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

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ABSTRACT

Fractionation of the deacetylated acetolyzate of the borate-soluble fraction of the dextran elaborated by *Leuconostoc mesenteroides* NRRL B-1299 gave, after chromatography on charcoal–Celite, preparative paper-chromatography, and paper electrophoresis, four trisaccharide fractions and four tetrasaccharide fractions. The isolated oligosaccharides were characterized by their paper-chromatographic mobility, examination of partial acid-hydrolyzates of the oligosaccharides and their corresponding alditols, and methylation analysis. These oligosaccharides were shown to be (*a*) kojitriose (**1**), (*b*) isomaltotriose (**2**), (*c*) a mixture of 2-*O*-α-isomaltosyl-D-glucose (**3**), 2^1-*O*-α-D-glucosylisomaltose (**4**), and 2-*O*-α-nigerosyl-D-glucose (**5**), (*d*) 6-*O*-α-kojibiosyl-D-glucose (**6**), (*e*) isomaltotetraose (**7**), (*f*) a mixture of 2-*O*-α-isomaltotriosyl-D-glucose (**8**) and 2^1-*O*-α-D-glucosylisomaltotriose (**9**), (*g*) 6-*O*-α-kojitriosyl-D-glucose (**10**), and (*h*) 6^3-*O*-α-D-glucosylkojitriose (**11**), respectively. Some of these oligosaccharides are newly isolated and characterized.

INTRODUCTION

In a previous paper[1], we reported on the fractionation of *Leuconostoc mesenteroides* NRRL B-1299 dextran. The dextran was fractionated by solubility in borate buffer into two components, borate-soluble and borate-insoluble fractions, which might correspond respectively to the "water-soluble (S)" and "less water-soluble (L)" fractions reported by other investigators[2]. These two fractions were characterized by periodate oxidation, partial acid-hydrolysis, controlled acetolysis, interaction with concanavalin A, and methylation analysis[1,3]. The same dextran was also analyzed by Bourne *et al.*[4], who reported on the types and percentages of secondary linkages in the fractions S and L of the dextran. They later reported[5] the isolation and characterization of some oligosaccharides from an enzymic digest of the partially acid-degraded fraction S. Recently, we have isolated and characterized some oligosaccharides from the deacetylated acetolyzate of the borate-insoluble fraction of the dextran[6].

Although isolation of the fragment oligosaccharides from fraction S of the *Leuconostoc mesenteroides* NRRL B-1299 dextran was attempted by Bourne *et al.*[5], most of the products were obtained only as mixtures, and individual oligosaccharides were not satisfactorily characterized. We now report the isolation and more-detailed characterization of the oligosaccharides from the deacetylated acetolyzate of the borate-soluble fraction of this dextran. Fragmentation analysis of another dextran by controlled acetolysis was attempted by Sakakibara *et al.*[7], who used *Leuconostoc mesenteroides* NRRL B-1397 dextran, which is known to have a structure similar to that of the dextran used in the present study.

RESULTS

Preliminary characterization of the isolated oligosaccharides by paper-chromatographic mobility. — Preliminary structural evidence for the isolated oligosaccharides (Table I) was obtained from the relation between the logarithm of a partition function α' and molecular size (d.p.), in which α' is defined[8] as $R_F/(1 - R_F)$. As may be seen from Fig. 1, a straight-line relationship is observed (line I) when logα' values of glucose, kojibiose, and the oligosaccharide I (**1**) are plotted against their d.p. values,

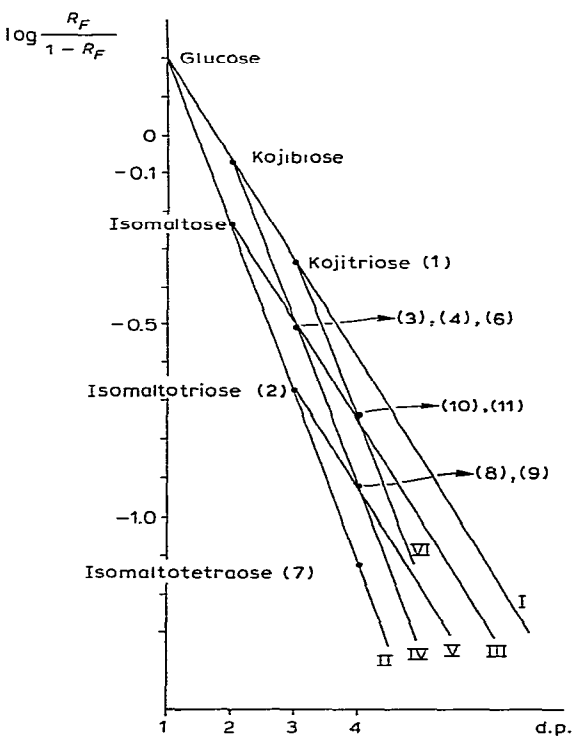


Fig. 1. Paper-chromatographic mobilities of oligosaccharides obtained from borate-soluble fraction. Developed three times by the ascending method with solvent system *A*.

TABLE I

FRACTIONATION OF THE ACETOLYZATE OF THE BORATE-SOLUBLE FRACTION BY CHARCOAL–CELITE COLUMN CHROMATOGRAPHY

Fraction no.	*Solvent used for elution*	*Sugar component*[a]	*Yield*[b] *(g)*
1–13	Water	Glucose	5.83
14–24	2.5% EtOH	Glucose, kojibiose	0.46
25–42	5% EtOH	Kojibiose, isomaltose	0.96
43–57	7.5% EtOH	Kojibiose, isomaltose, nigerose, oligo. II-S	1.08
58–71	10% EtOH	Nigerose, isomaltose, oligo. I, oligo. II-F, oligo. II-S, oligo. III-F	1.27
72–87	12.5% EtOH	Oligo. I, oligo. IV-F, oligo. IV-S	0.96
88–101	15% EtOH	Oligo. IV-S, oligo. V, unidentified oligo.	0.88
102–114	20% EtOH	Oligo. V, unidentified oligo., unidentified oligo.	0.91

[a]Oligo. = oligosaccharide. [b]Amorphous dry weight.

suggesting that these sugars belong to a homologous series. Thus, oligosaccharide I was assigned as kojitriose. Similarly, a straight-line relationship of the plot (line II) is also observed for glucose, isomaltose, the oligosaccharide II-F (**2**), and the oligosaccharide IV-F (**7**). Accordingly, oligosaccharides II-F and IV-F were assigned as isomaltotriose and isomaltotetraose, respectively.

When logα' of the oligosaccharides III-F (**6**) is plotted against its d.p. value, the point generated is at the intersection of the lines III and IV (Fig. 1), which start from the points of isomaltose and kojibiose and are parallel to line I (kojibiose series) and line II (isomaltose series), respectively. The oligosaccharide III-F was thus assigned as a trisaccharide having α-(1→2) and α-(1→6) linkages. The oligosaccharides II-S (**3**, **4**, **5**) showed almost the same paper-chromatographic mobility as that of oligosaccharide III-F and, hence, were presumed to have the same combination of linkages as oligosaccharide III-F.

When logα' of the oligosaccharide IV-S (**8** and **9**) is plotted against its d.p. value, the point generated is at the intersection of lines IV and V. The latter starts from the point of isomaltotriose and is parallel to line I. This result suggests that oligosaccharide IV-S is a tetrasaccharide having one α-(1→2) and two α-(1→6) linkages.

When logα' of oligosaccharide V-F (**10**) is plotted against its d.p. value, the point generated is at the intersection of lines III and VI. The latter starts from the point of kojitriose and is parallel to line II. Thus, the oligosaccharide V-F was presumed to have one α-(1→6) and two α-(1→2) linkages. The oligosaccharide V-S (**11**)

TABLE II

YIELDS AND PROPERTIES OF THE OLIGOSACCHARIDES ISOLATED BY CHARCOAL–CELITE COLUMN CHROMATOGRAPHY, PAPER CHROMATOGRAPHY, AND PAPER ELECTROPHORESIS

Oligosaccharide	*Fraction no.*	*Ethanol conc. (%)*	*Yield (mg)*	*D.p.*	$[\alpha]_D$ *(deg.)*	R_{Glc}[a]	M_G[b]	*TTC*[c]	*Hydrolyzates from methylated products*
I	67– 76	10 –12.5	18.7	2.9	+120	0.52	0.25	—	
II-F	56– 62	7.5–10	40.9	3.1		0.30	0.58	+	
II-S	56– 62	7.5–10	293.0	2.6	+138	0.41	0.22	—	2,3,4,6-tetra-*O*-Me-Glc 2,3,4-tri-*O*-Me-Glc 3,4,6-tri-*O*-Me-Glc 3,4-di-*O*-Me-Glc
III-F	63– 64	10	40.7	3.1	+140	0.38	0.63	+	
IV-F	79– 87	12.5	70.0		+149	0.11	0.55	+	
IV-S	79– 87	12.5	71.3	3.9	+156	0.18	0.25	—	2,3,4,6-tetra-*O*-Me-Glc 2,3,4-tri-*O*-Me-Glc 3,4,6-tri-*O*-Me-Glc 3,4-di-*O*-Me-Glc
V-F	92–101	15	15.0	4.2	+152	0.26	0.53	+	
V-S	92–101	15	18.6	4.4	+159	0.26	0.21	—	2,3,4,6-tetra-*O*-Me-Glc 2,3,4-tri-*O*-Me-Glc 3,4,6-tri-*O*-Me-Glc

[a]Paper-chromatographic mobility (solvent *A*, relative to glucose). [b]Paper-electrophoretic mobility (relative to glucose). [c]Triphenyltetrazolium chloride reaction.

TABLE III

HYDROLYSIS OF THE OLIGOSACCHARIDES OBTAINED FROM THE BORATE-SOLUBLE FRACTION OF DEXTRAN NRRL B-1299 BEFORE AND AFTER REDUCTION

Oligosaccharide	*Structure*	*Partial acid hydrolyzate*	
		Before reduction with $NaBH_4$	*After reduction with $NaBH_4$ (detected by AHP[a])*
I	**1**	Glucose, kojibiose, original oligosaccharide I	Glucose, kojibiose
II-F	**2**	Glucose, isomaltose, original oligosaccharide II-F	Glucose, isomaltose
II-S	**3,4,5,6**	Glucose, kojibiose, isomaltose, nigerose (faint), original oligosaccharide II-S	Glucose, nigerose (faint), isomaltose
III-F		Glucose, kojibiose, isomaltose, original oligosaccharide III-F	Glucose, kojibiose
IV-F	**7**	Glucose, isomaltose, isomaltotriose, original oligosaccharide IV-F	
IV-S	**8,9**	Glucose, kojibiose, isomaltose, isomaltotriose, trisaccharide having R_{Glc} 0.40, original oligosaccharide IV-S	Glucose, isomaltose, isomaltotriose
V-F	**10**	Glucose, kojibiose, isomaltose, kojitriose, trisaccharide having R_{Glc} 0.38, original oligosaccharide V-F	Glucose, kojibiose, kojitriose
V-S	**11**	Glucose, kojibiose, isomaltose, kojitriose, trisaccharide having R_{Glc} 0.40, original oligosaccharide V-S	Glucose, kojibiose, isomaltose, trisaccharide having R_{Glc} 0.40

[a]Aniline hydrogenphthalate.

TABLE IV

STRUCTURES OF OLIGOSACCHARIDES ISOLATED FROM BORATE-SOLUBLE DEXTRAN

Oligosaccharide	*Structure*[a]		
I	G→G→G 2 2	(1)	Kojitriose
II-F	G→G→G 6 6	(2)	Isomaltotriose
II-S	G→G→G 6 2	(3)	2-*O*-α-Isomaltosyl-D-glucose
	G→G 6↑2 G	(4)	2^1-*O*-α-D-Glucosylisomaltose
	G→G→G 3 2	(5)	2-*O*-α-Nigerosyl-D-glucose
III-F	G→G→G 2 6	(6)	6-*O*-α-Kojibiosyl-D-glucose
IV-F	G→G→G→G 6 6 6	(7)	Isomaltotetraose
IV-S	G→G→G→G 6 6 2	(8)	2-*O*-α-Isomaltotriosyl-D-glucose
	G→G→G 6 6↑2 G	(9)	2^1-*O*-α-D-Glucosylisomaltotriose
V-F	G→G→G→G 2 2 6	(10)	6-*O*-α-Kojitriosyl-D-glucose
V-S	G→G→G→G 6 2 2	(11)	6^3-*O*-α-D-Glucosylkojitriose

[a]G =)-α-D-Glc*p*-1-(.

had the same R_{Glc} value as that of oligosaccharide V-F and, hence, was presumed to have the same combination of linkages as oligosaccharide V-S.

Further characterization of the oligosaccharides. — The oligosaccharides were further characterized by examination of partial acid-hydrolyzates of the oligosaccharides and their corresponding alditols, and by methylation analysis. Mobility in paper electrophoresis and staining with triphenyltetrazolium chloride (TTC)[9] served to distinguish α-(1→6) and α-(1→2) linkages at the reducing end. The results of the structural analyses of the isolated oligosaccharides are summarized in Tables II–IV.

DISCUSSION

Preliminary examination of the borate-soluble dextran from *Leuconostoc mesenteroides* NRRL B-1299 has shown the preponderance of an α-(1→2)-linked, branched structure in this dextran[3]. Characterization of the water-soluble dextran elaborated by the same strain was also performed by ^{13}C- and proton-n.m.r. spectroscopy. The n.m.r. studies indicated that the α-(1→2)-linked side-chain branches have a comb-like structure[10].

Bourne *et al.*[5] have demonstrated that the average repeating-unit of NRRL B-1299 dextran S, containing fifteen D-glucose residues, possesses five branches. The branches consist mainly of single (1→2)-α-D-glucopyranosyl groups, and some appear to be terminated by an α-nigerosyl group.

Sakakibara *et al.*[7] have obtained 6-*O*-α-kojibiosyl-D-glucose, 2-*O*-α-isomaltosyl-D-glucose, and 2^1-*O*-α-D-glucosylisomaltose by acetolysis of the dextran of *L. mesenteroides* NRRL B-1397.

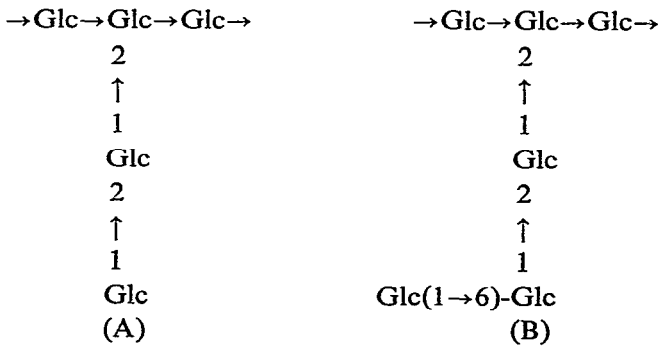
Acetolysis[11-13] of dextrans has been widely used to isolate oligosaccharides containing α-D-(1→2) and -(1→3) linkages, as these linkages are more stable to acetolysis but less stable to partial acid-hydrolysis. In this work, therefore, acetolysis was adopted for partial degradation of the borate-soluble fraction. Fractionation of the resulting, partial-degradation products of the borate-soluble dextran by charcoal–Celite chromatography yielded eleven kinds of oligosaccharides. Of these oligosaccharides, kojitriose, isomaltotriose, a mixture of 2-*O*-α-isomaltosyl-D-glucose, 2^1-*O*-α-D-glucosylisomaltose, and 2-*O*-α-nigerosyl-D-glucose, 6-*O*-α-kojibiosyl-D-glucose, isomaltotetraose, a mixture of 2-*O*-α-isomaltotriosyl-D-glucose and 2^1-*O*-α-D-glucosylisomaltotriose, 6-*O*-α-kojitriosyl-D-glucose, and 6^3-*O*-α-D-glucosylkojitriose were isolated and characterized.

Kojitriose, 2^1-*O*-α-D-glucosylisomaltotriose, 6-*O*-α-kojitriosyl-D-glucose, and 6^3-*O*-α-D-glucosylkojitriose were newly isolated and characterized.

These results demonstrate that some of the consecutive secondary linkages [α-(1→2)] are located in the linear portion or in part of the branch chains of the borate-soluble dextran. The branches consist mainly of single (1→2)-α-D-glucopyranosyl groups, as reported by Bourne *et al.*[5].

The occurrence of consecutive sequences of α-(1→2) linkages was suggested by methylation analysis, as 1.6–9.0% of 3,4,6-tri-*O*-methyl-D-glucose was detected[3]. These results were confirmed by isolation of kojitriose, 6-*O*-α-kojitriosyl-D-glucose, and 6^3-*O*-α-D-glucosylkojitriose.

From the results of structural analysis of the oligosaccharides isolated from borate-soluble dextran, it is demonstrated that fragments (A) and (B) exist in NRRL B-1299 dextran S, in addition to the average repeating-unit of this dextran reported by Bourne *et al.*[5].



MATERIALS AND METHODS

Preparation and fractionation of the dextran. — Preparation and fractionation of the *Leuconostoc mesenteroides* NRRL B-1299 dextran was performed essentially as reported previously[1]. The borate-soluble fraction was purified by reprecipitation with methanol and used without further treatment.

General methods. — 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 the multiple ascending or descending method with the following solvent-systems: (*A*) 6:4:3 (v/v) butanol–pyridine–water[14]; and (*B*) water-saturated butanone[15]. Preparative paper-chromatography was carried out on Toyo No. 526 thick filter-paper with solvent system *A*. The zone corresponding to the desired sugar was excised, eluted with deionized water, and the solution evaporated. Paper electrophoresis was performed on Toyo No. 50 filter paper at 15 V/cm with 0.1M sodium tetraborate (pH 9.2). Preparative paper-electrophoresis was conducted on Toyo No. 526 thick filter-paper at 13.3 V/cm with 0.1M sodium tetraborate. The zone corresponding to the desired sugar was excised, and eluted with deionized water. The eluate was treated with Amberlite IR-120 (H^+ form) resin and evaporated. Methanol was then evaporated repeatedly from the residue until borate ion was completely removed. Sugars were detected by aniline hydrogenphthalate[16] or silver nitrate reagent (dip method) [17]. The degrees of polymerization (d.p.) of the oligosaccharides were determined by the method of Peat *et al.*[18].

Partial acid-hydrolysis of the oligosaccharides and their corresponding alditols was performed by heating 1–2 mg of the sample with 1–2 mL of 0.1M hydrochloric acid for 90 min at 100°. The hydrolyzate was made neutral with silver carbonate and then treated with Amberlite IR-120 (H^+ form) resin. Reduction of the oligosaccharides to the corresponding alditols was performed according to a standard procedure[19], using 1–2 mg of the sample.

Acetylation[20] *of the dextran.* — A suspension of the purified dextran (5 g) in formamide (250 mL) was shaken overnight at room temperature. After addition of pyridine (110 mL), acetic anhydride (75 mL) was added dropwise with stirring. The temperature of the mixture was maintained at 20° until all of the acetic anhydride had been added. The mixture was agitated for an additional 6 h at 45–55° and then poured into water. The mixture was kept overnight at room temperature, and the precipitated product collected by filtration and washed with water. The acetylated dextran was resuspended in water, collected by centrifugation, washed successively with methanol and ether, and finally air-dried. Six such runs were made, and 38.0 g of acetylated dextran was obtained from 28.3 g of the purified dextran.

Acetolysis[11–13] *of the acetylated dextran and deacetylation of the acetolyzate.* — The acetylated dextran (38.0 g) was treated with a mixture of acetic anhydride, acetic acid, and sulfuric acid (24:16:3, v/v) (327 mL) for 24 h at 27° and the resulting mixture was poured into ice–water. The mixture was kept for 24 h at room tempera-

ture and then extracted with chloroform. The extract was dried with anhydrous sodium sulfate and evaporated to a syrup; yield 36.1 g. The acetolyzate was deacetylated twice with 0.05M sodium methoxide, and then treated with Amberlite IR-120 (H^+ form) resin, and evaporated; yield 14.5 g.

Fractionation of the deacetylated acetolyzate of the dextran by charcoal–Celite chromatography. — The deacetylated acetolyzate (14.5 g) was dissolved in 145 mL of deionized water. The solution was adjusted to pH 6.2 with sodium hydroxide and applied to a column (30 × 9.5 cm) containing 300 g of charcoal (Takeda Pharmaceutical Co.) and the same amount of Celite (No. 545). The sugars were eluted stepwise with water, and 2.5, 5, 7.5, 10, 12.5, 15, and 20% ethanol. The eluates were collected after each 1000 mL, evaporated, and examined by paper chromatography. The results are shown in Table I. Eleven kinds of oligosaccharides were detected, in addition to the disaccharides, kojibiose, isomaltose, and nigerose.

Further separation of the oligosaccharides. — Further separation of the oligosaccharides from the charcoal–Celite chromatography was performed by preparative paper-chromatography and paper electrophoresis. Four trisaccharide and four tetrasaccharide fractions were purified to chromatographic and electrophoretic homogeneity. Although several other oligosaccharides were detected by paper chromatography and paper electrophoresis, these were obtained in only minute amounts and were not examined further. Yields and some properties of the isolated oligosaccharides are listed in Table II.

Methylation analysis. — The oligosaccharides were methylated by the method of Hakomori[21]. Methanolysis of the methylated oligosaccharides was performed by an established procedure[22]. The methanolyzates were analyzed by gas–liquid chromatography, under conditions reported previously[3].

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