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STRUCTURE OF EXTRACELLULAR POLYSACCHARIDES OF *ESCHERICHIA COLI* STRAINS 36M, 72M AND 29M ISOLATED FROM COLIGRANULOMA OF CHICK INTESTINE: III. POLYSACCHARIDE FROM *E. COLI* 72M

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SUMMARY:

The antigenic polysaccharide of *Escherichia coli* 72M was isolated from the chick intestine, consisted of glucuronic acid (2 moles), mannose (3 moles), galactose (2 moles), glucose (2 moles) and pyruvic acid (about 0.9 moles). It has a core sugar-chain of mannose polymer and highly branched structure. Its repeating unit structure was postulated by methylation, Smith degradation, partial acid hydrolysis and methanolysis.

Ogawa *et al.* (1) isolated three types of *Escherichia coli* (termed 36M, 72M and 29M) from a coligranuloma of the chick intestine. They produced antigenic and type-specific polysaccharides, respectively, with immune activity to each antisera obtained from rabbits immunized with each *E. coli* organism.

The purpose of this work is to elucidate the structural difference among these three kinds of extracellular polysaccharide with immune activity. In the previous paper (2-4), we reported the postulated structure of polysaccharides from *E. coli* 36M and 29M. This paper concerns with the structural study of the polysaccharide from *E. coli* 72M.

MATERIALS AND METHODS:

The procedures for extraction and purification of the extracellular polysaccharide with immune activity from *E. coli* 72M were essentially the same as described previously (2), and the polysaccharide was referred to as Ps-II.

Total amounts of sugar were 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 determined by the method of Nogare *et al.*[2] (8). A reducing-end hexose and the molar ratios of component sugars of oligosaccharides were determined by means of gas chromatography and/or gas chromatography -mass spectroscopy (9).

Ascending paper chromatography was carried out on Whatman 3MM or Toyo No. 51A filter paper in the following solvent systems: a, pyridine-isoamyl-alcohol-benzene-water (5:5:3:2, v/v); b, pyridine-isoamylalcohol-water (7:7:6,

Abbreviation: Ps-II, the antigenic polysaccharide from *Escherichia coli* 72M.

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v/v); c, butanol-ethanol-water-28% ammonia (40:10:49:1, v/v); d, ethylacetate -pyridine-acetic acid-water (5:5:1:3, v/v). Thin layer chromatography on Avicel SF was performed using solvent (d). High-voltage paper electrophoresis was performed in the following buffers: a, pyridine-acetic acid-water (5:0.2:95, v /v), pH 6.0; b, 0.05 M sodium borate, pH 9.2. Sugars were detected on chromatograms and electrophoretograms with alkaline silver nitrate or aniline hydrogen phthalate. Sugars, products of Smith degradation and methylated sugars were identified on Shimadzu GC-5A gas chromatograph equipped with a digital integrator, ITG-4A, and a flame ionized detector using the following columns: a, 3% ECNSS-M on 100-120 mesh Gas Chrom Q, 0.3 x 300 cm; b, 15% butane-1,4-diol-succinate polyester on 80-100 mesh Celite, 0.3 x 200 cm; c, 5% SE-30 on 80-100 mesh Chromosorb W, 0.3 x 200 cm. Gel filtration chromatography was performed on columns of Sephadex G-150 (7 x 90 cm), Sephadex G-25 (2.6 x 130 cm) and Sephadex G-15 (2.6 x 133 cm) using 0.5 or 1.0 M NaCl.

Acid hydrolysis for preparation of oligosaccharides and monosaccharides was carried out using 0.1 to 0.5 M H_2SO_4 at 100°C for 1 to 42 hr in a closed tube depending on the purpose of the individual experiment. Analysis of resulting products was carried out by gas chromatography using column (a) and/or gas chromatography-mass spectroscopy (9).

Permethylation of Ps-II was performed by Hakomori's method (10). The ester carbonyl groups of permethylated Ps-II were reduced with $LiAlH_4$ according to the method of Sandford *et al.* (11). De-O-methylation of methylated monosaccharides was carried out by the method of Bonner *et al.* (12). The methylated sugars obtained from the permethylated products by acid hydrolysis (13) or methanolysis (14) were identified as the methylglycoside or the alditol acetate derivatives by gas chromatography using columns (a) and (b).

For Smith degradation, Ps-II (49.3 mg) was treated with 50 ml of 0.04 M $NaIO_4$ at 4°C for 120 hr in a dark according to Smith's method (15). The resulting products were detected by gas chromatography using column (c) according to the method of Yamaguchi *et al.* (16). The unoxidized sugars were identified as the alditol acetate derivative by gas chromatography using column (a).

Immunological assay was carried out by the microprecipitin technique of Davis (17), as follows. A constant volume (below 0.5 ml) of sample solution was mixed with 0.5 ml of rabbit antisera containing 1.75 mg of antibody as protein. The mixture was adjusted to a total volume of 1.0 ml with 0.15 M NaCl -0.01 M phosphate buffer, pH 7.3, incubated at 37°C for 1 hr and stored at 4°C for 24 hr. Then it was centrifuged at 1500 x g at 4°C for 1 hr. The precipitate obtained was washed twicw with the cold buffer used above. The amounts of protein precipitated by antigen-antibody reaction were measured by the method of Lowry-Folin (18) using rabbit IgG as standard. The antisera to Ps-II were prepared from rabbit immunized with the whole of *E. coli* 72M organism.

The experimental procedures for the isolation and characterization of pyruvic acid and pyruvic acid-carrying sugars from Ps-II were described in detail in the previous paper (2).

RESULTS AND DISCUSSION:

The extracellular polysaccharide obtained from the culture broth of *E. coli* 72M was separated into two major fractions on a Sephadex G-150 column (Fig. 1). The substance with high molecular weight (Fraction I) had a positive immune activity to rabbit antisera and was an acidic polysaccharide containing glucuronic acid and pyruvic acid. On the other hand, substance with lower molecular weight had no immune activity and consisted of hexose only, mainly mannose (Fraction II). Therefore, the lower molecular weight component of the

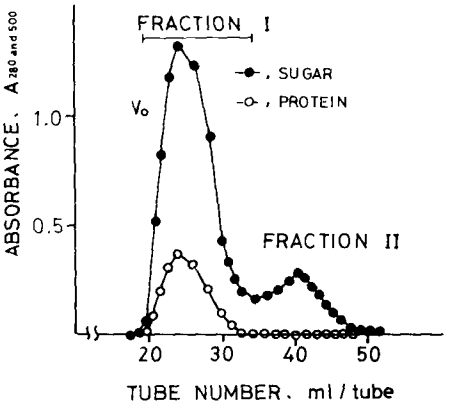

Fig. 1. Fractionation of the extracellular polysaccharide from *E. coli* 72M by Sephadex G-150 column chromatography. Tubes 23-36 were pooled and subjected to chromatography on a DEAE-cellulose column with a linear gradient of NaCl for further purification. Detection of carbohydrate was made using tryptophan-H_2SO_4 method. Antigenic activity of each fraction was assayed by the precipitin reaction with rabbit antisera. The contents of protein precipitated by the reaction were measured by the method of Lowry-Folin and by the absorbance at 280 nm using rabbit IgG as standard.

Table I. Chemical and Physical Properties of Ps-II

Molar ratio of Constituents*	D-GlcUA	D-Man	D-Glc	D-Gal	Pyruvic acid
	1.00	1.50	1.01	0.99	0.49
Acid Equivalent	520 (measured by titration with 0.02 M NaOH)				
$[\alpha]_D$	$+52.4^{\circ}$ (0.5% in water)				
ORD	(+) simple curve (1% in water)				
pKa	3.7				

* Molar ratios to glucuronic acid.

extracellular polysaccharide was not pursued further in this work. Fraction I was further purified by DEAE-cellulose column 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 I was referred to as Ps-II. Ps-II showed a single band with a mobility of 4.3 cm/40 min at 1 mA/cm by cellulose acetate paper electrophoresis (pyridine-acetic acid-water, 8:71:921, v/v, pH 3.5).

The chemical and physical properties of Ps-II were summarized in Table I. Acid hydrolysis of Ps-II in 0.5 M H_2SO_4 resulted in a progressive decrease in $[\alpha]_D$ value ($+30^\circ$ to $+20^\circ$) with the time of hydrolysis, indicating that Ps-II is a polysaccharide containing an α-D-glycosidic linkage.

The structure of Ps-II was postulated on the basis of the following experimental facts. a) *Methylation Analysis of Ps-II*-Methylated monosaccharides derived from permethylated polysaccharides were identified by gas chromatography and the results are shown in Table II. Each peak in Table II was identified by co-chromatography with authentic compounds and/or by making a comparison with the retention times reported by Aspinall (19) and Björndal (20). Monomethylated sugar corresponding to Peak IX, which was separated from the hydrolyzate of permethylated Ps-II by paper chromatography using solvent (c), was further characterized by de-O-methylation analysis as well as by periodate oxidation reaction. Peak IX was a substance with a R_G of 0.47 (R_G is relative to 2,3,4,6-tetra-O-methyl-α-D-glucose). The results of gas-chromatographic analysis concluded that Ps-II is composed of 1,3- and 1,2,6-linked glucosyl, 1,2-, 1,3- and 1,2,4,6-linked mannosyl and 1,3- and 1,4,6-linked galactosyl residues. Futhermore, the above results also show that the non-reducing termini appears to be glucuronic acid because 2,3,4,6-tetra-O-methyl-glucose was detected as Peak I from carboxy-reduced and then remethylated Ps-II. Calculation of the percentage compositions was made from individual peak areas of the gas chromatographic tracing using an integrator coupled to the gas chromatograph apparatus. b) *Analysis of Compositions of Oligosaccharides obtained from Ps-II*-The hydrolyzate of Ps-II with 0.5 M H_2SO_4 was separated into four neutral oligosaccharides and eight acidic oligosaccharides by gel filtration on a Sephadex G-25 column (Fig. 2), DEAE-cellulose column chromatography and then paper electrophoresis using buffers (a) (for acidic oligosaccharides) and (b) (for neutral oligosaccharides). If necessary, further separation was carried out by repeating paper chromatography using solvents (a) (for neutral oligosaccharides) and (b) (for acidic oligosaccharides). The sugar compositions and reducing terminal

Table II. Gas-Chromatographic Analyses of Partially Methylated Alditol Acetates derived from Permethylated Ps-II and Its Carboxy-Redeced, Remethylated Product

Peak	Relative Retention Time*		Mole% of Total Methylated Products		Tentative Assignment**
	(a)***	(b)****	(a)***	(b)****	
I	----	1.00	0	23.1	1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-glucitol
II	1.24	1.24	1.5	1.1	1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-galactitol
III	1.94	1.94	14.2	10.3	1,2,5-tri-O-acetyl-3,4,6-tri-O-methyl-mannitol
IV	1.95	1.95	14.3	11.0	1,3,5-tri-O-acetyl-2,4,6-tri-O-methyl-glucitol
V	2.09	2.09	14.2	11.7	1,3,5-tri-O-acetyl-2,4,6-tri-O-methyl-mannitol
VI	2.28	2.28	14.3	11.4	1,3,5-tri-O-acetyl-2,4,6-tri-O-methyl-galactitol
VII	5.86	5.86	14.3	10.9	1,2,5,6-tetra-O-acetyl-3,4-di-O-methyl-glucitol
VIII	6.78	6.78	13.0	9.4	1,4,5,6-tetra-O-acetyl-2,3-di-O-methyl-galactitol
IX	8.39	8.39	14.4	11.1	1,2,4,5,6-penta-O-acetyl-3-O-methyl-mannitol

* Relative to 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-glucitol.
** Tentative assignments were performed on the basis of the gas chromatography using columns 1 and 2, de-O-methylation studies and/or by periodate oxidation studies.
*** Permethylated Ps-II.
**** Carboxy-reduced, remethylated Ps-II.

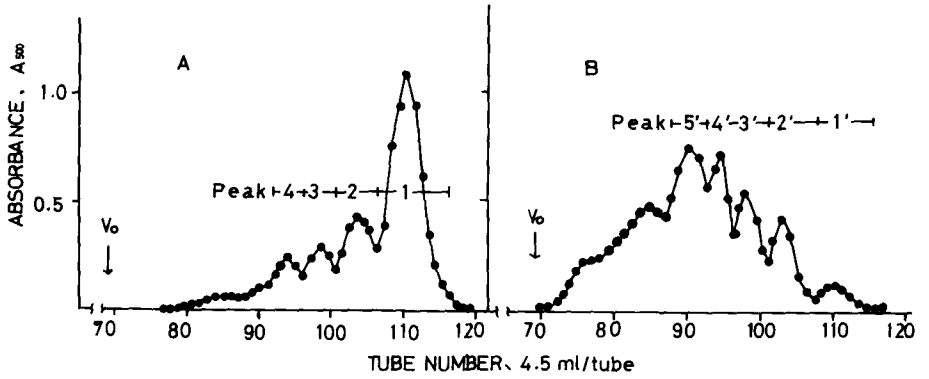

Fig. 2. Separation of neutral oligosaccharides and acidic oligosaccharides obtained from the hydrolyzate of Ps-II by DEAE-cellulose column chromatography. Separation of each oligosaccharide was carried out on a column of Sephadex G-25 using 1.0 M NaCl. The flow rate was maintained at about 20 ml/hr and 5 ml fractions were collected. Panels A and B show the elution patterns of neutral oligosaccharides and acidic oligosaccharides, respectively. Analysis of each fraction was made using tryptophan-H_2SO_4 method. Solid bars indicate the fraction which were combined for further purification. The molecular size of each oligosaccharide was estimated from its Kav to be mono- (glucose), di- (mannose) and trisaccharide (melezitose) as authentic standards.

Table III. Quantitative Analysis of Neutral(A) and Acidic(B) Oligosaccharides prepared from Ps-II

Samples	Mole% of Constituents				
	Glc	Gal	Man	GlcUA	Pyruvic acid
(A)					
Peak 2-a*	0	50.8	49.2**	0	0
Peak 2-b*	50.1	49.4**	0	0	0
Peak 3	33.6	32.8	33.6	0	0
Peak 4	24.7	25.3	50.0	0	0
(B)					
Peak 1'-b*	0	50.1	0	0	49.9
Peak 2'-a*	0	0	49.1	50.9	0
Peak 2'-b*	49.8	0	0	50.2	0
Peak 3'-a*	34.1	0	33.2	32.7	0
Peak 3'-b*	25.3	24.9	0	25.3	24.5
Peak 3'-c*	33.1	34.0	0	32.1	0
Peak 4'	0	24.6	51.3	24.1	0
Peak 5'	19.3	20.8	40.8	20.1	0

* Their peaks were separated from each fraction which was obtained by Sephadex G-25 column chromatography, by paper electrophoresis and/or by paper chromatography.
** Underlined sugar is the reducing-end in each oligosaccharide.

sugar of the resulting oligosaccharides were determined by gas chromatography-mass spectroscopy, as shown in Table III. Pyruvic acid-carrying sugars were obtained from Peaks 1'-b and 3'-b. These substances inevitably contained galactose. The substance of Peak 1'-b especialy consisted of equimolar amounts of pyruvic acid and galactose, which were determined by gas chromatography using columns (a) and (c), and by thin layer chromatography. These results suggest that the pyruvic acid-carrying sugar is galactose. c) *Smith degradation of Ps-II*-The products of controlled Smith degradation of Ps-II were determined by gas chromatography using columns (a) and (c), as shown in Table IV. For the identification of the unoxidized sugars, the other portion of the products of Smith degradation was submitted to gel filtration on a Sephadex G-15 column, where two fractions, tryptophan-H_2SO_4-reactive, were eluted at the void volume (Fraction 1) and at the position expected for trisaccharide (melezitose as a reference) (Fraction 2), as shown in Fig. 3. Fraction 1 was a polymer consisting of mannose only. Fraction 2 was composed of glucose, galactose and mannose

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

Conditions for Acid hydrolysis	Products	Mole%
0.1 M HCl at room temp. for 24 hr	Glycolaldehyde (oxime)	38.4
	Glyceraldehyde (oxime)	25.9
	Glycerol	24.4
	Erythritol	11.3
0.5 M H_2SO_4 at 100°C for 24 hr (for unoxidized sugars)	Mannose	50.4
	Glucose	25.2
	Galactose	24.4

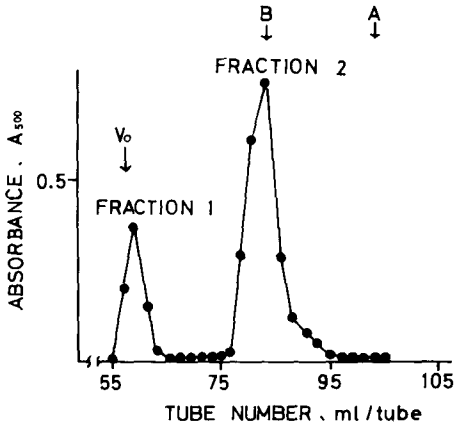

Fig. 3. Fractionation of the products of Smith degradation of Ps-II on a Sephadex G-15 column. 5 ml fractions were collected and assayed by tryptophan-H_2SO_4 method. Fractions 1 and 2 were sequentially pooled for further analysis. Arrows indicate the positions of glucose (A) and melezitose (B) as authentic standards.

as sugar in a molar ratio of 0.99:1.01:1.00, as measured by gas chromatography using column (a). These results indicate that Ps-II contains the core sugar-chain consisting of mannose and side sugar-chains extend from each mannose. Furthermore, the facts that the trisaccharide consisting of three kinds of sugar in Fraction 2 was detected, shows that these three sugars were linked one another through 1,3-bond formation, because the trisaccharide was resistant to periodate oxidation. The other types of sugar linkage can be decided by the

Table V. Gas-Chromatographic Analyses of Methylated Monosaccharides derived from Materials of Peaks 2'-a and 2'-b, and Fraction 1

Samples	Tentative Assignment (as Methylglycoside)	Retention Time*
Peak 2'-a	2,3,4-tri-O-methyl-mannose	2.70
	2,3,4-tri-O-methyl-glucuronic acid (methyl ester)	2.24, 2.88
Peak 2'-b	3,4,6-tri-O-methyl-glucose	2.73, 3.19
	2,3,4-tri-O-methyl-glucuronic acid (methyl ester)	2.24, 2.88
Fraction 1	2,3,6-tri-O-methyl-mannose	4.40

* Relative to methyl-2,3,4,6-tetra-O-methyl-β-D-glucopyranoside.

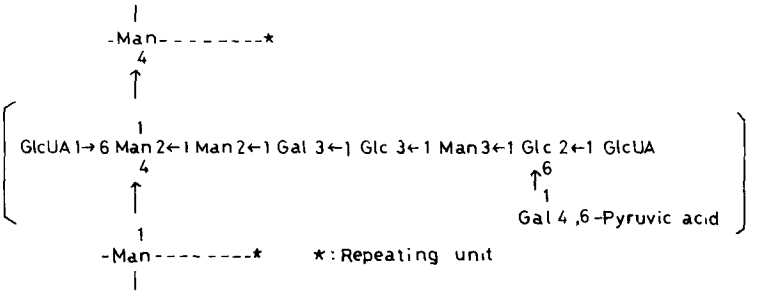

Fig. 4. Postulated structure for the extracellular polysaccharide with immune activity from *Escherichia coli* 72M.

type of Smith degradation products; *e. g.*, glyceraldehyde and glycerol should arise from non-reducing terminal pyranose unit, glyceraldehyde and glycerol from 1,2-glycosidic links and erythritol from 1,4-, 1,4,6- and/or 5-O-substituted glycosidic links. These results are compatible with those of methylation and partial acid hydrolysis. d) *Identification of Pyruvic Acid and Pyruvic Acid -carrying Sugar*-The general methods and the main results were reported in the previous paper (2). The pyruvic acid-carrying sugar obtained from Ps-II was 4,6-O-(1-carboxyethylidene)-D-galactopyranose, in which the galactose linked to the position 6 of glucose.

Furthermore, the type of sugar linkage can be assigned on the basis of the methylation study on the materials of Peaks 2'-a and 2'-b (cf. Table III), as well as on these of Fraction 1 (cf. Fig. 3) as shown in Table V.

A postulated structure for the antigenic polysaccharide from *E. coli* 72M is illustrated in Fig. 4. Based on the methylation and Smith degradation studies, it is estimated that a few percent of the terminal galactose residue do not bear pyruvic acid.

Though the antigenic polysaccharide from *E. coli* 36M has a straight chain structure, those from *E. coli* 72M and 29M have such a core sugar-chain as mannose polymer, and highly branched structure. It is proposed that either glucose or galactose is the pyruvic acid-carrying sugar in the polysaccharide of extracell which was produced by *Escherichia coli*.

REFERENCES

1. Ogawa, T. (1952) Nagoya Med. J. 12, 1248
2. Kamei, A., Nakazawa, K., Takeuchi, N., Akasi, S. ans Kagabe, K. (1977) J. Biochem. 82, 599-602.
3. Kamei, A., Takeuchi, N., Akashi, S. and Kagabe, K. (1978) J. Biochem. 89, 1009-1017.
4. Kamei, A., Takeuchi, N., Akashi, S. and Kagabe, K. Chem. Pharm. Bull. in press.
5. Badim, J., Jackson, C. and Schbert, M. (1952) Proc. Soc. Exp. Bio. Med. 84, 288-291.
6. Trevelyan, W. E. and Harrison, J. B. (1952) Biochem. J. 50, 298-303.
7. Bitter, T. and Muir, H. M. (1962) Anal. Biochem. 4, 330-334.
8. Nogare, S. D., Norris, T. O. and Nitchell, J. (1951) Anal. Chem. 23, 1473-1478.
9. Kamei, A., Yoshizumi, H., Akashi, S. and Kagabe, K. (1976) Chem. Pharm. Bull. 24, 1108-1110.
10. Hakomori, S. (1964) J. Biochem. 55, 205-207.
11. Sandford, A. and Conrad, H. E. (1966) Biochemistry 5, 1508.
12. Bonner, T. G., Bourne, E. K. and McNally, S. (1960) J. Chem. Soc. 2929.
13. Garegg, P. J. and Lindberg, B. (1960) Acta Chem. Scand. 14, 871.
14. Stoffyn, A., Stoffyn, P. and Mårtensson, E. (1968) Biochim. Biophys. Acta 152, 353.
15. Smith, F. and Van Cleve, J. W. (1955) J. Am. Chem. Soc. 77, 3091-3096.
16. Yamaguchi, H., Ikenaka, T. and Matsushima, Y. (1968) J. Biochem. 63, 553.
17. Davis, B. J. (1964) Ann. New York Acad. Sci. 121, 404.
18. Lowry, O. H., Rosebrough, H. J., Farr, A. and Randal, R. J. (1951) J. Biol. Chem. 193, 265.
19. Aspinall, G. O. (1963) J. Chem. Soc. 1676-1680.
20. Björndal, H., Lindberg, B. and Svensson, S. (1967) Acta Chem. Scand. 21, 1800-1804.