# MECHANISM OF ACTION OF PULLULANASE

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## 1. Introduction

Pullulanase, an extracellular carbohydrase from *Aerobacter aerogenes*, was found by Bender and Wallenfels [1] to cause an essentially quantitative conversion of pullulan into the trisaccharide, maltotriose. Pullulan, a linear α-glucan elaborated by *Pullularia pullulans* [1,2], consists of repeating units of α-maltotriose joined "head-to-tail" by 1→6-bonds. Pullulanase could attack pullulan by either of the two modes of action shown in fig. 1. These are: (a) an exo action in which hydrolysis is restricted to the 1→6-linkage nearest to the terminal non-reducing end (or reducing end), with the stepwise release of maltotriose as the only low-molecular-weight product of the reaction. (b) An endo action in which initial hydrolysis can occur at internal as well as external 1→6-linkages, with the intermediate production of hexa-, nona- and larger oligosaccharides, in addition to maltotriose. Our interest in pullulanase lies primarily in its usefulness as a tool for studying the structures of branched α-glucans [3]. Preliminary observations from our laboratory [4] indicated that it acts as an endo enzyme, but a recent report in conflict with this finding [5] prompted us to re-investigate the mechanism of action. Experimental evidence is presented that confirms our earlier observations, namely that pullulanase hydrolyses pullulan in an essentially random manner and therefore can be classed as an endo enzyme.

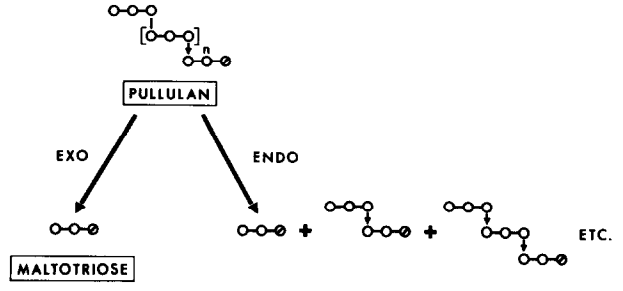


Fig. 1. Possible action patterns of pullulanase. Exo action leads exclusively to maltotriose, endo action to maltotriose with the intermediary production of $6^3$-α-maltotriosylmaltotriose etc. Key: O = α-glucopyranose; Ø = reducing terminus; = 1→4-bond; ↓ = 1→6-bond.

## 2. Materials and methods

Pullulan hydrolysates were separated by (i) descending chromatography on Whatman no. 3mm paper using ethyl acetate:pyridine:water (10:4:3, by vol.). The spots were revealed with silver nitrate-sodium hydroxide [6]. (ii) Qualitative thin-layer chromatograms on silica gel G/kieselguhr G (3:1) plates were run in propranol:nitromethane:water (5:2:3, by vol.) and the spots revealed as by Huber et al. [7]. (iii) Silica gel F 254 plates (E.Merck A.G., Darmstadt, Germany) were used for quantitative thin-layer chromatograms and, after separation in ethyl acetate: methanol:water (37:40:23, by vol.) and colour development as for the qualitative plates, the intensities of the spots were measured using a densitometer (Photovolt, New York, N.Y., USA) and integrator (Nester-Faust, Newark, Delaware, USA). Amounts from

1–8 μg of standard solutions of glucose and of maltotriose were applied to each plate before development in the solvent. In this weight range the integrator readings for the standard spots were directly proportional to the amount of sugar applied, and factors for each plate were derived. Each factor was used to calculate the amounts of glucose and maltotriose in the test samples separated on the same plate.

Pullulanase was prepared as by Wallenfels et al. [8] as modified by Frantz [9]; glucose oxidase (Grade II) and horseradish peroxidase (Grade II) were obtained from the Boehringer-Mannheim Corporation, New York, USA; a twice-crystallised preparation of sweet-potato β-amylase was purchased from the Worhington Biochemical Corporation, Freehold, N.J., USA. In all β-amylase digests, human serum albumin and reduced glutathione were included to stabilise the enzyme [10] and erythritol was included to inhibit an α-glucosidase impurity associated with the amylase preparation [11] Pullulan was prepared from *Pullularia pullulans* [2] and was fractionated on a Sephadex G-75 column. Only the excluded material (M.W. > 50,000) was used in the following experiments. The concentration of pullulan was determined by the method of Pirt and Whelan [12].

## 3. Experimental

The hydrolysis of pullulan by pullulanase was followed at 30° in a digest (25 ml) containing 80 mM-sodium citrate/phosphate buffer pH 5.0, 0.1% pullulan and pullulanase (0.064 units/ml [9]). Samples (9 ml) were withdrawn at intervals and pullulanase was inactivated by heating to 100° for 10 min. The reducing power of a sample (0.4 ml) was determined as by Nelson [13]. Standard glucose solution (0.1 ml of a 0.25% solution) was added to portions (8 ml) of each sample, which were then deionised using carbonated AG 501-X8 resin (Bio-Rad, Richmond, Calif., USA), freeze-dried and dissolved in water (2 ml). Amounts from 6–12 μl of each solution were applied to the quantitative and qualitative thin-layer plates. The added glucose acted as an internal standard in the quantitative chromatography procedure. Another portion of the pullulan digest (0.4 ml) was incubated at 35° in a digest (1 ml) containing 80 mM-sodium acetate, pH 5.0, human serum albumin (0.5 mg/ml), reduced glutathione (0.5 mM) erythritol (1 M) and β-amylase (1000 units/ml [14]). After 48 hr the glucose production was measured by the glucose oxidase method [15].

## 4. Results

The progress of hydrolysis of pullulan was measured by the following parameters. (A) Total reducing power [13]: this measured the total number of glucoside bonds broken. (B) Maltotriose, by thin-layer chromatography: the amount of maltotriose found in each portion of hydrolysate was normalized to the original digest via the glucose standard (see Experimental). The inclusion of the standard was to guard against adventitious losses during deionization and freeze-drying. (C) Maltotriose, by β-amylolysis: the sugar was determined by its hydrolysis to glucose and maltose and specific determination of the former [14]. Maltotriose is the only product of pullulan breakdown that could give rise to glucose.

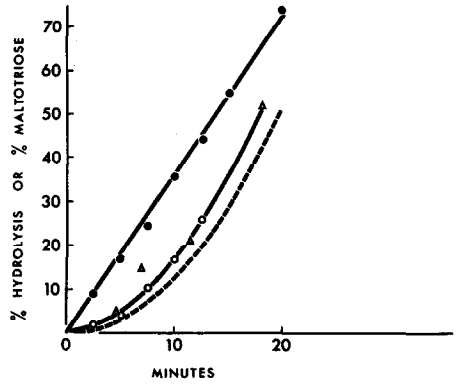


Fig. 2. Relation between degree of hydrolysis of pullulan by pullulanase and release of maltotriose. The curves are: release of reducing groups (●, A), maltotriose measured by thin-layer chromatography (○, B), maltotriose measured by β-amylolysis (△, C) and maltotriose calculated from A (– – –, D), assuming endo action by pullulanase (see text).

In fig. 2 are plotted the rates of release of reducing groups (A), and of maltotriose, as measured by methods B and C. Also plotted (D) is the theoretical amount of maltotriose that would be formed in a totally random (endo) hydrolysis of pullulan by

pullulanase, when the overall rate of hydrolysis is expressed by curve A. The fraction of monomer (maltotriose) at any degree of hydrolysis ($\alpha$) is $\alpha^2$ [16].

It will be noted from fig. 2 that (i) the production of maltotriose as measured by quantitative thin-layer chromatography (B) agrees with the measurements made by $\beta$-amylolysis (C), (ii) the total number of reducing groups always exceeds the number that can be accounted for as maltotriose (curve A versus B and C), and (iii) the actual amounts of maltotriose found corresponded closely with those expected from random hydrolysis (curves B and C versus D).

## 5. Discussion

If pullulanase is an exo enzyme, all the reducing groups set free should be found in maltotriose. That is, in fig. 2, curves A, B and C should be coincident. That there are always present substantial numbers of reducing groups not accountable for as maltotriose argues for an endo action in which oligomers of maltotriose should also be found, to account for the extra reducing groups. These oligomers were in fact revealed by qualitative thin-layer chromatography. Fig. 3 shows the presence of saccharides corresponding in $R_F$ value to hexa-, nona-, dodeca-saccharides etc. Discrete separation of the oligomers up to a degree of polymerization of 21 can be seen. Furthermore, the near coincidence of curves B, C and D indicates that pullulanase action on pullulan is, in essence, completely random.

There are two possible sources of erroneous conclusion in these experiments. It could be that pullulanase is an exo enzyme and that the oligomers of maltotriose seen in fig. 3 arise by transglycosylation. To test this possibility, the pullulanolysis was repeated in the presence of maltose and of Schardinger $\alpha$-dextrin (cyclomaltohexaose). If transglycosylation was occurring both these sugars could be expected to act as acceptors and give rise to extra oligosaccharides detectable by paper chromatography. None was found. Secondly, even an exo action will produce maltotriose oligomers as the chain eroding action nears its end. But since a high-molecular-weight fraction of pullulan (M.W. > 50,000) was used in these experiments, the amounts of chain-end material would be negligible. We therefore conclude that, contrary to the statement by Wallenfels et al. [5] that pullulanase acts by an exo mechanism, the action pattern is that of an endo enzyme.

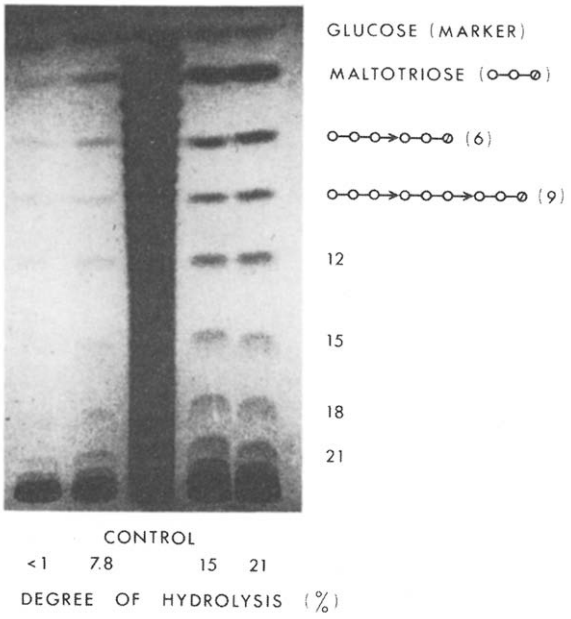


Fig. 3. Products of the partial hydrolysis of pullulan by pullulanase as revealed by thin-layer chromatography. Symbols are as in fig. 1 and the numbers at the right of the chromatogram refer to the degree of polymerization of the oligosaccharide. The glucose was not a product of the reaction but was added as an internal standard for quantitative determination of the maltotriose (see Experimental). The control was a mixture of glucose and a continuous series of maltosaccharides from maltose upwards.

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