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Purification and General Biochemical Properties of Thermostable Pullulanase from *Bacillus stearothermophilus* G-82

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ABSTRACT

Thermostable extracellular pullulanase, produced by *Bacillus stearothermophilus* G-82 was purified to homogeneity from supernatants of continuous culture by ultrafiltration, ammonium sulphate precipitation, chromatography on Sephadex G-100, and DEAE cellulose. A mol wt of 53,000 was determined by gel filtration and 56,000 by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The isoelectric point (pI) was 4.2. The pullulanase contained predominantly acidic amino acids. The enzyme was optimally active at a temperature of 60°C and pH 7.0. It preserved 100% of its activity after 10 min treatment at 60°C. The thermostability was considerably increased in the presence of pullulan. Ca^{2+} did not increase activity or thermostability. Enzyme activity was fully inhibited by *N*-bromosuccinimide and partially by phenylmethylsulfonyl fluoride. *Bacillus stearothermophilus* G-82 pullulanase was able to hydrolyze α_{1-6} as well as α_{1-4} glucosidic bonds in pullulan, amylopectin, amylose, glycogen, and dextrin. The enzyme showed highest affinity to pullulan ($Km = 0.14$).

Index Entries: Pullulanase; *Bacillus stearothermophilus;* purification; properties; thermostability.

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INTRODUCTION

Pullulanase (pullulan 6-glucanohydrolase, EC 3.2.1.41) hydrolyzes specifically α_{1-6} glucosidic linkages of pullulan, amylopectin, glycogen, and dextrin; and it is generally used for the enhancement of the starch saccharification process in industry as well as for structural studies of carbohydrates. A number of thermophilic aerobic and anaerobic bacteria that produce thermostable pullulanase were isolated during the last few years (*1*). There are few reports about thermostable pullulanase from thermophilic *Bacillus* (*2–4*). We have isolated from a hyperthermal spring in Bulgaria a thermophilic strain of *B. stearothermophilus* that produces thermostable pullulanase (*5*). This paper reports the purification procedure of the enzyme and its general physicochemical and catalytic properties that show evidence of its belonging to the new type of amylase-like pullulanase: amylopullulanase.

MATERIALS AND METHODS

Cultivation of Microorganisms

Bacillus stearothermophilus G-82 was cultivated in a liquid medium containing: 0.3% bactopeptone, 0.3% yeast extract, and 0.1% starch, pH 7.8. Continuous cultivation was carried out in a Bioflo Model C 30 fermentor (New Brunswick Co., Edison, NJ). The working vol employed was 0.350 L. A temperature of 55°C, air flow rate of 1.0 vvm, rotational speed of the impeller of 350 rpm, and dilution rate of 0.1 h^{-1} were maintained. The obtained culture liquid was centrifuged, and the supernatant was used for purification procedure.

Enzyme Purification

The supernatant was concentrated using a Millipore (Bedford, IN) ultrafiltration system (membrane with a pore size of 10,000). The concentrate was supplemented with ammonium sulphate to 60% final concentration. The precipitate was redissolved and dialyzed in 10 m*M* phosphate buffer and was applied (30 mg protein) to a 65×26 mm column LKB equilibrated with 10 m*M* phosphate buffer on Sephadex G-100. Flow rate was adjusted to 0.03 L/h. The active pullulanase peak fractions were applied (20 mg protein) directly to a DEAE cellulose column 35×16 mm. The column was washed with the same buffer and then eluted with a 160 mL linear NaCl (0–1*M*) gradient in the same buffer at a flow rate of 0.03 L/h. All procedures were carried out at room temperature. The chemicals used were of analytical grade.

Assay of Pullulanase Activity

Pullulanase activity was assayed by the method of Suzuki and Chishiro (2) at 60°C and pH 7.0. One unit of enzyme activity was defined as the amount of enzyme that is released by 1 μmol of reducing sugars per minute using glucose as a standard.

Determination of Protein Concentration

Protein concentration was estimated by the method of Lowry et al. (6) with bovine serum albumin (BSA) as a standard.

Polyacrylamide-Gel Electrophoresis

SDS-polyacrylamide gel electrophoresis of the proteins was carried out under denaturating conditions according to Laemmli (7). Protein samples (50 μg) were applied to a 10% porosity gel.

Isoelectric Focusing

Servalyt Precote gel (Serva) of pH range 3–6 was used for isoelectric focusing. This was performed on a Pharmacia 3000 apparatus. Gels were focused at a constant power of 3 W and 1700 V. The gel was stained with coomassie brilliant blue R-250.

Carbohydrate Analysis

Thin-layer chromatography of the reducing sugars, obtained after pullulanase action on certain oligosaccharides, was carried out on Silica gel-60 plates 20×20 (Merck, Darmstadt, Germany) with a solvent system of *n*-propanol/ethylacetate/deionized water (7/1/2, v/v). After the migration of the samples (10 μg), the plates were dried up and treated with staining solution containing ethanol/*p*-anisaldehyde/sulphuric acid/glacial acetic acid (89/5/5/1, v/v) and dried at 100°C for approx 10 min.

Quantitative analysis of sugars was done by Waters Liquid Chromatograph system equipped with a refractive index detector and Separone SGX RPS column (length 4×250 mm, ID 4 mm), particle size 7 μm (Tessek Ltd., Czechoslovakia). The mobile phase consisted of bidistilled water; the flow rate was 0.7 mL/min. Twenty microliters of the samples (10 mg/mL) were used for carbohydrate determination. D-glucose, maltose, and maltooligosaccharides from maltotriose to maltoheptaose (Boehringer) were used as standards in concentration from 1.25 to 1.65 mg/mL.

Amino Acid Analysis

Analysis of the amino acid composition was performed by Biotronic analyzer. The samples were hydrolyzed with 6*N* HCl for 24 h in a vacuum at 110°C. The hydrolysate was dried and redissolved in 1 mL citric buffer, pH 2.2.

Table 1
Purification of *Bacillus stearothermophilus* G-82 Pullulanase

Step	Total vol, mL	Total protein, mg	Total activity, U	Specific activity, U/mg	Yield, %	Purification factor
Cell-free supernatant	1200	1440.0	540.0	0.375	100.0	1.0
Ultrafiltration	130	1079.0	470.9	0.436	87.2	1.2
$(NH_4)_2SO_4$ precipitate	72	337.0	337.0	1.000	62.4	2.7
Sephadex G-100 eluate	250	92.5	309.4	3.345	57.2	8.9
DEAE cellulose eluate	202	24.2	197.6	8.165	36.6	21.8

RESULTS AND DISCUSSION

Purification

Pullulanase purification data are reported in Table 1. The purification factor was 21.8 and yield was 36.6%. The specific activity of the enzyme was 8.165 E/mg protein.

Molecular Properties of Purified Pullulanase

The mol wt of the purified pullulanase was estimated to be 53,000 by gel filtration on Sephadex G-100 (using a series of proteins of known mol wt as standards) and 56,000 by SDS-PAGE using Test Mixture 4 (Serva). In the literature, mol wt of bacterial pullulanases vary from 56,000 to 150,000 (*8*). The value for *B. stearothermophilus* G-82 pullulanase was similar to that of *Aerobacter aerogenes* (*9*) and significantly differs from thermophilic pullulanases, related to the high-mol-wt group.

Melasniemi (*10*) reported two forms of *Clostridium thermohydrosulfuricum* pullulanase with high mol wt of 370 and 330,000. The enzyme migrated as a single protein band on isoelectric focusing having an pI of 4.2 (Fig. 1), indicating that it is an acidic protein similar to those synthesized by *C. thermohydrosulfuricum* (*11*), *B. acidopullulyticus* (*8*), *B. stearothermophilus* KP 1064 (*12*), and *B. sp.* N 202-1 (*13*).

Amino Acid Analysis

Results of the amino acid analysis are presented in Table 2. The data show that the amino acid composition is similar to that of the pullulanases from other sources with a relatively high content of acidic amino acids.

Fig. 1. Isoelectric focusing of purified pullulanase. **1:** standard marker proteins (Serva, pI 9); **2:** purified pullulanase of *B. stearothermophilus* G-82.

Table 2
Amino Acid Composition
of *B. Stearothermophilus* G-82 Pullulanase

Amino acid	Residues
Aspartic acid	51
Glutamic acid	58
Serine	15
Glycine	33
Histidine	17
Arginine	10
Threonine	20
Alanine	40
Proline	23
Tyrosine	18
Valine	31
Methionine	8
Isoleucine	20
Leucine	30
Phenylalanine	21
Lysine	39

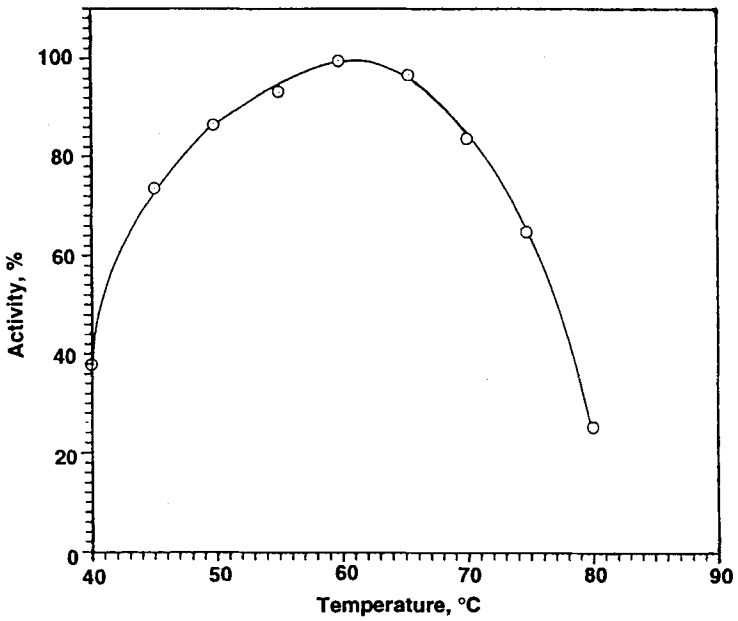

Fig. 2. Influence of temperature on pullulanase activity.

Physicochemical Properties of Purified Pullulanase

A number of investigations were performed concerning the temperature influence on enzyme activity and stability. The pullulanase was active in a large temperature (25–75°C) and pH (4.5–9.0) range. The temperature optimum for enzyme action (Fig. 2) was 60°C. The established value for pH optimum, 7.0 (Fig. 3), is different from most reported enzymes, these having pH optimum in the acidic area (5.0–6.0) (*1*). The enzyme from *B. stearothermophilus* G-82 preserves 100% of its activity after 10 min treatment at 60°C and 50% at 75°C (Fig. 4). In comparison with other pullulanases from thermophilic *Bacillus,* only the pullulanase from *B. stearothermophilus* KP 1064 (*12*) shows higher thermostability: It preserves 100% of its activity after 10 min treatment at 65°C.

The influence of substrate on the enzyme thermostability is shown in Fig. 5. Similar to the pullulanase from *C. thermohydrosulfuricum* (*14*), enzyme stability was significantly enhanced by the presence of pullulan. Investigations of pH influence on thermostability showed that it did not change in pH area from 6 to 8.

Influence of Certain Reagents on Pullulanase Activity

The data shown in Table 3 illustrate that Cu^{2+}, Fe^{2+}, Zn^{2+}, and Mn^{2+} are strong inhibitors. The presence of Na^+ and K^+ did not change enzyme

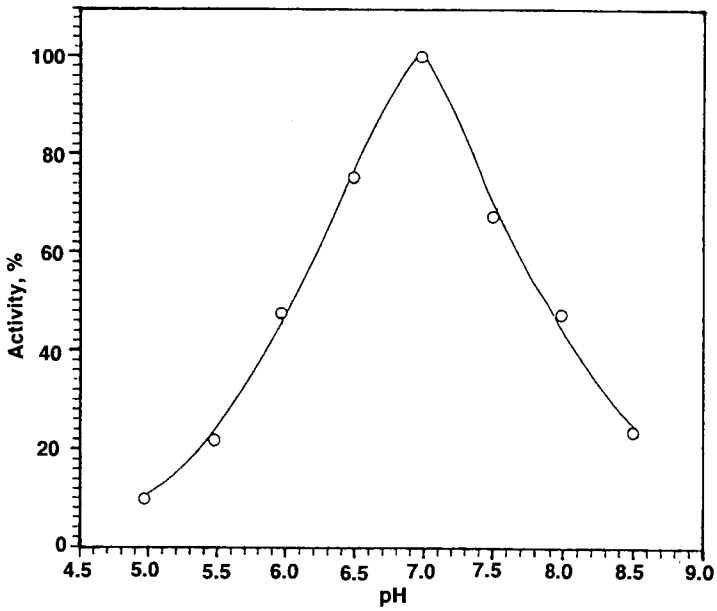

Fig. 3. Influence of pH on pullulanase activity.

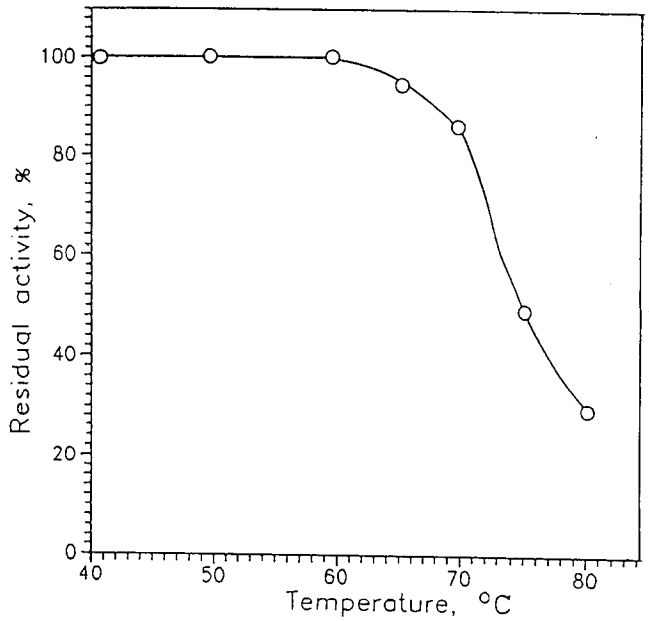

Fig. 4. Stability of pullulanase at different temperatures for 10 min at pH 7.0.

activity. Unlike the pullulanase from other thermophiles (*3,13,15*), the presence of Ca^{2+} did not increase activity as well as thermostability. According to the obtained results concerning the absence of stimulating effect of studied metal ions on the enzyme activity, the examined metal inhibitors did not change it. The enzyme was partially inhibited by PMSF, indicating the presence of serine in active center. Full inhibition of enzyme activity

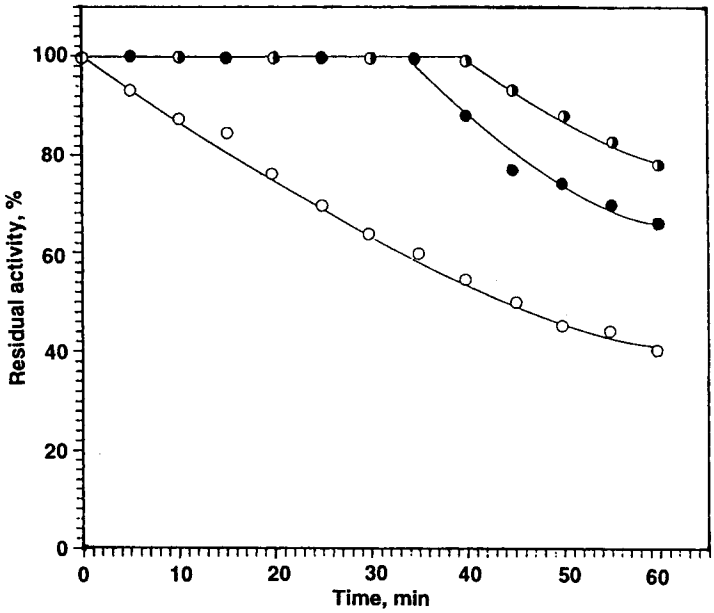

Fig. 5. Effect of pullulan on thermal stability of *B. stearothermophilus* G-82 pullulanase at 70°C. Pullulan percentage indicated are 0 (○), 1 (●), and 2 (◑).

was observed by NBS, and additional investigations are necessary to demonstrate the presence of tryptophan in the active center.

Catalytic Properties of Purified Pullulanase

The purified enzyme hydrolyzed the most of the investigated substrates: pullulan, glycogen, amylopectin, amylose, dextrin, maltotetraose, maltopentaose, maltohexaose, and maltoheptaose. Table 4 shows that glycogen and pullulan were almost fully hydrolyzed, whereas amylopectin and dextrin only partially and amylose very weakly. High-pressure liquid chromatography (HPLC) analysis of products resulted in pullulan hydrolysis, showing that the enzyme mainly attacks. $\alpha_{1\text{-}6}$ glucosidic bonds releasing maltotriose as the dominant product. However, a small amount of glucose, maltose, and maltopentaose were yielded, too, demonstrating enzyme action on $\alpha_{1\text{-}4}$ bonds in this substrate as well. The pullulanase ability to hydrolyze $\alpha_{1\text{-}4}$ bonds is confirmed by enzyme action on amylose, maltotetraose, maltopentaose, maltohexaose, and maltoheptaose.

This substrate specificity of *B. stearothermophilus* G-82 pullulanase permits enlisting it in a new class of pullulanases, tentatively named amylopullulanases by Saha and Zeikus (*1*) and pullulanases type II by

Table 3
Effect of Various Reagents
on Pullulanase Activity

Reagent	Relative activity, %
Metal ions, 2 m*M*	
None	100.0
NaCl	100.0
KI	100.0
$CaCl_2$	76.7
$MgSO_4$	74.8
$CoCl_2$	52.4
$MnSO_4$	38.8
$ZnSO_4$	21.1
$FeSO_4$	4.5
$CuSO_4$	0.0
Other reagents, 5 m*M*	
EDTA	100.0
Urea	100.0
Sodium laurylsulfate	100.0
Monoiodoacetate	100.0
p-Chloromercuribenzoate	92.0
o-Phenantroline	88.8
Phenylmethylsulfonyl fluoride	51.3
N-Bromosuccinimide	0.0

To investigate the influence of certain reagents on *B. stearothermophilus* G-82, pullulanase was dialyzed against 2 m*M* EDTA for 20 h. The reagents were added to the reaction mixtures in corresponding concentration, and enzyme activities were assayed.

Table 4
Products Derived from Varied Substrates by *B. stearothermophilus* G-82 Pullulanase

	Hydrolytic products, μg/mL					
Substrate	Glucose	Maltose	Maltotriose	Maltotetraose	Maltopentaose	Hydrolysis, %
Pullulan	117.1	489.3	7260.0	0.0	183.8	80.5
Amylopectin	844.5	554.0	152.2	traces	0.0	15.5
Amylose	198.3	103.7	40.2	0.0	0.0	3.4
Glycogen	497.4	4707.8	2885.1	traces	0.0	80.9
Dextrin	143.0	753.1	317.4	traces	0.0	12.1

Hydrolysis was performed at 60°C, pH 7.0; reaction time was 40 min.

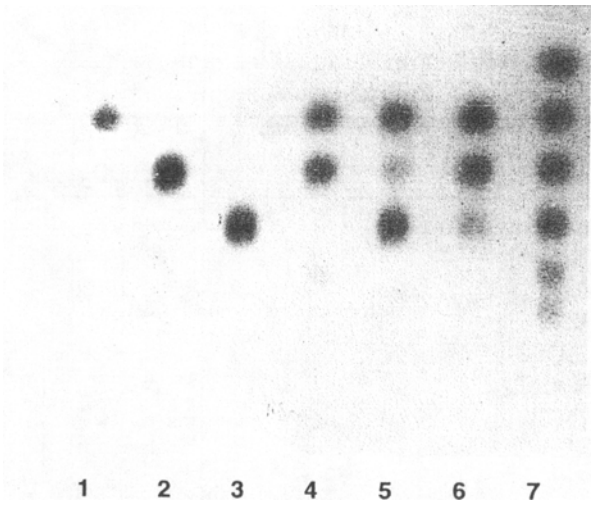

Fig. 6. Thin-layer chromatogram of products derived from pullulanase action on certain oligosaccharides. 1, maltose; 2, maltotriose; 3, maltotetraose; 4, maltopentaose; 5, maltohexaose; 6, maltoheptaose; and 7, standards G_1–G_7.

Spreinat and Antranikian (*16*). Dual specificity toward α_{1-6} and α_{1-4} glucosidic bonds in certain polysaccharides has been observed for pullulanases isolated by *C. thermosulfurogenes* EM1 (*16*), *B. sp.* 3183 (*4*), *Thermoanaerobium tok* 6-B1 (*17*), and *Thermoactinomyces vulgaris* (*18*).

Abdullah and French (*19*) have reported the participation of the pullulanase in the condensation process of maltose and maltooligosaccharides, but our efforts to detect condensation products were unsuccessful. Maltose and maltotriose were not used as substrates. Maltotetraose was the smallest substrate for this enzyme. Hydrolysis products yielded from maltotetraose, maltopentaose, maltohexaose, and maltoheptaose can be seen in Fig. 6. Maltooligosaccharides were hydrolyzed very slowly; the spots were visible after 18 h at 60°C. Substrate specificity of *B. stearothermophilus* G-82 pullulanase resembles that of *Thermoanaerobium tok* 6-B1 (*20*). The enzyme was hydrolyzed in highest degree glycogen and pullulan. Examination of the products obtained after different terms of enzyme action on pullulan demonstrated that enzyme cleaves this polysaccharide at terminal residues, which means the enzyme action followed a random exomechanism. A similar mechanism was observed with pullulanase from *B. sp.* (*3*).

Bacillus stearothermophilus G-82 thermostable pullulanase showed simple Michaelis-Menten kinetics with pullulan, amylopectin, glycogen, and dextrin as substrate. The Michaelis-Menten constants (*Km*, mg/mL) and the maximum initial rates (V, g/min per mg protein) of hydrolysis were respectively 0.14 and 1.52 for pullulan, 0.22 and 0.312 for dextrin, 8.3 and 0.455 for amylopectin, and 16.6 and 0.269 for glycogen. The enzyme showed the highest affinity and maximum value of reaction rate for pullulan.

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