirase, *Bull. Chem. Soc.* (Japan), **30**, 75 (1957).

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Figure 7 illustrated a postulated structure for the antigenic extracellular polysaccharide from *E. coli* 29M strain.

Though Ps-I from *E. coli* 36M reported in the previous paper[4b] has a straight chain structure, Ps-III has a core sugar-chain of mannose polymer and highly branched structure. We think Ps-III is a polysaccharide having a characteristic structure.

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