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ACETOLYSIS OF *Leuconostoc mesenteroides* NRRL B-1299 DEXTRAN. ISOLATION AND CHARACTERIZATION OF TETRASACCHARIDES CONTAINING SECONDARY LINKAGES FROM THE BORATE-INSOLUBLE FRACTION*

TOSHIYUKI WATANABE, KENICHI SHISHIDO, MIKIHIKO KOBAYASHI, AND KAZUO MATSUDA
Department of Agricultural Chemistry, Faculty of Agriculture, Tohoku University, Sendai (Japan)
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ABSTRACT

Fractionation of the deacetylated acetolyzate of the borate-insoluble fraction of the dextran elaborated by *Leuconostoc mesenteroides* NRRL B-1299 gave five tetrasaccharide fractions, isolated after chromatography on charcoal–Celite, paper chromatography, and paper electrophoresis. Examination of partial acid hydrolyzates of the tetrasaccharide fractions and their corresponding alditols, the relation between the logarithm of their partition functions (α') and molecular size, and methylation studies, showed them to be (*a*) 2^3-α-D-glucosyl-nigerotriose (**1**), (*b*) a mixture of 6-α-nigerotriosyl-D-glucose (**2**) and 6^1-α-D-glucosyl-nigerotriose (**3**) and/or 6^2-α-D-glucosyl-nigerotriose (**4**), (*c*) a mixture of 2^1-α-nigerosyl-isomaltose (**5**) and 3^2-α-isomaltosyl-kojibiose (**6**) and/or 6^2-α-nigerosyl-kojibiose(**7**), (*d*) 2-α-nigerotriosyl-D-glucose (**8**) and (*e*) nigerotetraose (**9**).

INTRODUCTION

The dextran elaborated by *Leuconostoc mesenteroides* NRRL B-1299 has been shown to be heterogeneous. It was first fractionated by Wilham *et al.*[1] into two major fractions, the less-soluble fraction L (insoluble in 39% ethanol) and the more-soluble fraction S (precipitated at a range of 39–42% ethanol concentration).

In our previous study[2,3], we used another fractionation procedure based on the solubility in borate buffer (pH 9.2). The borate-insoluble fraction and the borate-soluble fraction seemed to be very similar in their properties to the corresponding fractions reported by Wilham *et al.*[1]. Preliminary characterization of these two fractions was conducted by periodate oxidation, partial acid hydrolysis, acetolysis, interaction with concanavalin A, and methylation analysis.

Bourne *et al.*[4] reported on the types and percentages of secondary linkages in the dextran L (less water-soluble) and S (water soluble) elaborated by *L. mesenteroides*

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*Dedicated to Professor Dexter French on the occasion of his 60th birthday.

NRRL B-1299. These two fractions also seem to correspond to the borate-insoluble and borate-soluble fractions. They also reported[5] the isolation and characterization of oligosaccharides from water-soluble dextran (dextran S). However, fragmentation analysis of the less-soluble fraction L of the dextran elaborated by *L. mesenteroides* NRRL B-1299 has not been attempted.

In the present paper, we report the isolation and characterization of 2^3-α-D-glucosyl-nigerotriose (**1**), a mixture of 6-α-nigerotriosyl-D-glucose (**2**) and 6^1-α-D-glucosyl-nigerotriose (**3**) and/or 6^2-α-D-glucosyl-nigerotriose (**4**), a mixture of 2^1-α-nigerosyl-isomaltose (**5**) and 3^2-α-isomaltosyl-kojibiose (**6**) and/or 6^2-α-nigerosyl-kojibiose (**7**), 2-α-nigerotriosyl-D-glucose (**8**), and nigerotetraose (**9**) from the deacetylated products of the acetolyzate of the borate-insoluble fraction (less-soluble fraction L).

RESULTS

Fractionation of the deacetylated acetolyzates of borate-insoluble dextran by charcoal–Celite column chromatography yielded thirteen kinds of oligosaccharides. The elution profile is shown in Fig. 1.

Of these oligosaccharides, five tetrasaccharide fractions (I–V) containing α-(1 → 2) and/or α-(1 → 3) linkages were isolated, as shown in Table I.

Isolation and characterization of oligosaccharides. — *Oligosaccharide I* (2^3-α-D-*glucosyl-nigerotriose*, **1**). Evaporation of fractions 44–50 (10% ethanol eluate),

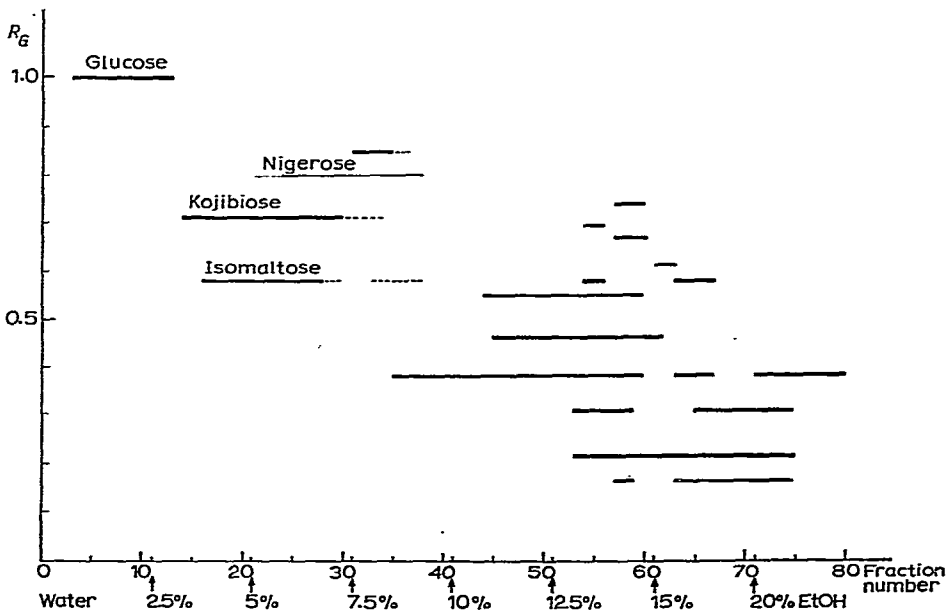

Fig. 1. Elution of the deacetylated acetolyzate of the borate-insoluble fraction by charcoal–Celite column chromatography.

TABLE I

YIELDS AND PROPERTIES OF OLIGOSACCHARIDES FROM BORATE-INSOLUBLE DEXTRAN[a]

Oligosaccharide fraction	*Yield (mg)*	*D.p.*	$[\alpha]_D$	R_G	M_G	*TTC*[b]	*Structure*[c]
I	12	4.12	—	0.38	0.63	+	G→^{2}G→^{3}G→^{3}G (1)
II	12.5	—	+133°	0.38	0.57	+	G→^{3}G→^{3}G→^{6}G (2)
							G→^{3}G→^{3}G (3) G→^{3}G→^{3}G (4)
III	15	—	+123°	0.38	0.28	—	G→^{3}G G→^{6}G→^{3}G→^{2}G (6)
							G→^{6}G (5) G→^{3}G→^{6}G→^{2}G (7)
IV	10	4.02	—	0.38	0.23	—	G→^{3}G→^{3}G→^{2}G (8)
V	7	3.84	—	0.46	0.64	+	G→^{3}G→^{3}G→^{3}G (9)

[a]Isolation procedure: purified dextran (25.0 g) → Acetylated dextran (34.3 g) → Acetolyzate (33.0 g) → Deacetylated acetolyzate (13.85 g) → Charcoal–Celite column chromatography (13.85 g). [b]Triphenyltetrazolium chloride reaction. [c]G =)-α-D-Glc*p*-1-(.

which showed three oligosaccharides (R_G 0.55, 0.46, and 0.38), gave 360 mg of amorphous powder. Each component was separated by preparative paper-chromatography. The zone having R_G 0.38 was excised and eluted with water. The eluate was evaporated to a syrup which, upon treatment with hot methanol followed by evaporation, gave 176 mg of amorphous powder. Paper electrophoresis of the latter in 0.1M sodium borate showed four oligosaccharides (M_G 0.63, 0.57, 0.28, and 0.23), each of which was separated by preparative paper electrophoresis.

The zone having M_G 0.63 was excised and eluted with water. The eluate was treated with Amberlite IR-120 (H^+) resin and then evaporated under diminished pressure with several intermediate additions of methanol to remove boric acid as the volatile methyl borate; 12 mg of amorphous powder was obtained. This product had d.p. 4.12, as determined by the method of Peat *et al.*[6].

Preliminary structural evidence was obtained from the relation between the logarithm of a partition function α' and molecular size[7], where α' is defined as $R_F/1-R_F$. A linear relationship is observed (Fig. 2) when log α' of glucose, nigerose, and nigerotriose are plotted against degree of polymerization (d.p.) (line I). This result indicates that line I represents the α-(1 → 3)-linked series. Similarly, a plot of log α' of glucose, kojibiose, and kojitriose* against d.p. is also linear (line II), suggesting that line II represents the α-(1 → 2)-linked series. When log α' of oligosaccharide I is plotted against d.p., the point generated is the intersection of lines III

*Isolated from the borate-soluble fraction. Details of isolation have not yet been reported.

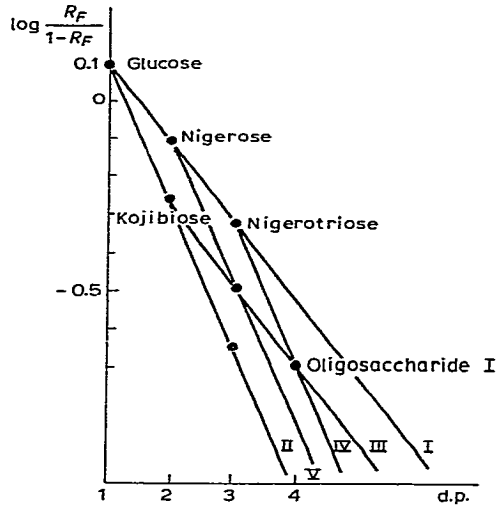

Fig. 2. Paper-chromatographic mobilities of oligosaccharide I (2^3-α-D-glucosyl-nigerotriose, **1**). Developed three times by the ascending method with solvent system *A*.

and IV, which are parallel to lines I (from the plot of kojibiose) and II (from the plot of nigerotriose), respectively.

Oligosaccharide I migrated faster (M_G 0.63) on paper electrophoresis, and was detected on a paper chromatogram by spraying with triphenyltetrazolium chloride (TTC)[8] and, therefore, does not possess a kojibiose reducing end-group.

The paper-chromatographic and paper-electrophoretic evidence thus suggests that oligosaccharide I is a tetrasaccharide containing one α-(1 → 2)- and two α-(1 → 3)-linkages, and that the reducing end of this sugar is α-(1 → 3)-linked.

Gas-chromatographic analysis of the methanolyzate of permethylated oligosaccharide I showed peaks corresponding in relative retention-times to the methyl glycosides of 2,3,4,6-tetra-, 3,4,6-tri-, and 2,4,6-tri-*O*-methyl-D-glucose.

These data indicate that oligosaccharide I is either 2^3-α-D-glucosyl-nigerotriose (**1**) or 2^2-α-nigerosyl-nigerose.

In order to ascertain the precise structure of this sugar, the following experiments were performed. Reduction of oligosaccharide I, followed by partial acid hydrolysis, gave glucose, nigerose, kojibiose, and a trisaccharide (R_G 0.53) as the sugars detectable by aniline hydrogen phthalate; nigerotriose was not detected. When log α′ of the trisaccharide (R_G 0.53) is plotted against d.p., the point generated is the intersection of lines III and V (Fig. 2), which are parallel to lines I (from the plot of kojibiose) and II (from the plot of nigerose), respectively. This trisaccharide, separated by preparative paper-chromatography, was detectable on paper chromatograms by spraying with TTC, and it migrated faster (M_G 0.65) on paper electrophoresis than authentic kojibiose.

These results suggest that the trisaccharide is 2^2-α-D-glucosyl-nigerose, and not

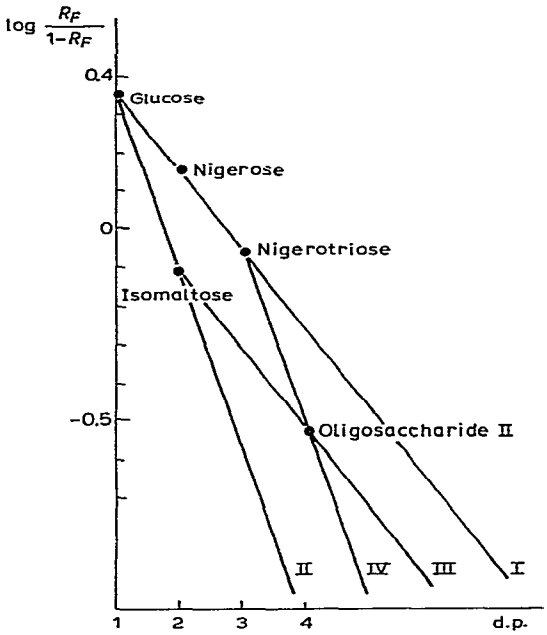

Fig. 3. Paper-chromatographic mobilities of oligosaccharide fraction II. Developed four times by the ascending method with solvent system *A*.

3^2-α-D-glucosyl-kojibiose. Thus, oligosaccharide I must be 2^3-α-D-glucosyl-nigerotriose (**1**).

Oligosaccharide fraction II. The zone having M_G 0.57 was excised and eluted with water. Sodium borate was removed as before and the solution evaporated to give 12.5 mg of amorphous powder, $[\alpha]_D$ +133° (*c* 0.4, water); it reacted with TTC on a paper chromatogram and thus does not possess a kojibiose reducing end-group.

As may be seen from Fig. 3, the relationship of log α′ and d.p. showed oligosaccharide fraction II to contain two α-(1 → 3)- and one α-(1 → 6)-linked D-glucose residues. Partial acid hydrolysis of this oligosaccharide fraction gave glucose and an oligosaccharide having R_G 0.50, together with the original oligosaccharide fraction II (papergram). The spot having R_G 0.50 and M_G 0.59 was chromatographically and electrophoretically identical with that of authentic 3-α-isomaltosyl-D-glucose[9], which was isolated and characterized from the deacetylated acetolyzate of *L. mesenteroides* B dextran. However, the paper-chromatographic behavior of this sugar is very similar to that of 6-α-nigerosyl-D-glucose and 3^1-α-D-glucosyl-isomaltose and it is very difficult to distinguish these three trisaccharides from each other. The exact structure of this trisaccharide fragment is, therefore, not still certain.

Gas-chromatographic analysis of the methanolyzate of the methylated product from fraction II indicated peaks having relative retention-times corresponding to the methyl glycosides of 2,3,4,6-tetra-, 2,3,4-tri- and 2,4,6-tri-*O*-methyl-D-glucose. Moreover, paper-chromatographic analysis of the hydrolyzate of the methylated product

of this sugar showed the presence of a dimethyl derivative having an R_F value close to that of 2,4-di-*O*-methyl-D-glucose, in addition to tetra- and tri-*O*-methyl derivatives.

Reduction of fraction II with sodium borohydride gave the alditol (II-ol), which on paper electrophoresis in 0.1M sodium molybdate (pH 5.0)[10] showed a major component migrating toward the anode (M_s = 0.65), together with a faint, immobile spot. This suggests that the oligosaccharide fraction II is a mixture of a tetrasaccharide having an α-(1 → 6) linkage at the reducing end (major) and a tetrasaccharide having an α-(1 → 3) linkage at the reducing end (minor).

From the results of methylation analysis and paper electrophoresis of the reduction product, the major tetrasaccharide of fraction II was assigned as to 6-α-nigerotriosyl-D-glucose (**2**) and the minor one was either 6^1-α-D-glucosyl-nigerotriose (**3**), 6^2-α-D-glucosyl-nigerotriose (**4**), or a mixture of both.

Oligosaccharide fraction III. The zone having M_G 0.28 isolated and deionized as before to give 15 mg of amorphous powder, $[\alpha]_D$ +123° (*c* 0.5, water). On partial acid hydrolysis, it gave glucose, a trisaccharide having the same R_G value as 3-*O*-α-isomaltosyl-D-glucose, small proportions of kojibiose and of nigerose, and the original oligosaccharide fraction III. Oligosaccharide fraction III migrated slowly (M_G 0.28) on paper electrophoresis, and was not detected on a paper chromatogram by spraying with TTC, suggesting that it contains α-(1 → 6), α-(1 → 3), and α-(1 → 2) linkages and that the reducing end has an α-(1 → 2) linkage.

Methylation analysis of oligosaccharide fraction III gave the methyl glycosides of 2,3,4,6-tetra-*O*-methyl glucose, and 2,3,4-, 2,4,6- and 3,4,6-tri-*O*-methylglucose, which were detected by g.l.c. Moreover, paper-chromatographic analysis of the hydrolyzate of the methylated sugar showed a dimethyl derivative having an R_F value very close to that of 3,4-di-*O*-methylglucose, as well as a tetra- and a small proportion of tri-*O*-methyl derivatives.

These results indicate that oligosaccharide fraction III may be a mixture of 2^1-α-nigerosyl-isomaltose (**5**, major) and 3^2-α-isomaltosyl-kojibiose (**6**) and/or 6^2-α-nigerosyl-kojibiose (**7**, minor).

Oligosaccharide IV (*2-O-α-nigerotriosyl-D-glucose*, **8**). The component having M_G 0.23 was isolated as before; yield 10 mg of amorphous powder. Partial acid hydrolysis of this compound (oligosaccharide IV) gave glucose, nigerose, kojibiose, nigerotriose, and the original oligosaccharide IV (papergram). Reduction of the oligosaccharide, followed by partial acid hydrolysis gave glucose, nigerose, and nigerotriose as the sugars detectable by aniline hydrogen phthalate. The original oligosaccharide (d.p. = 4.02) was not detected on a paper chromatogram sprayed with TTC.

As may be seen from Fig. 4, oligosaccharide IV was shown to contain two α-(1 → 3) linkages and one α-(1 → 2) linkage, from the relationship of log α′ and d.p. Peaks corresponding in relative retention-times to the methyl glycosides of 2,3,4,6-tetra-*O*-methyl glucose, and 3,4,6- and 2,4,6-tri-*O*-methylglucose were detected in the methanolyzate of methylated oligosaccharide IV.

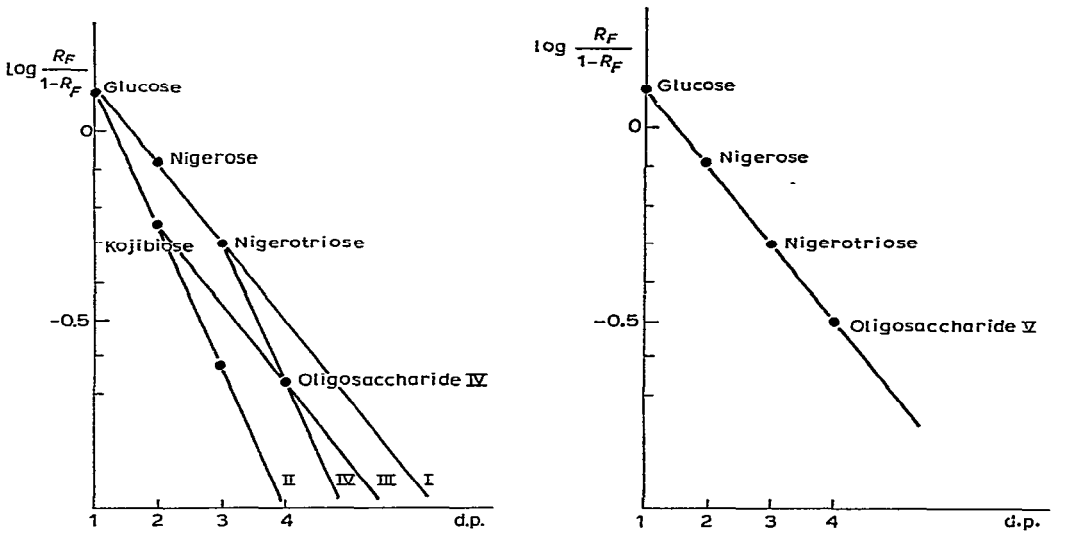

Fig. 4. Paper-chromatographic mobilities of oligosaccharide IV (2-α-nigerotriosyl-D-glucose, **8**). Developed three times by the ascending method with solvent system *A*.

Fig. 5. Paper-chromatographic mobilities of oligosaccharide V (nigerotetraose, **9**). Developed three times by the ascending method with solvent system *A*.

From these results, oligosaccharide IV was characterized as 2-*O*-α-nigerotriosyl-D-glucose (**8**).

Oligosaccharide V (nigerotetraose, **9**). The zone having R_G 0.46 (fractions 44–50, 10% ethanol eluate) was excised and eluted with water. The eluate was evaporated to a syrup which, upon treatment with hot methanol followed by evaporation, gave 66 mg of amorphous powder, which was examined by paper electrophoresis in 0.1M sodium borate. Four oligosaccharides (M_G 0.64, 0.58, 0.32, and 0.11) were detected. The zone having M_G 0.64 was separated by preparative paper-electrophoresis, and isolated and deionized as before; yield 7 mg of amorphous powder. This compound (oligosaccharide V, d.p. = 3.84), on partial acid hydrolysis, gave glucose, nigerose, nigerotriose, and the original oligosaccharide V. As may be seen from Fig. 5, a straight-line relationship is observed when the logarithm of α' of glucose, nigerose, nigerotriose, and oligosaccharide V are plotted against d.p. From these results, oligosaccharide V was identified as nigerotetraose (**9**).

DISCUSSION

Bourne *et al.*[4] reported on the types and percentages of secondary linkages in fractions S (more soluble) and L (less soluble) of the dextran elaborated by *L. mesenteroides* NRRL B-1299. They later reported[5] the isolation and characterization of oligosaccharides from the more-soluble dextran (dextran S). From the results of their fragmentation analysis, the average repeating-unit of NRRL B-1299 dextran S,

containing fifteen D-glucose residues, possesses five branches, which occur at each position 6 of such segments and at position 2. The branches consist mainly of α-D-glucopyranosyl groups and some appear to be terminated by the α-nigerosyl group. However, the less soluble dextran of *L. mesenteroides* NRRL B-1299 has not been studied by fragmentation analysis.

Acetolysis of dextrans has been widely used to isolate oligosaccharides containing (1 → 2)- and (1 → 3)-α-D-linkages, as these linkages are more stable to acetolysis but less stable to partial acid hydrolysis. Therefore, acetolysis was adopted for the partial degradation of the borate-insoluble dextran (less-soluble fraction). After fractionation of oligosaccharides, 2^3-α-D-glucosyl-nigerotriose (**1**), 6-α-nigerotriosyl-D-glucose (**2**), 6^1-α-D-glucosyl-nigerotriose (**3**, and/or 6^2-α-glucosyl-nigerotriose, **4**), 2^1-α-nigerosyl-isomaltose (**5**), 3^2-α-isomaltosyl-kojibiose (**6**, and/or 6^2-α-nigerosyl kojibiose, **7**), 2-*O*-α-nigerotriosyl-D-glucose (**8**), and nigerotetraose (**9**) were isolated and characterized.

In the borate-insoluble fraction of dextran NRRL B-1299, the amounts of oligosaccharides containing α-(1 → 3) linkage were larger than those containing α-(1 → 6) and α-(1 → 2) linkages, whereas in the borate-soluble fraction the amounts of oligosaccharides containing α-(1 → 6) and α-(1 → 2) linkages were larger than those containing the α-(1 → 3) linkage.

In earlier work[3], a considerable proportion of 2,4,6-tri- and 3,4,6-tri-*O*-methylglucose was detected upon methylation analysis of the borate-insoluble fraction. These data demonstrate the presence of significant amounts of unbranched (1 → 3) and (1 → 2)-linked residues in the fraction. Detection of only a small proportion of 2,4-di-*O*-methylglucose suggests that almost none of the (1 → 3) linkages are involved in branching.

Bourne *et al.*[4] also showed by methylation analysis that the less-soluble fraction had a higher content of unbranched α-(1 → 3)-linked residues.

The present work demonstrates that these residues occur in sequences, and lends further support to the relationship between lower solubility and sequences of α-(1 → 3)-linked residues.

EXPERIMENTAL

General methods. — All evaporations were conducted under diminished pressure below 40°. Optical rotations were measured with a Nippon Bunko Model DIP-SL polarimeter. Paper chromatography was performed on Toyo No. 50 filter paper by multiple ascending or descending methods with the following solvent systems: (*A*) 6:4:3 (v/v) 1-butanol–pyridine–water[11], and (*B*) water-saturated butanone. Preparative paper-chromatography was carried out on Toyo No. 526 thick filter-paper with solvent system *A*. Paper electrophoresis was performed on Toyo No. 50 filter paper at 15 V/cm with 0.1M sodium borate (pH 9.2), or 0.1M sodium molybdate (pH 5.0) buffers. Preparative paper-electrophoresis was conducted on Toyo No. 526

thick filter-paper at 13.3 V/cm with the former buffer. Aniline hydrogen phthalate[12] or silver dip[13] methods was used for the detection of sugars.

Preparation and fractionation of the dextran. — Dextran NRRL B-1299 was prepared by essentially the same procedure as reported previously[2]. The purified dextran was suspended in 0.1M sodium borate (pH 9.2) and stirred overnight at room temperature. The insoluble dextran, separated by centrifugation (7,000 r.p.m., 20 min), was suspended in water and reprecipitated with an equal volume of methanol. The precipitate was collected by centrifugation, washed with methanol and ether, and dried *in vacuo*. The borate-insoluble fraction was homogeneous by electrophoresis, as reported previously[2].

Acetylation of the purified dextran (borate-insoluble fraction). — A suspension of the purified dextran (5 g) in formamide (250 ml) was shaken overnight. Pyridine (110 ml) was added, followed by acetic anhydride (75 ml) dropwise with stirring. The temperature of the mixture was maintained at 20° until all of the acetic anhydride had been added, and then the mixture was agitated for 6 h at 45–55°. The acetylated dextran was precipitated by pouring the mixture into water and allowed it to remain overnight at room temperature. This product was filtered off, washed with water, and resuspended in water. After centrifugation, the acetylated dextran was washed with methanol, ether, and air-dried. This experiment was repeated five times. The purified dextran (25.0 g) gave 34.3 g of the acetyl derivative.

Acetolysis. — Acetolysis of the acetylated dextran (34.3 g) was performed by treating the sample with 24:16:3 (v/v) acetic anhydride–acetic acid–sulfuric acid for 24 h at 27°. The mixture was poured into ice–water, and after being kept for 24 h, the acetolysis product was extracted with chloroform. The chloroform layer was dried and evaporated to a syrup; yield 33.0 g. The acetolyzate of the acetylated dextran was subjected twice to deacetylation with 0.05M sodium methoxide, treated with Amberlite IR-120 (H^+) resin, and evaporated; yield 13.85 g.

Fractionation of the deacetylated acetolyzate of the borate-insoluble fraction by charcoal–Celite column chromatography. — The deacetylated acetolyzate (13.85 g) was dissolved in 140 ml of distilled water. The solution was adjusted to pH 6.2 with sodium hydroxide and applied to a column (28.5 × 9.5 cm) composed of 280 g of charcoal (Takeda Pharmaceutical Co. Ltd.) and the same amount of Celite (No. 545). The sugars were eluted stepwise with water, 2.5, 5, 7.5, 10, 12.5, 15, 20 and 25% ethanol. The eluates were collected after each 1,000 ml, evaporated, and examined by paper chromatography.

Methylation analysis. — The oligosaccharides were methylated by the method of Hakomori[14]. Conditions for methylation analysis and g.l.c. were the same as reported previously[3].

Partial acid hydrolysis of oligosaccharide was performed by heating 1–2 mg of sample with 1–2 ml of 0.1M hydrochloric acid for 90 min at 100°, and hydrolyzates were neutralized with silver carbonate followed by treatment with Amberlite IR-120 (H^+) resin.

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