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A Specific Pullulanase for α-1,6-Glucosidic Linkage of Glucan from *Thermus caldophilus*

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Abstract A thermostable pullulanase has been isolated and purified from *Thermus caldophilus* GK-24 to a homogeneity by gel-filtration and ion-exchange chromatography. The specific activity of the purified enzyme was 431-fold increase from the crude culture broth with a recovery of 11.4%. The purified enzyme showed M_r of 65 kDa on denaturated and natural conditions. The pI of the enzyme was 6.1 and Schiff staining was negative, suggesting that the enzyme is not a glycoprotein. The enzyme was most active at pH 5.5. The activity was maximal at 75℃ and stable up to 95℃ for 30 min at pH 5.5. The enzyme was stable to incubation from pH 3.5 to pH 8.0 at 4℃ for 24 hr. The presence of pullulan protected the enzyme from heat inactivation, the extent depending upon the substrate concentration. The activity of the enzyme was stimulated by Mn^{2+} ion, Ni^{2+}, Ca^{2+}, Co^{2+} ions. The enzyme hydrolyzed the α-1,6-linkages of amylopectin, glycogens, α, β-limited dextrin, and pullulan. The enzyme caused the complete hydrolysis of pullulan to maltotriose and the activity was inhibited by α, β, or γ-cyclodextrins. The NH_2-terminal amino acid sequence [(Ala-Pro-Gln-(Asp or Tyr)-Asn-Leu-Leu-Xaa-ILe-Gly-Ala(Ser)] was compared with known sequences of various sources and that was different from those of bacterial and plant enzymes, suggesting that the enzymes are structurally different.

Key words: Pullulanase, α-1,6-glucosidic linkage, (Thermus caldophilus GK-24)

Introduction

Pullulanase (pullulan 6-glucanohydrolase, EC 3.2.1.41) has an activity to hydrolyze the α-1,6-glucosidic linkages in pullulan and produces maltotriose as the end product [1]. The enzyme can be used together with several amlyases to improve the efficiency of starch saccharification for the production of glucose, maltose and high glucose syrups. This enzyme was first found in *Klebsiella pneumoniae* by Bender and Wallenfels [5]. In most cases, pullulanases can also split the α-1,6-glucosidic linkages of branched polysaccharides, such as amylopectin or glycogen. Isoamylase also hydrolyzes the α-1,6-glucosidic inter chain linkages of certain branched α-D-glucans but it cannot hydrolyze pullulan.

Since the discovery of *K. pneumoniae* pullulanase by Bender and Wallenfels [5], a number of microbial pullulanases have been purified from and characterized from mesophilic and thermophilic bacteria by many investigators [3,4,10,13-15,27,29-31,34,36,38,39]. However, little is known about the biochemical properties and substrate specificities of such enzymes from extremely thermophilic bacteria except for those from *Thermus aquaticus* [34] and *Thermus* sp. AMD33 [29]. Most enzymes from thermophilic bacteria are Type II pullulanases, which hydrolyze the α-1,6-glucosidic linkages of pullulan, as well as the α-1,4-linkages of other polysaccharides. Pullulanases of Type I, which exclusively hydrolyze the α-1,6-linkages of pullulan, are produced by *K. pneumoniae* [5], *Bacteroides thetaiotaomicron* 95-1 [38], alkaliphilic *Bacillus* sp. KSM-1876 [3], *Thermus aquaticus* YT-1 [34], alkaliphilic *Bacillus* sp. S-1 [13,14], alkaliphilic *Micrococcus* sp. Y-1 [14], and *Bacillus acidopullulyticus* [10]. To our knowledge, there are few reports on microbial thermostable pullulanases of Type I, except case of *T. aquaticus* YT-1 [34].

The genus *Thermus* can routinely grow at temperatures of 70 - 75℃, and some species can grow at 85℃ [7]. Various

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highly thermostable enzymes, which have been shown to be useful in high-temperature systems, have been isolated from thermophilic bacteria [33,34,41]. In particular, carbohydrates-related enzymes from extreme thermophiles have drawn interest because of their application to biomass bioconversions. However, there are a few report on formation and biochemical characteristics of thermophilic enzymes used in carbohydrate degradation from *Thermus* yet. Moreover, pullulanase from *Thermus* species has not been fully studied yet. Comparison of the properties and primary structures of the thermostable pullulanase with those of the enzyme from other sources will give insight into structure and function of this protein. The organisms produce pullulanases in intracellular space, in a similar manner to what is observed in other bacteria [17]. The evolutionary relationships of *Thermus* to prokaryotes and plant promptes a comparison of *Thermus* pullulanase properties to those of bacterial sources.

In this paper, we report a novel pullulanase from the thermophilic *T. caldophilus* GK-24 and compared molecular weight, NH_2-terminal amino acid sequence and heat stability of enzyme from *T. caldophilus* GK-24 to the enzymes from other bacteria.

Materials And Methods

Materials

Pullulan (M_r, 65,000), panose, and isopanose were from Hayashibara Co. Ltd. (Okayama, Japan). Maltooligosaccharides were purchased from Nihon Shokuhin Kako Co. (Tokyo, Japan), and α-, β-, and γ-cyclodextrins were purchased from Seikagaku Yakuhinn Co. (Osaka, Japan). Shimpak Ultron 300-C4 column (pore size of 5 μm, 4.6 mm × 150 mm) for reverse-phase high pressure liquid chromatography (HPLC) was purchased from Shinwa Co. Ltd. (Tokyo, Japan). All the other reagents were of the highest grade available.

Cultivation of thermophilic *T. caldophilus* GK24 and preparation of crude enzyme

T. caldophilus GK-24 cells (kindly supplied by professor T. Ohta, Faculty of Agriculture, The University of Tokyo, Tokyo, Japan) [33,41] was grown in a medium containing 0.8% polypeptone, 0.4% yeast extract, and basal salts with 1% soluble starch as a carbon source [32]. The pH of the medium was adjusted to 7.2. The cultivation was carried out at 75℃ for 2 days in a 5-liter stirred-tank fermentor (Korea Fermentor Co. Ltd, Inchon, Korea) [32]. The working volume was 3 liters and a speed of the impeller of 350 rpm was maintained. Previous seed culture media was made of 0.4% polypeptone, 0.2% yeast extract, and basal salts. The culture obtained was centrifuged, and the cells were used for purification procedure.

Enzyme assay and analytical methods.

Pullulanase and amylase activities were assayed by measuring the reducing sugar released from pullulan and soluble starch, respectively [16]. The reaction mixture (1.0 ml) containing pullulan or soluble starch (1%, w/v), 50 mM Tris-HCl (pH 7.0), and enzyme (5-20 μg) was incubated at 73℃ for 30 min. The reducing sugar was measured by the dinitrosalicylic acid (DNS) procedure [6,28]. One unit (U) of pullulanase or amylase activity was defined as the amount of enzyme that produced 1 μmole of reducing sugar as glucose per min under the conditions described above. Protein were measured by the method of Lowry *et al.* [23] with bovine serum albumin as the standard. The absorbance at 280 nm was used to monitor protein in column eluates.

Purification of the thermostable pullulanase from *T. caldophilus* GK24

Unless otherwise stated, all steps were performed at 4℃. The cells were grown at 75℃ in the medium as described (32) and harvested at the logarithmic phase (*ca.* 2×10^9 cells/ml). The frozen cells (50 g) of *T. caldophilus* GK24 were thawed and resuspended (about 5 ml buffer/g cells) into 50 mM Tris-Cl buffer, pH 8.0 (buffer A), containing 5 mM $MgCl_2$. The cells were disrupted by sonic oscillation in a Ultrasonic homogenizer (model Cole-parmer 4710 series, USA) and followed by centrifugation at 12,000 × g for 20 min. The supernatants are referred to as the crude extract. The crude enzyme was fractionated with 20 to 70% saturation of ammonium sulfate. The precipitates were dissolved in 120 ml of buffer A and dialyzed against the same buffer overnight. The dialysate was divided into portions, and each portion was chromatographed sequentially on a Sephacryl-S200 column (2.5 × 120 cm) that had been equilibrated with Buffer A. Fractions of 8 ml each were collected and enzyme activity was detected between 17-24 (Fig. 1A). The active enzyme fractions (tubes nos 44-58) were combined and concentrated by ultrafiltration (Amicon PM30; Amicon Co. Ltd., Danvers, MA, USA) and dialyzed against the same buffer overnight.

The concentrated enzyme solution (24 ml) was further dialyzed against buffer A and applied on a DEAE-Sephacel column (1.5 × 20 cm) that had been preequilibrated with the same buffer. After washing with 1.5 liters of the same buffer A, the enzyme was eluted with a linear NaCl gradient of 0.0 to 0.4 M in 300 ml of buffer A. Enzyme activity was detected in the region of 0.17 M- 0.25 M NaCl concentration and the active fractions (5 ml/fraction; Nos 21 to 30) were pooled (50 ml) and then concentrated in the manner indicated above (Fig. 1B).

Further purification of the pullulanase enzyme protein was achieved by high performance ion-exchange chromatography (HPLC) on a DEAE Toyopearl 5PW column (10 × 100 mm; Tosoh Co., Tokyo, Japan). The concentrated sample (5 ml) was applied to the column preequilibrated

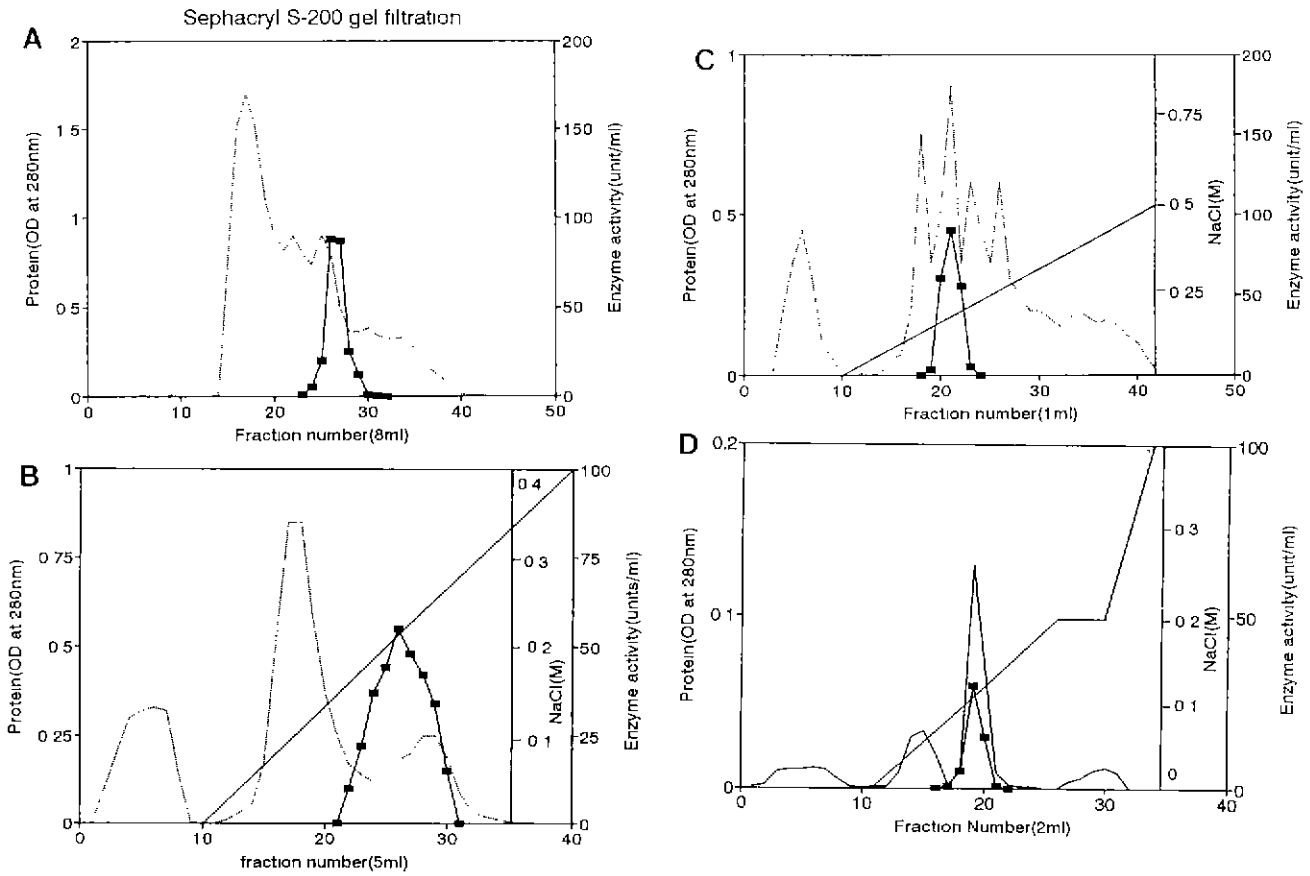

Fig. 1. Elution profile of the pullulanase activity after column chromatography.
Ezyme activity of the eluted fractions (■) was assayed, and protein absorbance was monitored at 280nm (----) with NaCl gradient (——).
A. Elution profile of gel filtration on a Sephacryl S200.
B. Elution profile of the pullulanase activity after chromatography on a DEAE-Sephacel.
C. Elution profile of the pullulanase activity after HPLC on a DEAE Toyopearl 5PW.
D. Elution profile of the pullulanase activity after FPLC on a Mono-Q column.

with buffer A. The column was washed with 5 column volumes of the same buffer and the enzyme activity was eluted with a linear NaCl gradient (100 ml, 0.0 - 0.5 M) in buffer A at a flow rate of 0.5 ml/min. Enzyme activity was detected in the region of 0.17 - 0.19 M NaCl concentration and the active fractions (tubes nos 19-23) of 1 ml were collected and those containing activity were pooled (Fig. 1C) and concentrated by ultrafiltration (Amicon PM30), and the concentrate was dialyzed against the same buffer. The collected fractions were reapplied to a FPLC Mono-Q HR 10/10 column (10 × 100 mm; Pharmacia LKB, Upssala, Sweden) and the pullulanase was recovered as a single peak which eluted at 0.11 - 0.13 M NaCl, where the enzyme activity was found to coincide with the protein peak (Fig. 1D). At this stage of purification the pullulanase was found to be pure from other contaminating proteins and the putified pullulanase was used for the next characterization. For analysis of the N-terminal amino acid sequence, the purified pullulanase-E was further ultra-purified by chromatography on a column of Ultron 300-C4, using an HPLC System Gold (Beckman, San Ramon, CA, USA).

Electrophoresis, measurement of molecular weight, activity staining, and Western blotting.

Native polyacrylamide gel electrophoresis (PAGE) was done using 7.5% (w/v) polyacrylamide gels, as described by Davis [9]. For Activity staining of pullulanase after PAGE, gels were soaked in a solution of 1% (w/v) starch in 50 mM Tris-HCl buffer (pH 7.0) at 50℃ for 30 min, rinsed with water, then stained for amylase or pullulanase activity by flooding with an iodine solution [12]. The bands of amylase activity were seen as white zones and those of pullulanase activity as blue zones, on the weak blue background.

Discontinuous sodium dodecyl sulfate (SDS)-PAGE was done essentially by the method of Laemmli [20]. The bands of protein were stained with Coomassie Blue R250 dye and destained with a solution of 5% methanol- 7.5% acetic acid

(v/v). Molecular mass markers (Bio-Rad) used were myosin (200 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (BSA, 68 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), and lysozyme (18.4 kDa). The molecular mass of pullulanase was also measured by calibration of a Sepharose 4B column (1.6 cm × 76 cm) with standard proteins which included ovalbumin, BSA, cytochrome C (12.4 kDa), catalase (232 kDa), and thyroglobulin (669 kDa).

Isoelectric point of the purified enzyme was determined by isoelectric focusing (IEF) gel electrophoresis using precast PhastGels with a pH gradient from 3.5 to 9.0 (Pharmacia LKB Biotechnology Inc.). The protein standards for IEF were trypsinogen (*p*I = 9.0), lentil lectin-basic band (*p*I = 8.65), lentil lectin-middle band (*p*I = 8.45), lentil lectin-acidic band (*p*I = 8.15), myoglobin- basic band (*p*I = 7.35), myoglobin-acidic band (*p*I = 6.85), human carbonic anhydrase B (*p*I = 6.56), bovine carbonic anhydrase B (*p*I = 5.86), β-lactoglobulin A (*p*I = 5.20), and soybean trypsin inhibitor (*p*I = 4.60).

Effects of pH and temperature on the pullulanase activity

For examination of effect of temperature on the activity and stability, the enzyme activities were measured as in the pullulanase assay, except that 0.087 U of enzyme was incubated with 2% pullulan at various temperatures (20 to 100℃). The maximum in each temperature-activty profile was taken as 100%. For thermal stability, the enzyme solutions were kept at various temperatures for 60 min in Tris-HCl (pH 6.0), and then the residual activity was measured.

For effect of pH on the activity and stability, the enzyme activities of the crude enzyme and purified PUL were measured as in the pullulanase assay, except that 0.023 U of enzyme was added and the pH was changed using 50 μl of one of the buffers at various pHs. Buffers used were 30 mM sodium acetate buffer (pH 3.0 - 6.0), 30 mM sodium phosphate buffer (pH 6.0 - 8.0), 30 mM Tris-HCl buffer (pH 8.0-10.0), and 30 mM glycine buffer (pH 9.0 - 12.0). The activity found at pH 9.0 was taken as 100%. For pH stability of the enzymes, enzyme solutions kept in 30 mM various buffers of KCl- HCl (pH 2.0 - 3.0) and the buffer systemdescribed above for 24 hr at 4℃, and assayed for remaining activity after twofold dilution with 1.0 M Tris-HCl (pH 6.0). The activity of untreated enzyme was expressed as 100%.

Sequencing of N-terminal amino acids

After ultra-purification of the final sample by reverse phase chromatography using an Ultron 300-C4 column, the N-terminal part of the protein was sequenced using a 470A gas phase protein sequencer equipped with a 120A on-line phenylthiohydantoin analyzer (Applied Biosystems, Warrington, Cheshire, U.K.)

Other analytical methods

Schiff staining for glycosylation of protein was done [21]. Oligosaccharides produced by enzymatic action were examined by thin layer chromatography (TLC), as described by Kim *et al.* [22].

Results and Discussion

Purification of pullulanase

T. caldophilus GK24 pullulanase was purified to homogeneity by sequencial chromatographic methods including Sephacryl S-200, DEAE-sephacel and Mono Q chromatography. pullulanase was purified 431-fold to homogeneity with a specific activity of 86.2 U/mg protein and 13.2% recovery (Table 1).

Of interest is that Affi-gel Blue, usually utilized for purification of proteins related diphosphate, was effective in purification of the enzyme. The specific activity (86.2 U/mg protein) of the purified enzyme is approximately similar to the values reported for the highly purified pullulanase from other bacteria. Pullulanases of the active fractions in gel filtration column were further purified by DEAE-Sephacel, DEAE Toyoperal 5PW of HPLC and Mono Q column of FPLC. The single peak of activity eluted was used as purified pullulanase. The purified enzyme migrated as a single protein band on SDS-PAGE (Fig. 2). To obtain the ultra-pure pullulanase for N-terminal amino acid sequencing, further purification was done by reverse phase HPLC using an Ultron 300-C4 column (data not shown).

Table 1. Purification summary of pullulanase

Purification step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yields (%)	fold
Crude enzyme	3250	783.4	0.2	100	1
$(NH_4)_2SO_4$ precipitate (30-70%)	3075	803.3	0.3	102.5	1.5
Sephacryl-S200	560	562.5	1.0	71.8	5.0
DEAE-Sephacel	187	531.2	2.8	67.8	14.0
DEAE-Toyopeal 5PW	40	482.7	12.1	61.6	60.5
Mono-Q	1.2	103.4	86.2	13.2	431.0

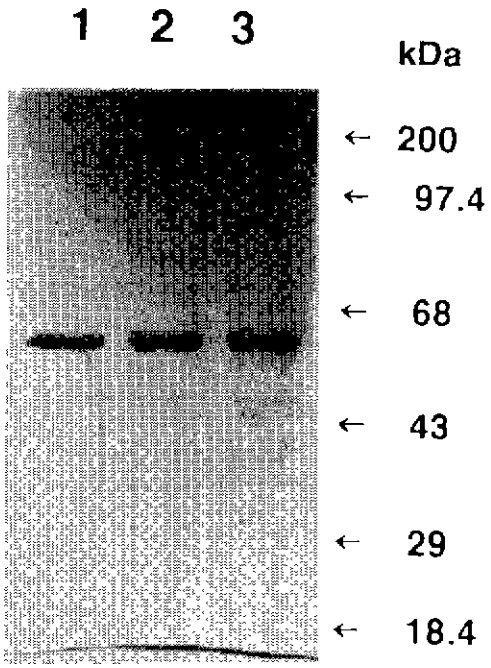

Fig. 2. SDS-PAGE of the purified pullulanase.
Approximately 10 μg of protein was put on each lane and 2 mA per lane was applied for 120 min. Arrowheads denote the position of pullulanase. Positions of standard proteins are indicated by (small) arrowheads. Lane 1, purified pullulanase (3 μg); lane 2, purified pullulanase (4 μg); lane 3, purified pullulanase (5 μg). Standard molecular weight markers: myosin (200 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), and β-lactoglobulin (18.4 kDa).

The molecular mass of the enzyme

The molecular mass of the pullulanase was 65 kDa. A calibrated column of Sepharose 4B gave a molecular mass of 63 kDa. The value was similar to those of pullulanases reported to date. The molecular masses of pullulanases reported thus far are range between 70 and 140 kDa: alkalophilic *Bacillus* sp. KSM-1876 (120 kDa) [3], *K. pneumoniae* (80 kDa) [5], *T. aquaticus* YT1 (83 kDa) [34], alkaliphilic *Bacillus* sp. S-1 (140 kDa) [13], *B. acidopullulyticus* (115 and 116 kDa) [10], *C. thermosulfurogenes* EM1 (102 kDa) [39], alkaliphilic *Bacillus* No. 202-1 (92 kDa) [30], *B. flavocaldarius* KP1228 (55 kDa) [40], alkalopsychrotrophic *Micrococcus* sp. (120 kDa) [18], thermophilic *Bacillus* sp. (100 kDa) [4], *Bacteroides thetaiotaomicron* 95-1 (72 kDa) [38], *B. stearothermophilus* (70 kDa) [19], *T. aquaticus* YT-1 (70 kDa) [34], *Thermus* sp. AMD33 [29], and *Thermoactinomycetes thalpophilus* (79 kDa) [31]. Some pullulanase-like enzymes, such as *B. circulans* F-2 amylase-pullulanse enzyme (220 kDa) [15], *B. subtilis* pullulanase-amylase complex (450 kDa) [42], *Thermoanaerobacter* B6A amylopullulanase (450 kDa) [36], and *C. thermohydrosulfuricum* α-amylase-pullulanase (165 kDa) [27], have been regarded as enzymes of high molecular masses. Furthermore, these enzymes are not true pullulanases in that they all have dual activities of amylase and pullulanase. Therefore, pullulanase from *T. caldophilis* GK24 can be identified as a real pullulanase or pullulanase Type I which has the smallest molecular mass.

Isoelectric point, carbohydrate content, and N-terminal amino acid sequence

The isoelectric point of pullulanase was estimated to be 6.1 by isoelectric focussing using a Pharmacia LKB IEF system (data not shown). The pullulanase, when resolved by SDS-PAGE, was negative for staining with the Schiff reagent, indicating that the enzyme is not glycosylated. The ultra-purified sample was treated by Edman degradation.

The N-terminal amino acid sequence of the purified native pullulanase is Ala-Pro-Gln-(Asp or Tyr)-Asn-Ser-Leu-Xaa-ILe-Gly-Ala. This sequence shows some similarity to pullulanases from *Thermoanaerobacterium saccharolyticum* B6A-R1 [35], *T. thermosulfurigens* EM1 [24], *Thermoanaerobacter ethanolicus* 39E [26], *K. pneumoniae* ATCC1505 [11] and *B. flavocaldarius* [40]. As shown in Fig. 3, Ala-Pro (positions 1-2), Asn (5), and ILe-Gly (9-10) sequences coincide with sequences from the similar, noted

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a   1AlaPro GlnAspAsnSer                    LeuXaaIleGlyAla(Ser)11
b   1AlaAspAsnAlaSerValValAlaAsnIleValGlyAspPheGlnAspGlnLeuGlyAspSerAsnTrpAsn24
c   1AlaAspAsnAlaSerValValAlaAsnIleValGlyGluPheGlnAspGlnLeuGlyAspSerAsnTrp23
d   1GluThrAspThrAlaProAlaIleAlaAsnValValGlyAspPheGlnSerLysIleGlyAspSerAspTrp24
e   1GluThrAspThrAlaProAlaIleAlaAsnValValGlyAsnPheGlnSerLysLeuGlyAspSerAspTrp24
f   1AlaTrpTyrGluGlyAla PhePheTyrGlnIlePheProAspTyrPhePheTyrAlaGlySerAsnTrpAsnIle25
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Fig. 3. N-terminal amino acid sequence comparison of pullulanases from various procaryotic sources. Sequences from (a) *T. caldophilus* GK24 (this study), (b) *T. saccharolyticum* B6A-R1 [35], (c) *T. thermosulfurigens* EM1 [24], (d) *Thermoanaerobacter ethanolicus* 39E [26], (e) *K. pneumoniae* ATCC1505 [11] and (f) *B. flavocaldarius* [40] arew aligned. Identical amino acid sequences between pullulanase and other pullulanases are indicated by dark-shadow boxes.

pullulanases.

Effects of temperature and pH on the activity and stability

The optimum temperature for the enzyme reaction was around 75℃ at pH 6.0 (Fig. 4A). The purified pullulanase was stable up to 90℃, after incubation for 60 min at pH 5.5, and the activity was lost completely on heating at 95℃ (data not shown). However, crude enzyme was stable up to 98℃. The enzyme was stabilized by the addition of the substrate, pullulan. As the temperature for optimal activity rose with increasing substrate added, the thermostability of this enzyme seems more enhanced at higher substrate levels. The enzyme was active at 93℃ in 3.0% (w/v) pullulan. Pullulan protected the enzyme from heat inactivation, and this effect was dependent on the added concentrations. In the absence of pullulan, pullulanase rapidly lost its pullulanase activity at temperatures above 85℃, and the half-life

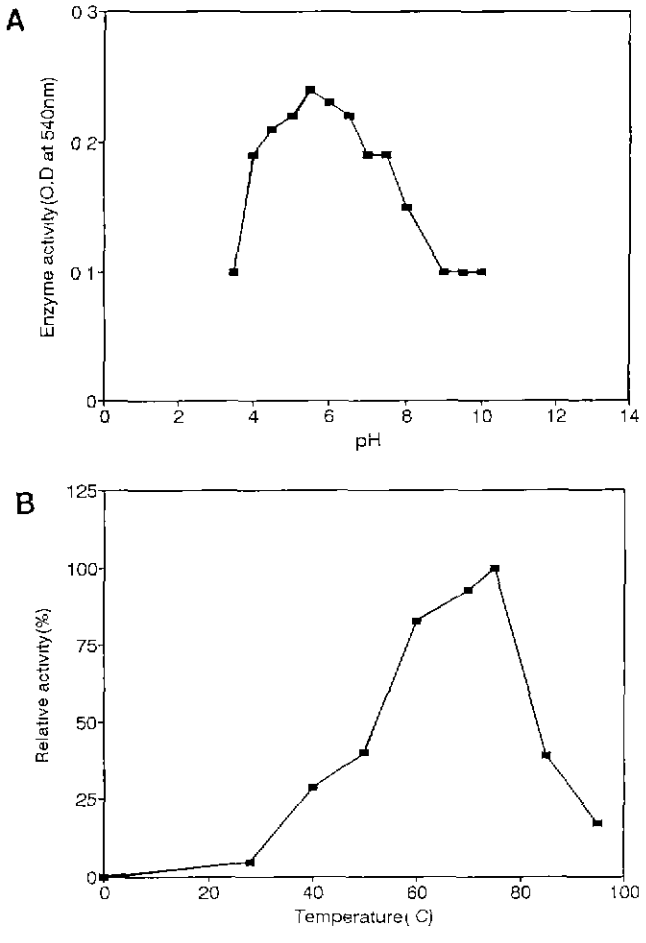

Fig. 4. Effects of temperature and pH on the purified pullulanase. Experimental details are described in Materials and Methods. Symbols: ■, activity of purified pullulanase. The activity of untreated enzyme was expressed as 100%. A) Temperature effect. B) pH effect

of the enzyme was 72 hr when incubated at pH 6.0 and at 78℃. In the presence of 5% (w/v) starch, pullulanase activity was completely stable at 80℃, and 45% of the original activity remained after heating at 80℃ for 30 min. This property and stability will give it potential as a debranching enzyme in the starch-processing industry [2].

Pullulanase was most active at around pH 5.5 (Fig. 4B). This optimum pH is in good qgreement with those of other pullulanases from *K. pneumoniae* (pH 6.5) [5] and *B. acidpullulyticus* (pH 5.0) [10], etc, except for enzymes from alkaliphilic bacteria [3,13,14,30]. pullulanase was stable from pH 4.0 to 8.0 after incubation at 4℃ for 24 hr (data not shown). Thus, the pullulanase from thermophilic *T. caldophilus* GK24 is mesophilic.

Effects of chemical reagents.

Divalent cations, such as Zn^{2+}, Cu^{2+}, and Fe^{2+} ions, inhibited the enzyme activity (Table 2). Ni^{2+}, Co^{2+}, K^{+}, and Na^{+} ions, each at 1 mM, were neither inhibitory nor stimulatory. Ca^{2+}, Mg^{2+}, and Mn^{2+} ions stimulated the pullulanase activity and EDTA was not inhibitory. The purified pullulanase was dialyzed against H_2O or EDTA for 24 hr. The effects of Mn^{2+} ions and EDTA on the pullulanase activity were then examined 9data not shown). Dialysis against EDTA did not inhibit the activity, when compared with the control (dialyzed against H_2O). EDTA and EGTA, each at 15 mM, were also not inhibitory under our assay conditions, suggesting that these chemicals did not chelate a possible divalent cation(s) required for the pullulanase activity of pullulanase. Such insensitivity to EDTA and EGTA is also observed with *B. flavocaldarius* and alkaliphilic *Bacillus* sp. S-1 pullulanases [13,40]. However, dialysis against EDTA stimulated of the enzymatic activity of pullulanase in the presence of Mn^{2+} or Co^{2+}, suggesting that Mn^{2+} and Co^{2+} ions are required for full activity of the pullulanase. Similarly, in most cases of the reported pullulanases, Ca^{2+} ions are required for their full activity [4,5,18,31]. It was also known that Ca^{2+} ions strongly enhance both the activity and stability of pullulanases from *T. aquaticus* [34] and *T. thalpophilus* [4] at a high temperature. Ca^{2+} ions are without effect on the activities of pullulanases from thermophilic *T. caldophilus* GK24 (Table 2) and from *B. flavocaldarius* [40]. The activity of pullulanase was inhibited by α, β, and γ-cyclodextrins, indicating that α-, β- and γ-cyclodextrins, known as possible competitive inhibitors of pullulanase [25], had the same effect. These results show that pullulanase is similar to other bacterial pullulanases.

Substrate specificity and action pattern of pullulanase enzyme on various polysaccharides

Of the carbohydrate substrates tested, the enzyme hydrolyzed pullulan efficiently. α,β-Limited dextrin, β-limited dextrin, soluble starch, amylopectin, potato starch, and glycogen were also hydrolyzed to some extent. Dextran and

Table 2. Comparison of enzyme effectors on purified pullulanase Activity

Reagent	Concentration (mM)	Relative activity(%)	Reagent	Concentration (mM)	Relative activity(%)
No addition	-	100	$CuCl_2$	1	31
KCl	1	107	$FeSO_4$	1	72
NaCl	1	110	$CuSO_4$	1	41
$CaCl_2$	1	107	$FeCl_3$	1	35
$CoCl_2$	1	102	$MnCl_2$	1	134
$NiCl_2$	1	103	EDTA	1	105
$NiSO_4$	1	105	α-CD	1	12
$MgCl_2$	1	132	β-CD	1	15
$ZnCl_2$	1	56	γ-CD	1	8

α-, β- or γ -cyclodextrins were practically unaffected by the enzyme (Table 3). Maltotriose was detectable as the sole product of hydrolysis of pullulan, indicating that the enzyme had an absolute specificity directed at the α(1→6) glucosidic linkages of pullulan (Fig. 5). Low levels of glucose, maltose, and maltotriose were also detectable in the hydrolysis products of amylopectin and soluble starch. This observation is consistent with activity being directed at the α(1→6) branch points of amylopectin and starch. Maltose, maltotriose, maltotetraose, and dextran were not hydrolyzed by pullulanase like *B. acidpullulanaseluliticus* [10], *K. pneumoniae* [5], *T. aquaticus* YT-1 [34], and *Micrococcus* sp. [30]. In addition to pullulanase (pullulanase type I), pullulan-hydrolyzing activities have been demonstrated in a glucoamylase that hydrolyze pullulan at the nonreducing end to produce glucose; an isopullulanase that cleaves the α -1,4-glucosidic linkages of pullulan to generate isopanose [37]; and a neopullulanase [19] and a pullulan-hydrolyzing α-amylase [8], both of which act on the α-1,4-glucosidic linkages of pullulan to panose. Like pullulanases from *K. pneumoniae* [5], alkaliphilic *Bacillus* KSM-1876 [3], and alkaliphilic *Bacillus* sp. S-1 [13], the enzyme from thermophilic *T. caldophilus* GK24 generated maltotriose, and not panose and isopanose from pullulan. Unlike pullulanases from alkaliphilic *Bacillus* KSM-1876 [3], and *K. pneumoniae* [5], our thermostable pullulanase, as well as the enzymes from alkaliphilic *Bacillus* No 202-1 [30] and *Bacillus* sp.S-1 [13], hydrolyzed glycogens.

In conclusion, our pullulanase is characteristically stable in the high temperature range. Current work is focussing on the cloning of the gene encoding pullulanase and aimed at applications of the enzyme in the detergent and starch industries.

Table 3. Substrate specificity of pullulanase

Substrate (2%, w/v)	Relative activity (%)	Substrate (2%, w/v)	Relative activity (%)
Pullulan	100	Dextran	N.D
Soluble starch	10.6	β-Limited dextrin	16.7
Potato starch	2.4	Amylopectin	21.0
α,β-Limited dextrin	36.3	Oyster glycogen	14.2
Amylose	N.D	Liver glycogen	13.5

N.D., Not Detected.

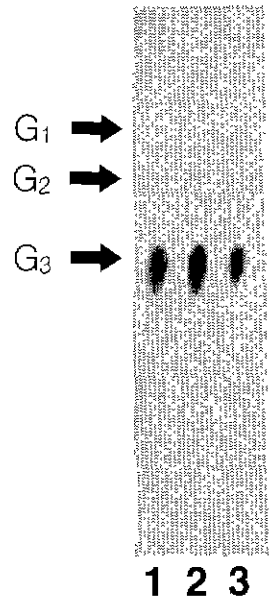

Fig. 5. Thin-layer Chromatogram (TLC) of the Reaction Products Produced by the Purified pullulanase.
One-half ml of the purified pullulanase solution was incubated with 0.5 ml of 2% pullulan at 50℃. Ten μl portions of the reaction mixture were withdrawn at intervals and tested to TLC as described in Materials and Methods. Lane 1, standard of maltotriose (G3); lane 2, 5-min; lane 3, 10-min incubation.

References

1. Abdullah, M. and French, D. 1966. Reverse action of pullulanase, Nature **210**, 200.
2. Allen, W. G. and Dawson, H. G. 1975. Technology and uses of debranching enzymes. Food Technol. **29**, 71-80.
3. Ara, K., Igarashi, K., Saeki, K., Kawai, S. and Ito, S. 1992. Purification and some properties of an alkaline pullulanase from alkalophilic *Bacillus* sp. KSM-1876. Biosci. Biotech. Biochem. **56**, 62-65.

4. Balayan, A. M. and Markossian, L. S. 1989. Some properties of intracellular pullulanase from the thermophilic strain of *Bacillus* sp. Biokhimiya **54**, 112-117.
5. Bender, H. and allenfeld, K. W. 1961. Untersuchungen an pullulan. II. Spezifischer abbau durich ein balterielles Enzym. Biochem Z, **334**, 79-94.
6. Bernfeld, P. 1955. Amylases α and β. *Methods Enzymol.* **1**, 149-150.
7. Brock, T. D. 1986. Thermophiles (Brock,T.D., ed.) pp.27-28, John Wiley & Sons, Inc., New York.
8. Brown, S. H. and Kelly, R. 1993. Characterization of amylolytic enzymes, having both α-1,4 and α-1,6 hydrolytic activity, from the thermophilic archaea *Pyrococcus furiosus* and *Thermococcus litoralis. Appl. Environ. Microbiol.* **59**, 2614-2621.
9. Davis, B. J. 1964. Disk electrophoresis. II. Methods and application to human serum proteins. Ann. N. Y. Acad. Sci. **121**, 404-427.
10. Imanaka, T. and Kuriki, T. 1989. Pattern of action of Bacillus stearothermophilus neopullulanase on pullulan. *J. Bacteriol.* **171**, 369-374.
11. Katsuragi, N., Takizawa, N. and Murooka, Y. 1987. Entire nucleotide sequence of the pullulanase gene of *Klebsiella pneumoniae* W70. J. Bacteriol. **169**, 2301-2306.
12. Kim, C. H. 1994. Specific detection of pullulanase type I in polyacrylamide gels. FEMS Microbiol. Lett. **116**, 327-332.
13. Kim, C. H., Choi, H. I. and Lee, D. S. 1993. Purification and biochemical properties of an alkaline pullulanase from alkalophilic *Bacillus* sp. S-1. *Biosci. Biotechnol. Biochem.* **57**, 1632-1637.
14. Kim, C. H., Choi, H. I. and Lee, D. S. 1993. Pullulanases of alkaline and broad pH range from a newly isolated alkalophilic *Bacillus* sp.S-1 and a *Micrococcus* sp. Y-1. *J. Industrial Microbiol.*, **12**, 48-57.
15. Kim, C. H. and Kim, Y. S. 1995. Substrate specificity and detailed characterization of a bifunctional amylase- pullulanase enzyme by from *B. circulans* having two different active sites on one polypeptide. *Eur. J. Biochem.* **227**, 687-693.
16. Kim, C. H. 1992. Kwon, S. T. and Lee, D. S. : Proteolytic modification of rsda from *Bacillus circulans* with subtilisin: Separation of hydrolytic domain and raw substrate adsorption domain *Biochim. Biophys. Acta* 1122, 243-250.
17. Kim, C. H., Nashiru, O. and Ko, J. H. 1996. Purification and biochemical characterization of pullulanase type I from *Thermus caldophilus* GK24. *FEMS Microbiol. Lett.* 138, 147-152.
18. Kimura, T. and Horikoshi, K. 1990. Characterization of pullulan-hydrolyzing enzyme from alkalopsychrotrophic *Micrococcus* sp. Appl. Microbiol. Biotechnol. **34**, 52-56.
19. Kuriki, T. and Imanaka, T. 1989. Nucleotide sequence of the neopullulanase gene from *Bacillus stearothermophilus.* J. Gen. Microbiol. **135**, 1521-1528.
20. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4, *Nature* **227**, 680-685.
21. Leach, B. S., Collawn, S. F. and Fish, W. W. 1980. *Biochemistry*, **19**, 5738-5742.
22. Lee, M. J., Lee, Y. C., and Kim, C. H. 1997. Intracellular and extracellular forms of pullululanases of an alkaliphilic *Bacillus* sp. S-1. *Arch. Biochim. Biophys. In Press.*
23. Lowry, O. H., Rosenbrough, N. H., Farr, A. L. and Randall, A. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**, 265-275.
24. Machuschek, M., Burchhardt, G., Sahm, K. and Bahl, H. 1994. Pullulanase of *Thermoanaerobacterium thermosulfurigenes* EM1 (*Clsotridium thermosulforogenes*): Molecular analysis of the gene, composite structure of the enzyme, and a common model for its attachment to the cell surface.J. Bacteriol. **176**, 3295-3302.
25. Marshall, J. J. 1973. Inhibition of pullulanase by schardinger dextrins. *FEBS Lett.*, **37**, 269-273.
26. Mathupala, S. P., Lowe S. E., Podkovyrov, S. M. and Zeikus, J. G. 1993. Sequencing of the amylopullulanase *(apu)* gene of *Thermoanaerobacter ethanolicus* 39E, and identification of the active site by site-directed mutagenesis. J. Biol. Chem. **268**, 16332-16344.
27. Melaswien, H., Paloheimo, M. and Hemio, L. 1990. Nucleotide sequence of the α-amylase-pullulanase gene from *Clostridium thermohydrosulfurium.* J. Gen. Microbiol. **136**, 447-454.
28. Miller, G. L. 1959. Use of dinitrosalicylic agent for determination of reducing sugars. *Anal. Chem.*, **31**, 426-428.
29. Nakamura, N., Sashihara, N., Nagayama, H. and Horikoshi, K. 1989 : Characterization of pullulanase and α-amylase activities of a *Thernus* sp. AMD33. Starch/ Starke **41**, 112-117.
30. Nakamura, N., Watanabe, K. and Horikoshi, K. 1975. Purification and some properties of alkaline pullulanase from a strain of *Bacillus* No.201-1, an alkalophilic microorganism. Biochim. Biophys. Acta **397**, 188-193.
31. Obido, F. J. C. and Obi, S. K. C. 1988. Purification and characterization of a thermostable pullulanase from *Thermoactinomyces thalpophilus.* J.Industrial Microbiol. **3**, 343-350.
32. Ono, M., Matsuzawa, H., and Ohta, T. 1990. Nucleotide sequence and characteristics of the gene for L-lactate dehydrogenase of *Thermus aquaticus* YT-1 and the deduced amino acid sequence of the enzyme. *J. Biochem.* **107**, 21-26.
33. Park,, J H., Kim, J. S., Kwon, S., and Lee, D. S. 1993. Purification and characterization of *Thermus caldophilus* GK-24 DNA polymerase. *Eur. J. Biochem.* **214,** 135-140.
34. Plant, A. R., and Morgan, H. W. and Daniel, R. M. 1986. A highly stable pullulanase from Thermus aquaticus YT-1. Enz. Microb. Technol. **8**, 668-672
35. Ramesh, M. V., Podkovyrov, S. M., Lowe, S. E. and Zeikus, J. G. 1994. Cloning and sequencing of the *Thermoanaerobacterium saccharolyticum* B6A-RI *apu* gene and purification and characterization of the amylopullulanase from *Escherichia coli.* Appl. Environ. Microbiol. **60**, 94-101.
36. Saha, B. C., Lamed, R., Lee, C. Y. 1990. Mathupala, S. P. and Zeikus, J. G., Characterization of an endo-acting amylopullulanase from Thermoanaerobacter strain B6A. Appl. Environ. Microbiol. **56**, 881-886.
37. Sakano, Y., Higuchi, M. and Kobayashi, K. 1972. Pullulan 4-glucan-hydrolase from *Aspergillus niger.* Arch. Biochem. Biophys. **153,** 180-187.
38. Smith, K. A. and Salyers, A. A. 1989. Cell-associated pullulanase from *Bacteroides thetaiotaomicron*: cloning, characterization, and insertional mutagenesis to determine role in pullulan utilization. J. Bacteriol. **171**, 2116-2123.
39. Spreinat, A. and Antranikian, G. 1990. Purification and properties of a thermostable pullulanase from Clostridium

thermosulfurogenes EM1 which hydrolyzes both α-1,6- and α-1,4-glycosidic linkages. Appl. Microbiol. Biotechnol. **33**, 511-518.

40. Suzuki, Y., Hatagaki, K. and Oda, H. 1991. A hyperthermostable pullulanase produced by an extreme thermophile *Bacillus flavocaldarius* KP 1228, and evidence for the proline theory of increasing protein thermostability. *Appl. Microbiol. Biotechnol.* **34**, 707-714.
41. Taguchi, H., Yanashita, M., Matsuzawa, H., and Ohta, T. 1982. Heat-stable and fructose 1,6-bisphosphate-activated L-lactate dehydrogenase from an extremely thermostable bacterium. *J. Biochem.* (Tokyo) **91**, 1343-1348.
42. Takasaki, Y. 1987. Pullulanase-amylase complex enzyme from *Bacillus subtilis*, Agric. Biol. Chem. **51**, 9-16.