Purification and characterization of a highly thermostable novel pullulanase from *Clostridium thermohydrosulfuricum*

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Clostridium thermohydrosulfuricum mutant Z 21-109 produced intracellular thermostable pullulanase and glucoamylase activities. The glucoamylase activity was inactivated by treating *C. thermohydrosulfuricum* cells with 10 % (v/v) propan-1-ol at 85 °C in the presence of 5 mM-CaCl₂. Pullulanase activity was selectively solubilized from cells by treatment with detergent and lipase. The solubilized pullulanase was purified by treatment with streptomycin sulphate and (NH₄)₂SO₄ and by DEAE-Sephacel, octyl-Sepharose and pullulan-Sepharose chromatography. Pullulanase was purified 3511-fold and displayed homogeneity on SDS/polyacrylamide-gel electrophoresis. The pullulanase was a monomeric glycoprotein with an apparent M_r of about 136 500, and it displayed a pI of 5.9. The enzyme was enriched in both acidic and hydrophobic amino acids. The purified pullulanase was stable and optimally active at 90 °C. The optimum pH for activity and pH-stability ranges were 5.0–5.5 and 3.0–5.0 respectively. The enzyme was inhibited by cyclodextrins, EDTA and *N*-bromosuccinimide, but not by *p*-chloromercuribenzoate and acarbose. The pullulanase displayed a relative substrate specificity for hydrolysis of pullulan (100 %) versus 75 % for glycogen and 50 % for soluble starch. The apparent K_m , V_{max} and K_{cat} values for enzyme activity on pullulan at 60 °C were 0.675 mg/ml, 122.5 μ mol of reducing sugar formed/min per mg of protein and 16240 min⁻¹ respectively. The novel properties of this extremely thermostable pullulanase are discussed in relation to other purified starch-debranching enzymes.

INTRODUCTION

Pullulanase (pullulan 6-glucanohydrolase, EC 3.2.1.41) is a debranching enzyme that specifically cleaves certain α -1,6 links in starch, amylopectin, pullulan and related oligosaccharides (Abdullah *et al.*, 1966). It is an industrially important enzyme, which is generally used in combination with saccharifying amylases such as glucoamylase, fungal α -amylase and β -amylase for the production of various sugar syrups because it improves saccharification and yield (Norman, 1982). Moreover, it has gained significant attention as a useful tool for structural studies of carbohydrates (Whelan, 1971).

High value is placed on the extreme thermostability and thermoactivity of pullulanase in the bioprocessing of starch. Pullulanase is produced by mesophilic organisms such as *Aerobacter aerogenes* (Bender & Wallenfels, 1961), *Bacillus* sp. (Norman, 1982; Nakamura *et al.*, 1975), *Bacillus polymyxa* (Fogarty & Griffin, 1975), *B. macerans* (Adams & Priest, 1977), *Bacillus cereus* var. *mycoides* (Takasaki, 1976) and *Streptococcus mitis* (Walker, 1968), but their respective enzymes lack extreme thermal stability and activity. Extreme thermophiles often possess thermostable enzymes, and interest in thermoanaerobes has increased in recent years for their potential value in the production of thermoactive and thermostable amylases or as gene sources for cloning these enzymes into aerobic industrial hosts (Zeikus, 1979).

Hyun & Zeikus (1985a) have found that *Clostridium thermohydrosulfuricum* produces highly thermoactive and thermostable cell-bound pullulanase and glucoamylase activities. They studied the general mechanism for regulation of the synthesis of pullulanase and

glucoamylase activities in this organism, and developed a hyperproductive mutant, Z 21-109 (Hyun & Zeikus, 1985b). The purpose of the present paper was to purify and characterize the molecular, physicochemical, stability and catalytic properties of pullulanase from this organism. We report on the general and novel properties of this extremely thermostable and active enzyme.

MATERIALS AND METHODS

Cultivation of organism

The mutant strain Z 21-109 of *C. thermohydrosulfuricum* 39E (A.T.C.C. 33223), deposited with the American Type Culture Collection (Rockville, MD, U.S.A.), was grown in 16 litres of TYE medium with 0.5 % soluble starch under anaerobic condition without shaking for 20 h at 65 °C (Zeikus *et al.*, 1980). The cells were then collected by centrifugation (12000 *g* for 30 min), washed with 50 mM-acetate buffer (pH 6.0)/5 mM-CaCl₂, and stored at –20 °C.

Enzyme assays

The standard assay procedure used for measurement of pullulanase activity entailed incubating a reaction mixture (1 ml) that consisted of pullulan (1 %) in acetate buffer (50 mM, pH 6.0) and the enzyme source at 60 °C for 30 min. The reducing sugars formed were determined by the dinitrosalicylic acid method (Bernfeld, 1955). One unit of pullulanase activity is the amount of enzyme that produces 1 μ mol of reducing sugar (with glucose as standard)/min under the above assay conditions.

The standard assay procedure used for measurement of glucoamylase activity entailed a reaction mixture

Table 1. Protocol used to purify pullulanase from *C. thermohydrosulfuricum*

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg of protein)	Recovery (%)	Purification (fold)
Crude enzyme extract	4400	602	0.14	100	1.0
Heat-treated extract	3259	590	0.18	98	1.3
Streptomycin sulphate treatment	2881	524	0.18	87	1.3
(NH ₄) ₂ SO ₄ treatment (30–60 %-satd.)	1406	450	0.32	75	2.3
DEAE-Sephacel	560	328	0.59	55	4.3
Octyl-Sepharose	154	278	1.81	46	13.2
Pullulan-Sepharose	0.52	250	481.0	42	3511

(1 ml) containing boiled soluble starch (2%, w/v) solution, acetate buffer (50 mM, pH 6.0) and the enzyme source. After incubation at 60 °C for 30 min, the reaction mixture was kept in a steam bath for 15 min and centrifuged (12000 *g* for 5 min) to remove insoluble materials. The released glucose was measured with a glucose analyser (model 27, Yellow Spring Instrument Co.). One unit of glucoamylase activity is defined as the amount of enzyme that produces 1 μ mol of glucose/min under the assay conditions described above.

Purification of pullulanase

All operations were performed under aerobic conditions and at 4 °C, unless otherwise mentioned. The frozen cells were thawed and suspended in 50 mM-acetate buffer (pH 6.0)/5 mM-CaCl₂ and treated with lipase (0.2%, from *Candida cylindracea*; Sigma Chemical Co.) and sodium cholate (0.2%) overnight at 37 °C. The resulting suspension was centrifuged at 48000 *g* for 1 h. The supernatant was used as a crude enzyme extract.

The crude enzyme extract was heated at 75 °C in a water bath for 10 min, cooled and then centrifuged at 48000 *g* for 30 min to remove insoluble materials.

Streptomycin sulphate was added to the crude extract (10 ml of a 10% solution/g of protein), stirred and kept for 30 min. The precipitate formed was removed by centrifugation (48000 *g* for 30 min).

Solid (NH₄)₂SO₄ was slowly added to the resulting supernatant at 30% satn. and the mixture was kept for 2 h. The precipitate formed contained mostly glucoamylase activity and was collected by centrifugation (48000 *g* for 30 min). More (NH₄)₂SO₄ was added to the supernatant to give a final concentration of 65% satn. and the mixture was left overnight. The precipitate formed was collected by centrifugation, dissolved in 50 mM-acetate buffer (pH 6.0)/5 mM-CaCl₂ and dialysed overnight against the same buffer.

The dialysed enzyme solution was applied to a DEAE-Sephacel column (2.6 cm $\times$ 38 cm) pre-equilibrated with 50 mM-acetate buffer (pH 6.0)/5 mM-CaCl₂. The column was washed with the same buffer until the *A*₂₈₀ returned to the baseline, and was then eluted with a 600 ml linear NaCl (0–1 M) gradient in the same buffer at a flow rate of 20 ml/h. The active pullulanase peak fractions were pooled, concentrated with an Amicon cell (model 202; Amicon Corp., Lexington, MA, U.S.A.) equipped with a PM 10 membrane, under nitrogen pressure of 20 lb/in² (138 kPa) and dialysed against the same buffer.

The dialysed enzyme solution was applied directly to a

column (2.6 cm $\times$ 35 cm) of octyl-Sepharose pre-equilibrated with 30% (NH₄)₂SO₄ in 50 mM-acetate buffer (pH 6.0)/5 mM-CaCl₂. The column was washed with the same buffer, and the enzyme was eluted with a 600 ml linear gradient of 0–50% (v/v) ethylene glycol in 50 mM-acetate buffer (pH 6.0)/5 mM-CaCl₂. The active protein peak fractions were pooled, dialysed against the elution buffer without ethylene glycol, and concentrated by ultrafiltration (PM 10).

Pullulan-Sepharose affinity matrix was prepared by coupling pullulan to epoxy-activated Sepharose 6B by a standard procedure described below (Affinity Chromatography, Pharmacia Fine Chemicals, Uppsala, Sweden). The epoxy-activated Sepharose 6B (15 g) was washed with distilled water (1.5 litres) and re-swelled in 0.1 M-carbonate buffer, pH 11.0. To this gel suspension (100 ml), pullulan solution (4%, w/v; 100 ml) in 0.1 M-carbonate buffer (pH 11.0) was added and mixed. The mixture was shaken at 40 °C for 16 h. The excess ligand was washed away with carbonate buffer (pH 11.0), distilled water, 0.1 M-carbonate buffer (pH 8.0) and finally with 0.1 M-acetate buffer (pH 4.0). The remaining epoxy groups were blocked with 1 M-ethanolamine overnight at 30 °C. The resulting pullulan-Sepharose gel was washed with water and kept at 4 °C. The concentrated enzyme solution after octyl-Sepharose chromatography was applied to a pullulan-Sepharose column (2.5 cm $\times$ 12 cm) pre-equilibrated with 50 mM-acetate buffer (pH 6.0)/5 mM-CaCl₂. The column was washed with the same buffer, then with the same buffer containing 1 M-NaCl, and finally eluted with 50 mM-acetate buffer (pH 6.0)/5 mM-CaCl₂ plus 1% pullulan. The active peak fractions were pooled, incubated overnight at 60 °C, concentrated by ultrafiltration and dialysed extensively against the same buffer and used as purified pullulanase.

Other methods

Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. SDS/polyacrylamide-gel electrophoresis was performed by the method of Laemmli (1970). The *M_r* of native enzyme was estimated by gel filtration on Sephacryl S-200 as described by Andrews (1964). For pI determination, a Servalyt Precote isoelectric-focusing gel (pH 3–10) was used. Samples were run at constant power of 1 W in a LKB Ultraphore isoelectric-focusing apparatus, and the gel was stained with Serva Blue W. For determination of amino acid composition, the samples were prepared and analysed by the PICO.TAG method (Waters Associates,

Fig. 1. Analysis of purified pullulanase by SDS/polyacrylamide-gel electrophoresis

The purified enzyme was electrophoresed at pH 8.3 on 10%-acrylamide gel and stained with Coomassie Brilliant Blue R-250. Lanes: 1, purified pullulanase; 2, standard marker proteins.

Table 2. Amino acid composition of pure pullulanase

Amino acid	Molar proportions
Aspartic acid	476
Glutamic acid	326
Serine	271
Glycine	337
Histidine	45
Arginine	113
Threonine	242
Alanine	228
Proline	187
Tyrosine	143
Valine	238
Methionine	35
Isoleucine	28
Leucine	182
Phenylalanine	114
Lysine	184

Milford, MA, U.S.A.). The K_m was determined by the Lineweaver-Burk (1934) method.

The reaction products of pure pullulanase were analysed by h.p.l.c. The separation system consists of a multi-solvent delivery system (600; Waters Chromatography Division, Millipore Corp., Milford, MA, U.S.A.) equipped with an autosampler (712, WISP; Waters), a refractive-index detector (410 differential refractometer; Waters), data module (740; Waters) and a saccharide-analysis column (Aminex HPX-42A; Bio-Rad Laboratories, Richmond, CA, U.S.A.) fitted with a carbohydrate de-ashing system (Bio-Rad) or a Supelcosil LC-NH₂ column (Supelco, Bellefonte, PA, U.S.A.). The Aminex HPX-42A column was used to detect oligosaccharides. The column was maintained at 85 °C, and sugars were eluted with distilled water. Maltotriose was distinguished from panose or isopanose by using the Supelcosil LC-NH₂ column and acetonitrile/water (3:1, v/v) solvent system.

RESULTS

Purification and characterization of pullulanase activity

To initiate purification of pullulanase, cell suspensions were treated in 50 mM-acetate buffer (pH 6.0)/10% (v/v) propan-1-ol and heated at 85 °C in the presence and the absence of CaCl₂ (5 mM). By this treatment, the glucoamylase activity was destroyed within 10 min, whereas pullulanase activity was essentially maintained (95% of initial activity) in the presence, but not in the absence, of CaCl₂.

Various procedures were examined in order to develop a procedure that would selectively release pullulanase from other cell-bound enzymes. With the non-ionic detergent Triton X-100 glucoamylase was largely released, but not pullulanase. With lipase, pullulanase was released from the cells. The conditions for maximum solubilization of pullulanase from cells were then established by using a lipase-based treatment (results not shown), and this was the source of crude enzyme extract.

Table 1 summarizes the steps used to purify pullulanase by the lipase-solubilized cell-treatment procedure. The final enzyme solution was purified 3511-fold over the crude enzyme extract with an overall recovery of 42% and had a specific activity of 481 units/mg of protein. The purified pullulanase showed a single band by SDS/polyacrylamide-gel electrophoresis when stained with Coomassie Brilliant Blue (Fig. 1). It also showed a single band when stained with periodic acid/Schiff's reagent, which indicates that the enzyme was glycoprotein in nature (results not shown).

Molecular properties of purified pullulanase

The M_r of the purified pullulanase was estimated to be 140 000 by SDS/polyacrylamide-gel electrophoresis and 133 000 by gel filtration on Sephacryl S-200. The purified enzyme migrated as a single protein band on isoelectric focusing (Servalyt polyacrylamide gel) and had a pI of 5.9, by comparison with commercial standards (results not shown). Analysis of the amino acid composition of purified pullulanase indicated that it was enriched in acidic and hydrophobic amino acids (Table 2).

Fig. 2. Effect of temperature on stability and activity of *C. thermohydrosulfuricum* pullulanase

(a) Thermal stability. The enzyme was placed in acetate buffer (50 mM, pH 6.0) with 5 mM- CaCl_2 and preincubated at various temperatures for 30 min, and then residual pullulanase activities were assayed. (b) Effect of heat on activity. The enzyme activity was assayed at various temperatures by the standard assay method (30 min incubation).

Fig. 3. Effect of pH on stability and activity of *C. thermohydrosulfuricum* pullulanase

(a) Effect of pH on stability. Purified pullulanase solutions were incubated for 1 h at 60 °C at various pH values. After adjustment of pH, the residual activity was assayed by the standard method. (b) Effect of pH on activity. The activity of purified pullulanase was measured by the standard assay method by changing the buffer to obtain the desired pH.

Physicochemical stability of purified pullulanase

The thermal stability and activity of *C. thermohydrosulfuricum* pullulanase were remarkably high (Fig. 2). The enzyme was quite thermostable in the absence of substrate even up to 90 °C, with essentially no loss of activity in 30 min. However, the enzyme lost about 40 % of its original activity when incubated at 95 °C for 30 min.

The optimum temperature for the action of the purified enzyme on pullulan was 90 °C. Purified pullulanase activity rapidly decreased on incubation at 95 °C, to only 38 % of maximal.

Fig. 3 shows that the pH-stability and activity of *C. thermohydrosulfuricum* were rather broad. The enzyme was stable at pH 3.0–5.0, and it was gradually inactivated at pH values above 5.5. The enzyme showed optimal activity at pH 5.5, with about 95 % and 75 % activity at pH 5.0 and 4.5 respectively.

Catalytic properties of purified pullulanase

The purified enzyme did not contain other amylase activities, as judged by h.p.l.c. analysis of reaction products on pullulan. The purified pullulanase produced only maltotriose, but not panose or isopanose, as hydrolysis products of pullulan. Table 3 compares the initial reaction velocity for hydrolysis of various substrates by purified pullulanase from *C. thermohydrosulfuricum* with that of *Bacillus* pullulanase (Promozyme; Novo Industri A/S, Denmark), in order to assess the relative substrate specificities of these two enzymes. *C. thermohydrosulfuricum* pullulanase displayed 3-fold higher specificity towards soluble starch and amylopectin and 7-fold higher specificity towards glycogen than did the *Bacillus* enzyme.

The general enzyme kinetic features of *C. thermohydrosulfuricum* pullulanase were determined on pullulan at 60 °C. The apparent Michaelis constant (K_m) for pul-

Table 3. Comparison of substrate specificity of pure pullulanase from *C. thermohydrosulfuricum* with that of a commercial enzyme source

Sufficient enzyme was added to cause linear release of product during the first 15 min of reaction. The substrate specificity is expressed as the initial hydrolysis velocity as a percentage of that obtained with pullulan substrate.

Substrate	Relative substrate specificity (% hydrolysis)	
	<i>C. thermohydrosulfuricum</i>	<i>Bacillus</i> sp.
Pullulan	100	100
Amylopectin	62	22
Glycogen (oyster)	75	10
β -Limit dextrin from glycogen (oyster)	77	33
Soluble starch	52	16

Table 4. Comparison of various reagents as inhibitors of pure pullulanase from *C. thermohydrosulfuricum*

Reagent	Concn. (mM)	Relative activity*
None	—	100
CaCl ₂	10	105
MgCl ₂	10	105
MnCl ₂	10	110
CoCl ₂	10	122
ZnCl ₂	10	34
FeCl ₃	10	42
EDTA	10	61
<i>p</i> -Chloromercuribenzoate	0.02	95
Dithiothreitol	10	127
Acarbose	20 mg	93
α -Cyclodextrin	10	74
β -Cyclodextrin	10	25
γ -Cyclodextrin	10	34

* 100% activity corresponds to 0.35 unit of pullulanase under standard conditions.

lulan, the maximum initial reaction rate ($V_{\max}$) and K_{cat} were 0.675 mg/ml, 122.5 μmol of reducing sugar formed/min per mg of protein and 16240 min^{-1} respectively. The K_m for *Bacillus* pullulanase was also determined at 60 °C on pullulan, and was 0.664 mg/ml.

Table 4 compares the effects of various metal ions and other inhibitors on pullulanase activity. The enzyme was inhibited by cyclodextrins, EDTA or *N*-bromosuccinimide, but not by acarbose. Purified pullulanase was not inhibited by *p*-chloromercuribenzoate, suggesting that cysteine was not required for enzyme activity.

The reversion products of pullulanase activity were examined by incubating maltose or maltotriose solutions (5%) with purified enzyme for 24 h, after which the reaction products were analysed by h.p.l.c. With maltose, a tetrasaccharide and a hexasaccharide peak appeared.

With maltotriose, a hexasaccharide and a peak of higher saccharides (not determined, possibly nonasaccharide) were obtained.

DISCUSSION

In general, these results represent the first purification to homogeneity of an extremely thermostable pullulanase. General characterization of the enzymes indicate that it differs from well-described thermolabile pullulanases. *C. thermohydrosulfuricum* produces highly thermoactive pullulanase and glucoamylase activities, from which purified pullulanase has been described here. The exact enzyme composition of glucoamylase activity remains to be identified. It could be a real glucoamylase, α -glucosidase, or other enzymes and in combination.

The lipase-solubilized pullulanase was purified to homogeneity by treatment with streptomycin sulphate and $(\text{NH}_4)_2\text{SO}_4$, and by DEAE-Sephacel, octyl-Sepharose and pullulan-Sepharose chromatography. We did not detect any multiplicity of solubilized pullulanase during the course of purification, unlike with other pullulanase preparations (Eisele *et al.*, 1972; Mercier *et al.*, 1972; Ueda & Ohba, 1973; Ohba & Ueda, 1973). High purity and specific activity of pullulanase were made possible by the use of pullulan-Sepharose chromatography.

The enzyme had an M_r of 140000 as estimated by SDS/polyacrylamide-gel electrophoresis, and 133000 determined by gel filtration on Sephacryl S-200. These values are close. Taking the average of the two values, the M_r for pullulanase from *C. thermohydrosulfuricum* was 136500. The M_r of pullulanases from other sources ranges from 56000 to 150000 (Fogarty, 1983). However, this pullulanase is very similar to that of *Aerobacter aerogenes* with respect to M_r (143000) and having a single polypeptide chain (Eisele *et al.*, 1972). Periodic acid/Schiff staining indicated that the enzyme might be glycoprotein in nature. This is contrary to the finding that *Klebsiella pneumoniae* extracellular pullulanase is a lipoprotein (Pugsley *et al.*, 1986), although further studies are needed to confirm the glycoprotein nature of our enzyme. The enzyme had a pI of 5.9, which indicates that it is an acidic protein.

The amino acid composition of the purified pullulanase showed a high degree of similarity to pullulanase from other sources (Eisele *et al.*, 1972; Ohba & Ueda, 1975; Brandt *et al.*, 1976), differing significantly only in having less methionine [except that from *A. aerogenes* (Ohba & Ueda, 1975), which has much lower amounts], leucine and isoleucine and more hydrophobic amino acids.

The enzyme was stable up to 90 °C and at pH 3.0–5.0, and was optimally active also at 90 °C and at pH 5.5. The pH optima of most pullulanases occur between pH 5.0 and 7.0, and temperature optimum is between 45 to 60 °C (Fogarty, 1983). At present it is not clear whether or not the presence of a carbohydrate moiety lends better thermostability to this pullulanase. The enzyme acts on pullulan and makes only maltotriose and not panose and isopanose, which indicates that it cleaves pullulan at α -1,6 linkages.

Schardinger dextrins inhibited the pullulanase activity, and β -dextrin was a stronger inhibitor than α -dextrin. This is true with other pullulanases (Enevoldsen *et al.*, 1977). The enzyme was not inhibited by *p*-chloromercuribenzoate, although pullulanase from *B. cereus*

(Takasaki, 1976) and *B. polymyxa* (Fogarty & Griffin, 1975) are inhibited by it. This indicates that there is no thiol-group involvement for its catalytic activity. The enzyme did not require Ca^{2+} for its activity. However, it is inhibited by the chelating agent EDTA, which indicates that Ca^{2+} is probably important for its stability. Ca^{2+} played an important role in thermal stability of the enzyme, and may maintain the conformation of the enzyme. Acarbose, a potent inhibitor of glucoamylase (Truscheit *et al.*, 1981), has almost no effect on pullulanase activity even at high concentration. The enzyme was inhibited by *N*-bromosuccinimide, which indicates that tryptophan residues may be involved in catalysis. This pullulanase, like other reported pullulanases (Abdullah & French, 1970), produced condensation products.

Thermostable pullulanase activities from other sources, e.g. *Thermus aquaticus* YT-1 (Plant *et al.*, 1986), *Thermoanaerobium* Tok 6-B1 (Plant *et al.*, 1987), *Thermoanaerobium brockii* (Coleman *et al.*, 1987), *Clostridium* sp. (Madi *et al.*, 1987) and *C. thermohydrosulfuricum* E101-69 (Melasniemi, 1987), have been reported. *T. brockii* pullulanase has been cloned in *Escherichia coli* and *B. subtilis*. The cloned enzyme cleaved all of the α -1,6 glucosidic linkages (and none of the α -1,4 bonds) in pullulan; it hydrolysed mostly α -1,4 and very few of the α -1,6 linkages of starch (Coleman *et al.*, 1987). *Thermoanaerobium* Tok 6-B1 pullulanase was also able to hydrolyse α -1,4 glycosidic bonds in starch (Plant *et al.*, 1987). Melasniemi (1987) characterized α -amylase and pullulanase activities of a different *C. thermohydrosulfuricum* strain, and suggested that the two activities are properties of the same protein.

Preliminary data at hand from detailed active-site and substrate-specificity studies on the purified pullulanase from *C. thermohydrosulfuricum* indicate that the single enzyme can hydrolyse both α -1,6 and α -1,4 glycosidic bonds in an endo-cleaving fashion, and hence it is novel in activity compared with other purified pullulanases previously described.

Takasaki (1987) reported a novel pullulanase-amylase complex enzyme from *B. subtilis*, which hydrolyses pullulan to maltotriose as well as starch to maltose and maltotriose as the main products. It is not clear why a pullulanase cleaves α -1,4 linkages in starch. It is most likely a new type of enzymic mechanisms for a pullulanase-amylase activity.

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