J. Biochem. Mol. Biol. Vol. 30, No. 1, pp. 46~54 January 31, 1997

Processing of an Intracellular Immature Pullulanase to the Mature Form Involves Enzymatic Activation and Stabilization in Alkaliphilic *Bacillus* sp. S-1

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(Received November 2, 1996)

Abstract : Alkaliphilic *Bacillus* sp. S-1 secretes a large amount (approximately 80% of total pullulanase activity) of an extracellular pullulanase (PUL-E). The pullulanase exists in two forms: a precursor form (PUL-I: M_r 180,000), and a processed form (PUL-E: M_r 140,000). Two forms were purified to homogeneity and their properties were compared. PUL-I was different in molecular weight, isoelectric point, NH_2-terminal amino acid sequence, and stabilities over pH and temperature ranges. The catalytic activities of PUL-I were also distinguishable in the K_m and V_{max} values for various substrates, and in the specific activity for pullulan hydrolysis. PUL-E showed 10-fold higher specific activities than PUL-I. However, PUL-I is immunologically identical to PUL-E, suggesting that PUL-I is initially synthesized and proteolytically processed to the mature form of PUL-E. Processing was inhibited by PMSF, but not by pepstatin, suggesting that some intracellular serine proteases could be responsible for processing of the PUL-I. PUL-I has a different conformational structure for antibody recognition from that of PUL-E. It is also postulated that the translocation of alkaline pullulanase (AP) in the bacterium possibly requires processing of the NH_2-terminal region of the AP protein. Processing of the precursor involves a conformational shift, resulting in a mature form. Therefore, precursor processing not only cleaves the signal peptide, but also induces conformational shift, allowing development of active form of the enzyme.

Key words : activation, alkaliphilic *Bacillus* sp. S-1, conformation, processing, pullulanase.

Pullulanase (pullulan 6-glucanohydrolase, EC 3.2.1.41) is capable of hydrolyzing the α-1,6-glucosidic linkages of pullulan, producing maltotriose as an end product (Bender and Wallenfeld, 1961). pullulanases can also split the α-1,6-glucosidic linkages of branched polysaccharides, such as amylopectin and glycogen (Abdullah and French, 1966). To date, a number of pullulanase producers have been reported, however, most enzymes from 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. Type I pullulanases, which exclusively hydrolyze the α-1,6-linkages of pullulan, are produced by *K. pneumoniaee* (Bender and Wallenfeld, 1961), *Bacteroides thetaiotaomicron* 95-1 (Smith and Salyers, 1989), alkaliphilic *Bacillus* sp. KSM-1876 (Ara *et al.*, 1992), *Thermus aquaticus* YT-1 (Plant *et al.*, 1986), *T. caldophilus* (Kim *et al.*, 1996) and *Bacillus acidopullulyticus* (Kusano *et al.*, 1988). Pullulanases from *K. pneumoniae* and *B. acidopullulyticus* are active in a narrow pH range between 4.0 and 6.0, and have no stability at a high pH. There are apparently few reports on microbial Type I alkaline pullulanases (Bender and Wallenfeld, 1961; Ara *et al.*, 1992) with a broad pH stability. Furthermore, no intracellular Type I enzyme

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has been purified from microorganisms yet.

Recently, an alkaliphilic *Bacillus* sp. S-1, which secretes an extremely high concentration of Type I pullulanase, has been isolated (Kim *et al.*, 1993a; Kim *et al.*, 1993b). Up to 80% of the total enzyme was detected in the culture fluid and the remaining approximately 20% was detected in the cell extract. Western analysis with mouse anti-serum against an extracellular PUL-E showed that PUL-E is produced as a single enzyme form. Purified PUL-E has a molecular mass of approximately 140 kDa under denaturating and natural conditions, and also shows a broad pH stability, ranging from pH 4.0 to pH 11.0. Furthermore, the enzyme exhibits a single specificity by hydrolyzing α-1,6-glucosidic linkages in various glucose polymers (Kim *et al.*, 1993b). This investigation of the purification and properties of intracellular enzymes was carried out to determine whether or not the intracellular enzyme is similar to the extracellular enzyme. Such a confirmatory study has not as yet been done. Two forms of AP were purified from cells and designated PUL-I and PUL-E.

Present results indicate that the intracellular form consists of a 180 kDa precursor form that is converted to a extracellular form of 140 kDa. Based on immunological and enzymological analysis, PUL-I is identical to PUL-E, and PUL-I is similar to PUL-E in its antigenicity, optimum pH and temperature, substrate specificities, and patterns of inhibition. However, differences exist in the apparent molecular weight, kinetic parameters, and biophysical properties, such as isoelectric point, stabilities over pH and temperature ranges, and the NH_2-terminal amino acid sequence.

Materials and Methods

Materials and bacterium

Pullulan (M_r=65,000), panose, isopanose, pepstatin, bestatin, phenylmethane sulfonyl fluoride (PMSF), α-, β-, γ-cyclodextrins, and α, β-limited dextrin (LD) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Maltooligosaccharides were purchased from Nihon Shokuhin Kako Co. (Tokyo, Japan), and Sepharose-4B for FPLC was purchased from Pharmacia Co. (Upssala, Sweden). All other reagents were standard commercial preparations. The bacterial strain used in this study was alkaliphilic *Bacillus* sp. S-1 (Kim *et al.*, 1993a; Kim *et al.*, 1993b).

Cultivation of alkaliphilic *Bacillus* sp. S-1 and preparation of a total cell extract

Pullulanase-hyperproducing *Bacillus* sp. S-1 was isolated (Kim *et al.*, 1993a). It was cultivated aerobically at 50°C and pH 9.0 for 2 days in a 10 liter stirred tank fermentor (Korea Fermentor Co. Ltd, Inchon, Korea). The working volume was 8 liters and an impeller speed of 350 rpm was maintained. The medium consisted of (w/v): 1% potato starch, 0.5% yeast extract, 0.1% K_2HPO_4, 0.1% $MnCl_2$, 0.2% $MgSO_4 \cdot 7H_2O$, and 1% Na_2CO_3 (pH 9.0). For preparation of a total cell extract after cultivation, harvested cells were sonicated with the addition of 10 mM PMSF (f.c) then centrifuged at 15,000 rpm for 30 min (Chun and Chung, 1995). The whole cell extract was fractionated using ammonium sulfate (30~70%) after centrifugation (15,000 rpm, 30 min). The precipitate formed was collected by centrifugation, dissolved in 50 mM Tris-HCl buffer/0.05% Tween 80/10 mM PMSF/0.05% NaN_3 (pH 8.0; 5 ml/gram of cells) with 1 mM $MnCl_2$, and dialyzed overnight against the same buffer. The dialyzed enzyme solution was used as a crude PUL-I preparation.

Electrophoresis, measurement of molecular weight, activity staining, and immunological analysis

Native polyacrylamide gel electrophoresis (PAGE) was performed using 7.5% (w/v) polyacrylamide gel, as described (Davis, 1964). Discontinuous sodium dodecyl sulfate (SDS)-PAGE was performed according to the method of Laemmli (Laemmli, 1970). Protein bands were stained with Coomassie Blue R250 dye and destained with a solution of 5% methanol and 7.5% acetic acid (v/v). Molecular mass markers (Bio-Rad) were myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (BSA, 67 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), and lysozyme (15 kDa). The molecular mass of pullulanase was also measured by calibration of a Sepharose 4B column (1.6 cm×76 cm^2) with standard proteins, which included bovine serum albumin (BSA, 68 kDa), aldolase (158 kDa), catalase (240 kDa), and ferritin (450 kDa).

Mouse antiserum was prepared against purified PUL-E (M_r=kDa) (Kim *et al.*, 1993b). Western immunoblotting of protein was done by the method of Gooderham *et al.* (1983) using a biotinyl-anti IgG (Vector Laboratories Inc., CA, U.S.A) goat and streptavidin-horseradish peroxidase system (Promega, Madison, WI, USA).

pH and temperature profiles

For effects of pH on activity and stability, the enzyme activities of PUL-I and PUL-E were measured as in the pullulanase assay, except that 0.023 U of PUL-E and 0.031 U of PUL-I enzyme were added, respectively, and the pH value was changed using 50 μl of

one of the buffers at various pH values. 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, enzyme solutions were kept in 30 mM KCl-HCl buffers (pH 2.0~3.0) as described above for 24 hr at 4°C. Remaining activity was assayed after a two-fold dilution with 1.0 M Tris-HCl (pH 9.0). The activity of the untreated enzyme was expressed as 100%.

For effects of temperature on activity and stability, enzyme activities were measured as in the pullulanase assay, except that 0.092 U of PUL-E and 0.07 U of PUL-I enzyme were incubated with 2% pullulan at various temperatures (20 to 95°C). The maximum point in each temperature-activity profile was taken as 100%. For thermal stability, enzyme solutions were maintained at various temperatures for 60 min in Tris-HCl (pH 9.0), then the residual activity was measured.

Enzyme assay and analytical methods

Pullulanase activity was assayed by measuring the amount of reducing sugar released from pullulan ((Kim, 1994). A reaction mixture (1.0 ml) containing pullulan (1%, w/v), 50 mM Tris-HCl (pH 9.0), and enzyme (0.2~0.5 μg) was incubated at 50°C for 30 min. The amount of reducing sugar was measured by the dinitrosalicylic acid procedure (Bernfeld, 1955). One unit (U) of pullulanase or amylase activity was defined as the amount of enzyme that produced 1 μmol of reducing sugar as maltotriose per min under the experimental conditions. Protein amounts were measured by the method of Lowry *et al.* (1951) with bovine serum albumin as the standard. The absorbance at 280 nm was used to monitor proteins in column eluates.

Detection of the glycosylation of proteins was done after SDS-PAGE (Bhattacharya *et al.*, 1993). Oligosaccharides produced by enzymatic action were examined by thin layer chromatography (TLC), as described by Kim *et al.* (1990). Isoelectric foccusing was performed analytically (Kim *et al.*, 1993b). Standards (Sigma) had *p*I values of 3.55 (amyloglucosidase), 4.55 (soybean trypsin inhibitor), 5.13 (beta-lactoglobulin), and 5.85 (carbonic anhydrase B). Proteins were stained with Coomassie Blue. NH_2-terminal sequencing analysis was performed using a 470A gas phase protein sequencer equipped with a 120A on-line phenylthiohydantoin analyzer (Applied Biosystems, Warrington, Cheshire, U.K.).

Purification of PUL-I and PUL-E

Unless otherwise stated, all procedures were perfomred with addition of 10 mM PMSF (f.c) at 4°C. Culture broth was centrifuged (4,000×*g*, 15 min) and the bacterial pellet and supernatant were used as PUL-I and PUL-E sources, respectively. PUL-E was purified as described previously (Kim *et al.*, 1993b). The pellet in 270 ml of 50 mM Tris-HCl (pH 9.0) containing 10 mM PMSF, was sonicated and the cell extract was centrifuged (15,000×*g*, 30 min) at 4°C. At this stage the fraction containing the insoluble form of membrane-bound pullulanase was eliminated (Lee *et al.*, 1997). Then, the supernatant solution was fractionated with 30 to 70% saturation of ammonium sulfate. The precipitates were dissolved in 40 ml of 50 mM Tris-HCl buffer (pH 8.0) and dialyzed overnight against the same buffer containing 1 M ammonium sulfate. The dialysate was divided into portions, and each portion was chromatographed on a Phenyl TSK gel column (2.3 cm×25 cm; Tosoh Co. Ltd., Tokyo, Japan) interfaced with a FPLC system (Pharmacia) which was equilibrated with 50 mM Tris-HCl buffer (pH 8.0) containing 1 M ammonium sulfate. After washing the column with the equilibration buffer, proteins were eluted in 2 ml fractions at a flow rate of 20 ml/h with a gradient of 1 to 0 M ammonium sulfate. Pullulanase activity was eluted in two peaks. The first peak, which was eluted near 0.75 M ammonium sulfate (fraction numbers 44~58), was collected as PUL-I. The active enzyme fractions were concentrated 10-fold at 4°C by ultrafiltration (Amicon PM30; Amicon Co. Ltd., Danvers, MA, USA) with a cut off of M_r=30,000 and dialyzed overnight against the same buffer and then each 5 ml was subjected to a Mono-Q column (1.2 cm×12 cm; Pharmacia LKB, Upssala, Sweden) equilibrated with the same buffer. After washing the column with the same buffer (30 ml), proteins were eluted with a gradient of 0.1~0.45 M NaCl at a flow rate of 0.5 ml/min. The fraction size was 0.3 ml. After PUL-I was applied to the column the active fractions (tubes nos 31~34) (Fig. 1), which were eluted near 0.24 M ammonium sulfate, were pooled and concentrated by ultrafiltration (Amicon PM30), and the concentrate was dialyzed against the same buffer containing 0.15 M NaCl. When PUL was applied to the Mono-Q column under the same conditions, pullulanase activity was eluted near 0.37 M ammonium sulfate. The active fractions (tube nos 44~48) were collected, concentrated, and dialyzed.

Each dialyzed sample (1 ml) was applied to a Sepharose 4B column (FPLC, Pharmacia LKB, Upsala, Sweden) connected to a FPLC and equilibrated with 50 mM Tris-HCl buffer (pH 8.0) containing 0.15 M NaCl.

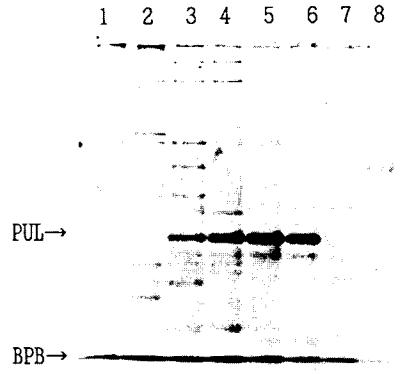

Fig. 1. Electrophoresis pattern of the eluted PUL-I fractions from Mono-Q column. Sample was subjected to a Mono-Q column (1.2 cm×12 cm; Pharmacia LKB, Upssala, Sweden) and proteins were eluted with a gradient of 0.1~0.45 M NaCl. The fraction size was 0.3 ml. The active fractions (tube numbers 31~34) were analysed using a 10% native-PAGE. Numbers correspond to fractions from 29 to 36. Arrowheads denote the positions of PUL-I and BPB.

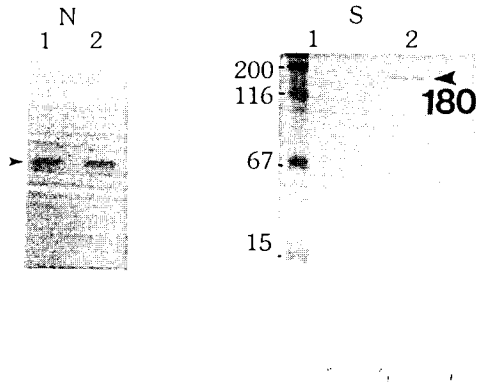

Fig. 2. Electrophoresis of the purified PUL-I from *Bacillus* sp. S-1. (N) Native PAGE of purified PUL-I. Lane 1, purified PUL-I (10 μg); lane 2, purified PUL-I (8 μg). (S) SDS-PAGE of purified PUL-I. Lane 1, molecular maker; lane 2, purified PUL-I (5 μg). Proteins were stained with Coomassie blue. Arrowheads denote the positions of PUL-I. Positions of standard proteins are indicated.

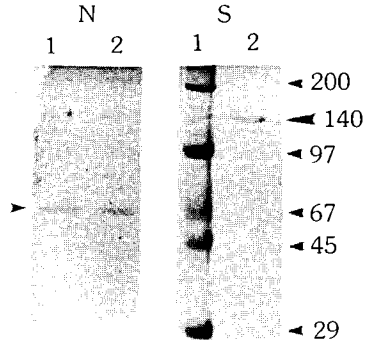

Fig. 3. Electrophoresis of the purified PUL-E from *Bacillus* sp. S-1. (N) Native PAGE of purified PUL-I. Lane 1, purified PUL-I (8 μg); lane 2, purified PUL-I (10 μg). (S) SDS-PAGE of purified PUL-I. Lane 1, molecular marker; lane 2, purified PUL-E (5 μg). Proteins were stained with Coomassie blue. Arrowheads denote the positions of PUL-I. Positions of standard proteins are indicated.

The active enzyme fractions (tubes 36~41 for PUL-I and tubes 57~62 for PUL-E) were independently collected, concentrated, and dialyzed. Each dialyzed enzyme was finally applied to a 2nd gel filtration on a Sepharose 4B column (FPLC, Pharmacia LKB, Upssala, Sweden) pre-equilibrated with 50 mM Tris-HCl (pH 8.0) containing 0.15 M NaCl. The active enzyme peaks were collected, concentrated, and dialyzed against 50 mM Tris-HCl.

Inhibition studies

All inhibitors were diluted from 1 M stock solutions in 50 mM Tris-HCl (pH 9.0) to final concentrations of 1 mM, 10 mM, or 50 mM, except SDS which was diluted to 10%, then further diluted in Tris buffer. The pullulanase concentration was adjusted such that its activity corresponded to 0.03 U in a regular assay. Pullulanase (approximately 0.3 μg) was incubated with a substrate and the inhibitors for 30 min at 50°C.

Results and Discussion

Purification of the intracellular immature and extracellular mature PUL forms

The great majority (approximately 80%) of pullulanase synthesized was released into the culture broth, and cell washing with high-ionic-strength solutions released the remaining pullulanase activity. The sum of both fractions was labelled as "extracellular pullulanase".

To examine the catalytic properties of AP, each enzyme was purified. The PUL-1 was usually detected only when high concentrations of PMSF (above 10 mM) were included in the cell extract, indicating that conversion of the PUL-I form to the PUL-E form is sensitive to proteases. What is remarkable is that the precursor form (180 kDa) is accumulated intracellularly in large amounts. This is in contrast to most other secreted proteins. Generally, precursor forms of secreted proteins containing a signal peptide are rapidly secreted and processed and only low amounts of precursor forms are observed (Smith and Salyers, 1989).

PUL-I and PUL-E showed single bands on SDS-PAGE (Fig. 2S and Fig. 3S). The active fractions of PUL-I and PUL-E were homogeneous on native-PAGE (Fig. 2N and Fig. 3N). The results of purification of PUL-I and PUL-E are shown in Table 1. The specific

Table 1. Summary of AP purification

A) PUL-I

Purification step	Volume (ml)	Total activity (U)	Protein (mg)	Specific activity (U/mg)	Yield (%)	Purification factor (fold)
Crude enzyme	270	99,219	23,545	4.2	100	1
Ammonium sulfate	130	41,088	6,420	6.4	41.1	1.5
Phenyl TSK 650M	28	20,421	665	30.7	20.6	7.3
Mono Q	16	9,490	248	38.3	9.6	9.1
Sepharose 4B, 1st	8	8,726	125	69.7	8.8	16.6
Sepharose 4B, 2nd	6	7,825	109	71.4	7.9	17.0

B) PUL-E

Purification step	Volume (ml)	Total activity (U)	Protein (mg)	Specific activity (U/mg)	Yield (%)	Purification factor (fold)
Crude enzyme	270	99,219	23,545	4.2	100	1
Ammonium sulfate	130	41,088	6,420	6.4	41.1	1.5
Phenyl TSK 650M	32	12,150	942	12.9	12.3	3.1
Mono Q	22	10,250	101	101.5	10.3	24.2
Sepharose 4B, 1st	11	8,628	11	719.4	8.7	171.2
Sepharose 4B, 2nd	5	7,217	9.9	736.4	7.2	175.3

The activity of AP could not be divided into those of PUL-I and PUL-E in the initial crude fractions because of the enzyme mixture. The culture was grown in a 10 liter fermentor as described in Materials and Methods.

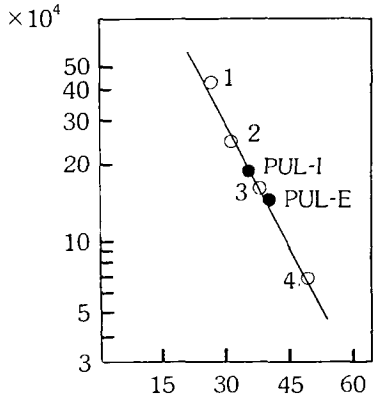

Fig. 4. Plot of logarithm of molecular weight of PUL-I and PUL-E on Sepharose 4B column. Standard proteins: 1, ferritin (450,000); 2, catalase (240,000); 3, aldolase (158,000); 4, albumin (bovine serum: 68,000).

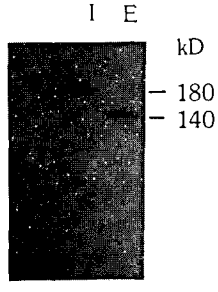

Fig. 5. Western analysis of the purified PUL-I and PUL-E using anti-mouse PUL-E polyclonal antibody. SDS-PAGE-western immunoblotting of protein was done using a biotinyl-anti IgG goat and streptavidin-horseradish peroxidase system with a mouse antiserum against purified PUL-E as described previously (Kim *et al.*, 1993b).

activity (U/mg protein) of PUL-I was 71.4 representing a purification of 17-fold with 1.7% recovery. PUL-E was purified approximately 175-fold with a specific activity of 736.4 U/mg protein. These results indicate that the specific activity of PUL-E is 10-fold greater than the activity of PUL-I.

General characteristics of the purified enzymes

The molecular mass of PUL-I was approximately 180 kDa under denaturing conditions (Fig. 2) and the M_r value of PUL-E was estimated to be approximately 140 kDa (Fig. 3), and a calibrated Sepharose 4B column chromatography of PUL-I indicated a molecular mass of 185 kDa (Fig. 4). Western analysis of purified PUL-I differed in the apparent M_r value of PUL-E (Fig. 5). Thus, there was an apparent decrease in the size of the polypeptide. However, the M_r values of the two enzymes were higher than the M_r values of any pullulanases reported to date (Bender and Wallenfeld, 1961; Nakamura *et al.*, 1975; Hyun and Zeikus, 1985; Suzuki and Imai, 1985; Obido and Obi, 1988; Balayan and Markossian, 1989; Kimura and Horikoshi, 1989; Kuriki and Imanaka, 1989; Nakamura *et al.*, 1989; Smith and Salyers, 1989; Kimura and Horikoshi, 1990; Suzuki *et al.*, 1991; Ara *et al.*, 1992; Kambourova and

Emanuilova, 1992; Kim *et al.*, 1996; Plant *et al.*, 1996). Some pullulanase-like enzymes, such as *B. circulans* amylase-pullulanase enzyme (220 kDa) (Kim and Kim, 1995), *B. subtilis* pullulanase-amylase complex (450 kDa) (Takasaki, 1987), *Thermoanaerobacter* B6A amylopullulanase (450 kDa) (Saha *et al.*, 1990), and *C. thermohydrosulfuricum* α-amylase-pullulanase (165 kDa) (Melaswien *et al.*, 1990), have been regarded as high molecular mass enzymes. However, these enzymes are not true pullulanases in that they all have dual amylase and pullulanase activities. Therefore, PUL-I and PUL-E from alkaliphilic *Bacillus* sp. S-1 can be regarded as a real Type I pullulanase, which has the highest molecular mass.

The isoelectric point of PUL-I was estimated to be 5.97 by isoelectric focussing using a Pharmacia LKB IEF system. When resolved by SDS-PAGE, PUL-I was negative for staining with the periodic acid/Schiff's reagent, indicating that the enzyme is not glycosylated (data not shown). When purified PUL-I was subjected to Edman degradation, the N-terminal amino acid sequence of the enzyme was completely blocked (data not shown). However, the isoelectric point of PUL-E was estimated to be 5.7 (data not shown) and the NH_2-terminal amino acid sequence was Phe-Leu-Asn-Met-Ser, which is the same as PUL-E (Kim *et al.*, 1993b).

The optimum reaction pH for PUL-I was 9.0 and the enzyme was stable in a pH range of 6.0 to 10.5 after incubation at 4°C for 24 hr. PUL-I and PUL-E both had the same optimum pH value (Fig. 6A). However, PUL-I exhibited a narrower range of pH stability and was also more unstable than PUL-E. The optimum reaction temperature for PUL-I was 60°C and the enzyme was stable up to 65°C after incubation for 60 min at pH 9.0. However, it was almost completely inactivated at 75°C after 30 min (Fig. 6B). These results indicate that PUL-I is more unstable than PUL-E. The optimum pH for these two enzymes is higher than the optima of other pullulanases from *K. pneumoniae* (pH 6.5) (Bender and Wallenfeld, 1961), *B. acidopullulyticus* (pH 5.0) (Kusano *et al.*, 1988), alkaliphilic *Bacillus* No 202-1 (pH 8.5-9.0) (Nakamura *et al.*, 1975), and an alkalopsychrotrophic *Micrococcus* sp. (pH 8.0) (Kimura and Horikoshi, 1989).

PUL-I and PUL-E were stabilized by the addition of the substrate pullulan. As the temperature for optimum activity rose with an increasing amount of added substrate, the thermostability of these enzymes seemed to be enhanced with more substrate. The enzymes were active at 75°C in 3.0% (w/v) pullulan. Pullulan protected the enzymes from heat deactivation. This effect was dependent on the concentration of the added substrate. In the absence of pullulan the enzymes rapidly lost their pullulanase activities at temperatures above 70°C (Fig. 7). The half-life of the enzymes was 50 hr when incubated in 3.0% pullulan at pH 9.0 and at 70°C. In the presence of 5% (w/v) starch, the AP activities were stable at 70°C, and 45% of the original activity remained after heating at 75°C for 30 min. This

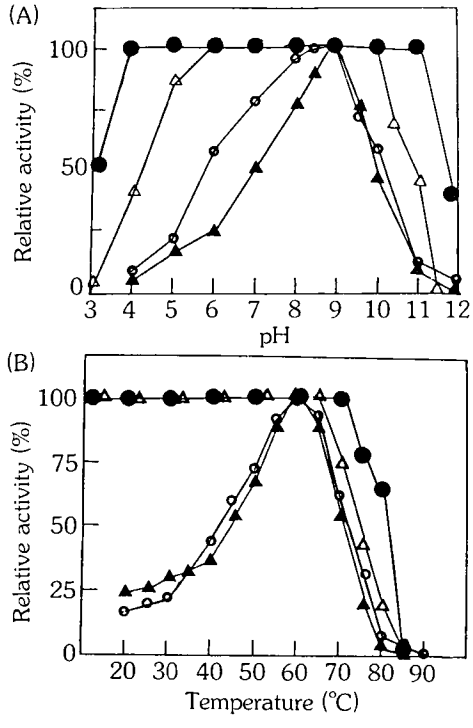

Fig. 6. Effects of pH and temperature on the activities of PUL-I and PUL-E from alkaliphilic *Bacillus* sp. S-1. (A) Effects of pH on activity and stability. (B) Effects of temperature on activity and stability. Symbols: ●, stability of purified PUL-E; △, stability of PUL-I; ○, activity of purified PUL-E; ▲, activity of PUL-I.

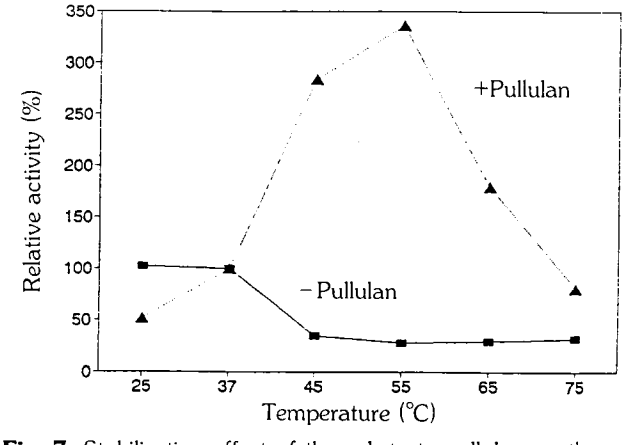

Fig. 7. Stabilization effect of the substrate pullulan on the purified PUL-E activity. ▲, incubation with 3.0% (w/v) pullulan; ■, incubation without pullulan.

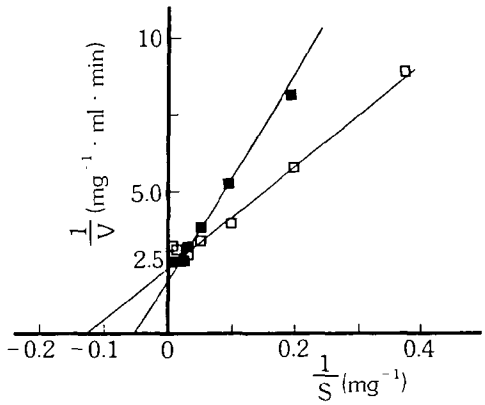

Fig. 8. Lineweaver-Burk plot of PUL-I and PUL-E from *Bacillus* sp. S-1 for the determination of K_m and V_{max} values for pullulan. Symbols: □, PUL-I; ■, PUL-E.

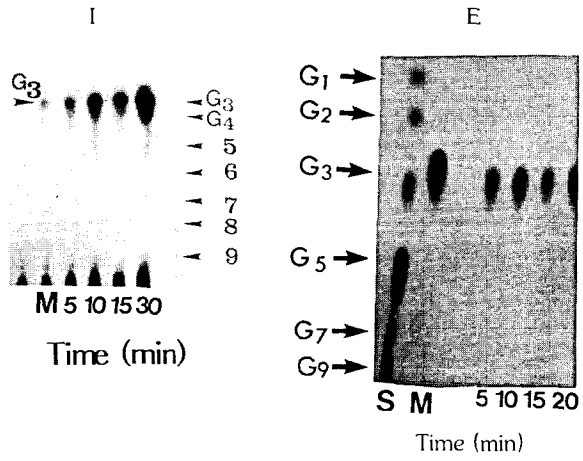

Fig. 9. Digestion pattern of the purified PUL-I and PUL-E on pullulan as a substrate. Each enzyme preparation was incubated with 2% pullulan and aliquots were taken with a time courses of 5 to 30 min as described in Materials and Methods. (I) Action pattern of PUL-I. (E) Action pattern of PUL-E. M, maltotriose; S, Standard markers including a series of maltooligosaccharides (G_1 to G_9).

heat stability is similar to that of PUL-E (data not shown).

The K_m value of PUL-I for pullulan from a Lineweaver-Burk plot was 16.4 mg/ml. and the value of PUL-E was 7.96 mg/ml (Fig. 8). The V_{max} value for PUL-I was 512 mg/min/ml. whereas values for PUL-E were 396 mg/min/ml. respectively. Therefore. the K_m value of PUL-I is higher than the values of PUL-E (7.92 mg/ml) (Kim *et al.*, 1993b). This possibly suggests that the affinity of PUL-I for the substrate decreased because of a conformation shift in the molecule. The K_m and V_{max} values of PUL-I were also determined to be a K_m of 2.34 mg/ml for amylopectin with a V_{max} of 46.8 mg/min/ml. and a K_m of 4.9 mg/ml for α, β-LD with a V_{max} of 72.9 mg/min/ml. These values are in contrast to the values of PUL-E. which were a K_m of 1.63 mg/ml for amylopectin with a V_{max} of 37.8 mg/min/ml and a K_m of 3.1 mg/ml for α, β-LD with a V_{max} of 39.6 mg/min/ml.

The substrate specificities of the two enzymes were also examined. All enzymes showed the same specificities. Maltotriose was the sole product of hydrolysis of pullulan. indicating that the enzymes attacked the α (1→6) glucosidic linkages of pullulan (Fig. 9). Like pullulanases from *K. pneumoniae* (Bender and Wallenfeld. 1961) and alkaliphilic *Bacillus* KSM-1876 (Ara *et al.*, 1992). the enzymes from alkaliphilic *Bacillus* sp. S-1 generated maltotriose. and not panose or isopanose from pullulan. Unlike pullulanases from alkaliphilic *Bacillus* KSM-1876 (Ara *et al.*, 1992) and *K. pneumoniae* (Bender and Wallenfeld. 1961), these two APs. as well as an intracellular pullulanase from alkaliphilic *Bacillus* No 202-1 (Nakamura *et al.*, 1975), hydrolyzed glycogens.

The effects of metal ions on the activities of PUL-I and PUL-E were examined. While divalent cations, such as Zn^{2+}, Cu^{2+}, and Fe^{2+}, inhibited the activities of both enzymes (Table 2), 1 mM concentrations of Ca^{2+}, Mg^{2+}, Ni^{2+}, Co^{2+}, K^+, and Na^+, were neither inhibitory nor stimulatory. Whereas Mn^{2+} greatly stimulated PUL-I and PUL-E activities. EDTA was not inhibitory. The effects of Mn^{2+}, EGTA, and EDTA on the AP activities of both enzymes were then determined. Dialysis against EDTA and EGTA did not inhibit the AP activities. when compared to a control (dialyzed against H_2O). EDTA and EGTA. each at 15 mM, were also not inhibitory under the assay conditions. These results suggest that these chemicals do not chelate a divalent cation (s) which is possibly required for the activities of the two APs. Such insensitivity to EDTA and EGTA is also observed in *B. flavocaldarius* pullulanase (Suzuki *et al.*, 1991). However, dialysis with EDTA and Mn^{2+} stimulates the enzymatic activities of PUL-E and PUL-I. suggesting that Mn^{2+} ions are required for the full activities of the two APs. It is generally known that Ca^{2+} strongly enhances both the activities and stabilities of pullulanases from *T. aquaticus* (Plant *et al.*, 1986) and *T. thalpophilus* (Obido and Obi, 1988) at a high temperature. Ca^{2+} has no effect on the activities of the two APs from alkaliphilic *Bacillus* sp. S-1 (Table 2), and also no effect on the pullulanase from *B. flavocaldarius* (Suzuki *et al.*, 1991). However, Hg^{2+} and *N'*-bromosuccinimide, both of which can act on SH groups, affect the activities, indicating either that thiol groups are presented in the active site of the enzymes, or that these groups are important in maintaining the enzyme structure.

The activities of PUL-I and PUL-E are not inhibited by α-, β- or γ-CDs, possible competitive inhibitors of

Table 2. Comparison of the effects of various chemical agents on the activities of purified PUL-E and PUL-I from *Bacillus* sp. S-1

Reagent	Concentration	Relative activity (%)		Reagent	Concentration	Relative activity (%)	
		PUL-E	PUL-I			PUL-E	PUL-I
None	–	100	100	$CuCl_2$	1 mM	31	27
KCl	1 mM	108	110	$CuSO_4$	1 mM	43	39
NaCl	1 mM	122	128	$FeCl_3$	1 mM	56	48
$CaCl_2$	1 mM	102	108	$FeSO_4$	1 mM	103	112
$CoCl_2$	1 mM	108	107	$MnCl_2$	1 mM	152	167
$NiCl_2$	1 mM	102	100	EDTA	1 mM	107	117
$NiSO_4$	1 mM	107	112	α-CD	1 mM	107	112
$MgCl_2$	1 mM	112	116	β-CD	1 mM	52	67
$ZnCl_2$	1 mM	62	57	γ-CD	1 mM	74	56
$HgCl_2$	10 mM	7	6	METOH[b]	2000 mM	101	106
	1 mM	27	22		50 mM	121	112
BSM[a]	10 mM	1	0	SDS	1%	105	87
	1 mM	2	0		0.1%	110	92

[a]*N*-bromosuccinimide.
[b]β-Mercaptoethanol.

pullulanase (Ara *et al.*, 1992). These results differentiate PUL-I and PUL-E from other bacterial pullulanases.

Differential inhibitory effects of polyclonal antibodies to PUL-E and PUL-I, and conversion of PUL-I to PUL-E

Purified antibodies were used to study inhibition effects on AP activities. As shown in Fig. 10, antibodies inhibited approximately 88% of PUL-E activity with incubation of 5 μg and 10 μg of enzyme, respectively. Only 62% of PUL-I activity was inhibited with incubation of 5 μg of the enzyme. These results suggest that PUL-I has a different conformational structure for antibody recognition from that of PUL-E, and also that processing of the precursor involves an enzyme-to-enzyme conformational shift, resulting in a mature form. Therefore, precursor processing not only cleaves the signal peptide, but also produces a conformational shift, allowing development of mature active forms of the enzymes (Kim *et al.*, 1991). PUL-I was converted to PUL-E by incubation. The conversion was inhibited by PMSF, but not by pepstatin, suggesting that some intracellular serine proteases could be responsible for processing of the PUL-I. There was a correlation between the conversion ratio of PUL-I and an increase of activity. The reason for this is unknown, but it may take some time to develop stable enzyme activity after processing, or some other, as yet unknown process which allows a conformational shift toward the stable form may be acting.

On the other hand, PMSF inhibited conversion of PUL-I to PUL-E, suggesting that some intracellular proteases could be responsible for processing of the PUL-I. Incubation of the crude cell extract caused a gradual increase of total AP activity (Fig. 11) with concomitant conversion of the 180-kDa form to 140-kDa form as ascertained by immunoblot analysis (data not shown). However, total enzyme activity decreased rapidly after 12 hrs incubation, indicating that endogeneous proteases process PUL-I. Further work is needed to study the effects of endogeneous proteolytic enzymes on PUL-E formation at the molecular level. Current work is focusing on the elucidation of processing, translocation

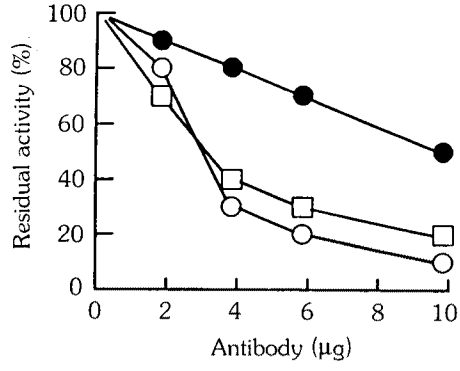

Fig. 10. Differential inhibitory effects of antibodies against PUL-E and PUL-I. Purified (0.5 μg) PUL-E and PUL-I enzymes were incubated with varying amounts of polyclonal antibody in 50 mM Tris-HCl buffer (pH 9.0) containing 0.02% bovine serum albimun in a total volume of 0.3 ml at 4°C for 1 h, and then the remaining activity was assayed. The values represent the percentages of the residual activities in the supernatants to the enzyme activities determined in the presence of a control mouse serum. Symbols: ○, PUL-E; ●, PUL-Ia; □, PUL-Ib.

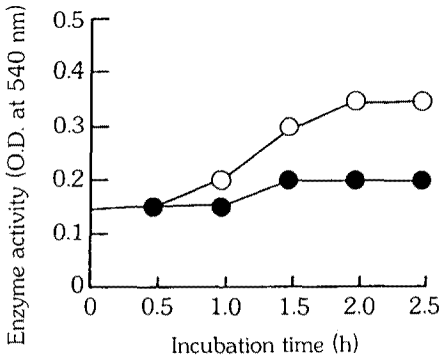

Fig. 11. Increase of total enzyme activity during the incubation of cell extract without (●) or with 10 mM PMSF (○). Bacterial cell extract (24 μg) were incubated with 10 mM PMSF or not, and then enzyme activity was assayed.

of PUL, and molecular cloning of the gene.

Acknowledgement

This work was in part supported by a grant of Ministry of Education, Korean Government.

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