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OBSERVATIONS ON THE STRUCTURE OF THE FUNGAL EXTRACELLULAR POLYSACCHARIDE, PULLULAN

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ABSTRACT

Hydrolysis of pullulan, derived from strain P50 of *Aureobasidium pullulans*, by amyloglucosidase leaves a major portion of the glucan untouched, suggesting that the polysaccharide does not contain the simple linear sequence of α-(1→6)-linked maltotriosyl residues hitherto ascribed.

INTRODUCTION

Pullulan, an extracellular polysaccharide elaborated by the polymorphic fungus *Aureobasidium pullulans*, can be described as a linear α-D-glucan comprising a regular repeating trisaccharide residue that can be presented in a number of ways, *e.g.*, maltotriosyl residues terminally linked by (1→6) bonds, 6^2-α-D-glucosylmaltose linked by (1→4) bonds, *etc.* (Fig. 1)[1]. Occasionally, maltotriosyl residues are replaced by maltotetraosyl in an apparently random fashion[2]. If the structure is the simple linear sequence depicted in Fig. 1, then all the bonds should be hydrolysed by a glucosidase capable of completely hydrolysing glycogen, which possesses a more compact structure composed of (1→4)- and (1→6)-linked α-D-glucosyl residues.

In the course of determining the specific activities of D-glucosyl residues of partially radio-labelled pullulan, it became apparent that the fungal amyloglucosidase from *Aspergillus niger* [(1→4)-α-D-glucan glucohydrolase EC 3.2.1.3] was not releasing all of the glucosyl residues from the polysaccharide (Fig. 2), a major portion of which appeared to be resistant. A similar observation was reported[3] when the source of glucanase was *Cladosporium resinae.*

We now report on the resistance of pullulan to hydrolysis by amyloglucosidase.

EXPERIMENTAL

Organism and culture conditions. — The two strains of *Aureobasidium pullulans* (ATCC9348 and P50, Departmental collection) were grown[4] in liquid

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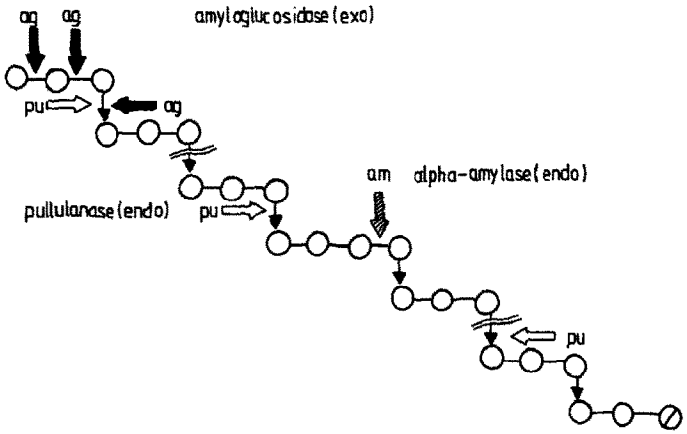

Fig. 1. The linear structure of pullulan, showing maltotriosyl residues with the occasional replacement by maltotetraose: ○, α-D-Glc; —, (1→4) linkages; and ↓, (1→6) linkages. Typical sites of enzymic attack are shown by large arrows. Amyloglucosidase (ag) acts on both (1→4) and (1→6) linkages, sequentially from the non-reducing end; pullulanase (pu) acts randomly on (1→6) linkages; and porcine alpha-amylase (am) acts randomly on the terminal (1→4) linkage of maltotetraosyl residues.

culture but with the substitution of yeast extract (Oxoid, 0.04%) for yeast nitrogen base.

Isolation of pullulan. — Cells were harvested after 3 days of growth and centrifuged at 13,000*g* for 10 min at 20°, and the pellet was discarded. The following isolation was performed at 0°. Ethanol was added with continuous stirring to the clear supernatant fluid, during 15 min, to a final concentration of 66%. After storage for 18 h, the precipitate was collected by centrifugation at 1000*g* for 5 min, and washed thrice with aqueous 66% ethanol followed by ethanol (twice) and methanol. The methanol was decanted and the residual white powder was dried *in vacuo* at room temperature over anhydrous calcium chloride, to give pullulans P50 and 9348.

Enzymic digestions. — A solution of pullulan (4 mg) in acetate buffer (pH 5.0; 100 μmol) was mixed with *A. niger* amyloglucosidase (50 nkat) in a final volume of 2.0 mL and incubated for 24 h at 37°. More enzyme (50 nkat) was then added and the digestion was continued for another 2 h. Pullulanase (25 nkat) was then added and the incubation terminated after 2 h. The D-glucose released was determined by the D-glucose oxidase method[5] and reducing power by the copper reductometric assay[6]. For digestion commencing with amylolysis, pullulan was first exposed to porcine alpha-amylase (3.3 μkat) for 24 h at 37° followed by inactivation of the enzyme at 100° for 5 min prior to the addition of amyloglucosidase. All three enzymes were purchased from Boehringer.

Gel-permeation chromatography. — Pullulan and pullulan products (2 mg, 1 mL) were eluted from a column (1 × 50 cm) of Sepharose 4B with water at 6 mL/h. Fractions (2.5 mL) were monitored for carbohydrate by the phenol–sulphuric acid procedure[7]. For the preparation of fractions of high, medium, and

low M_r, pullulan (10 mg, 3 mL) was fractionated on a column (1.6 × 85 cm) of Sepharose 4B.

Ion-exchange separation. — Pullulan or D-glucose 6-phosphate (2 mg, 1 mL) was applied to a column (0.8 × 20 cm) of DEAE-Sephadex A-50 equilibrated with Tris-HCl (pH 7.0) and washed with water prior to fractionation. The first eluate was water (30 mL) followed by 0.01M HCl containing 0.2M NaCl (30 mL). Carbohydrate was monitored by the phenol–sulphuric acid procedure[7].

RESULTS

The purity of pullulan produced by the two strains of *Aureobasidium pullulans* was established by conventional means, namely, that maltotriose and maltotetraose (resolved by p.c.) were the major and minor oligosaccharides, respectively, formed on digestion with pullulanase and that there was little or no macromolecular residue

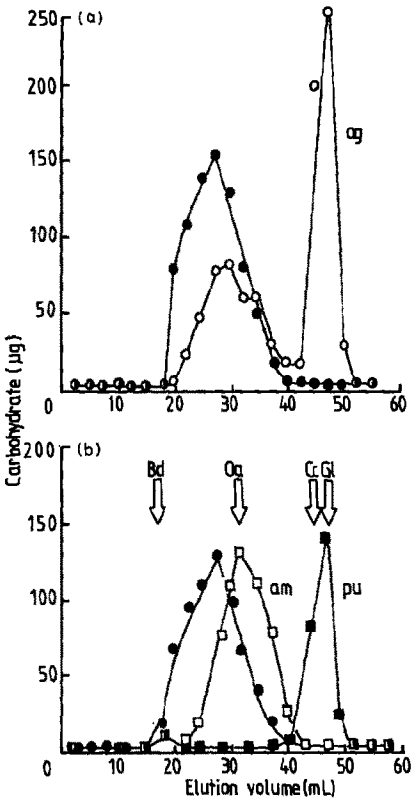

Fig. 2. Gel-permeation chromatography on a column (1 × 50 cm) of Sepharose 4B at 6 mL/h: (*a*) pullulan (●) and pullulan digested with amyloglucosidase (○); (*b*) pullulan (●), pullulan digested with alpha-amylase (□), and pullulan digested with pullulanase (■). Elution volumes of Blue Dextran (Bd), ovalbumin (Oa), cytochrome c (Cc), and glucose (G1) are indicated by arrows. Carbohydrate was monitored by the phenol–sulphuric acid procedure. Pullulan was derived from strain P50.

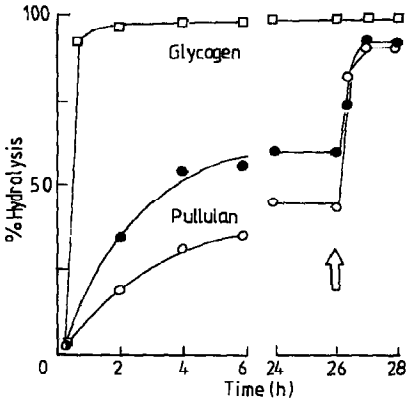

Fig. 3. The release of D-glucose by amyloglucosidase from glycogen (□), pullulan P50 (○), and pullulan P50 pre-treated with alpha-amylase (●). The addition of pullulanase is shown by the arrow.

when the digest was chromatographed on Sepharose 4B. Paper and gel chromatography of pullulan 9348 have already been reported[2]. The gel-filtration characteristics of pullulan P50 are shown in Fig. 2. The small peak of polysaccharide eluted in the void volume was also present in the fractionation of the products of digestion with alpha-amylase and was considered to be a contaminant. It was also apparent from chromatographic data (not shown) that this pullulan contained 1% of maltotetraose (*cf.* 6.6% for pullulan 9348).

There was no precipitation when equal volumes of aqueous 1% cetyltrimethylammonium bromide and polysaccharide were mixed and stored, thus demonstrating the absence of acidic polysaccharide[8].

The action of amyloglucosidase on equal amounts of glycogen and pullulan P50 is shown in Fig. 3. Digestions for periods of 72 h (not shown) suggested that the plateau reached after 24 h may not represent a complete cessation of pullulan hydrolysis, but the increase in glucose released during this period indicated only a further 10% hydrolysis. The addition of pullulanase had no effect on glycogen but

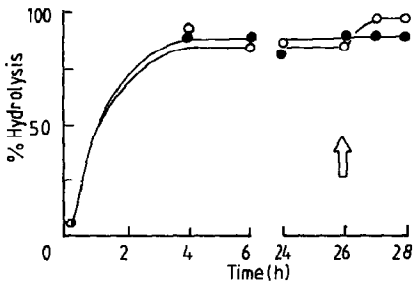

Fig. 4. The release of D-glucose by amyloglucosidase from pullulan 9348 (○) and pullulan 9348 pre-treated with alpha-amylase (●). The addition of pullulanase is shown by the arrow.

acted on pullulan to liberate the remaining maltotriosyl residues, thus rendering them accessible to amyloglucosidase (Fig. 3). Digestion of pullulan 9348 with amyloglucosidase in the absence and presence of pullulanase is shown in Fig. 4. In contrast to pullulan P50, only a small change was observed.

The combined effects of amyloglucosidase and pullulanase resulted in quantitative hydrolysis of all the glucosidic bonds in the polysaccharides derived from both strains.

Gel filtration of pullulan on Sepharose 4B produced the usual broad spectrum of polysaccharide sizes (Fig. 2ab and ref. 2). Using pullulan P50, the peak was arbitrarily divided into fractions of high, medium, and low M_r, corresponding to elution volumes of 20–26, 27–32, and 33–40 mL, and their susceptibilities to amyloglucosidase digestion were found to be 49, 52, and 70%, respectively.

Digestion of either pullulan with porcine alpha-amylase for 18 h released neither glucose nor reducing power. However, the susceptibility of pullulan P50 to amyloglucosidase was increased (Fig. 3). Gel-permeation chromatography on Sepharose 4B demonstrated that the M_r spectrum had been decreased (Fig. 2b and ref. 2).

DISCUSSION

There is reasonable evidence in the literature to support the periodic structure of pullulan shown in Fig. 1. Methylation analysis yielded methyl 2,3,4- and 2,3,6-tri-*O*-methyl-α-D-glucopyranoside in yields of 33% and 66%, respectively[8]. Pullulan consumed[8] 1.31 mol of periodate and liberated 0.32 mol of formic acid per "anhydroglucose" unit, results later confirmed[9] with values of 1.30 and 0.30 mol, respectively. Enzymic hydrolysis of pullulan by pullulanase[1] (EC 3.2.1.41) yielded maltotriose, by a bacterial amylase[10] (EC 3.2.1.57) yielded panose (6^2-α-D-glucosylmaltose), and by a fungal amylase[10] (EC 3.2.1.57) yielded isopanose (6^1-α-maltosyl-D-glucose), all in yields of >90%. That a maltotriosyl residue is occasionally replaced, apparently randomly, by a maltotetraosyl residue[2,9] (Fig. 1) does not alter the linear structure. The question now raised is whether this simple structure is sufficient to describe the whole molecule. The occasional modification or replacement of a residue that occurs in an otherwise regular periodic structure produces local alterations in the configuration of the polysaccharide that may be relevant to its biological function[11]. The detection of these minor changes is often difficult and trace components in hydrolysates or in methylation analyses may be viewed with scepticism. However, the use of exo-acting carbohydrases, enzymes which remove units from one end of a chain until they reach a bond or area of a molecule made inaccessible by local structural configurations, provides less equivocal evidence of minor changes. That an exo-acting glucanase, amyloglucosidase from *A. niger,* cannot hydrolyse all of the (1→4) and (1→6) linkages in pullulan P50 suggested that the polysaccharide might contain some minor structural modifications. The only observed difference between

pullulans P50 and 9348 is the maltotetraosyl content, which, as discussed below, may explain the difference in susceptibility to attack by amyloglucosidase.

The obstructing modification need only be minor in occurrence to have major consequences. Replacement of a glucosyl residue by another hexose, esterification, a change in the configuration of a glycosidic linkage, or a change in the distribution of (1→4) and (1→6) linkages may produce a domain inaccessible to the enzyme. Polysaccharides esterified by dibasic acids have been reported as products of *A. pullulans,* e.g., malic[12] and sulphoacetic[13] esters of (1→3),(1→6)-β-D-glucans. Potato amylopectin carries phosphate groups at positions 3 and 6[14–16], which block the action of *A. niger* amyloglucosidase. A phosphate content of 2.4% has been reported[8] in analyses of extracellular polysaccharides produced by *A. pullulans,* but this disappeared after complexation with cetyltrimethylammonium hydroxide. Although no precipitation occurred with this cation and the pullulan samples studied in this investigation, a further check for the absence of anionic moieties was carried out by chromatography on DEAE-Sephadex A-50, using water, and then 0.2M NaCl in 0.01M HCl, a procedure used to isolate beta-limit phosphodextrins of amylopectin[14]. Pullulan was eluted in the non-acidic breakthrough fractions, whereas D-glucose 6-phosphate, used as a control, was retarded and desorbed from the column on change to the acidic eluate. It is presumed, from this evidence and the observation that no sugars other than glucose have been detected in acid hydrolysates of pullulan, that the glucosyl residues in the polysaccharide are not covalently modified or replaced.

The presence of other than α-(1→4) and α-(1→6) linkages has been severally described. Sowa *et al.*[17], on the basis of i.r. and periodate-oxidation data, reported the presence of 6% of α-(1→3)-linked glucosyl residues and Bouveng *et al.*[8] reported 2% of periodate-resistant residues. Polymaltotriosyl chains covalently attached to a (1→3)-β-D-glucan backbone have been described[18] and evidence for a glycogen-like structure terminating the regular pullulan structure at the non-reducing end has been provided[19].

Sowa *et al.*[17] and Bouveng *et al.*[8] showed that partial acid hydrolysis under conditions of minimum reversion produced, in addition to the expected fragments, a trisaccharide tentatively identified as 6^1-α-D-glucosylmaltose, suggesting the presence of branching in the parent molecule. It seems likely, in the absence of evidence for substituted or covalently modified glucosyl residues, that occasional branching and local compact clustering of maltotriosyl chains is the cause of resistance to the action of amyloglucosidase. Digestion of the chromatographically isolated, amyloglucosidase-resistant pullulan with randomly acting pullulanase produced maltotriose and, in the presence of amyloglucosidase, complete hydrolysis to glucose occurred, confirming that the basic polymaltotriosyl structure exists on both sides of the structural modification. Conclusions about the location of this restriction are tentative. It appears that whether pullulan P50 of high (10^6) or low M_r (10^5) is taken, then approximately the same proportion of bonds, namely 1/2 to 1/3, are still unavailable to the exo-acting enzyme. An explanation may lie in

an examination of the effect of pre-treating pullulan with porcine alpha-amylase. This endo-acting hydrolase cleaves[2] pullulan at the randomly placed maltotetraosyl residues.

The percentage of these residues in pullulan derived from different strains of *A. pullulans* has been reported as both constant[21] and variable[22]. Pullulan examined in the majority of investigations reported in this communication was derived from a Departmental strain of *A. pullulans* designated P50 and estimated to have 1% of the trisaccharide units replaced by tetrasaccharide. Another strain (ATCC 9348), more commonly used in investigations in this laboratory, elaborates pullulan containing 6.6% of maltotetraose[9] and is therefore more susceptible to amylolysis than the P50-derived polysaccharide. The elution pattern from Sepharose 4B of the products of amylolysis of pullulan P50 is shown in Fig. 2 and demonstrates an average M_r with respect to the internal standard ovalbumin higher than those[2] derived from pullulan 9348. The actions of amylase, amyloglucosidase, and pullulanase on pullulan 9348 are shown in Fig. 4 and suggest that either there are fewer restriction points to amyloglucosidase action or that the smaller fragments derived from the larger number of cleavages by alpha-amylase provide greater accessibility to the glucanase.

The conclusion drawn is that structural restrictions are distributed throughout the molecule. The reported[3] presence of alpha-amylase activity in *A. niger* amyloglucosidase, albeit small, is probably sufficient to cleave the pullulan at the tetrasaccharide residues, thus allowing the glucanase to attack beyond the first restriction encountered in its sequential hydrolysis from the non-reducing end.

If branching does occur within the molecule, then more than one non-reducing glucosyl terminus must be present. In the methylation analysis reported by Bouveng *et al.*[8], 0.4% of methyl-2,3,4,6-tetra-*O*-methyl-α-D-glucopyranoside was identified, which could correspond to a linear molecule of M_r ~4×10^4. Gel-filtration studies[20] indicated considerably larger molecules (M_r 10^5–10^6), suggesting that there may be more than one non-reducing end in each molecule of pullulan and therefore branching.

Whatever the real structure of pullulan, the evidence now provided shows that, whereas the polymaltotriosyl representation describes most but not all of the structure, the molecule derived from some strains may also contain randomly distributed, tightly packed branch-points where additional polymaltotriosyl chains are linked to the central or terminal residues of a maltotriosyl residue. Alternatively, maltosyl or glucosyl groups may replace the maltotriosyl residue.

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