PULLULAN, A RELATIONSHIP BETWEEN MOLECULAR WEIGHT AND FINE STRUCTURE

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Received 14 July 1970

1. Introduction

The structure of pullulan, an extracellular α-glucan elaborated by *Pullularia pullulans,* has been shown to be a polymaltotriose polymerized through α-1, 6-bonds on the terminal glucose residues of the trisaccharide [1, 2]. Wallenfels et al. [2] have presented evidence for 6^3-α-glucosylmaltotriose and 6^1-α-maltotriosylglucose as minor tetrasaccharide components of pullulan, and presumed their location to be solely at the termini of the polymer (fig. 1a). Catley et al. [3] proposed the pesence of a small number of maltotetraose units located within the basic polymaltotriose structure, and showed them to be linked through α-1, 6-bonds on their terminal residues (fig. 1b). It had been observed [3] that there is, perhaps, no unique structure for pullulan, since it is the term used to describe any extracellular α-glucan elaborated by *P. pullulans* cultured under a variety of conditions on a variety of substrates. The location and content, therefore, of the tetrasaccharide described by Wallenfels et al. [2] and Catley et al. [3] are not held to be in conflict. However, these differences in the fine structure of pullulan prompted an investigation, presented in this letter, into the possibility of the modification of extracellular polysaccharides during the course of *P. pullulans* growth.

2. Materials and methods

Quartermaster strain no. 3092 of *P. pullulans,* kindly supplied by Dr. Elwyn T. Reese of the U.S. Army Natick Laboratories, Natick, Massachusetts, was grown at 25–27° on a gyrotory shaker at 200 rpm.

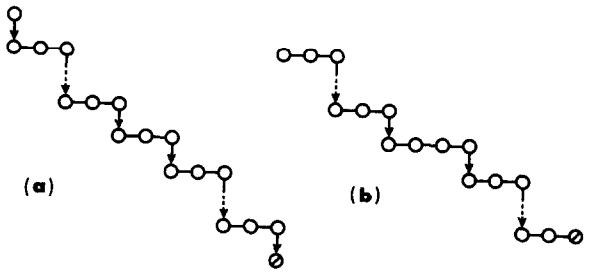

Fig. 1.(a) The structure of pullulan proposed by Wallenfels et al. [2] with terminal tetrasaccharide units.

(b) The structure proposed by Catley et al. [3] with maltotetraose units located within the polymer.

α-1,6-Linkages are represented by ↓, α-1,4-linkages by ——. Non-reducing glucosyl units are shown by ○, and reducing units as ⊖.

The medium is that used by Ueda et al. [4] with a 5% sucrose carbon source. Extracellular polysaccharide was precipitated from the supernatant with a 66% concentration of ethanol, washed with methanol and dried *in vacuo* over calcium chloride at 40°.The phenol-sulphuric acid procedure [5] served for detection of carbohydrate in the effluent from column fractionations. Examination of polysaccharide isolated after 80 and 160 hr of culture showed 90% of the carbohydrate to be composed of glucose, as judged by acid hydrolysis [6] followed by estimation of liberated glucose with glucose oxidase reagent [7]. All of this glucose could be accounted for as a maltotriose and tetrasaccharide after digestion with pullulanase, an enzyme specific for the hydrolysis of α-1,6-glucosidic bonds [8]. The extracellular polysaccharide was, therefore, essentially pullulan [1, 2].

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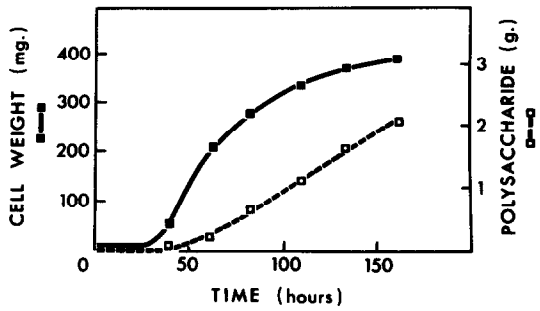

Fig.2. The production of extracellular polysaccharide from shake cultures of *P. pullulans* utilizing a 5% sucrose carbon scource Dry cell weight per 100 ml culture ▪——▪; weight of isolated polysaccharide per 100 ml culture □– – –□.

Pullulanolysis was conducted at pH 5.0 and 37° [9] until no further increase in reducing power was observed. The products were chromatographed by descending paper chromatography (Whatman No. 1) irrigated with ethyl acetate-pyridine-water (10:4:3, v/v) [10] and visualized with a silver nitrate-sodium hydroxide dip [11].

Molecular weights of isolated pullulan were determined using a column of Sepharose 4B calibrated with dextrans ranging in molecular weight from 2×10^6 to 1.5×10^5. Labelled oligosaccharides, applied to a 10 cm base line, were fractionated on paper (Whatman 3MM) as described above. The developed chromatogram was cut into 1 × 10 cm strips which were immersed in scintillation fluid and counted by standard procedures of scintillation spectrometry. Scintisol (Isolab Inc.) was used for counting 1 ml aqueous samples.

3. Results and discussion

Pullulan was isolated at 80 and 160 hr (fig. 2) and its molecular weight distribution examined by gel filtration on Sepharose 4B (fig. 3). A pullulan initially of a maximum molecular weight 2.1×10^6 appears to be converted to a species with less than one tenth of the original size. The weights of polysaccharide isolated at 80 and 160 hr (fig. 2) and the molecular weight distribution profile (fig. 3), showed that the phenomenon could not be accounted for by synthesis of an excessive amount of lower molecular weight pullulan

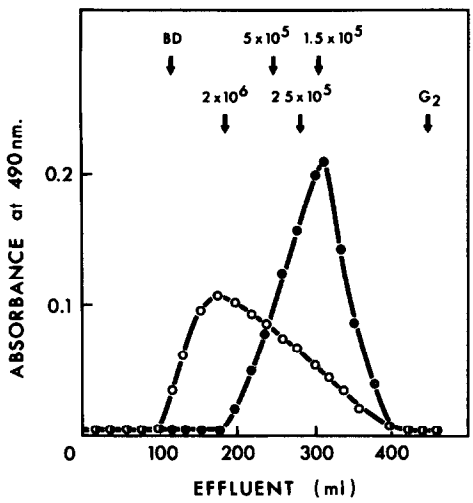

Fig.3. The molecular weight distribution of pullulans fractionated on a Sepharose 4B column (2.5 × 80 cm) in 50 mM tris-HCl, 100 mM NaCl. pH 7.0. Vertical arrows indicate the elution volumes of dextrans of MW. $2 \times 10^6 - 1.5 \times 10^5$; blue dextran (BD) and maltose (G_2). Polysaccharide isolated after 80 hr o——o, and 160 hr •——• is monitored by the phenol-sulphuric acid procedure (absorbance 490 nm).

at a later stage of culture and it was suspenced that the secreted pullulan was subject to extracellular modification. Further confirmation of this hypothesis was sought.

Labelled pullulan was isolated after five days growth of *P. pullulans* utilizing ^{14}C-sucrose as carbon source. A sample of this polysaccharide was introduced aseptically into a three day old shake culture of *P. pullulans* utilizing cold sucrose. Growth was allowed to proceed for a further six days, after which time polysaccharide was isolated from the supernatant, and the washed cells examined for radioactivity. No incorporation was found.

Samples of labelled polysaccharide isolated from both cultures were digested with crystalline pullulanase and the products separated by paper chromatography. The developed chromatograms were cut into strips and counted for carbon-14. The profiles are seen in fig. 4. The proportion of tetrasaccharide yielded by pullulanolysis is approximately the same whether polysaccharide is isolated from early or late stages of culture, however, the original maltotetraose component has apparently been transformed in part to a slower moving tetrasaccharide. This is probably

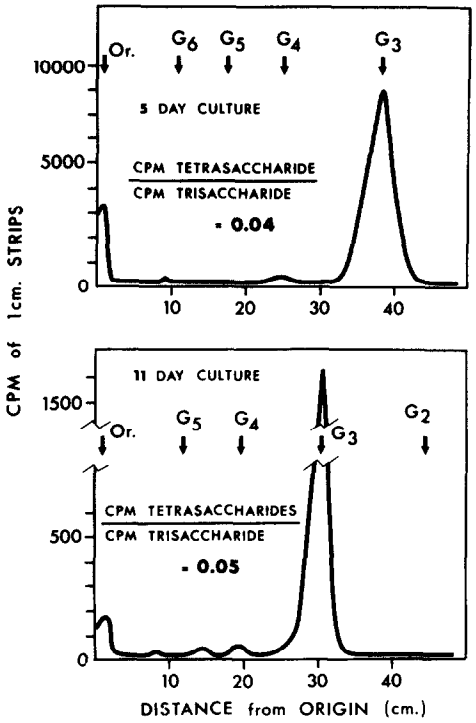

Fig. 4. Paper chromatography of labelled oligosaccharides produced by exhaustive pullulanolysis of ^{14}C-pullulan isolated after 5 days and 11 days culture. 1 cm strips of developed chromatogram were immersed in scintillation fluid and counted by standard procedures. Positions of maltodextrins: maltose G_2, maltotriose G_3 etc., included as chromatographic standards are indicated at the top of the figure; Or, origin.

the minor component observed by Wallenfels et al. [2].

Samples of labelled polysaccharide fractionated on a column of Sepharose 4B, (fig. 5), present a carbon-14 profile similar to the carbohydrate distribution observed in fig. 3.

The combination of these changes in molecular weight and fine structure of polysaccharide suggest that the maltotetraose present in the newly synthesized pullulan provides the site of attack for an extracellular, or cell surface, endoamylase that is produced during culture growth. Using amylopectin as substrate, an endo-carbohydrase activity has been detected in the supernatant of *P. pullulans* cultures. The proposed amylase would cleave the polysaccharide at these maltotetraose locations, decreasing the molecular weight and providing a modified pullulan structure.

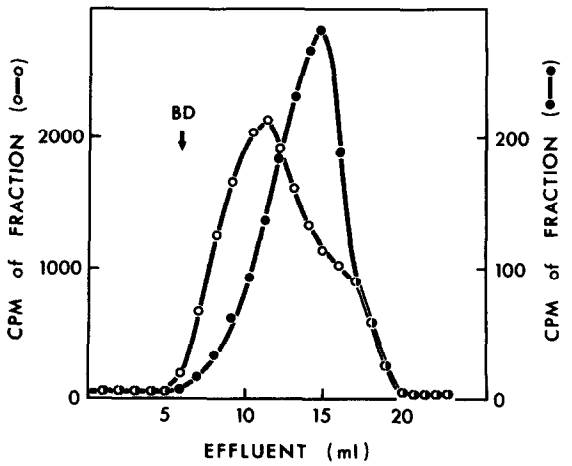

Fig. 5. Gel filtration of labelled pullulans, (isolated after 5 days o——o, and 11 days •——•), on a Sepharose 4B column (0.9 X 30 cm). 1 ml fractions were counted by standard scintillation procedures. Elution volume of blue dextran: BD.

This would yield glucosylmaltotriose, or maltotriosylglucose, upon debranching with pullulanase (fig. 6), since single α-1,6-linked glucose residues cannot be cleaved by this enzyme [9].

Indeed, it has already been observed [3] that the action of human salivary α-amylase on pullulan occurs at just such a point within the molecule, cleaving the maltotetraose at a unique bond (fig. 6).

The values of molecular weight of pullulan which appear in the literature e.g. Ueda et al., 250,000, [4] and Wallenfels et al. 235,000 [2] fall in the range noted in these studies.

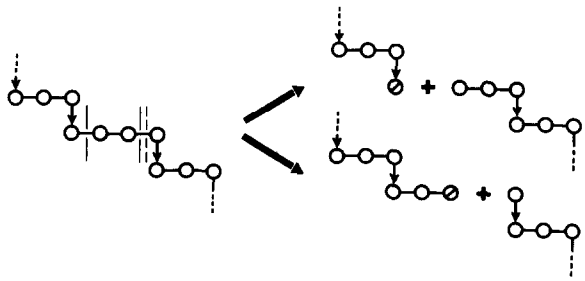

Fig. 6. The generation of pullulan structure (a) from (b) of fig. 1 with concomitant decrease in molecular weight. The solid vertical lines indicate sites of the proposed carbohydrase action leading to products of glucosylmaltotriose and maltotriosylglucose after pullulanase digestion of the modified pullulan. The broken vertical line indicates the site of action of human salivary α-amylase.

Acknowledgements

This work was supported by funds from a National Science Foundation grant to the University of Miami (grant GU3302) and performed whilst the author was an Investigator of the Howard Hughes Medical Institute. Thanks are due to professor W.J.Whelan for encouragement and advice and to Pharmacia Fine Chemicals, Inc. for a gift of dextrans used in calibration.

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