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MODES OF ACTION OF INTRACELLULAR DEXTRANASE AND THREE OLIGOGLUCANASES FROM *Pseudomonas UQM733**†

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ABSTRACT

The action patterns have been studied of a purified, intracellular dextranase and three intracellular α-D-glucosidases from *Pseudomonas UQM733* on pure isomalto-oligosaccharides. The glucosidases have optimal activity on isomaltotetraose and are therefore classified as oligoglucanases. They have been used to determine the structure of two branched isomalto-oligosaccharides obtained by enzymic degradation of dextran.

INTRODUCTION

In the previous paper of this series[1a], we described the purification and general properties of an intracellular dextranase (D_4) and of three intracellular glucosidases (G_1, G_2, and G_3) from *Pseudomonas UQM733*. This paper further describes the mode of action of these enzymes towards isomalto-oligosaccharides. Throughout this paper, the abbreviation IM_n refers to an oligosaccharide of the isomaltose series containing *n* D-glucosyl residues.

EXPERIMENTAL

All materials and methods, including enzyme assays, have been described previously[1a]. The isomalto-oligosaccharides were prepared in this laboratory by M. Streamer. The branched isomalto-oligosaccharides B_5 and B_6 (for preparation, see ref. 1a) showed $[\alpha]_D^{23}$ +177° and +180°, respectively (*c*, 0.12, water).

RESULTS AND DISCUSSION

Mode of action of dextranase D_4 *on isomalto-oligosaccharides.* — The increase in reducing power of the oligosaccharides as a result of hydrolysis of these substrates

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by dextranase D_4 is shown in Fig. 1. The initial rates of hydrolysis increase with increasing degree of polymerisation (d.p.) from IM_7–IM_9, but are very low for the lower oligosaccharides. The hydrolysis of IM_6 gave rise to approximately equimolar proportions of IM_4 and IM_2, with no IM_3 detectable by t.l.c. until 5 h of hydrolysis. The quantity of IM_3 formed (Fig. 2) at 5 h was approximately 10% of that of IM_4 or IM_2. Thus, an asymmetrical scission of IM_6 is favoured. This behaviour is similar to the results described earlier[1b] of hydrolysis of IM_6 by the extracellular dextranase D_1 of the same bacterium. Oligosaccharides having a degree of polymerisation (d.p.) greater than that of the substrate, namely IM_7–IM_9, were present in trace amounts in samples taken at 1 (IM_9 only), 5, and 9 h (IM_7–IM_9). Glucose was not detected in any of the hydrolysates. To account for the products, the following scheme is proposed:

$$E + IM_6 \rightarrow EIM_6 \rightarrow EIM_2 + IM_4 \qquad (1)$$

$$EIM_2 + IM_6 \rightarrow EIM_8 \rightarrow E + IM_2 + IM_3 + IM_4 + IM_5 + IM_6 \qquad (2)$$

(E represents the enzyme, and EIM_2 the enzyme–substrate complex). Reactions (*1*) and (*2*) would account for the small surplus of IM_4 as compared to IM_2. The alternative formation of EIM_4 in reaction (*1*) may account for the traces of IM_7 and IM_9

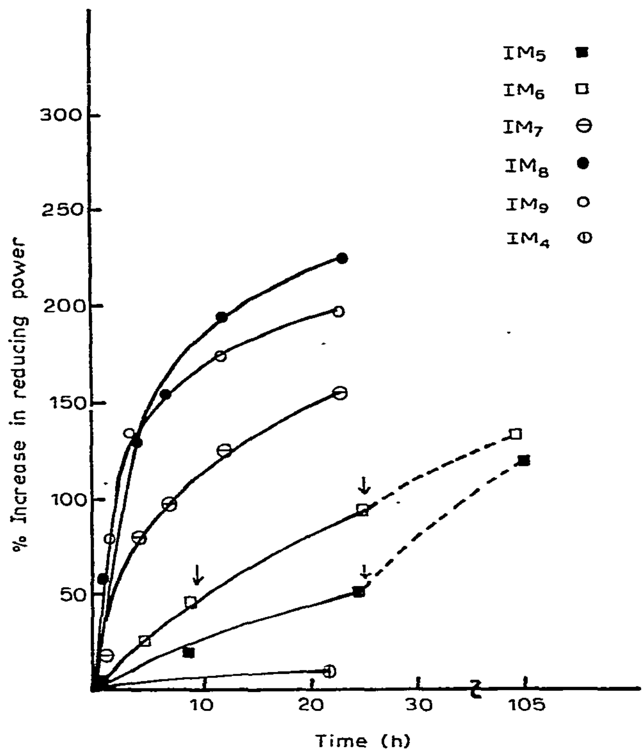


Fig. 1. Hydrolysis of oligosaccharides (4.5mM) with Dextranase D_4 at 33°. Initial enzyme 0.01 unit for IM_5 and IM_6, 0.02 unit for remainder. Arrows indicate further additions of 0.01 unit of enzyme.

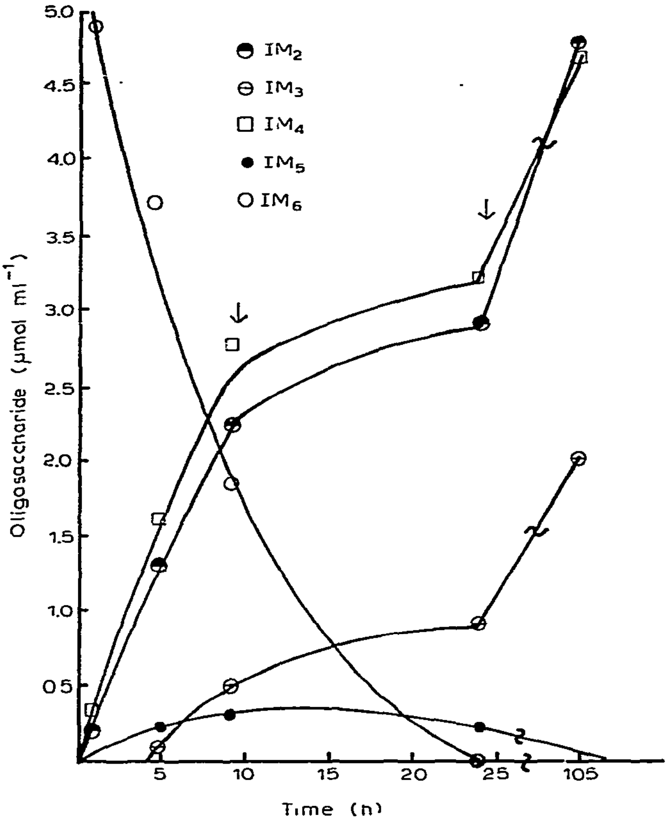


Fig. 2. Products of action of dextranase D_4 (0.01 unit) on IM_6 (5.3mM) at 33°. Arrows indicate further additions of 0.01 unit of enzyme.

detected in the hydrolysates following recombination of EIM_4 with IM_6. All of the products and rates observed in the action of dextranase D_4 on IM_7 and IM_8 (Figs. 3–5) may be explained similarly.

As isomalto-oligosaccharides having fewer than six glucosyl residues are hydrolysed only slowly, the binding-site of the enzyme appears to favour a sequence of at least six α-D-(1→6)-linked glucosyl residues in order to form an active enzyme–substrate complex. In this, the enzyme is similar to the extracellular endo-dextranase D_1. The active site appears to be asymmetrically placed within the binding-site, as such oligosaccharides having even numbers of D-glucosyl residues, as IM_6 and IM_8 were more readily hydrolysed at the glucosidic linkage adjacent to the central glucosidic linkage than at the central glucosidic linkage itself. It is not possible from the foregoing evidence to say whether the favoured mode of hydrolysis occurs nearer the reducing or non-reducing end of the oligosaccharides. In general, the hydrolysis proceeds more rapidly with increasing substrate size, at least up to d.p. 9, and recombination of some of the products with the original substrate is frequently observed.

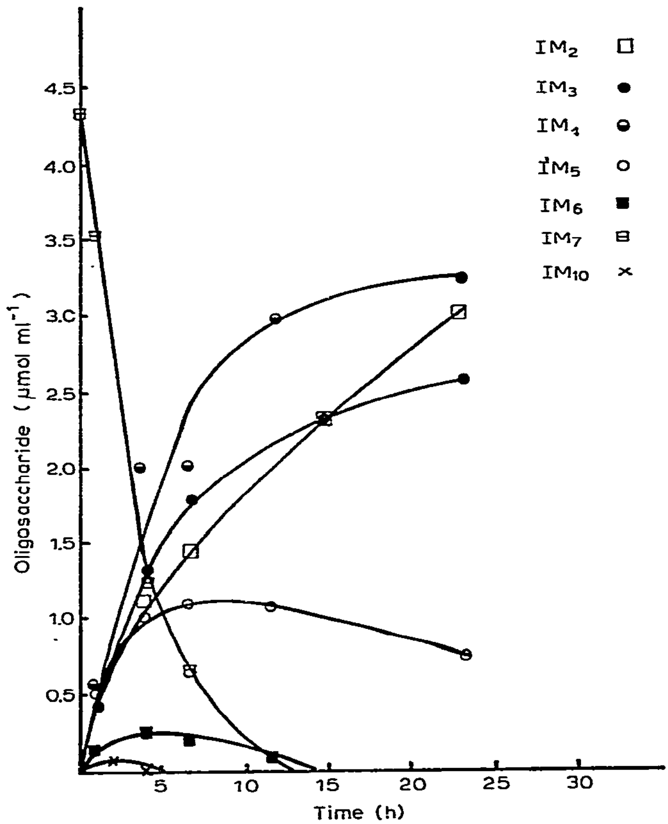


Fig. 3. Products of action of dextranase D_4 (0.02 unit) on IM_7 (4.5mM) at 33°.

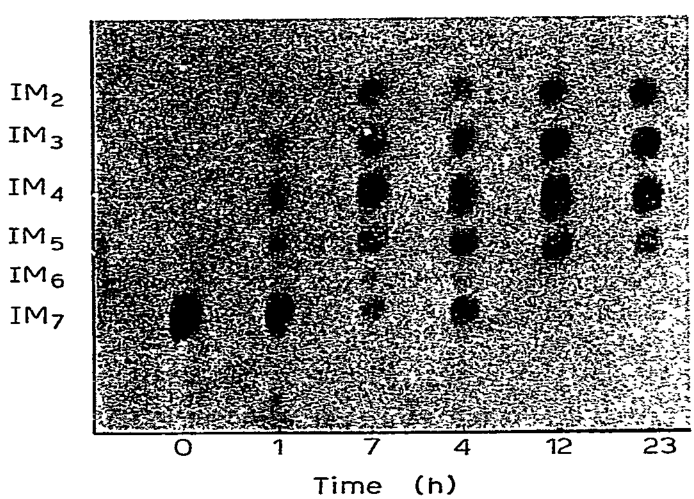


Fig. 4. Thin-layer chromatogram of products of action of dextranase D_4 on IM_7 (4 h at 28° with solvent *A*).

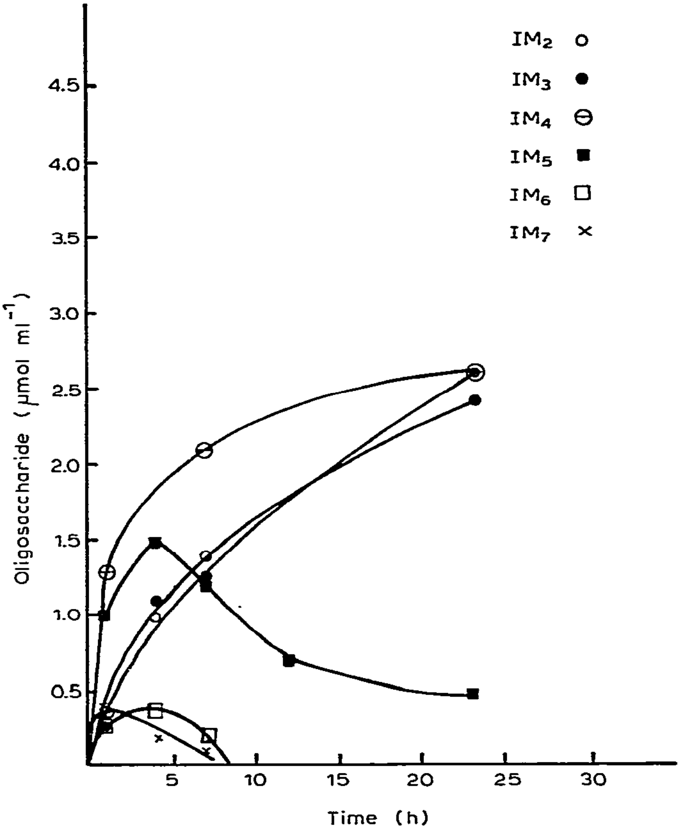


Fig. 5. Products of action of dextranase D_4 (0.02 unit) on IM_8 (3.8mM) at 33°.

All of the foregoing observations are compatible with our earlier tentative conclusion[1a] that the extracellular dextranase D_1 (ref. 1a) is the same enzyme as the intracellular D_4. It is therefore classified as (1→6)-α-D-glucan 6-glucohydrolase, E.C.3.2.1.11.

Mode of action of the glucosidases **1**, **2**, *and* **3** *towards isomalto-oligosaccharides.* — The relative activities of the glucosidases towards IM_2–IM_6 are shown in Fig. 6. The glucosidases all had similar relative activities on the isomalto-oligosaccharides examined, with each exhibiting maximal activity towards IM_4. Thus, these enzymes cannot be classified as α-D-glucosidases according to the criteria of Reese *et al.*[2], who suggested that the best criterion for distinction between an α-D-glucosidase and an exo-glucanase is the relative rate of attack of these enzymes on disaccharides and tetrasaccharides, with the rate of attack of an α-D-glucosidase on a disaccharide being greater than its rate of attack on a tetrasaccharide and the reverse applying for an exo-glucanase.

The glucosidases **1** and **2** were also found to be specific in the type of linkage they hydrolyse, exhibiting activity towards α-(1→6)-linkages and no activity towards

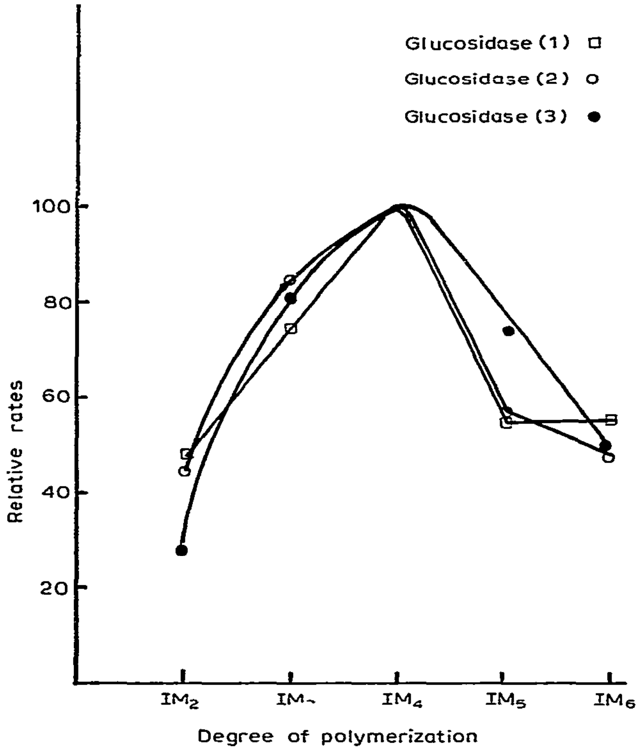


Fig. 6. Relative activities (rate of D-glucose formation) of glucosidases (0.001 unit) on isomalto-oligosaccharides (5.9mM) at 33°.

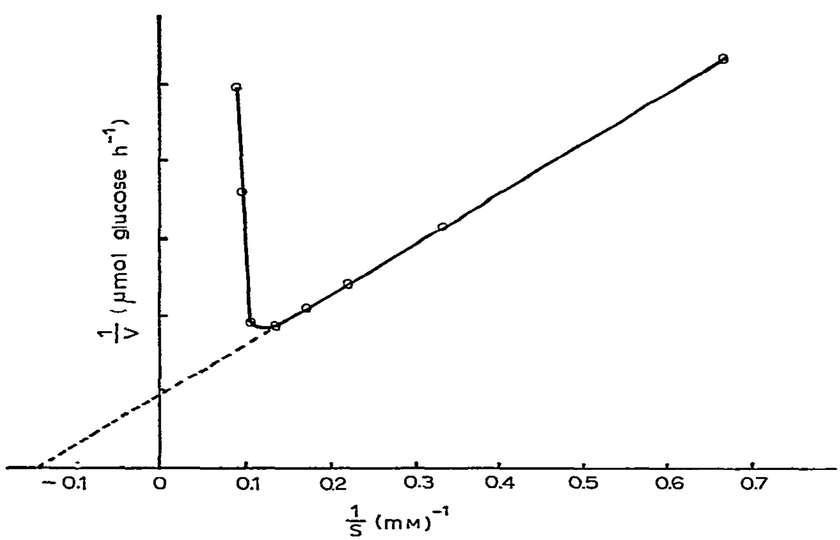


Fig. 7. Hydrolysis of IM_4 with glucosidase 1 at 33°.

α-(1→4)-linkages. This is also a characteristic of exo-glucanases according to the foregoing criteria[2]. Glucosidase 3, however, exhibited equal affinity for α-D-(1→6)- and α-D-(1→4)-linkages (disaccharides)[1a].

These glucosidases are similar in their relative activities to the cytoplasmic glucosidases isolated by Janson[3]. They also had a greater affinity for IM_4 than for any other isomalto-oligosaccharide.

The determination of K_m and V_{max} for the hydrolysis of IM_4 with the glucosidases was carried out with glucosidase **1** only. The Lineweaver–Burke plot (Fig. 7) showed that substrate inhibition occurred when the concentration of substrate was greater than 10mM, and gave K_m 6.9mM and V_{max} 0.41 μmol of D-glucose per h. The overall rate of formation of D-glucose was shown to be linear during the 2 h that the hydrolyses were conducted for the kinetic experiment. As the hydrolysis of IM_4 occurs via a multi-chain mechanism (see later), the K_m value obtained does not represent the true affinity of glucosidase **1** for IM_4, as further hydrolysis of the products IM_3 and IM_2 will also yield D-glucose. The substrate inhibition in hydrolysis of IM_4 by glucosidase **1** is similar to the effect of substrate concentration previously detected in the hydrolysis of maltose with acid α-D-glucosidase[6].

From our present studies, there remains the possibility that the onset of substrate inhibition with oligosaccharides greater than IM_4 may occur at much lower concen-

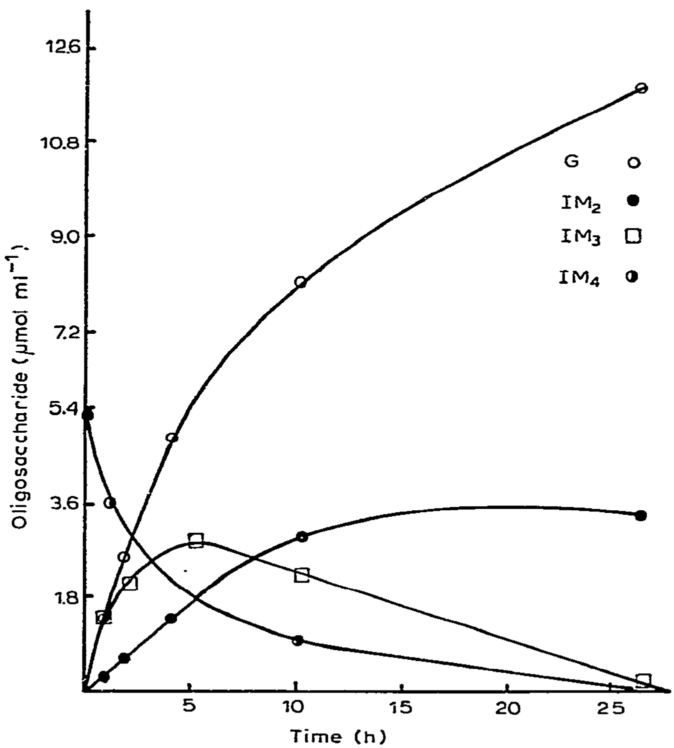


Fig. 8. Products of action of glucosidase **1** (0.003 unit) on IM4 (5.9mM) at 33°.

trations than 10mM, and this could explain the form of Fig. 6. Such a variation in onset of substrate inhibition seems rather unlikely with such similar substrates (compare ref. 6), but the possibility cannot be eliminated without further investigation of the kinetics of the system.

The mode of action of the three glucosidases on IM_4 was determined by quantitative t.l.c. of the enzyme hydrolysates. The results for enzyme **1** are shown in Fig. 8, and enzymes **2** and **3** gave very similar patterns. This information shows that, in each case, the initial reaction involves cleavage of one of the α-D-(1→6) linkages to release Glc and IM_3. This is indicated after 1 h of hydrolysis by the equimolar yields of Glc and IM_3, both of which were present in higher concentration than IM_2. The production of IM_3 reached a maximum at 5 h in the case of glucosidase **1**, approximately 15 h with **2**, and 10 h with **3**. The concentration of IM_2 continued to rise in the hydrolysates until the concentration of IM_3 fell to zero.

Thus, these glucosidases hydrolyse IM_4 in a multi-chain manner, giving rise to IM_3, IM_2, and D-glucose (Glc) initially. The following reactions indicate the hydrolytic process:

$$IM_4 \xrightarrow{E} IM_3 + Glc \qquad (1)$$

$$IM_3 \xrightarrow{E} IM_2 + Glc \qquad (2)$$

$$IM_2 \xrightarrow{E} 2Glc \qquad (3)$$

The alternative possibility of single-chain attack would have caused an increase in D-glucose corresponding to the decrease in IM_4, and no significant, intermediate build-up of IM_3 and IM_2. A similar observation (namely, no build-up of IM_3 and IM_2) would have resulted had the lower homologues been attacked more rapidly than IM_4, so that the results in Fig. 8 also support the conclusion summarised in Fig. 6 that the relative rates of reaction are **1** > **2** > **3**.

The foregoing results confirm our classification[1a] of glucosidases **1** and **2** as (1→6)-α-D-oligoglucan glucohydrolases, and glucosidase **3** is classified as an α-D-oligoglucan glucohydrolase.

Action of the glucosidases on mixed (1→3), (1→6)-α-D-gluco-oligosaccharides. — The branched oligosaccharides were prepared by exhaustive hydrolysis of B-512 native dextran with a mixture of two endodextranases (D_1 and D_2, ref. 1b) and amyloglucosidase (see ref. 1a). The major products were D-glucose and two oligosaccharides, B_5 and B_6, whose t.l.c. behaviour[4] indicated the presence of five and six glucose residues, respectively. ^{1}H-n.m.r. spectroscopy (Fig. 9 for B_5) showed the presence of (1→6)- and (1→3)-α-D-glucosidic linkages in the ratios 3:1 and 4:1, respectively, for B_5 and B_6, and evidently these oligosaccharides constitute fragments derived from the regions around the branch points in the original dextran.

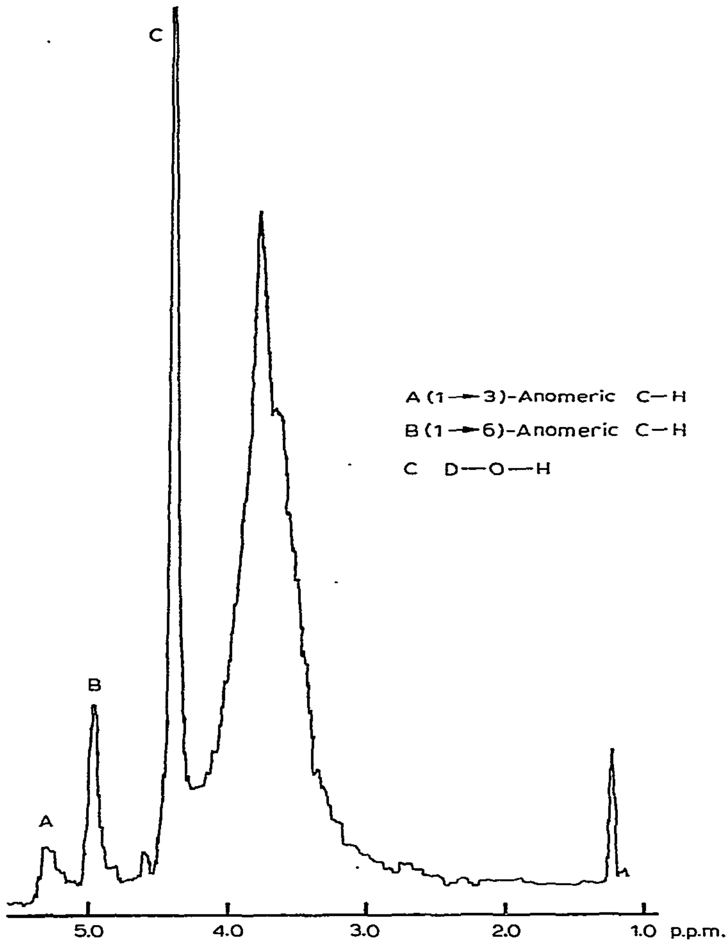


Fig. 9. [1]H-N.m.r. spectrum of oligosaccharide B_5, 3% in D_2O at 60°.

TABLE I

RELATIVE RATES OF HYDROLYSIS OF OLIGOSACCHARIDES BY GLUCOSIDASES

Substrate	*Glucosidase*	
	*G*1	*G*3
IM_4	100	100
B_5	14.4	13.6
B_6	10.1	13.3

TABLE II

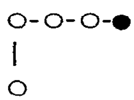
PRODUCTS OF HYDROLYSIS OF B_5 AND B_6 BY GLUCOSIDASES 1 AND 3

Enzyme/substrate	*Products*
(1)/B_5	Glc, IM_2^a, IM_3^a, IM_4^a, and B_5
(1)/B_6	Glc, IM_2^a, IM_3^a, IM_4^a, B_5, and B_6
(3)/B_5	Glc, IM_2^a, IM_3^a, IM_4^a, and B_5
(3)/B_6	Glc, IM_2^a, IM_3^a, IM_4^a, B_5, and B_6

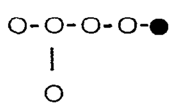
[a]Trace amounts only.

The glucosidases 1 and 3 each hydrolysed B_5 and B_6 at approximately equal rates (Table I). Inadequate yields of G_2 precluded similar experiments with this enzyme.

To account for these products, the following structures and modes of degradation for B_5 and B_6 are proposed:



B_5



B_6

$$B_5 \xrightarrow[E]{\text{slow}} IM_4 + Glc$$

$$IM_4 \xrightarrow[E]{\text{fast}} IM_3 + Glc \qquad B_6 \xrightarrow[E]{\text{slow}} B_5 + Glc$$

$$IM_3 \xrightarrow[E]{\text{fast}} IM_2 + Glc \qquad B_5 \xrightarrow[E]{\text{slow}} IM_4 + Glc$$

$$IM_2 \xrightarrow[E]{\text{fast}} 2Glc$$

[O-O indicates an α-(1→6)-link;

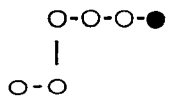
O-● indicates an α-(1→6)-link at the reducing end-group; and

O
| indicates an α-(1→3)-link]
O

According to this scheme, the rate-determining step for B_5 is the removal of the α-(1→3)-link to give IM_4 which is then rapidly degraded through IM_3→IM_2→Glc. The attachment of the α-(1→3)-link at the third or second glucose residue from the reducing end-group is unlikely, as this would make possible the removal of an α-(1→6)-linkage from the nonreducing end, which would give rise to further branched products (such as B_4). As no branched oligosaccharides of d.p. less than 5 were detected in t.l.c. of the hydrolysates, this type of structure may be eliminated. The appearance of trace amounts of IM_4 indicates that the glucosidases **1** and **3** are able to hydrolyse the α-(1→3)-linkage in B_5.

The appearance of B_5 in the enzyme hydrolysates of B_6 indicates that the branch point in B_6 occurs at the fourth glucosyl residue of the oligosaccharide from the reducing end-group. The removal of the fifth α-D-(1→6)-linked glucosyl residue probably occurs at a higher rate than the removal of the α-D-(1→3)-linked glucosyl residue. This step yields B_5 which, in turn, is slowly hydrolysed to IM_4 and D-glucose, with IM_4 meanwhile undergoing further, rapid hydrolysis.

The foregoing results may also be interpreted to indicate an alternative structure for B_6 thus:



In the preparation of B_5 and B_6, the endo-dextranases produce a wide range of branched oligosaccharides, probably including B_5 and B_6 (compare ref. 5). These are subsequently attacked by amyloglucosidase to remove D-glucosyl groups successively from the nonreducing end until only B_5 and B_6 remain. It may be concluded, therefore, that the branched oligosaccharides produced by action of dextranases D_1 and D_2 on B-512 dextran all have a branch point that is three glucosyl residues removed from the reducing end-group. This result, in turn, indicates the closest point of enzymic scission of the dextran in relation to the branch points.

ACKNOWLEDGMENTS

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