Carbohydrate Research, 127 (1984) 331–337
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

Note

Structures of three α-D-(1→2)-branched oligosaccharides isolated from *Leuconostoc mesenteroides* NRRL B-1299 dextran

YASUSHI MITSUISHI, MIKIHIKO KOBAYASHI, AND KAZUO MATSUDA
Department of Agricultural Chemistry, Faculty of Agriculture, Tohoku University, Sendai, Miyagi 980 (Japan)

(Received October 25th, 1983; accepted for publication, November 18th, 1983)

We earlier reported[1] that two different types of dextranase are produced in the culture fluid of a Gram-negative *Flavobacterium* sp. M-73. The first enzyme had a strict specificity for α-D-(1→2)-glucosidic linkages at the branch points of dextrans, and produced D-glucose as the only reducing sugar[2]. The second enzyme hydrolyzed the α-D-(1→6) linkages of virtually linear dextrans[3], and produced mainly isomaltotriose[4]. We now report isolation and structural determination of the three oligosaccharides that were used as substrates in our previous study[2] on the action pattern of the α-D-(1→2)-debranching enzyme.

EXPERIMENTAL

Dextran from *Leuconostoc mesenteroides* strain NRRL B-1299 was prepared by the procedure reported previously[5]. The water-soluble fraction of the purified B-1299 dextran (2.0 g) was dissolved in 50mM acetate buffer, pH 6.0 (400 mL), and digested with 3.0 units of *Flavobacterium* α-D-(1→2)-debranching enzyme[6] for 30 min at 40°. The reaction was terminated by heating for 5 min at 100°. The degree of hydrolysis was 3% as D-glucose, determined by the Nelson–Somogyi method as described previously[6]. The reaction mixture was dialyzed against distilled water to remove D-glucose. The dialyzate was concentrated to 100 mL, and digested with 66 units of endo-dextranase from *Chaetomium gracile*[7]. After incubation for 24 h at 40°, the degree of hydrolysis reached 10%. To the digest was added an equal volume of methanol, and high-molecular-weight materials were removed by centrifugation at 18,000*g* for 10 min. The oligosaccharide mixture was obtained from the supernatant liquor (yield 854 mg, 42.7%).

Paper-chromatographic analysis indicated the presence of several oligosaccharides corresponding in their R_G values to branched oligosaccharides. Therefore, the mixture of oligosaccharides was subjected to gel filtration in a column of

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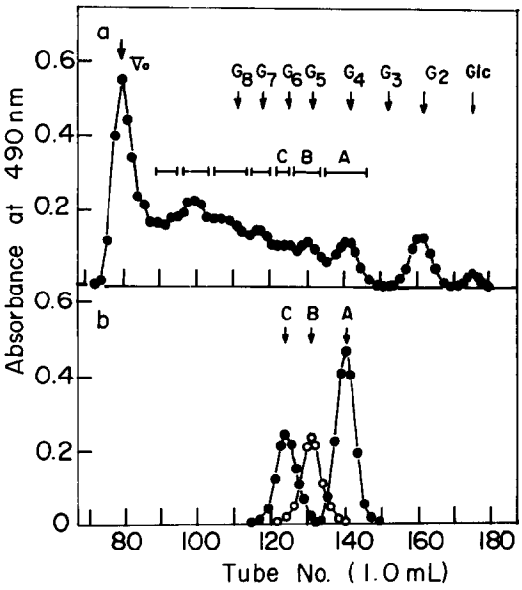

Fig. 1. Elution pattern of the endo-dextranase digest of B-1299 dextran from a Bio-Gel P-2 column. (a) The mixture of oligosaccharides, prepared by the consecutive action of α-D-(1→2)-debranching enzyme and endo-dextranase on B-1299 soluble dextran. (b) Composite patterns of the purified oligosaccharides A, B, and C. [Each oligosaccharide was purified to homogeneity by rechromatography on the same column. Sample (170 mg/0.8 mL of water) was applied to the column (1.5 × 140 cm), and eluted with water. Sugar was determined by the phenol–sulfuric acid method[8]. Abbreviations Glc to G_8 represent the elution volumes of D-glucose to isomalto-octaose. V_0: void volume of the column.]

Bio-Gel P-2, as shown in Fig. 1a. More than ten peaks were detected, and paper-chromatographic analysis showed that the last two peaks (Nos. 154–168 and 172–180) corresponded to isomaltose and glucose, respectively. Three oligosaccharide fractions (A, B, and C in Fig. 1a) were pooled, and purified further by repeated chromatography on the same column. Purified oligosaccharides A, B, and C were eluted at the positions of d.p. (degree of polymerization) 4, 5, and 6, respectively (see Fig. 1b).

The three oligosaccharides gave a single spot on paper-chromatographic (see Fig. 2 A, B, and C; lane 0) and paper-electrophoretic analysis. Some properties of these oligosaccharides are summarized in Table I, together with the yields of the sugars. The respective yields of oligosaccharides A, B, and C were 30.7, 11.9, and 18.8 mg. The d.p. values were almost compatible with those estimated from the gel-filtration chromatography. The mobility in paper electrophoresis and the staining property with triphenyltetrazolium chloride (TTC) indicated that the glucosidic linkages at the reducing end of these three sugars were not α-D-(1→2). The methyl glycosides of 2,3,4,6-tetra- and 2,3,4-tri-*O*-methyl-D-glucoses were detected from all of the sugars by methylation analysis. Moreover, 3,4,6-tri-*O*-methylglucose was detected from methylated sugars A and C, and 3,4-di-*O*-methylglucose detected

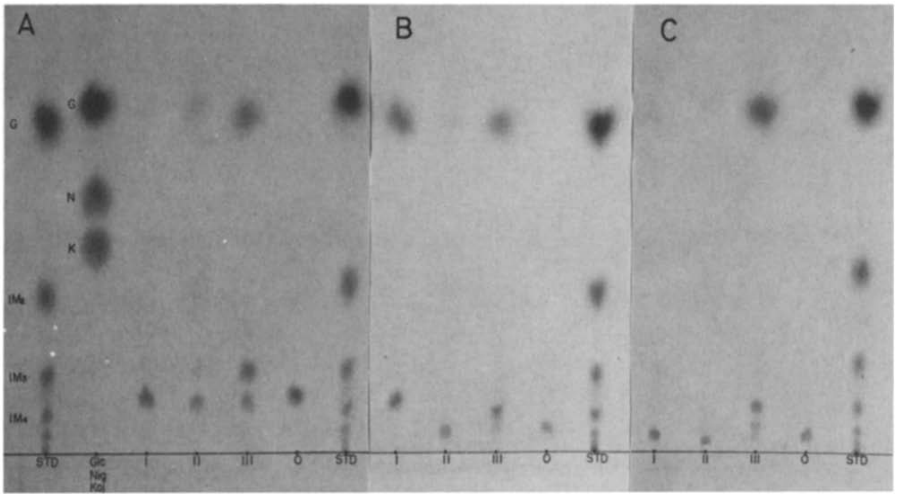

Fig. 2 Paper chromatograms of hydrolyzates of α-D-(1→2)-branched oligosaccharides with glucoamylase, endo-dextranase, and α-D-(1→2)-debranching enzyme. (A), Branched tetrasaccharide A; (B), branched pentasaccharide B; (C), branched hexasaccharide C. [STD corresponds to the standard sugars. G, D-glucose; N, nigerose; K, kojibiose; IM_2–IM_4, isomaltose to isomaltotetraose. Lane I, digested with glucoamylase; lane II, digested with endo-dextranase; lane III, digested with α-D-(1→2)-debranching enzyme; lane 0, original, untreated sample. The mixture (1.0 mL) consisted of the branched oligosaccharide (600 μg) and enzyme (0.14 unit) in water. Incubation was performed for 24 h at 40°. After concentration, each digest was subjected to ascending paper-chromatography, using 6:4:3 (v/v) 1-butanol–pyridine–water. Spots were detected as described previously[2].]

TABLE I

YIELDS AND PROPERTIES OF THE OLIGOSACCHARIDES A, B, AND C OBTAINED FROM THE B-1299 SOLUBLE DEXTRAN[a]

Fraction	*Oligosaccharide*		
	A	*B*	*C*
Yield (mg)	30.7	11.9	18.8
d.p.[b]	4.0	4.9	5.6
$[\alpha]_D$ (degrees)[c]	139.5	147.6	149.2
R_G[d]	0.46	0.32	0.24
M_G[e]	0.44	0.47	0.44
TTC[f]	+	+	+

[a]Three oligosaccharides were isolated from the B-1299 dextran by gel filtration on a column of Bio-Gel P-2 after consecutive, partial hydrolysis with α-D-(1→2)-debranching enzyme and endo-dextranase as described in the text. [b]The degree of polymerization (d.p.) of the oligosaccharides was determined by the method of Peat *et al.*[9]. [c]$[\alpha]_D$ was measured with a Nippon Bunko Model DIP-SL polarimeter. [d]Paper-chromatographic mobility [solvent: 13:7 (v/v) 1-propanol–water], relative to D-glucose as unity. [e]Paper-electrophoretic mobility (relative to D-glucose). Electrophoresis was conducted as described previously[10]. [f]Triphenyltetrazolium chloride reaction[11].

from methylated B and C. Thus, the following structures are suggested: together with α-(1→6) linkages, oligosaccharide A contains linear α-(1→2) linkages; oligosaccharide B contains branched α-(1→2) linkages; and oligosaccharide C contains both linear and branched α-(1→2) linkages.

Further studies on the structure of these three oligosaccharides were conducted by enzymic analysis. Because we had previously demonstrated that glucoamylase could hydrolyze the α-D-(1→6) linkage of dextran in an *exo* type of action[12], the action of this enzyme on oligosaccharides A, B, and C was examined,

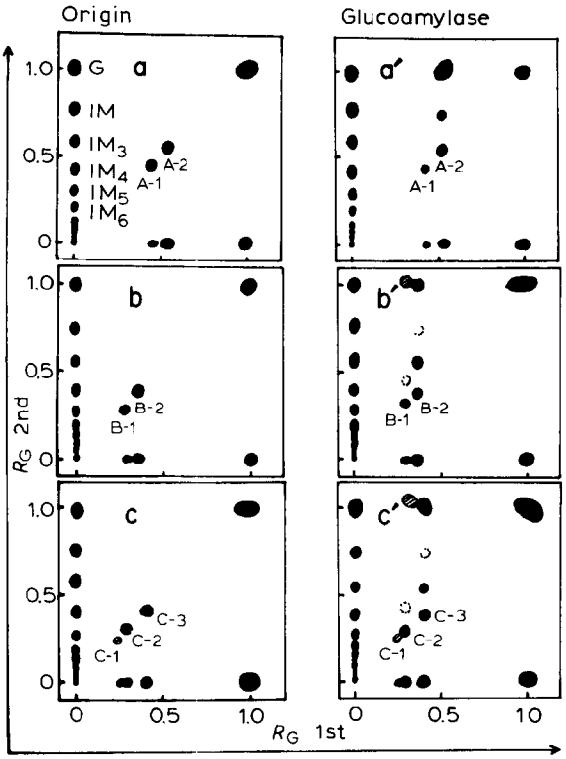

Fig. 3. Diagrammatic representation of the two-dimensional, paper chromatogram of the hydrolyzate of branched oligosaccharides with the combination of α-D-(1→2)-debranching enzyme and glucoamylase. [Origin (a), (b), and (c): branched oligosaccharides A, B, and C were digested with α-D-(1→2)-debranching enzyme as described in the legend to Fig. 2. Each sample was spotted on the filter paper, and developed in the 1st and 2nd directions. Glucoamylase (a'), (b'), and (c'): each oligosaccharide was digested with the debranching enzyme as just described. After development in the 1st direction, the filter paper was completely air-dried, glucoamylase solution (0.1%) was sprayed on the sample area, and the moistened paper was incubated for 2 h at 30°. The air-dried paper was then developed in the 2nd direction. (a), (a'): oligosaccharide A; (b), (b'): oligosaccharide B; (c), (c'): oligosaccharide C. Intensity of the spot ●, strong; ◍, moderate; ◌, weak. G to IM_6 correspond to the standard sugars, D-glucose to isomaltohexaose.

The following assignments of the fragment were based on the R_G of the products and the results shown in Fig. 2.

in order to confirm the presence of unsubstituted α-(1→6)-linked D-glucosyl groups at the nonreducing terminal. When the branched saccharides were digested with glucoamylase (*Rhizopus niveus*)[12], only oligosaccharide B was susceptible to this enzyme. The hydrolysis products were D-glucose and an oligosaccharide having the same R_G value as oligosaccharide A (see Fig. 2B, lane I).

The endo-dextranase from *Chaetomium gracile*[7] scarcely attacked these three oligosaccharides, supporting the presence of α-(1→2)-branched structures in these sugars (see Fig. 2A, B, and C; lane II). The purified, dextran α-D-(1→2)-debranching enzyme from *Flavobacterium* sp. M-73 attacked the three sugars. D-Glucose and isomaltotriose were produced from A, and D-glucose and isomaltotetraose from B (see Fig. 2A and B; lane III). On the other hand, D-glucose, isomaltotet-

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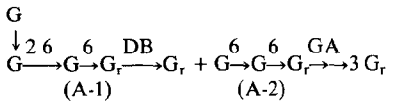
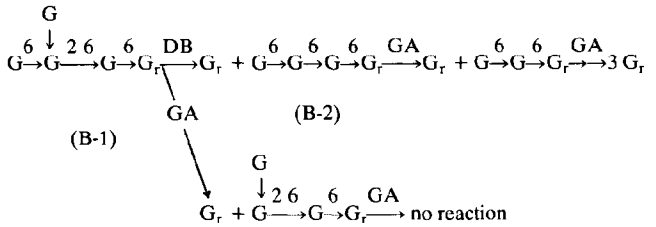
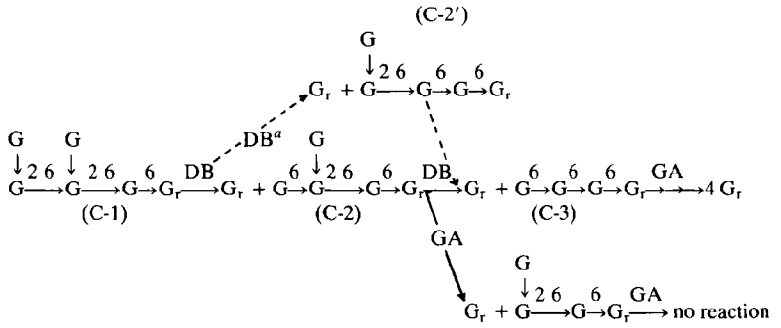
Oligosaccharide A:



Oligosaccharide B:



Oligosaccharide C:



DB, α-D-(1→2)-debranching enzyme; GA, Glucoamylase; G, α-D-glucopyranosyl unit; G$_r$, reducing D-glucose unit.

[a]Action of debranching enzyme on the internal α-D-(1→2)-branch may also be probable if fragments (C-2) and (C-2′) are indistinguishable with respect to their R_G values.]

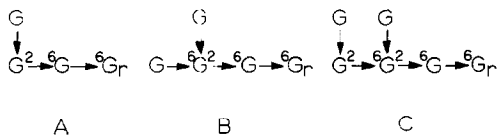


Fig. 4. Structure of the branched oligosaccharides A, B, and C. [G, α-D-glucopyranosyl unit; G_r, reducing D-glucose units.]

raose, and a branched tetraose having the same R_G value as sugar B were produced by the action of the debranching enzyme on sugar C (see Fig. 2C, lane III).

To determine the positions of the branch points, two-dimensional paper-chromatography was conducted by using samples that had been digested with a combination of the α-D-(1→2)-debranching enzyme and glucoamylase. As shown in Fig. 3, several oligosaccharide fragments were detected on the chromatograms. All of the fragments from sugars A, B, and C could be assigned as isomalto-saccharides or α-D-(1→2)-branched oligosaccharides (see Fig. 3, legend).

Based on the results of chemical and enzymic analysis, the structures of the three oligosaccharides are as shown in Fig. 4. Oligosaccharide A contained one α-D-(1→2)-glucosidic linkage at the nonreducing end, and its structure was determined to be 2^3-*O*-α-D-glucosylisomaltotriose. Oligosaccharide B contained one α-D-(1→2)-branch point adjacent to the nonreducing end, and the structure was 2^3-*O*-α-D-glucosylisomaltotetraose. Oligosaccharide C contained two adjacent α-D-(1→2) linkages at the nonreducing terminal, and C was identified as 2^3,2^4-di-*O*-α-D-glucosylisomaltotetraose.

Although these oligosaccharides could not be isolated from the acetolyzate of the B-1299 soluble-dextran[13], Bourne *et al.*[14] identified a pentasaccharide (2^4-*O*-α-D-glucosylisomaltotetraose) which was prepared by the action of *Penicillium lilacinum* endo-dextranase on acid-degraded, B-1299 soluble-dextran. Prior to the fragmentation analysis with endo-dextranase, Bourne *et al.*[14] adopted partial hydrolysis with acid to diminish the α-(1→2) branch points from the B-1299 dextran, whereas we have shown the effectiveness of the action of the α-D-(1→2)-debranching enzyme[2]. Enzymic preparation of branched oligosaccharides is more useful for obtaining a well processed form of branched sugars, because of the definite substrate-specificity of enzymes. Therefore, homogeneous preparations of the aforementioned, three branched saccharides were obtained by rechromatography on a Bio-Gel column, whereas tetra- and penta-saccharide fractions prepared by acid hydrolysis and subsequent dextranase hydrolysis were mixtures of more than two components[14].

Although Bourne *et al.*[14] isolated the tetrasaccharide A fraction, it was a mixture of tetrasaccharide A and another sugar. Tetrasaccharide A had the same structure as our sugar A, but we isolated sugar A as a homogeneous preparation. Moreover, oligosaccharide B (2^3-*O*-α-D-glucosylisomaltotetraose) and C (2^3,2^4-di-*O*-α-D-glucosylisomaltotetraose) have been newly isolated, and characterized, herein. The latter branched saccharide, C, provides good evidence for the occurr-

ence of two adjacent α-D-(1→2) branch-points on the α-D-(1→6)-linked backbone, as described previously[14]. It may also support a highly branched structure for the water-soluble dextran from[15] *L. mesenteroides* NRRL B-1299.

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