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THE LOCATION OF TETRASACCHARIDE UNITS IN PULLULAN

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ABSTRACT

The structure of pullulan, the extracellular α-D-glucan elaborated by the yeast-like fungus *Aureobasidium pullulans*, may be described as a polymaltotriose, the trisaccharides being linked by (1→6)-α-D-glucosidic bonds on the terminal D-glucosyl residues. Depending on the strain of the organism used, up to 6% of the trisaccharide units are replaced by maltotetraosyl residues. Exploiting the susceptibility of these tetrasaccharides to hydrolysis catalysed by porcine alpha-amylase, pullulan has been cleaved at these points and the fragments have been fractionated by gel-permeation chromatography. The heterogeneous size of the fragments indicates that there is no apparent, regular distribution of maltotetraosyl residues throughout the glucan.

INTRODUCTION

The presence of minor structural modifications in a polysaccharide of otherwise regular, periodic structure may have a profound influence on the physical properties of the polymer in solution. Thus, occasional replacement of 3,6-anhydro-D-galactose 2-sulphate by D-galactose 2,6-disulphate in iota-carrageenan, or the addition of L-arabinose to a xylan, are known[1] to affect conformation and to contribute substantially towards the effective role of the polymer *in vivo*.

Pullulan, the extracellular polysaccharide elaborated by the polymorphic fungus *Aureobasidium pullulans*, is an α-D-glucan comprising α-maltotriosyl residues linked by (1→6)-α-D-glucosidic bonds on the reducing and non-reducing termini[2]. The occurrence of the larger maltotetraosyl residues as an integral part of the structure, and not as terminal groups or derived from a concurrently elaborated polymaltotetraosyl polymer, was demonstrated by isolation of 6^4-α-maltotriosylmaltotetraose and 6^3-α-maltotetraosylmaltotriose from partial hydrolysates of pullulan[3]. Additional evidence of a mixed-linkage structure was seen in the action of human-salivary alpha-amylase which, although catalysing the hydrolysis of (1→4)-α-D-glucosidic bonds located in the maltotetraosyl residue, did not release any mixed (1→4)/(1→6)-linked tetrasaccharide that would have resulted from a regular polymaltotetraosyl repeating-structure[3].

The high proportion (33%) of (1→6)-α-D-glucosidic linkages in the molecule makes it unlikely that pullulan will assume a regular shape, *e.g.*, a helix or ribbon,

since the three degrees of rotational freedom present in the (1→6) linkage must confer a high degree of flexibility on the molecule in solution. It seems unlikely that the infrequent introduction of an extra (1→4)-α-D-glucosidic linkage located in the tetrasaccharide would alter the chemical or physical properties of the polymer and thus contribute to a change in its biological function, whatever that might be. The possibility of a less than reproducible biosynthetic process must also be entertained. Thus, a hypothesis proposing the occasional coalition of panosyl and isopanosyl residues during the final phase of assembly has been advanced[4] to account for the introduction of the maltotetraosyl residue. As part of an attempt to account for this apparently rogue residue, its distribution within the polymer has been explored. We now present evidence for a random distribution throughout the glucan.

EXPERIMENTAL

Micro-organisms and culture conditions. — *Aureobasidium pullulans* (ATCC 9348) was grown in liquid culture as described previously[5], but with the substitution of yeast extract (Oxoid) 0.04% (w/v) for yeast nitrogen base.

Isolation of pullulan. — Cells were harvested after 3 and 5 days of growth and centrifuged at 13,000*g* for 10 min at 20°, and the pellet was discarded. Ethanol (2 vol. at 0°) was added with constant stirring during 15 min to the clear, cold supernatant fluid, and the precipitate was stored overnight, collected, and washed (thrice) with cold 66% ethanol in water followed by cold ethanol (twice) and methanol. The methanol was decanted and the white powder dried *in vacuo*.

Enzymic digestion of pullulan. — A solution of pullulan (150 mg) in phosphate buffer (pH 6.9; 200 μmol containing NaCl, 75 μmol) was mixed with porcine alpha-amylase (4 μkat) in a final volume of 10 mL and incubated at 37°. The viscosity, measured using an Ostwald viscometer, rapidly dropped to a steady value after ~15 min, but the digestion was continued for a further 45 min before inactivation by heating at 100° for 3 min. Paper chromatography of the α-limit dextrins was performed by using descending irrigation with the mobile phase of ethyl acetate–pyridine–water[6] (10:4:3), and detection with alkaline silver nitrate[7].

The enzyme-catalysed hydrolysis of (1→6)-α-D-glucosidic bonds, either in pullulan or the fragments derived from the alpha-amylolysis thereof, was effected by dissolving carbohydrate (5 mg) in 0.03M citrate–phosphate buffer (pH 5.0, 0.5 mL) containing 20 nkat of pullulanase[8]. The hydrolysis was monitored by following the release of reducing power, as measured by the modified copper reagent of Somogyi[9], and allowed to proceed for twice the time taken to reach the maximum release of reducing groups. Both alpha-amylase and pullulanase were purchased from the Boehringer Corporation (London) Ltd.

Gel permeation of pullulan and fragments of pullulan. — Carbohydrate samples (5 mg, 1 mL) were chromatographed (eluant, 2nM sodium azide) on columns of Sepharose 4B (2 × 90 cm) at a flow-rate of 6 mL.h^{-1}, Ultrogel AcA34 (2 × 45 cm) at 10 mL.h^{-1}, and Bio-Gel P-2 (2 × 90 cm) at 9 mL.h^{-1}. The fractionations on

Sepharose 4B and Ultrogel AcA34 were at room temperature, whereas that on Bio-Gel was[10] at 60°. Void volumes were determined by using Blue Dextran 2,000, and an internal standard of ovalbumin (M_r 43,000) was incorporated into fractionations on Sepharose 4B and Ultrogel AcA34. Fractions were monitored for carbohydrate by the phenol–sulphuric acid procedure[11], for protein by absorbance at 280 nm, and for Blue Dextran by absorbance at 620 nm. Sepharose 4B was obtained from Pharmacia, Ultrogel AcA34 from LKB-Products AB, and Bio-Gel P2 from Bio Rad Laboratories.

RESULTS AND DISCUSSION

All three bonds of the trisaccharide moiety of pullulan are uniquely susceptible to enzyme-catalysed hydrolysis. Pullulanase[12] (EC 3.2.1.41) attacks the (1→6)-α-D-glucosidic bond, yielding maltotriose, and carbohydrases (EC 3.2.1.57) prepared from *Aspergillus niger*[13], *Athrobacter globiformis*[14], and *Thermoactinomyces vulgaris*[15] cleave the two (1→4)-α-D-glucosidic bonds, producing isopanose[13,14] (6^1-α-maltosyl-D-glucose) or panose[15] (6^2-α-D-glucosylmaltose). Alpha-amylases (EC 3.2.1.1.) from human saliva or porcine pancreas are generally assumed to be without action on pullulan. The specificity of salivary alpha-amylase is such that the minimum structure that is required before catalysed hydrolysis can take place is not present in the regular polymaltotriosyl structure[16]. However, the insertion of an extra (1→4)-α-D-glucosidic linkage to enlarge the maltotriosyl to a maltotetraosyl residue produces the required minimum structure, and the adjacent (1→4)-α-D-glucosidic linkage on the non-reducing side of the (1→6)-α-D-glucosidic linkage now becomes susceptible to catalysed hydrolysis[3] (Fig. 1). The proportion of maltotetraosyl residues present in pullulan, which varies little from strain to strain[17], is usually between 1 and 3%, but has never been observed to exceed 6% (B. S. Enevoldsen, personal communication). Reductometric assays of cleavage by alpha-amylase are therefore not likely to register significant measurements, since, even for a 6% substitution, only 1 in 34 glucosidic bonds would be hydrolysed. By contrast, a viscometric assay of bond cleavage is more responsive to monitoring the fragmentation of a polymer, and was therefore chosen to monitor the action of alpha-amylase on pullulan. It also follows that, in any viscometric assay of pullulanase using pullulan as substrate[18], care must

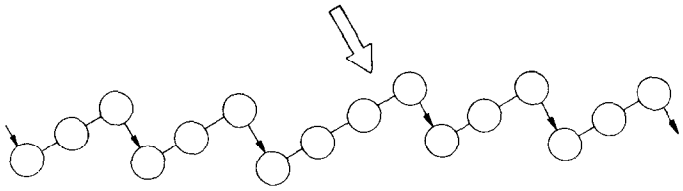

Fig. 1. A section of pullulan structure illustrating the inserted maltotetraosyl residue with the site of hydrolysis ⇩ catalysed by pancreatic alpha-amylase. D-Glucosyl residues are represented by O, (1→4)-α-D-glucosidic bonds by -, and (1→6)-α-D-glucosidic bonds by ↓.

be taken to ensure that there are no maltotetraosyl residues in the substrate structure if it is suspected that alpha-amylase might be present.

Paper-chromatographic evidence for the internal location of the maltotetraosyl residues present in the pullulan used in these investigations is shown in Fig. 2. The rationale for these conclusions has been summarised in the Introduction and has been presented fully elsewhere[3]. Briefly, the use of a sequential treatment by alpha-amylase followed by pullulanase allows the distinction to be made between a polymer possessing maltotriosyl residues with maltotetraosyl residues scattered through it, and a mixture of two polysaccharides, the one a maltotriosyl polymer and the other a maltotetraosyl polymer. An initial cleavage of the mixed-linkage polymer with alpha-amylase does not release small oligosaccharides that migrate in the paper-

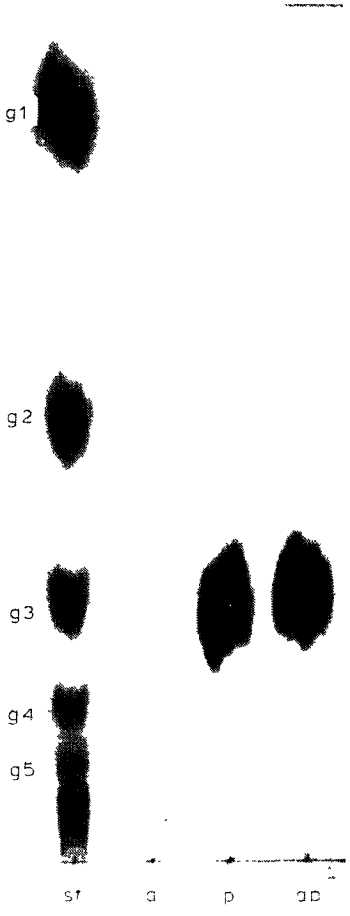

Fig. 2. Paper-chromatographic evidence that the location of the maltotetraosyl residue of pullulan isolated after 3 days of growth is part of the polymaltotriosyl molecule, and located within the structure (see ref. 3 for rationale). Standards (st) are D-glucose (g1), maltose (g2), maltotriose (g3), *etc*. Pullulan treated with alpha-amylase (a), with pullulanase (p), and with alpha-amylase that was subsequently inactivated and followed by pullulanase (ap) (see ref. 3 for experimental details).

chromatographic system used (Fig. 2, chromatogram "a"). In contrast, a polymaltotetraose would have yielded branched tetrasaccharides at this stage, because of the susceptibility of the (1→4)-α-D-glucosidic bond to alpha-amylase (Fig. 1). Only on subsequent treatment with pullulanase of the alpha-amylase-digested polymer containing the scattered tetrasaccharide residues, thus hydrolysing the (1→6)-α-D-glucosidic bonds, are any oligosaccharides released, *viz.*, the major product of maltotriose and the minor branched-tetrasaccharide (Fig. 2, chromatogram "ap"). Pullulanase will not remove a single D-glucosyl residue linked by a (1→6)-α-D-glucosidic bond to a maltodextrin[19]. Treatment of the original, intact polymer with pullulanase releases the major maltotriosyl product and the minor, linear maltotetraose (Fig. 2, chromatogram "p"). The different chromatographic mobilities of the linear and branched tetrasaccharides are clearly seen when comparing chromatograms "ap" and "p".

The question as to whether the maltotetraosyl residues are distributed in a regular or random manner throughout the molecule can be answered by exploiting the susceptibility of pullulan containing these residues to alpha-amylase. A regular distribution would give rise to an oligosaccharide of a particular size no matter what the length of the original polymer. An irregular distribution would yield oligomeric products of different molecular weights. Based on gel-chromatography profiles of pullulanase-treated pullulan, the ratio of maltotriose to maltotetraose in the present study indicated a 1% substitution. A regular distribution throughout the polymer would, after alpha-amylolysis, yield fragments of d.p. 300 with a M_r of 48,600. Gel-

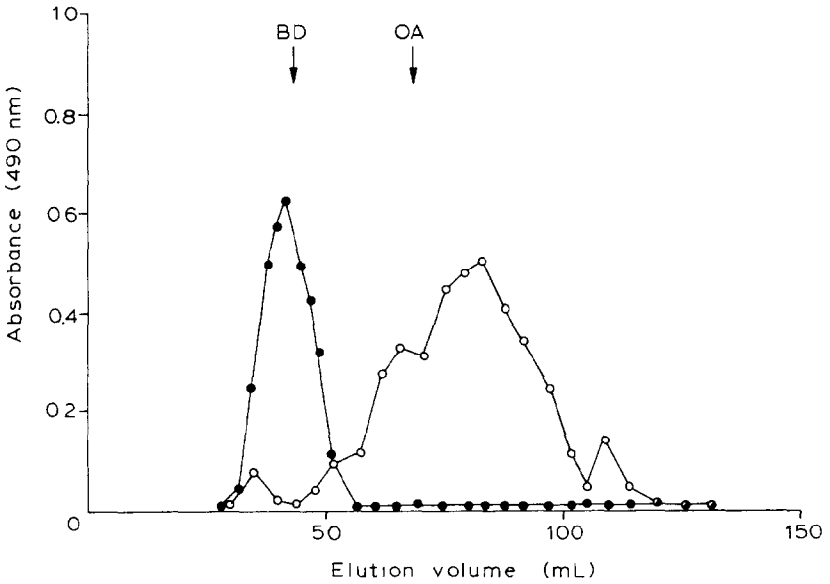

Fig. 3. Gel-permeation chromatography on a column (2 × 90 cm) of Sepharose 4B at a flow rate of 6 mL.h^{-1}; pullulan (—●—) and pullulan exhaustively digested with hog-pancreatic alpha-amylase (—○—). The elution volumes of Blue Dextran (BD) and ovalbumin (OA) are indicated by ↓.

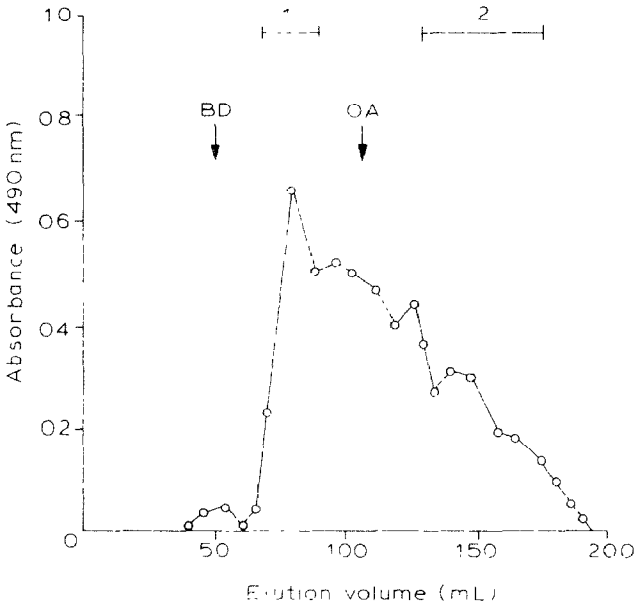

Fig. 4. Gel-permeation chromatography of pullulan, exhaustively digested with hog-pancreatic alpha-amylase, on a column (3 × 45 cm) of Ultrogel AcA34 at a flow rate of 10 ml h^{-1}. The elution volumes of Blue Dextran (BD) and ovalbumin (OA) are indicated by ↓

permeation chromatography of pullulan, isolated after 3 days of growth, subjected to exhaustive alpha-amylolysis, and fractionated on Sepharose 4B, is shown in Fig. 3. This profile is to be compared with that of the polysaccharide before exposure to alpha-amylase, also shown in Fig. 3. The combined fractions of alpha-amylolytic fragments were re-chromatographed on a column of Ultrogel AcA34 (Fig. 4) and the resulting fractions arbitrarily combined into 2 groups. These groups were re-chromatographed on Sepharose 4B, to demonstrate that their apparent high and low molecular weights, shown in the Ultrogel chromatogram, were real and did not arise through association phenomena (confirmatory data not shown). Another difference between groups 1 and 2 should be the different ratios of the tri- to tetra-saccharides displayed after digestion with pullulanase. Each oligosaccharide derived from alpha-amylolysis should now be terminated at the non-reducing end of the molecule by a 6^3-α-D-glucosylmaltotriosyl group. The smaller the d.p. of the oligosaccharide the greater should be the ratio of this tetrasaccharide to maltotriose. Catalysed hydrolysis of the (1→6)-α-D-glucosidic bonds by pullulanase released these tetra- and tri-saccharides, the (1→6)-α-D-glucosyl group of the tetrasaccharide remaining attached to the maltotriosyl residue, since the specificity of pullulanase will not allow the removal of such a D-glucosyl stub[19]. The tri- and tetra-saccharides were fractionated by gel-permeation chromatography on Bio Gel P2. The fragments derived from the fraction of lower molecular weight showed both trisaccharides and tetrasaccharides,

whereas there was no detectable tetrasaccharide derived from the fraction of higher molecular weight.

Analyses of pullulan isolated after growth of the organism for 5 days provided results similar to those reported above, *i.e.*, a random distribution of the maltotetraosyl residue. If the conventional, linear structure of pullulan is accepted, then, as discussed in the Introduction, it is reasonable to assume that the occasional tetrasaccharide residue would make little, if any, change to the physical or chemical properties of the polymer in solution. However, if the polysaccharide has a branched structure, with the conventional polymaltotriosyl chains disposed about some backbone, then the maltotetraosyl residues may act as linkage regions between the maltotriosyl chains of various lengths and the backbone. Just such a branched structure has been proposed for an extracellular polysaccharide elaborated by *A. pullulans*, but possessing mixed α- and β-D-glucosidic linkages[20]. If the tetrasaccharide residue acts as a linkage region, an alternative mechanism of biosynthesis must be found to that proposed for the internally located residues in a linear structure[4].

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