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Note

Degradation of barley glucan by a purified (1→4)-β-D-glucanase from the snail, *Helix pomatia*

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Previous papers[1,2] have described the purification and partial characterization of an endo-acting (1→4)-β-D-glucan hydrolase from the intestinal juice of the snail, *Helix pomatia*. Preliminary studies[2] on the substrate specificity of this enzyme indicated that it degrades mixed-linkage (1→3; 1→4)-β-D-glucans in a manner different from that of other known endo-(1→4)-β-D-glucanases, as evidenced by differences in the extents of hydrolysis and the chromatographic mobilities of the oligosaccharides resulting from enzyme action. This communication describes a study of the degradation of barley β-glucan by purified *Helix pomatia* (1→4)-β-D-glucanase, and characterization of the major low-molecular-weight product produced by the enzyme. The results are discussed with reference to the structure of barley glucan and the specificity of the enzyme.

Barley glucan (100 mg), isolated from Zephyr barley by the method of Preece and MacKenzie[3], was incubated at 37° with purified *Helix pomatia* endo-(1→4)-β-D-glucanase (1 unit) in a digest of composition similar to that used previously[2]. After 30 h, by which time release of reducing sugars (as measured by reduction of alkaline copper reagent[4]) had ceased, the enzyme was inactivated by heating the digest for 5 min at 100°. Eleven percent of hydrolysis of the substrate occurred during the enzymic degradation, giving products having an average degree of polymerization 9. Examination of the products by descending paper-chromatography in 10:4:3 ethyl acetate–pyridine–water (v/v) showed a single, major, chromatographically-mobile oligosaccharide having a mobility in the tetrasaccharide region of the chromatogram, and a large proportion of material of low mobility near the origin.

The major low-molecular-weight product in the digest was isolated by chromatography on a column (2.5 × 10 cm) of charcoal–Celite[5] that was eluted with a gradient of ethanol, with subsequent preparative paper-chromatography on Whatman 3MM paper that was developed with the solvent used for the analytical paper

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chromatography. We previously suggested[2], on the basis of chromatographic mobility, that the oligosaccharide is a tetrasaccharide, and this has now been confirmed by reduction with sodium borohydride to give the corresponding alditol[6]. The oligosaccharide alditol produced approximately 75% of the color of the unreduced oligosaccharide in the phenol–sulfuric acid reaction[7], indicating it to have a degree of polymerization of 4.

The structure of the oligosaccharide was established by use of three purified β-D-glucan hydrolases of well established specificity. In each instance, the products of enzyme action were identified by paper chromatography with use of suitable standard oligosaccharides. The action of Basidiomycetes QM 806 exo-(1→3)-β-D-glucanase[8–10], at a concentration of 50 units/ml (as measured by release of D-glucose from laminaran[8]) for 4 h, produced glucose and a sugar having the chromatographic mobility and characteristic behavior (elongated, tailing spot) of cellotriose. As the Basidiomycetes enzyme removes terminal β-(1→3)-linked D-glucose units and has no action on β-(1→4)-linked D-glucose residues, this finding suggested that the tetrasaccharide contains a terminal (1→3)-linked β-D-glucose unit. The production of cellotriose as the other product of enzyme action indicated that the other three D-glucose residues in the oligosaccharide are joined by β-D-(1→4) linkages. Thus the oligosaccharide appeared to be *O*-β-D-glucopyranosyl-(1→3)-*O*-β-D-glucopyranosyl-(1→4)-*O*-β-D-glucopyranosyl-(1→4)-D-glucopyranose. That this is indeed the correct structure was confirmed by the action of *Rhizopus arrhizus* (1→3)-β-D-glucanase[11,12]. During incubation for 4 h, low concentrations of this enzyme [0.03 unit/ml, as measured by the release of reducing sugars from laminaran[12]] converted the oligosaccharide into a mixture of laminarabiose and cellobiose in what appeared visually to be approximately equimolar amounts. The *Rhizopus arrhizus* enzyme cleaves (1→3) or (1→4)-β-D-glucosidic linkages adjacent to a β-D-(1→3) linkage[13,14]. Action on a tetrasaccharide to give laminarabiose and cellobiose requires that the oligosaccharide be *O*-β-D-glucopyranosyl-(1→3)-*O*-β-D-glucopyranosyl-(1→3)-*O*-β-D-glucopyranosyl-(1→4)-D-glucopyranose or *O*-β-D-glucopyranosyl-(1→3)-*O*-β-D-glucopyranosyl-(1→4)-*O*-β-D-glucopyranosyl-(1→4)-D-glucopyranose. The former structure is ruled out in view of the action of exo-(1→3)-β-D-glucanase on the oligosaccharide; only the latter can be converted into D-glucose and cellotriose by removal of terminal (1→3)-linked β-D-glucose units. In addition, adjacent (1→3)-β-D-glucosidic linkages are known to be virtually absent in barley glucan[15]. If the tetrasaccharide has the structure suggested, it should be resistant of the action of "lichenase"[16], an enzyme that cleaves (1→4)-β-D-glucosidic linkages adjacent to non-terminal (1→3)-β-D-glucosidic linkages in mixed-linkage polysaccharides and oligosaccharides[17]. Even extended incubation (4 h) at high concentration of enzyme (1.9 unit/ml, as measured by the release of reducing sugars from lichenan[17]) did not result in hydrolysis by this enzyme. It is, therefore, clear that the major, chromatographically mobile product resulting from the action of *Helix pomatia* (1→4)-β-D-glucanase on barley glucan is *O*-β-D-glucopyranosyl-(1→3)-*O*-β-D-glucopyranosyl-(1→4)-*O*-β-D-glucopyranosyl-(1→4)-D-glucopyranose. The actions of Basidiomycetes exo-(1→3)-β-D-glucanase and *Rhizopus*

arrhizus (1→3)-β-D-glucanase on the tetrasaccharide resulted in complete conversion into the stated products in both instances, ruling out the presence of any other tetrasaccharide(s) of similar chromatographic mobility. The action of the various enzymes on this oligosaccharide is illustrated diagrammatically in Fig. 1.

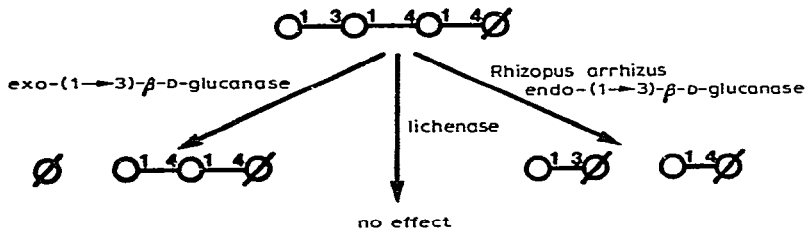

Fig. 1. Diagrammatic representation of the enzymic degradation of the tetrasaccharide product formed by action of *Helix pomatia* (1→4)-β-D-glucanase on barley glucan.
Key: ○, D-glucose residue; ∅, reducing D-glucose residue; —, β-D-glucosidic linkage.

Barley glucan is a linear polysaccharide in which 70–75% of the β-D-glucosidic linkages are (1→4) and 25–30% are (1→3). The arrangement of the different types of linkages in the polysaccharide is such that it may be considered to be constructed essentially of cellotriose and cellotetraose units joined by (1→3)-β-D-glucosidic linkages, there being about 2.5 cellotriose segments for every cellotetraose segment[18]. The structure is shown diagrammatically in Fig. 2. It is appropriate to consider the action of *Helix pomatia* endo-(1→4)-β-D-glucanase on barley glucan in relation to the fine structure of the polysaccharide. As the *Helix pomatia* enzyme cannot act on free cellotriose, it would seem unlikely that it should act on the cellotriose units in barley glucan. However, cleavage of one bond in each cellotetraose segment, as takes place in free cellotetraose, would account approximately for the measured extent of

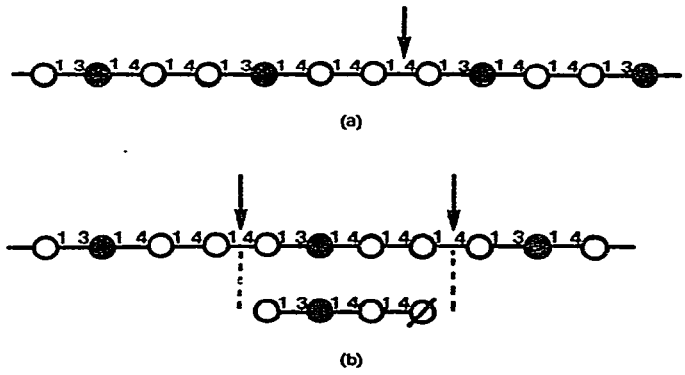

Fig. 2. (*a*) Diagrammatic representation of the structure of barley β-glucan (symbols as in Fig. 1). D-Glucose residues substituted at position 3 are shown in black for contrast[18]. Cleavage by *Helix pomatia* (1→4)-β-D-glucanase takes place at the position arrowed. (*b*) Showing the production of the oligosaccharide characterized here, from chain segments where two cellotetraose units are adjacent.

hydrolysis (11%, as compared with a calculated value of 9–10%). Thus it seems likely that the specificity ascribed here to the enzyme—the requirement for a series of 3 contiguous (1→4)-β-D-glucosidic linkages–holds in the case of action on the mixed-linkage glucans as well as for oligosaccharide substrates. Furthermore, the close agreement between the measured and calculated extents of hydrolysis indicates that the enzyme has a specificity that is directed towards the linkage split, as well as towards the linkages in the substrate involved in enzyme–substrate binding. Cleavage of (1→3) linkages by the enzyme, in a manner analogous to that whereby *Rhizopus arrhizus* (1→3)-β-D-glucanase acts on (1→4)-β-D-glucosidic linkages[13,14,18] would have been apparent by the measured extent of hydrolysis being markedly greater than the expected value, and the appearance of cellotriose in the hydrolysis products. Although the enzyme acts on free cellotetraose, splitting preferentially the middle (1→4)-β-D-glucosidic linkage, the same is not true during action on the cellotetraose units in barley glucan. In the latter case, the linkage split corresponds to that joining the free, reducing D-glucose and the penultimate residue in free cellotetraose. This action is presumably the result of constraints imposed by the (1→3)-substitution of the D-glucose residue corresponding to the non-reducing terminal unit in free cellotetraose.

The tetrasaccharide product that has been characterized can only arise from portions of the polysaccharide molecule where there are two adjacent cellotetraose segments joined by a (1→3)-β-D-glucosidic linkage (Fig. 2). Thus it can be concluded that the cellotetraose and cellotriose segments in barley glucan are more likely to be arranged randomly than regularly in this polysaccharide. A regular arrangement of those two types of (1→4)-linked segments would result in product(s) of enzyme action of rather higher degree of polymerization. Had the amount of tetrasaccharide been quantitated, it would have been possible to determine what proportion of the cellotetraose units in the polysaccharide are adjacent, instead of being separated by one or more non-susceptible cellotriose residues. Although barley glucan and lichenan contain almost the same proportions of (1→3) and (1→4)-β-D-glucosidic linkages, the extent of hydrolysis of the latter (6.5%) is rather lower than the former[2]. This indicates that the proportion of cellotetraose segments in lichenan is probably rather smaller than in barley glucan. The same conclusion has been reached by Perlin and Suzuki[19], by measurement of the relative amounts of the various oligosaccharide products produced from barley glucan and lichenan by *Streptomyces* cellulase and *Rhizopus arrhizus* (1→3)-β-D-glucanase.

The more restricted action on barley glucan of *Helix pomatia* (1→4)-β-D-glucanase than, for example, *Streptomyces* cellulase[18] reflects a more rigid specificity for the former enzyme. The use of highly purified, rigorously characterized enzymes is a most useful method for investigation of polysaccharide fine structures[20], and the *Helix pomatia* enzyme promises to be of considerable value for the further characterization of the fine structures of mixed-linkage glucans.

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