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Purification and characterization of a thermostable pullulanase from *Thermoactinomyces thalpophilus*

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SUMMARY

Thermoactinomyces thalpophilus No. 15 produced an extracellular pullulanase in an aerobic fermentation with soluble starch, salts, and complex nitrogen sources. Acetone fractionation, ion-exchange chromatography, and gel filtration purified the enzyme from cell-free broth 16-fold to an electrophoretically homogeneous state (specific activity, 1352 U/mg protein; yield, 4%). The purified enzyme (estimated MW 79 000) was optimally active at pH 7.0 and 70°C and retained 90% relative activity at 80°C (30 min) in the absence of substrate. The enzyme was activated by Co^{2+}, inhibited by Hg^{2+}, and exhibited enhanced stability in the presence of Ca^{2+}. The enzyme hydrolyzed pullulan (K_m 0.32%, w/v) forming maltotriose, and hydrolyzed amylopectin (K_m 0.36%, w/v), amylopectin beta-limit dextrin (K_m 0.45%, w/v) and glycogen beta-limit dextrin (K_m 1.11%, w/v) forming maltotriose and maltose.

INTRODUCTION

The majority of starches used in the manufacture of glucose syrup contain 75–85% amylopectin [11]. Amylopectin is a highly branched polysaccharide consisting of a linear chain of α-1,4-linked D-glucose residues joined together by α-1,6-glucosidic linkages. Beta-limit dextrins are formed when glycogen and amylopectin are hydrolyzed by β-amylase which is unable to by-pass the α-1,6-linkages of the polysaccharides [8]. Pullulan, however, is a linear polysaccharide consisting of 480 maltotriose repeating units that are polymerized through α-1,6-glucosidic linkages [26].

Pullulanase (EC 3.2.1.41, pullulan 6-glucanohydrolase) is a debranching enzyme which hydrolyzes the α-1,6-glucosidic linkages in pullulan and other amylaceous polysaccharides. It was first isolated from a strain of *Aerobacter aerogenes* by Bender and Wallenfels [4] and has since been reported from other bacteria: *Streptococcus mitis* [26], *Klebsiella aerogenes* [13], *Bacillus cereus* var. *mycoides* [25], *Bacillus macerans* [1], *Klebsiella pneumoniae* [12], among others.

Pullulanase is used in combination with the saccharifying α-amylase to raise the saccharification rates and yields in the production of fructose

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syrups. In combination with β-amylases, it is used to achieve yield enhancement during maltose production [3,19].

Industrial starch saccharification processes are usually carried out at temperatures above 60°C in order to obtain a higher substrate solubility and reduce microbial contamination [9]. However, most of the reported pullulanases are unstable at such temperatures [11,22]. Thus, it seems expedient to identify suitable thermoactive, thermostable pullulanases for the benefit of saccharification processes.

To this end, Suzuki and Chishiro [24], Jensen and Norman [11], and Hyun and Zeikus [9] reported the production of thermostable pullulanases from thermophilic bacteria. This paper reports the purification and characterization of a thermostable pullulanase from *Thermoactinomyces thalpophilus* No. 15.

MATERIALS AND METHODS

Organism used

Thermoactinomyces thalpophilus No. 15 used in this study was isolated and identified as described in an earlier report by Obi and Odibo [20] with the speciation being based on the recommendations of Cross [6].

Preliminary test for pullulanase production

The procedure was based on that described by Morgan et al. [18] and involved culturing the organism on a plate of pullulan agar (0.3% w/v pullulan) at 55°C until the appearance of visible growth. The culture was then flooded with ethanol, resulting in the formation of a transparent halo around the culture. This was indicative of pullulanase activity.

Production of pullulanase

Three loops (3-mm diameter) of cells from slant cultures of *T. thalpophilus* stored at 4°C were transferred into 500 ml Erlenmeyer flasks each containing 150 ml of medium N (pH 7.0) composed of 0.1% (w/v) K_2HPO_4, 0.4% (w/v) NaCl, 0.1% (w/v) $MgSO_4 \cdot 7H_2O$, 2% (w/v) soluble starch (British Drug House), 2% (w/v) bacteriological peptone (Difco Laboratories) and 0.2% (w/v) beef extract (Oxoid). The flasks were incubated at 55°C with rotary shaking at 180 rpm for 36 h, after which the cultures were centrifuged (3000 × *g*, 4°C, 15 min). The resultant supernatant was used as crude pullulanase.

Purification of pullulanase

Two volumes of cold (−10°C) acetone were added dropwise with stirring to 300 ml of crude enzyme. The resulting precipitate was resuspended in 70 ml of 20 mM phosphate buffer, pH 7.0 (hereafter, simply 'buffer') and the suspension was dialyzed overnight against distilled water. Dialyzed enzyme was chromatographed on DEAE-cellulose DE (32) equilibrated with buffer (column: 2 × 35 cm; gradient: NaCl in buffer, 0–0.5 M, linear). Fractions with high pullulanase activity were pooled and acetone was added as before. Precipitated enzyme was dissolved in 55 ml of buffer and 50 ml of enzyme solution was subjected to gel filtration (see legend to Fig. 1). Fractions with high specific activity were combined and acetone was added. Precipitated enzyme was redissolved in 30 ml of buffer for characterization.

Pullulanase assays

Pullulanase was assayed by the method of Miller [17]. Unless otherwise stated, each reaction mixture contained 0.5% pullulan, enzyme solution and buffer (for the volume and enzyme amounts, see the table and figure legends). Unless otherwise indicated, reactions were terminated after 10 min incubation at 55°C and the amount of maltotriose formed was then measured [17]. One unit (U) of enzyme is the amount required to release 1 mg maltotriose per min under these conditions. Polysaccharide substrate solutions were prepared in buffer. Unless otherwise stated, buffer was 20 mM phosphate buffer, pH 7.0. In assays in which the effect of additives on enzyme activity was to be determined, buffer with additive was added to the reaction mixture prior to enzyme to achieve the final additive concentration indicated in the text. To determine optimal pH, 0.2 M Universal buffer was used [5]. To

measure optimal reaction temperatures, 10 min reactions (volume 1.0 ml, enzyme 22.5 U/mg protein) were carried out at 50, 60, 70, 80, 90 and 100°C. To measure enzyme stability, the pullulanase was incubated in buffer or buffer with 1 mM $CaCl_2$ for 30 min, cooled rapidly to 55°C, and assayed as described above. To measure K_m values [14] for polysaccharides (pullulan and potato amylopectin, Sigma; rabbit liver glycogen, Merck; amylopectin and glycogen beta-limit dextrins, prepared in our laboratory following the method of Adams and Priest [1]), each polysaccharide was used in turn as substrate at concentrations ranging from 0 to 1% (w/v). Amounts of reducing sugars released from polysaccharides during a 4-h incubation with pullulanase were determined by the DNS method of Miller [17].

Protein assays

Protein was determined using the method of Lowry et al. [15] or, for purified enzymes, by measuring optical density at 280 nm. Bovine serum albumin was used as standard.

Electrophoresis

Gel electrophoresis was performed as described by Davis [7] using the following conditions: buffer, 3 mM Tris-glycine buffer, pH 8.3; current, 40 mA for eight tubes; duration, till tracking dye (0.001% w/v bromophenol blue) emerged. Protein was visualized with Coomassie Brilliant Blue R-250.

Product identification

Reducing sugars released from polysaccharides were determined, after 3 h of enzymatic hydrolysis (substrate, 1% w/v; 9 U/mg protein of pullulanase), by paper chromatography (paper: Whatman No. 1; solvent: pyridine/*n*-butanol/water, 4:6:3, v/v). Each reaction was terminated by adding $HgCl_2$ to 5% (v/v). Samples (20 μl) of each hydrolysate were chromatographed alongside standards of glucose, maltose, and maltotriose. Sugars were located with aniline hydrogen phthalate reagent [23].

Estimation of relative molecular weight

The method of Andrews [2] was employed, using a Sephadex G-150 column (2 × 45 cm) equilibrated with buffer for 2 days. The void volume was determined with blue dextran (see legend to Fig. 3 for markers).

RESULTS

Homogeneous T. thalpophilus pullulanase obtained for characterization

Pullulanase was purified by sequential application of acetone precipitation, ion-exchange chromatography and gel filtration (Table 1). The purified pullulanase appeared homogeneous according to two criteria: protein and activity profiles were identical in the gel filtration (Fig. 1) and only one

Table 1

Purification of pullulanase of *T. thalpophilus*

Step	Vol. (ml)	Total activity (U)	Total protein (mg)	Spec. act. (U · mg protein^{-1})	Yield (%)	Purification
Supernatant from culture broth	300.0	30450.0	360.0	84.6	100	1.0
Acetone precipitation	70.0	6277.6	61.4	102.0	21.0	1.2
Ion-exchange chromatography on DEAE-cellulose	55.0	2532.0	2.25	1125.0	8.0	13.3
Gel filtration on Sephadex G-75	30.0	1095.0	0.81	1352.0	3.6	16.0

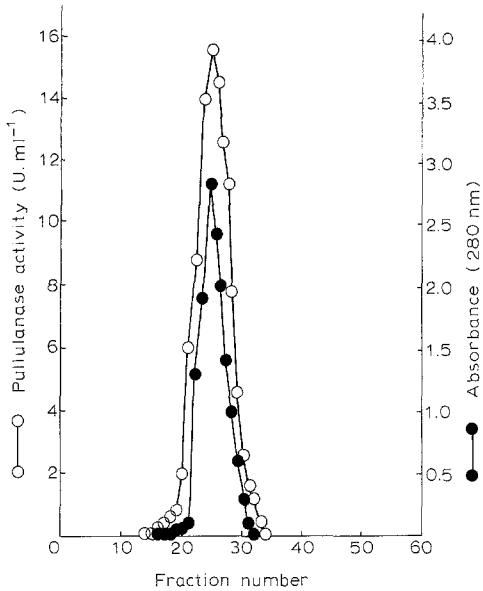

Fig. 1. Sephadex G-75 column chromatography of pullulanase of *T. thalpophilus* No. 15. Symbols: (○) pullulanase activity; (●) protein absorbance. One unit of pullulanase is equivalent to the amount of enzyme liberating 1 mg of maltotriose per min from pullulan under the assay conditions.

protein band was obtained when the purified enzyme was subjected to gel electrophoresis (Fig. 2).

Relative molecular weight of pullulanase

The relative molecular weight of the purified enzyme was estimated from the elution volumes of standard proteins in the same column. The results illustrated in Fig. 3 give the value of 79 000 Da for the enzyme.

T. thalpophilus pullulanase is optimally active and stable at 70°C and neutral pH

Purified pullulanase of *T. thalpophilus* had a broad pH profile, in which it retained 80% maximal activity at pH 6 and pH 11. It was, however, optimally active at pH 7.0. In a 10-min reaction, pullulanase activity was optimal at a reaction temperature of 70°C; 90% of its maximal activity was observed at 80°C. The enzyme was completely stable when incubated at 50 or 60°C for 30 min in the absence of polysaccharide substrate. Two and six percent decreases occurred when preincubation was

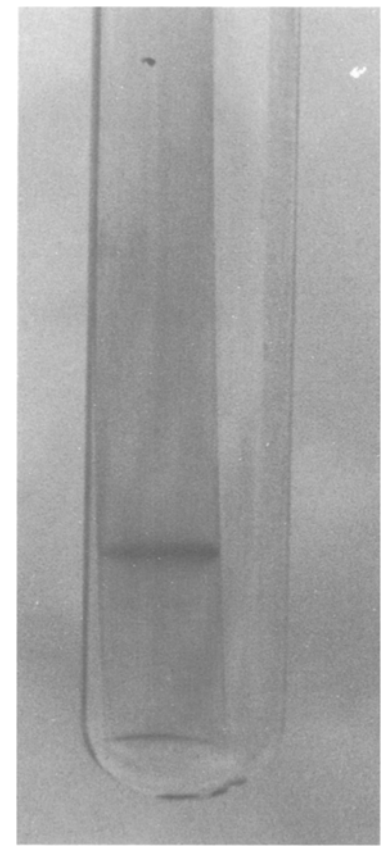

Fig. 2. Gel electrophoresis of pullulanase of *T. thalpophilus* No. 15. Electrophoresis was carried out as described in Materials and Methods. 20 μg of purified pullulanase per gel was applied and a current of 5 mA per tube was used for 2 h. Protein was stained with Coomassie Brilliant Blue R-250.

at 70 and 80°C, respectively. When preincubation was at 90 and 100°C, decreases were dramatic, i.e., 45 and 68%, respectively. However, when preincubation was in the presence of 1 mM Ca^{2+} stability was enhanced; for example, 72% of the original activity was retained after 30 min incubation at 100°C.

Although *T. thalpophilus* pullulanase was relatively stable to heat, the pullulanase reaction was sensitive to oxidation as judged by the 37% inhibition observed in the presence of 1 mM $KMnO_4$. The pullulanase was also sensitive to detergent (47% inhibition by 1 mM sodium dodecyl sulfate).

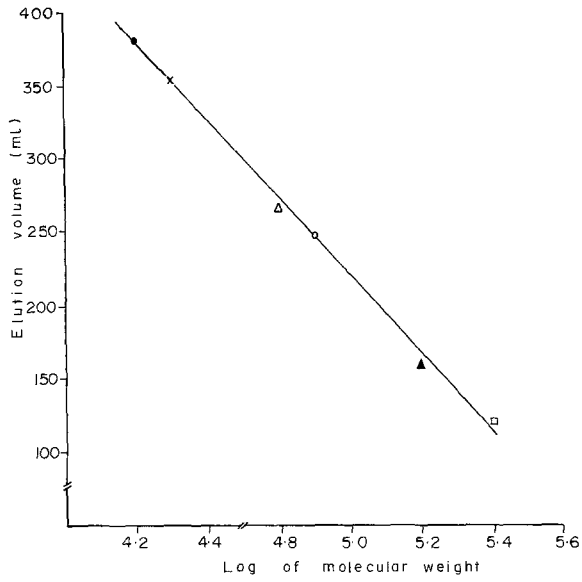

Fig. 3. Estimation of the molecular weight of the pullulanase of *T. thalpophilus* No. 15. Standard proteins used were: (●) myoglobin; (×) trypsin; (△) bovine serum albumin; (▲) lactate dehydrogenase; (□) pyruvate kinase. The open circle denotes pullulanase.

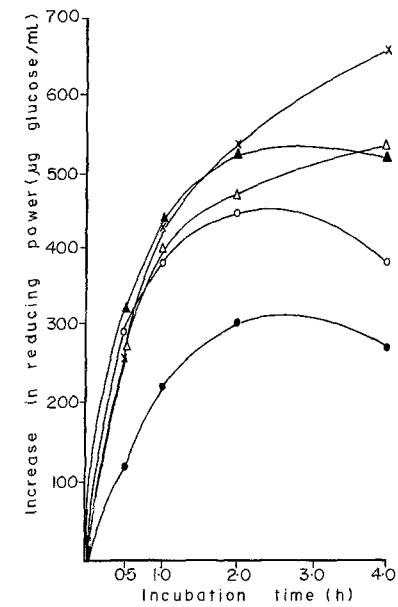

Fig. 4. Hydrolysis curves of polysaccharides incubated with *T. thalpophilus* No. 15 pullulanase. Symbols: (○) amylopectin; (▲) amylopectin beta-limit dextrin; (●) glycogen; (△) glycogen beta-limit dextrin; (×) pullulan.

Influence of metal ions and chelating agents

The activity of the purified pullulanase in the presence of metal salts at 1 mM was determined. $CaCl_2$ and $MnCl_2$ had no effect. Inhibition (expressed as %) occurred with the following salts: $HgCl_2$, 46%; Pb (acetate), 42%; $MgCl_2$, 33%; $AgNO_3$, 23%; $ZnSO_4$ or $BaCl_2$, 22%; $BiSO_4$, 20%; $FeSO_4$, 18%; $CuSO_4$ or $SrSO_4$, 15%. It is noteworthy that 1 mM $CoSO_4$ stimulated enzyme activity by 61%. The chelators EDTA and *o*-phenanthroline, each at 1 mM, inhibited the enzyme 42 and 48%, respectively. This indicates the probable involvement of bound metal ions in the structure of *T. thalpophilus* pullulanase.

Pullulan is the preferred substrate of T. thalpophilus pullulanase

Comparison of the amounts of reducing sugar released from five different amylaceous polysaccharides was made in five separate 4-h reactions catalyzed by the pullulanase (Fig. 4). During the first 2 h, reducing sugars were released from pullulan, amylopectin, amylopectin beta-limit dextrin, and glycogen beta-limit dextrin at approximately equivalent rates. For pullulan and, to a lesser extent, with glycogen beta-limit dextrin, further significant release occurred between 2 and 4 h. With amylopectin, amylopectin beta-limit dextrin, and glycogen, the amount of reducing sugar released did not increase after 2 h. The relative maximal amounts of reducing sugars released were: pullulan, 1.00; glycogen beta-limit dextrin, 0.81; amylopectin beta-limit dextrin, 0.77; amylopectin, 0.68; and glycogen, 0.43. Paper chromatography demonstrated the production of only maltotriose from pullulan, and maltotriose and maltose (as major products) from amylopectin, amylopectin beta-limit dextrin, glycogen beta-limit dextrin and soluble starch. For glycogen, the major product was maltotriose with a trace of unidentified maltooligosaccharide (Fig. 5).

The Michaelis constants (K_m) of the enzyme were 0.32%, 0.36%, 0.45% and 1.11% for pullulan, amylopectin, amylopectin beta-limit dextrin and

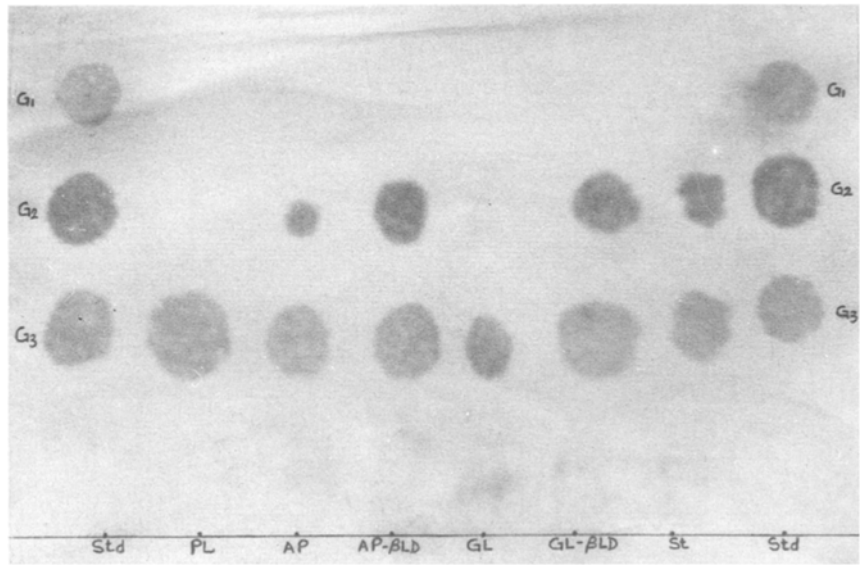

Fig. 5. Paper chromatogram of the hydrolysis products of *T. thalpophilus* No. 15 pullulanase. Symbols: AP (amylopectin); AP-βLD (amylopectin beta-limit dextrin); GL (glycogen); GL-βLD (glycogen beta-limit dextrin); PL (pullulan); Std (standard sugars); St (starch); G_1 (glucose); G_2 (maltose); G_3 (maltotriose).

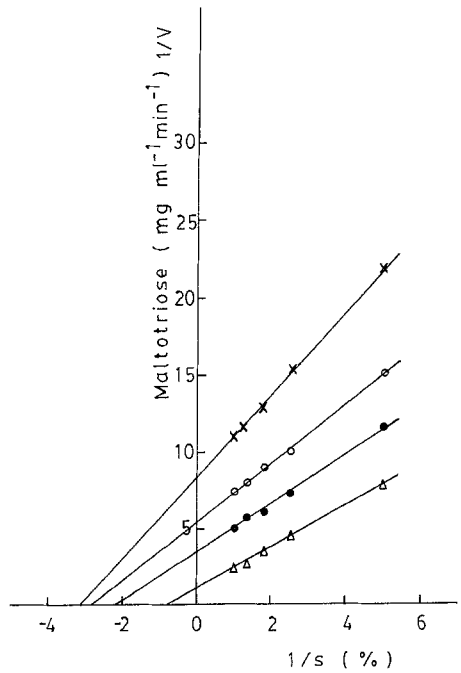

Fig. 6. Lineweaver-Burk plot for the determination of the K_m values of pullulanase. Symbols: (○) amylopectin; (●) amylopectin beta-limit dextrin; (△) glycogen beta-limit dextrin; (×) pullulan.

glycogen beta-limit dextrin, respectively (Fig. 6). The values indicate that among the listed polysaccharides, affinity for pullulanase is highest with pullulan.

DISCUSSION

We believe that this report is the second to demonstrate a thermostable pullulanase. The pullulanase of *Bacillus stearothermophilus* KP 1064 [24] and that (Promozyme) of *B. acidopullulyticus* [11] were only thermoactive. The thermostable pullulanase from *Clostridium thermohydrosulfuricum* has a higher temperature (85°C) for optimal activity [9] than the enzyme from *T. thalpophilus*. Since saccharification of starch is usually carried out industrially at temperatures above 60°C [9], and pullulanases from sources which produce thermolabile enzyme [8,22] have been employed to aid saccharification via their debranching activity, the pullulanases of *T. thalpophilus* and *C. thermohydrosulfuricum* by virtue of their thermostability and ther-

moactivity, deserve evaluation as aids in saccharification processes. Comparative utility of the two enzymes in the process cannot be assessed from the limited data currently available.

The pH activity profile of *T. thalpophilus* pullulanase is similar to that reported for the enzyme from *B. polymyxa* [8] and potato [10] but differs from those of *Streptomyces* sp. No. 280 [27], *B. cereus* var. *mycoides* [25], *B. acidopullulyticus* [11], and *C. thermohydrosulfuricum* [9], where optimal activity occurred between pH 5 and 6.

The observed enhancement of thermostability of *T. thalpophilus* pullulanase by Ca^{2+} was previously reported for the thermolabile pullulanase of *B. cereus* var. *mycoides* [25]. This observation was, however, not reported for the pullulanase of *C. thermohydrosulfuricum* [9]. Likewise, stimulation of pullulanase activity by Co^{2+}, observed in this study for *T. thalpophilus* pullulanase, was earlier reported by Ishizaki et al. [10] for the enzyme from potato but not the enzyme from *B. cereus* [25].

The relative molecular weight of *Aerobacter aerogenes* pullulanase (81 000 [20]) resembles that of *T. thalpophilus* pullulanase (79 000, this report). Both differ markedly from the values of 5600 and 500 000 reported for two forms of pullulanase from another strain of *Aerobacter aerogenes* by Mercier et al. [16]. Information on the relative molecular weights of thermoactive pullulanases of *B. acidopullulyticus* [11] and *B. stearothermophilus* [24] and the thermostable pullulanase of *C. thermohydrosulfuricum* [24] is not available for comparison.

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