Pullulan Elaboration by *Aureobasidium pullulans* Protoplasts

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Protoplasts of *Aureobasidium pullulans* are capable of producing pullulan. Biosynthesis of the polymer pullulan required induction with kinetics similar to those of whole cells. The protoplasts also produced a heteropolysaccharide component containing mannose, glucose, and galactose. The relative proportions of the pullulan and heteropolysaccharide fractions were a function of glucose concentration, with the pullulan content of the total polysaccharide rising from 20% at 2.5 mM glucose to 45% at 20 mM glucose. Elaboration of pullulan by both cells and protoplasts was sensitive to 0.6 M KCl, which was present as the osmotic stabilizer in protoplast experiments. The presence of KCl resulted in a shift in the pH optimum to a more acidic value. The molecular weight of the protoplast-derived pullulan was sharply reduced from the molecular weight of the whole-cell-derived product. Exposure of the protoplasts to proteolytic enzymes had no effect on polysaccharide elaboration.

The elaboration of fungal polysaccharide has been the subject of increasingly intense study over the last two decades. Significant progress has been achieved in the analysis of the formation of chitin and β-glucan, the main polysaccharides responsible for the structural integrity of fungal cells (for reviews of this subject, see the work of Cabib [4] and Farkas [15]). These studies have been aided by the development of techniques for the production and maintenance of viable fungal protoplasts (for reviews, see the work of Necas [28] and Peberdy [29]). By manipulating environmental parameters and utilizing various drugs and chemical reagents, much insight has been gained into the mechanisms and control of both chitin and β-glucan deposition (14, 30).

The area of nonstructural, extracellular polysaccharide synthesis has not received as much attention, probably because of its lesser significance for the central problem of morphogenesis. Nonetheless, the ability of a large variety of fungi and yeasts to secrete extracellular glucans has been widely observed and reported (3, 5, 12, 19, 20, 26). Among these, the synthesis of pullulan, an α-glucan, by *Aureobasidium pullulans*, a member of the *Fungi Imperfecti*, is a particularly interesting phenomenon.

Pullulan is a linear homopolymer of glucose comprised mainly of repeating maltotriosyl groups linked by α-(1 → 6) glucosidic bonds (31). A small percentage (6.6%) of the polymer consists of randomly distributed maltotetraosyl groups (11). Pullulan elaboration by *A. pullulans* has been the subject of a recent review by Catley (8). The elaboration by *A. pullulans* of glucans at three levels (intracellularly, glycogen; structurally, β-glucans; extracellularly, pullulan) offers a particularly useful model system for the study of the mechanisms and control of fungal polysaccharide synthesis.

Previously, only whole cells of *A. pullulans* have been shown to elaborate pullulan (5, 9). In this report, we describe the results of an investigation of pullulan biosynthesis by protoplasts.

**MATERIALS AND METHODS**

**Organism and culture methods.** *A. pullulans* ATCC 42023 was maintained and grown as described by Finkelman and Vardanis (17).

**Reagents.** Unless otherwise stated, all reagents employed in this study were of analytical grade. [U-14C]glucose (329 mCi/mmol) was obtained from New England Nuclear Corp., Boston Mass. Sources for enzymes were as follows: trypsin, pepsin, and chymotrypsin were from Worthington Diagnostics, Freehold, N.J.; *Streptomyces griseus* neutral protease, subtilopeptidase, and pullulanase were from Sigma Chemical Co., St. Louis, Mo.; papain was from General Biochemicals Div. (Mogul Corp.), Chagrin Falls, Ohio; Driselase, a lytic preparation from *Irps lacteus* (21), was obtained from Kyowa Hakko Kagyo Co. Ltd., Tokyo, Japan. Sepharose 4B was obtained from Pharmacia Fine Chemicals, Piscataway, N.J.

**Protoplast formation.** Protoplasts were prepared by a modification of the technique of Finkelman et al. (18). Cells grown for 24 h were harvested, suspended in distilled water containing 50 mM 2-mercaptoethanol, and incubated with shaking at 25°C for 30 min. The cells were recovered by centrifugation and suspended in a solution of Driselase (30,000 × g supernatant of a 0.2-g/ml suspension, containing 5.0 mg of
protein per ml) in 0.6 M KCl (final pH, 5.0). The cell suspension was agitated gently until protoplast formation was complete (2 h). The protoplasts were recovered by centrifugation at 500 x g for 5 min and washed twice with 0.6 M KCl. Incubation medium A consisted of 0.1 M sodium citrate, 0.2 M sodium phosphate (dibasic), 0.6 M KCl, and 10 to 90 mM glucose. Incubation medium B consisted of 25 mM sodium citrate, 50 mM sodium phosphate (dibasic), 0.6 M KCl, and 20 mM glucose. The final pellet was suspended in 0.6 M KCl to a concentration of 1 x 10^6 to 2 x 10^6 protoplasts per ml.

**Polysaccharide elaboration.** Polysaccharide synthesis and pullulan estimation were performed according to Finkelman and Vardanis (17), except that some incubation mixtures contained 0.6 M KCl. Because pullulanase activity declines below pH 4.0, incubation mixtures at pH 4.0 or lower were adjusted to pH 4.5 to 5.0 with 6 M NaOH.

**Chromatography.** The molecular weight profiles of the extracellular polysaccharides were obtained by using a column (1 by 48 cm) of Sepharose 4B. The eluant was distilled water containing 0.002% thimerosal to inhibit microbial growth. Fractions of 1 ml were collected at a flow rate of 5.25 ml/h. Polysaccharide for the chromatography experiments was prepared from scaled-up (1.0-ml) incubation mixtures. The supernatant for mixtures incubated for 6 h was precipitated by the addition of 2 volumes of ethanol. The resulting precipitate was washed three times in 66% ethanol and finally with acetone. The dried product was dissolved in distilled water.

**Acid hydrolysis and thin-layer chromatography.** Polysaccharide was dissolved or suspended in 2 N HCl at 2% (wt/vol). Hydrolysis was performed under vacuum for 2 h at 105°C. The resultant solutions were dried under a stream of air and dissolved in 50 μl of water. Samples were chromatographed for 16 h on Polygram CEL 300 precoated plastic sheets (0.1-mm cellulose; Machery-Nagel, Düren, West Germany) in a solvent system containing ethyl acetate, pyridine, and water (10:4:3). Spots were visualized by spraying with p-anisidine–phthalic acid reagent (0.1 M p-anisidine–0.1 M phthalic acid in 95% ethanol) followed by heating at 105°C for 10 min.

**Protease treatment.** Protoplasts, actively secreting polysaccharide, were exposed sequentially to trypsin (150 μg/ml) and soybean trypsin inhibitor (660 μg/ml). The pH of the incubation mixture was 6.8. Five other proteases were also tried at concentrations of 500 μg/ml.

**RESULTS**

The elaboration of extracellular polysaccharides by *A. pullulans* protoplasts is shown in Fig. 1. The time course of elaboration demonstrated the typical lag phase required for the induction of the synthetic apparatus as reported by Catley for whole cells (7). In this experiment, the protoplasts were incubated in the presence of the lytic complex Driselase (22). An identical time course of elaboration was observed in the absence of Driselase (data not shown). The pullulan content of the polymer after incubation for 3 h was 43% as measured by pullulanase sensitivity. Thus, the elaboration of both the pullulan and nonpullulan extracellular polysaccharides was unaffected by the absence of cell wall. The experiment further demonstrated that these polysaccharides were not sensitive to the enzymes of the whole Driselase complex. The insensitivity of pullulan to several fractions of the Driselase complex has been reported by Kawai et al. (22).

The elaboration of extracellular polysaccharide by protoplasts showed high sensitivity to pH in incubation medium A (Fig. 2). The effects of pH on the elaboration of the pullulan and nonpullulan components were different. The synthesis of the pullulan component had a sharp optimum at pH 3.8 and declined rapidly on either side of this value. The nonpullulan component had an optimum at pH 3.2 and declined rapidly at more acid values but only gradually on the alkaline side. A striking difference between this pattern and that observed with whole cells was the shift from a broad optimum at pH 5.0 to 5.5 (17) to a narrow one at pH 3.8.

This shift was investigated next. The results of an experiment testing the response of whole cells incubated in the protoplast medium in the presence and in the absence of 0.6 M KCl are shown in Fig. 3. The addition of 0.6 M KCl caused both a sharp narrowing of the optimum and a shift to pH 3.8. Thus, the pH shift was produced by the osmotic stabilizer; and it was not an intrinsic property of the elaborative mechanism in the absence of cell wall. It is interesting to note that with whole cells, both the pullulanase-sensitive and pullulanase-insensitive
components responded similarly with respect to pH, unlike their response with protoplasts.

The molecular weight profile of polymer produced by protoplasts, incubated at pH 4.0, was analyzed using Sepharose 4B column chromatography. Figure 4A illustrates the profile of polysaccharide secreted into the incubation medium with an initial glucose concentration of 12.5 mM. Only the second included peak showed pullulanase sensitivity. The average molecular weight for this peak was approximately 10^6. This is in marked contrast to the molecular weight of the cell-derived polysaccharide, which appeared to be at least 2 x 10^7 based upon coelution with blue dextran from columns of Sepharose 4B (Fig. 5A) and of Bio-Gel A-5m (data not shown). The pullulan content of the protoplast polysaccharide was 65%. Increasing the glucose concentration to 83 mM resulted in increases both in the proportion of pullulan in the extracellular polymer and in its average molecular weight (Fig. 4B). In this experiment, the average molecular weight of the pullulan increased to approximately 4 x 10^5, and its proportion increased to 92%. The high pullulan content of the polysaccharide samples used for chromatography resulted from the lengthening of the incubation time for bulk preparations to 6 h from the usual 3 h. The filter paper technique also allowed the recovery of a higher proportion of pullulanase-insensitive material (~5%), reducing the apparent pullulan content of the 3-h filter paper-recovered samples.

The pullulanase-insensitive polysaccharide appearing at the void volume was subjected to strong acid hydrolysis, and the products were analyzed by paper chromatography. Two fractions were evident. The first was soluble in cold 2 N HCl, and the second was insoluble. Fraction I contained roughly similar amounts of galactose, mannose, and glucose. Fraction II contained mainly glucose, with traces of mannose. Thus, preliminary analysis indicates that the pullulanase-insensitive material consisted of heteropolysaccharides of complex composition.

The effect of pH upon the elution profiles of polymer from whole-cell incubations is shown in Fig. 5A. No significant differences were observed between the polysaccharides elaborated at pH 4, the pH of the protoplast incubations, and those elaborated at pH 5.0, the pH optimum for cellular polysaccharide elaboration.

The effect of KCl upon the elution profile of the cell product is shown in Fig. 5B. The inclusion of 0.6 M KCl in the whole-cell incubation did not result in a reduction in molecular weight. The total polymer was eluted at the void volume. Thus, the molecular weight reduction was not the result of including 0.6 M KCl as the osmotic stabilizer.

The effect of glucose concentration on extracellular polysaccharide synthesis was more closely investigated. The preparation of protoplasts in 0.6 M KCl resulted in the approximation of the starved-cell state. Catley has shown that starved cells are incapable of extracellular polysaccharide synthesis and must be reinduced (7). Protoplasts prepared in 0.6 M KCl showed
the same induction kinetics found in starved whole cells (Fig. 1). Incubation of such starved protoplasts in limiting concentrations of glucose is instructive with respect to the fate of exogenous glucose. Figure 6A shows the results of incubating fresh protoplasts in glucose solutions of various concentrations. The elaboration of extracellular polysaccharide was linear with respect to glucose concentration within this range. Thus, the percent conversion of available substrate to extracellular polysaccharide was about the same at all glucose levels tested. Analysis of the proportion of pullulan in the total polymer revealed a different pattern (Fig. 6B). The pullulan content rose from a low of 20% to a high of about 45%, at which point it remained constant. Thus, at low glucose concentrations the proportion of pullulan was reduced in favor of the pullulanase-insensitive heteropolysaccharide.

The location of the elaborative apparatus of glycan synthesis in yeasts and fungi has been studied by a number of investigators (13, 21, 30). Holbein and Kidby (21) have localized the mannosyl transferase complex responsible for the
mannosylation of invertase. Their technique involved limited trypsinization of *Saccharomyces* protoplasts and subsequent analysis of the mannosylation pattern of newly synthesized invertase. Their results indicated that trypsinization eliminates mannan synthetase activity from the yeast plasma membrane and uncouples the synthesis and secretion steps in invertase production. A similar approach to the localization of the pullulan elaborative apparatus was attempted with *A. pullulans* protoplasts. The addition of trypsin (final concentration, 150 μg/ml) to the protoplast suspension affected no diminution in the polysaccharide elaboration as compared with the control. Additional experiments with α-chymotrypsin, subtilopeptidase, papain, pepsin, and *S. griseus* protease at concentrations of 500 μg/ml (data not shown) also failed to interfere with polysaccharide synthesis.

**DISCUSSION**

The synthesis and secretion of extracellular polysaccharides, including pullulan, β-glucans, and heteroglucans, by *A. pullulans* are well established (8). In this report, it has been demonstrated that protoplasts of *A. pullulans* are also capable of producing extracellular polysaccharides, including pullulan. Recently, Catley and Hutchison (9) have suggested that, in addition to the plasma membrane, the intact cell wall and periplasmic space of *A. pullulans* are requirements for pullulan elaboration. These suggestions were based upon the conclusion that treatment with Zymolase, a wall-lytic preparation from *Arthrobacter luteus*, impaired pullulan biosynthesis. The results described here oppose this conclusion, for they show that the elaborative apparatus can function without the cell wall and periplasmic space. The impairment of pullulan biosynthesis by Zymolase treatment (9) is probably unrelated to wall damage, and it may be the result of some effect upon the plasma membrane.

The pullulan content of the polysaccharides produced in the manner described in this report averaged about 50% (at a glucose concentration of 20 mM with 3-h incubations), a value somewhat lower than the average values (60 to 70%) reported for the whole-cell form (8). Both the pullulan and the heteropolysaccharide required the same induction period and were synthesized simultaneously rather than in a sequential fashion.

The synthesis of polysaccharides by protoplasts has been extensively studied with respect to cell wall regeneration (3). In many studies, protoplasts of various yeast and fungal genera have been shown to synthesize glucan, mannan, and chitin associated with wall regeneration. The protoplasts are generally held to be able to secrete these components into the surrounding medium (16, 25). The secretion of pullulan, a nonstructural polysaccharide, by *A. pullulans* protoplasts is unique in that it appears to be unrelated to cell wall regeneration. Other yeasts and fungi have been shown to secrete nonstructural polysaccharides (19), but it is not known whether their protoplasts maintain this capability.

The fact that the *A. pullulans* protoplasts elaborate polysaccharide not related to cell wall regeneration is indicative of a lack of tight control over the type of polysaccharide synthesized during protoplast regeneration. Some control over the level of pullulan produced does, however, appear to be operating under conditions of substrate limitation. The proportion of pullulan in the extracellular polysaccharide rose from a low of around 20% to a high of approximately 50% as the glucose level was raised (3-h incubations).

The influence of the osmotic stabilizer upon
the synthesis of pullulan is striking. A shift to a narrow and more acid pH optimum was observed. This sensitivity of the elaborative apparatus to high ion concentrations may be related to the negative response of the process to neutral and alkaline pHs (6). Decreased hydrogen ion concentrations have been shown to effect a similar inhibition of polysaccharide synthesis by resting cells of *Saccharomyces cerevisiae* (1). The molecular weights of pullulan elaborated by whole cells and by protoplasts of *A. pullulans* were quite different. The low-molecular-weight product of the protoplasts was not due to pH or to the inclusion of KCl as the osmotic stabilizer. This result indicates that the environment provided by the complete cell envelope is important for the normal function of the elaborative process. This may be due to simple mechanical protection of the nascent polymer as it lengthens.

The high-molecular-weight, pullulanase-insensitive material which formed a significant proportion of the protoplast-derived polymer was not released in significant amounts by whole cells incubated in the presence of 0.6 M KCl. This material became predominant in the extracellular polysaccharide of protoplasts as the glucose concentration was lowered. Its significance is uncertain, but it may be an important component of the regenerating protoplast wall. Brown and Lindberg (2) have described a heteropolysaccharide of similar but not identical composition obtained by hot (100°C) water extraction of the cell walls of *A. pullulans* (yeast form). This heteropolysaccharide contained D-mannose, D-galactose, D-glucose, and D-glucuronic acid in the proportions 43:45:10:12. The proposed structure consisted of an α-(1→6)-linked mannan backbone, variously substituted with the other constituents. Kikuchi et al. (23) have analyzed two cell wall polysaccharides obtained by phenol extraction of *A. pullulans*. These were found to contain glucose, mannose, and galactose, with glucose as the major component in both cases. The qualitative similarity of the heteropolysaccharides described in these reports to the protoplast heteropolysaccharide described here indicates that they may be related. The dominance in synthesis of the protoplast heteropolysaccharide under glucose-limiting conditions suggests that this polymer plays a central role in cell wall development. Investigation of this polymer is continuing.

The location of the synthetic apparatus also remains unknown. The failure of proteolytic attack to inhibit elaboration may reflect either non-susceptibility to the range of proteases used or an inaccessible location in the membrane. A similar lack of effect of proteases on the wall-synthesizing apparatus of yeasts has been reported by Kopecka et al. (24). Recent work by Catley and McDowell (10) indicating the probable involvement of lipid intermediates argues for a membrane location for the synthetic apparatus. The failure of recent efforts to find maltosyl or maltotriosyl nucleotides in *A. pullulans* further supports the membrane location hypothesis (27). The finding of such higher sugar nucleotides would support a cytoplasmic location for the elaboration of at least part of the polysaccharide.

Substantial progress in the analysis of pullulan synthesis awaits the development of an in vitro assay. In the absence of such a technique, the use of protoplasts offers a new approach to the study of extracellular polysaccharide synthesis by *A. pullulans*.

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