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A Possible Mechanism Responsible for Translocation and Secretion of an Alkaliphilic *Bacillus* sp. S-1 Pullulanase

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The secretion of the alkaliphilic *Bacillus* sp. S-1 extracellular pullulanase involves translocation across the cytoplasmic membrane of the Gram-positive bacterial cell envelope. Translocation of the intracellular pullulanase PUL-I, was traced to elucidate the mechanism and pathway of protein secretion from an alkaliphilic *Bacillus* sp. S-1. Pullulanase could be slowly but quantitatively released into the medium during growth of the cells in medium containing proteinase K. The released pullulanase lacked the N-terminal domain. The N-terminus is the sole membrane anchor in the pullulanase protein and was not affected by proteases, confirming that it is not exposed on the cell surface. Processing of a 180,000 M_r pullulanase to a 140,000 M_r polypeptide has been demonstrated in cell extracts using antibodies raised against 140,000 M_r extracellular form. Processing of the 180,000 M_r protein occured during the preparation of extracts in an alkaline pH condition. A modified rapid extraction procedure suggested that the processing event also occured *in vivo*. Processing apparently increased the activity of pullulanase. The western blotting analysis with mouse anti-serum against 140-kDa extracellular pullulanase PUL-E showed that PUL-I is processed into PUL-X via intermediate form of PUL-E. Possible explanations for the translocation are discussed.

Key words: Alkaliphilic *Bacillus* sp. S-1, intracellular pullulanase, secretion, translocation

Extracelluar proteins play key roles in their interactions with their environment or with other organisms. Gram-positive bacteria secrete relatively a lot of extracellular proteins. Proteins targeted to the milieu of a Gram-positive bacterium must cross the membrane enveloping the cell. The export of proteins through the membranes by the universal and highly conserved Sec machinery has been extensively studied in mesophilic Gram-positive and Gram-negative bacteria (17). Much less is known about the secretion of proteins across the membrane (3).

Pullulanase (pullulan 6-glucanohydrolase, EC 3.2.1.41) have been found in various organisms (1, 2, 11, 16, 18, 19, 21, 23, 28), but most work on pullulanases involved *Klebsiella pneumoniae* (10, 20, 25, 26, 29). The enzyme possesses an amino-terminal signal peptide and is secreted from *K. pneumoniae* via an extended Sec pathway in which a signal peptide-containing preprotein is exported across the inner membrane, processed, and then translocated as a mature protein across the outer membrane (3, 24). Little is known about the features of these proteins that target them to the Sec-dependent pathway or the mechanism by which the outer membrane is traversed, although the process clearly differs among bacteria.

In previous papers, we isolated an alkaliphilic *Bacillus* sp. S-1, which secreted extremely high concentrations (up to 80% of the total enzyme) of pullulanase in medium and about 20% of the enzyme was detected in cell extracts (6-8). The western blotting analysis showed that only PUL-E was detected in culture medium. Therefore, pullulanase is synthesized as a precursor with a signal peptide which is processed by some signal peptidase (13). Several additional lines of evidence suggest that the initial stage of pullulanase export is via the general export pathway by which proteins are exported across the cytoplasmic membrane to the cell envelope. The PUL-E had a molecular mass of about 140 kDa and also showed a broad pH stability, ranging from pH 4.0 to pH 11.0. The purified enzyme exhibited single specificity by hydrolyzing α-1,6-glucosidic linkage in various glucose polymers (18).

The aim of this study is to determine how extracellular proteins are translocated across the cel-

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luar membrane. We are using model system based on the alkaline pullulanase (AP) of alkaliphilic *Bacillus* sp. S-1. The mature AP polypeptide is extracellular, but it remains anchored to the cell surface by the possible N-terminal domain of the processed precursor, and is slowly and probably spontaneously released into the growth medium only after an exponential growth (13). The secretion mechanism has been studied using anti-AP antibody. The role of N-terminal domain connected to the AP in anchoring PUL-I and translocation to the cell envelope was assumed, but it is not clear whether they actively participate in the translocation of the enzyme through the cell envelope. In previous studies, the N-terminal amino acid sequence of PUL-E was determined, but that of PUL-I was completely blocked.

This investigation of the translocation and processing of the pullulanase was carried out to determine the pathway of translocation of the enzyme in alkaliphilic bacterium. By immunological analysis, we have shown that PUL-I is processed to a smaller form of PUL-E and then released into the outer medium. This event is carried out by some bacterial endogeneous proteases and also facilitate the increasing of the enzyme activity, resulting in formation of mature enzyme.

Materials and Methods

Materials

Pullulan (M_r=65,000), phenylmethanesulfonyl fluoride (PMSF) and pestatin were purchased from Sigma Co. (St. Louis, MO., USA). Maltooligosaccharides were purchased from Nihon Shokuhin Kako Co. (Tokyo, Japan), and Sepharose-4B for FPLC was purchased from Pharmaica Co. (Upssala, Sweden).

Cultivation of alkaliphilic *Bacillus* sp. S-1 and preparation of cell extracts

The pullulanase-hyperproducing alkaliphilic *Bacillus* sp. S-1 was isolated in our laboratory (7). It was cultivated aerobically at 50°C and at 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 a speed of the impeller 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). After cultivation, the harvested cell was sonicated with addition of 10 mM PMSF (f.c) and then centrifugated at 15,000 rpm for 30 min. The whole cell extract was fractionated by 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 dialysed overnight against the same buffer. The dialysed enzyme solution was used as crude PUL-I preparation.

Fractionation of bacterial components

Protoplasts were prepared from stationary-phase bacteria by a slight modification of the method of Petit-Glatron *et al.* (22). The routine procedure used to quench cellular biochemical events was as follows : 5 ml of the cell suspension was mixed in 30 ml of 100 mM Tris-HCl buffer (pH 9.0) containing 50 mM PMSF and 5 mM EDTA at 0°C, and vigorously stirred. The cell suspension was then centrifuged and the bacteria were washed twice with a cold buffer containing 2 M KCl and the protease inhibitors mentioned above. Next, the cells were resuspended in a small volume of sample buffer for SDS-PAGE and disrupted by five periods of sonication, 30 sec each (this was the total cell extract fraction).

To obtain membrane and cytoplasmic fractions, cells were resuspended in a small volume of 10 mM Tris-HCl buffer and lysed in the presense of a lysozyme/DNase mixture (2 mg/ml lysozyme, 1 g/ml DNase, 10 mM PMSF, 20 μg/ml $MgCl_2$). The cells were gently mixed and allowed to stand for 20 min at 0°C. The protoplasts were then pelleted at 3,800× g for 15 min and the supernatant was saved (this was the trapped fraction of the cell surface, i.e., the fraction between the cytoplasmic membrane and the cell wall). The pellet was washed twice with Tris-HCl buffer (pH 9.0) and vigorously resuspended in 1 ml of 10 mM Tris-HCl buffer (pH 9.0) containing 1 mM EDTA and 10 mM PMSF to lyse the protoplasts. The suspension was allowed to stand for 15 min, then was centrifuged at 32,000× g for 30 min. The supernatant was saved (this was the cytoplasmic fraction), and the pellet was washed with 1 ml of 10 mM Tris-HCl buffer containing 10 mM PMSF, then resuspended in 1 ml of 10 mM Tris-HCl buffer (pH 9.0) containing 1 mM EDTA, 10 mM PMSF, and 2% Triton X-100 (vol/vol). This suspension was allowed to stand for 15 min, and then centrifuged at 50,000× g for 1 hr. The supernatant was saved (this was the membrane fraction). All fractions were stored at -70°C.

Electrophoresis, measurement of molecular weight, and immunological analysis

Native polyacrylamide gel electrophoresis (PAGE) was done using 7.5% (w/v) polyacrylamide gels, as

described by Davis (4). Discontinuous sodium dodecyl sulfate (SDS)-PAGE was done essentially by the method of Laemmli (12). The bands of protein were stained with Coomassie brilliant 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, 67 kDa), ovalbumin (45 kDa), and carbonic anhydrase (29 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 bovine serum albumin (BSA, 68,000), aldolase (158,000), catalase (240,000), and ferritin (450,000).

Preparation of mouse antiserum against the purified PUL-E (M_r=140,000) was described previously (8). For immunoblot analysis proteins were transferred to nitrocellulose filters (0.45 um, Schleicher and Schuel) at 4 C for 120 min in a TransBlot Unit (BioRad) at 400 mA by the method of Gooderham *et al.* (5). The blots were blocked for 1 h in 20 mM Tris-HCl (pH 7.2), 150 mM NaCl, 2% skim milk and developed with a biotinyl-anti IgG (Vector Laboratories Inc., CA, U.S.A) of goat and streptavidin-horseradish peroxidase system (Promega, Madison, WI, USA) according to the instructions of the supplier.

Enzyme assay and analytical methods

Pullulanase activity was assayed by measuring the reducing sugar released from pullulan, respectively (9). The reaction mixture (1.0 ml) containing pullulan (1%, w/v), 50 mM Tris-HCl (pH 9.0), and enzyme (5~20 μg) was incubated at 50°C for 30 min. The reducing sugar was measured by the dinitrosalicylic acid procedure (2). One unit (U) of pullulanase 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.* (15) with bovine serum albumin as the standard. The absorbance at 280 nm was used to monitor protein in column eluates. NH_2-terminal sequencing analysis was tried 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

Unless otherwise stated, all procedures were performed with addition of 10 mM PMSF (f.c) at 4°C. After bacterial cultivation, the bacterial pellet and supernatant were used as PUL-I and PUL-E sources, respectively. PUL-E from the supernatant was then purified as described (8). The pellet, which was dissolved in 270 ml of 50 mM Tris-HCl (pH 9.0) containing 10 mM PMSF, was sonicated to obtain the cell extract. Then the cell extract was centrifuged (15,000× g, 30 min). The supernatant solution was fractionated with 30 to 70% saturation of ammonium sulfate. The precipitate, which was dissolved in 50 mM Tris-HCl buffer (pH 8.0) and dialyzed, was chromatographed on a Phenyl TSK gel column (2.3 cm × 25 cm; Tosoh Co. Ltd., Tokyo, Japan) interfaced with a fast protein liquid chromatography system (Pharmacia) which was equilibrated with 50 mM Tris-HCl buffer (pH 8.0) containing 1 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 second peak, which was eluted near 0.2 M ammonium sulfate (fraction numbers 82-105) was collected as PUL-E. The active enzyme fractions were combined and concentrated approximately 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. Each concentrated pool (5 ml) was subjected to a Mono-Q column (1.2 cm × 12 cm; Pharmacia LKB, Upssala, Sweden) equilibrated with 50 mM Tris-HCl buffer (pH 8.0) and proteins were eluted with a gradient of 0.1~0.45 M NaCl. After PUL-I was applied to the column the active fractions were pooled, concentrated and dialyzed. Each dialyzed sample was applied to a Sepharose 4B column (FPLC, Pharmacia LKB, Upsala, Sweden). The active enzyme fractions were collected, concentrated, and dialyzed against 50 mM Tris-HCl buffer (pH 8.0). Dialyzed enzyme was finally applied to a 2nd gel filtration on a Sepharose 4B column. The active enzyme peak was collected, concentrated, and dialysed against 50 mM Tris-HCl. The dialysed (6 ml) PUL-I was used for further study.

Results and Discussion

Action of exogeneous proteases on pullulanase

If pullulanase is indeed fully exposed on the surface of the bacterium, exogeneous protease might be able to degrade it or to release it in almost PUL-E form. To test this hypothesis, alkaliphilic *Bacillus* sp. S-1 was grown in minimal medium containing different proteases. Initially, cell-associated and extracellular pullulanase activities were assayed after 12 hours of incubation, and a sample of culture medium supernatant was applied to a 10%

Table 1. Effects of proteases on extracellular and cell-associated pullulanase activities produced by alkaliphilic *Bacillus* sp. S-1[a]

Portease	Concentration		Pullulanase activity (U)			
	mg/ml	U/ml	Intracellular	Cell-associated	Extracellular	Total
–	–	–	6	5	35	46
Proteinase K	1	10	5	0.7	40	45.7
Pronase E	1	44	7	1	11	19
Trypsin	1	21	7	0.5	21	28.5
Pepsin	1	11	7	1	23	31

[a] Induction medium was inoculated with a 1/100 dilution of a saturated culture of alkaliphilic *Bacillus* sp. S-1, and incubated for 24 hours at 30°C with vigorous shaking. Cells were harvested, washed twice and resuspended in unsupplemented induction medium solution. Pullulanase was assayed by the release of reducing sugar from pullulan substrate as described above. Surface-exposed activity was determined using whole cells, and total cellular activity was determined with cells lysed by sonication. Heat-inactivated samples were used in controls to compensate for reducing sugars present in the medium. The proteases had no effect on cell growth.

SDS-PAGE to determine whether secreted pullulanase was the mature form. In control cultures without protease, up to 75% of the pullulanase activity was detected in the medium in an entirely processed form, the remainder being cell-associated (Table 1). Most proteases had little or no effect on cell-bound or cell-free pullulanase (data not shown). However, pullulanase was totally absent from the cells grown in medium containing trypsin or pepsin, but the medium contained small amounts of apparently 100 kDa pullulanase. For reasons which will be discussed below, the amount of pullulanase activity recovered from the proteinase K-treated cultures was actually higher than that from the control (untreated) cultures.

Neither of these proteases caused growth inhibition or detectable lysis except at very high concentrations. Control experiments in which cells harvested from the cultures were extensively washed in 20 mM Tris-HCl buffer (pH 9.0) containing 5 mM phenylmethylsulfonyl fluoride(to present further proteolysis) and then examined by SDS-PAGE showed that pullulanase polypeptide was totally absent from the protease-grown cells. Thus the results of the protease accessibility assays confirm that at least part of the pullulanase polypeptide is exposed at the surface of the cells.

To study the effects of proteinase K on AP in greater detail, cells were grown in medium containing different concentrations of the protease; and samples were analysed at various stages of growth. Higher concentrations of proteinase K (1~5.0 mg/ml)

Table 2. Effect of proteinase-K concentration on pullulanase activity and release from alkaliphilic *Bacillus* sp[a]

Incubation time (h)	Protease (mg/ml)	Total activity (U)	% Extracellular
5	–	32	76
	0.05	35	78
	0.1	37	80
	0.2	42	80
	0.5	32	82
	2	12	84
24	–	48	76
	0.05	60	78
	0.1	72	80
	0.2	81	80
	0.5	64	82
	2	39	84

[a] Cells were incubated as indicated in Table 1 in induction medium containing different amounts of pronase. Inoculation was carried out with a 1/100 dilution of a saturated culture of alkaliphilic *Bacillus* sp. S-1, and incubated for 24 hours at 30°C with vigorous shaking. Cells were harvested, washed twice and resuspended in unsupplemented induction medium solution. Pullulanase was assayed by the release of reducing sugar from pullulan substrate as described above. Surface-exposed activity was determined using whole cells, and total cellular activity was determined with cells lysed by sonication. Total activity is the sum of the activities present in the medium and in lysed cells. Cell growth was inhibited at 3 mg/ml pronase. Heat-inactivated samples were used in controls to compensate for reducing sugars present in the medium. The proteases had no effect on cell growth.

caused the loss of AP activity, while cells growing medium containing low amounts of proteinase K retained some cell associated AP (Table 2). The most attractive explanation for the effects of proteinase K was that proteolytic cleavage close to the N-terminus released enzymatically active pullulanase from the cell surface. Although proteolysis will be necessary for the release of the AP from the cell, it is not clear whether specific peptidases are involved, or whether the non-specific intracellular proteases which are also produced by the bacteria perform this function.

Total AP activity was consistently higher in stationary phase proteinase K-treated cultures of alkaliphilic *Bacillus* sp. S-1 than in control cultures. Thus, proteinase K seemed to stabilize or to activate secreted AP. To distinguish between these two possibilities, alkaliphilic *Bacillus* sp. S-1 was grown four generations in pullulan-containing medium (to induce AP synthesis and exposition) and then washed and resuspended at the same density in induction medium containing 2% glucose (to repress further AP synthesis). Cell-associated and secreted AP activities were measured at time intervals over a period of 24 hours of further in-

cubation with or without 0.5 mg/ml proteinase K. AP activity in the control culture declined gradually to levels that were 40~70% lower than in the inoculum, whereas the activity recovered from the proteinase K-treated culture remained almost constant throughout. Bovine serum albumin (5 mg/ml) or heat-inactivated proteinase K (1 mg/ml) were without effect. Prolonged incubation of the medium containing extracelular AP with 0.2 mg/ml proteinase K resulted in decreasing its enzymatic activity; in fact, a 10~15% decline in AP activity was consistently observed after 16 hours of incubation, presumably because the AP was slightly degraded by the protease. Thus, proteinase K stabilize rather than activate AP.

Chromatographic behavior of processing stages

To examine the ion-strength of the processed polypeptides on anion exchange resin, cell extracts of alkaliphilic *Bacillus* sp. S-1, which was incubated with time course, were subjected to chromatography separation using DEAE-Toyopearl M 650 column. Sonicated cell extract of the alkaliphilic *Bacillus* sp. S-1 showed two different peaks of AP activities: one big major and one minor peak. Of them, the first major peak of AP corresponded to PUL-I polypeptide, as determined by western analysis (Fig. 1A). However, during incubation for 10 min in 30°C without PMSF, the enzyme activities of the right peak increased to about 2-fold than that of the control with concomitant decreasing of the first peak, indicating that the PUL-I polypeptide is processed to the PUL-E form. When it was incubated to trace the phenomenon for further details, PUL-I was almost converted to the second PUL-E form (Fig. 1B and 1C). These results are essentially similar to those of western analysis.

Immunological analysis of PUL-E and PUL-I: PUL-I is converted to the final PUL-X form via PUL-E, an intermediate form having pullulanase activity

To determine whether the intracellular pullulanase form is directly processed to PUL-E of extracellular form or not, cell extracts were incubated without PMSF with time course. PUL-I was processed to PUL-E form after 6 hours of incubation. But the amounts of PUL-E in solution was much less than that of 0 time incubation (Fig. 2, lane 3), indicating the PUL-E was cleaved by some signal peptidase which specifically recongizes and degrades the objective site to produce PUL-E, and also was proteolytically attacked

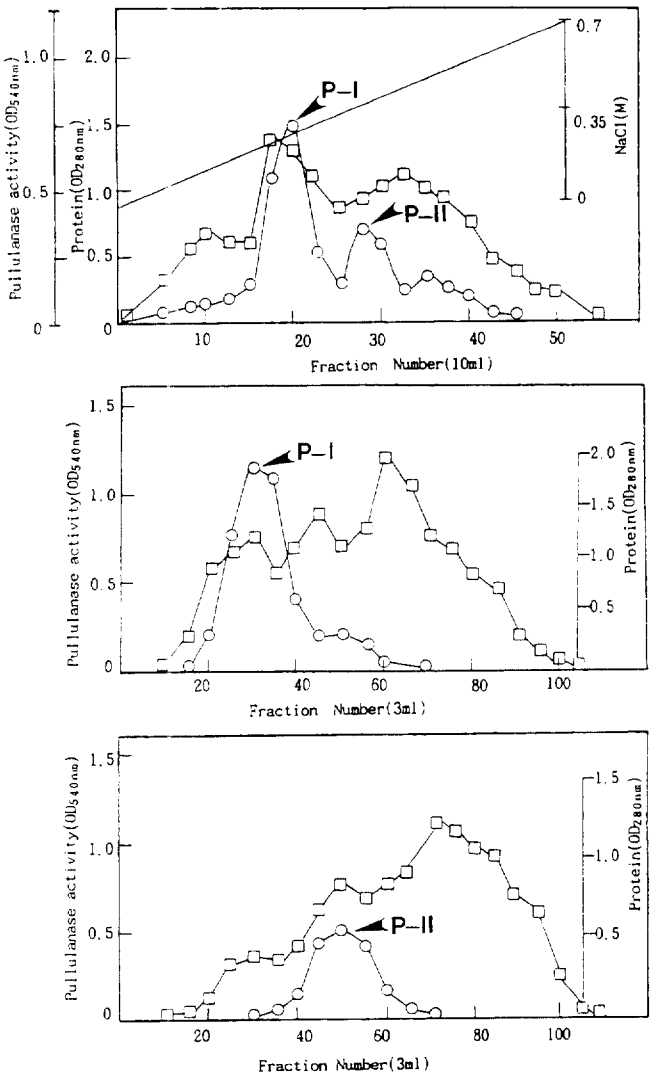

Fig. 1. Chromatographic profiles showing conversion reaction of PUL-I form to PUL-E form using whole cell lysates of the *Bacillus* sp. S-1 by DEAE Toyopearl M650 column. Chromatogram of the intracellular fraction prepared by sonication. Pullulanase activity was expressed as optical density at 540 nm. Symbols: —, NaCl gradient; □, protein concentration at 280 nm; ○, pullulanase activity at 540nm. Peaks corresponding to intracellular and extracellular pullulanase were indicated as P-I and P-II, respectively. A, Chromatogram showing co-existence of PUL-I and PUL-E just after preparation of total cell lysates with 10 mM PMSF. B, sample which was incubated for 30 min at 37°C after sonication without PMSF. C, sample which was incubated for 12 hr at 37°C after sonication without PMSF.

by other endogeneous proteases that have broad substrate specificities. The PUL-I was finally processed to about 100 kDa form of PUL-X with further incubation, even though its enzymatic activity was not defined. These results clearly suggested that the PUL-I was converted to PUL-X via PUL-I form.

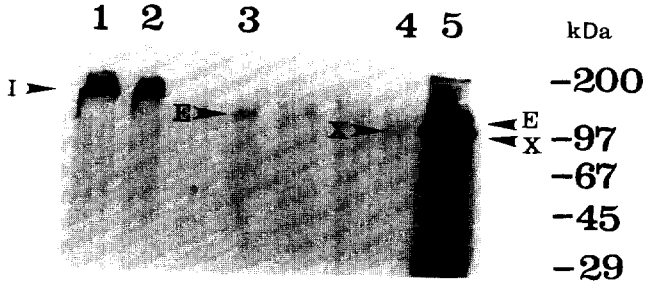

Fig. 2. Immunoblot analysis showing conversion reaction of PUL-I form to PUL-E form using whole cell lysates of the *Bacillus* sp. S-1 by anti-pullulanase serum. Intracellular fraction was prepared from the exponentially grown cells by sonication. Pullulanase activity was assayed as described above by reducing sugars released from pullulan as substrate. Bands corresponding to intracellular and extracellular pullulanase were indicated as I and E, respectively. X indicates 100 kDa protein fragment derived from PUL-I. Total and specific pullulanase activities during conversion of PUL-I form to PUL-E form activities are shown. Lanes 1 and 2, intracellular fraction prepared just after preparation of total cell lysates with 10 mM PMSF; lane 3, sample which was incubated for 2 hr at 37°C after sonication without PMSF; lane 4 (2 μg) and 5 (50 μg), sample which was incubated for 12 hr at 37°C after sonication without PMSF.

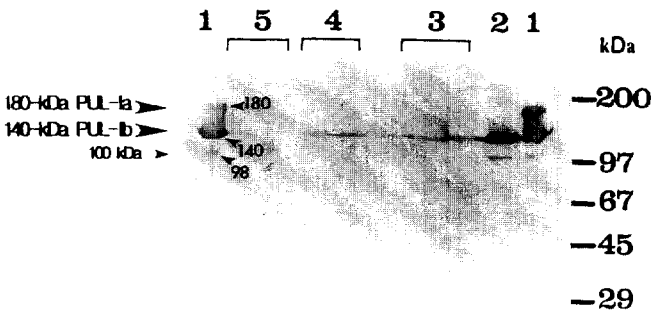

Fig. 3. Immunoblot analysis of intracellular, cell-associated, and extracellular pullulanases detected by anti-PUL serum from *Bacillus* sp. S-1. Samples were dissolved in SDS buffer containing beta-mercaptoethanol at 30°C prior to loading on the SDS-PAGE gel. Size standards (kDa) are shown on the right. Lane 1, intracellular fraction; lane 2, membrane fraction as cell-associated enzyme; lane 3, pronase-treated whole cell fraction; lane 4, extracellular media; lane 5, pronase-treated medium (PUL-E band was degraded). Proteins in the medium were precipitated with 20% trichloracetic acid and dissolved in 2% SDS sample buffer containing 0.5% beta-mercaptoethanol and heated to 100°C for 5 min. The equivalent of 0.2 ml of medium was loaded onto the gel. The arrowheads indicate pullulanase. Note that extracellular pullulanase is proteolytically modified.

Processing of PUL-I into PUL-E facilitates increasing enzymatic activity

The K_m value for pullulan of PUL-I by Lineweaver-Burk plot was 16.4 mg/ml. The Vmax value for PUL-I was 512 mg/min/ml, whereas value for PUL-E was 396 mg/min/ml. The K_m value of PUL-I was 2-fold higher than that of PUL-E (7.92 mg/ml) (19). The increase in AP activity could be attributed to "folding" of the AP protein involving cleavage of 40-kDa peptide, thus, increasing apparent enzyme activity. These peptides are not found in the extracellular form of the protein, which implies the involvement of a specific or non-specific proteolysis in the release from the membrane. However, the absence of this pre-peptide does severely affect the cell's ability to secrete AP into the medium. We cannot rule out the possibility that the prepeptide is involved in a temporary anchoring of the protein in the membrane or is protecting against proteolysis. Alternatively, the appearance of the shortened form (X) of the protein in the membrane may simply be due to the action of another intracellular protease. It can be thus considered that the affinity to substrate of PUL-I decreased because of the increase in its molecular weight and "unfolding" of the enzyme.

Immunoblot analysis and localization of intracellular, cell-associated, and extracellular pullulanases detected by anti-PUL-E serum from *Bacillus* sp. S-1

It was confirmed that pullulanase is initially incorporated mainly into the alkaliphilic *Bacillus* sp. S-1 cytoplasmic membrane and is then released into the growth medium. Full AP activity was detected with whole cells of alkaliphilic *Bacillus* sp. S-1. This result indicates that AP is probably located on the outer face of the cell membrane in alkaliphilic *Bacillus* sp. S-1. The association of AP with the envelope was apparently loose. Approximately 25% of the AP polypeptide was released by sonication (i.e., failed to sediment when centrifuged at 125,000×g for 90 min). Furthermore, less than 5% of the pullulanase remained insoluble when cell envelopes were treated with Triton X-100 plus Mg^{2+}, i.e., under conditions which failed to localize all other cell membrane proteins (data not shown).

To identify localization of AP form in cellular fractions, samples which was fractionated, were loaded on the SDS-PAGE gel, followed by immunoblotting analysis. As shown in Fig. 3 (Lane 1), intracellular fraction clearly had two distinct forms of popypeptides, one for 148 and the other for 140 kDa; and a also smaller minor polypeptide corresponding to about 100 kDa was detected; whereas membrane fraction showed one major polypeptide (140 kDa) as cell-associated enzyme with 100 kDa form (lane 2). However, when whole cell fraction was treated with proteinase K, only 140 kDa form was detected (lane 3). On the other hand, when pro-

teins in the medium were precipitated with 20% trichloracetic acid and analysed with anti-PUL-E serum, only 140 kDa form was detected in extracellular medium (lane 4). However, when the medium fraction was treated with proteinase K, PUL-E band was totally degraded without any difference in its molecular form (lane 5). These results indicate that the intracellular pullulanase form of 148 kDa was initially synthesized and translocated into cytoplasmic membrane which involved cleavage of 40 kDa polypeptide, and then the processed form being released to the medium.

As described above, the precursor form of the cell treated with PMSF was only inefficiently processed and remained cell-associated. To determine its subcellular location, cells producing this form of the enzyme were lysed in French pressure cell, and the enzyme contents of the soluble (cytoplasm) and envelope (pellet) fractions was analyzed. Over 85% of the enzyme activity was present in the membrane fraction, from which it could not be removed by 1 M NaCl or 2 M urea. This indicates that prepullulanase is firmly membrane-anchored. In contrast, as little as 30% of wild type pullulanase was present in the membrane fraction.

Effects of PMSF and incubation time on processing of PUL-I and PUL-E forms

APs were each found as two major polypeptide bands in the intracellular fraction as judged by SDS-PAGE gel. Only 140 kDa form was specifically secreted. The precursor form appeared to remain entirely intracellular, although trace amounts of an intracellular form may not have been detected in cell membrane. To see kinetics of processing and effects of PMSF on conversion, cells and medium were separated after cultivation. The cells were treated as described, and the proteins were separated on SDS-PAGE gel, and immunoreacted with anti-PUL-E serum. Large PUL-I form was processed to intermediate form of PUL-E in concentrations less than 1 mM PMSF. With decreasing concentrations of PMSF, processing yield increased, resulting in concomitant conversion of intermediate and small forms of the polypeptides. The intracellular form appeared to be less stable, as its gradual disappearance coincided with appearance of increased amounts of the PUL-E form (Fig. 5). Taken together, these results indicate that the PUL-I form is rapidly and efficiently converted into the PUL-E form, while the cell-associated PUL-E form is apparently stable and is only very slowly converted into a smaller form (labeled X in Fig. 5) that migrates slightly more slowly than the extracellular form (labeld E in Fig. 5).

On the other hand, Fig. 6. kinetic experiment of PUL-E and PUL-X formation in *Bacillus* sp. S-1 was carried out by western blot of AP extracts prepared. After cultivating with time intervals, cells were sonicated in the presence of 20 mM PMSF (f.c) to prevent further processing. Then cell extracts were analysed by western blotting. Results showed that PUL-I had begun to be produced after 4 hours of cultivation. Maximum production was reached after a 12 hour cultivation with gradual accumulation of PUL-I and concomitant increasing of extracellular and X forms.

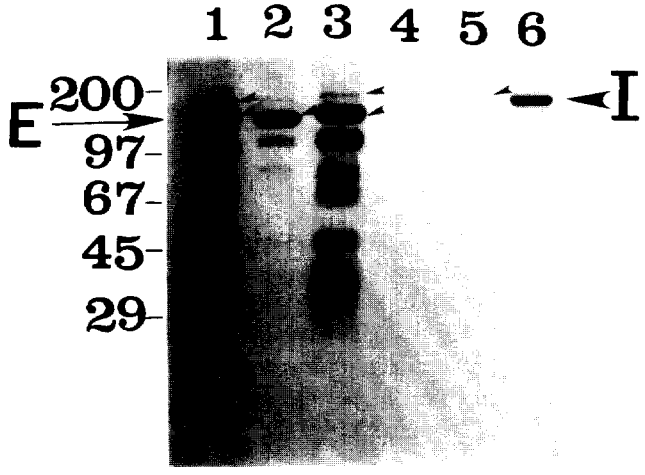

Fig. 4. Coomassie brilliant blue-stained SDS-polyacrylamide gel showing whole cell lysate and concentrated culture supernatants (S) prepared from exponentially growing (A_{660} nm=0.8~1.0) *Bacillus* sp. S-1. Molecular sizes are indicated. The positions of the intracellular and extracellular pullulanase forms are indicated by the *arrows*. Culture supernatants were concentrated 10-fold relative to whole cell lysates. Lane 1, whole cell lysate with 10 mM PMSF; lane 2, membrane fraction without PMSF; lane 3, whole cell lysate without PMSF; lane 5, purified PUL-I (0.5 μg); lane 6, purified PUL-I (10 μg).

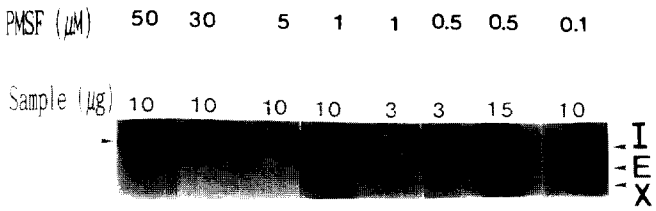

Fig. 5. Processing of PUL-I into the PUL-E and X forms produced by *Bacillus* sp. S-1. After cultivation, cells and medium were separated. The Cells were treated with various concentrations of PMSF as described in Materials and Methods, and proteins were separated on a 30-cm-long 7% acrylamide, 0.1% bisacrylamide gel, which was then blotted to Immobilon membrane (Millipore), and then immunoreacted with anti-PUL-E serum. Large, intermediate, and small forms of the polypeptides are indicated by the letters I, E, and X, respectively. Intracellular and extracellular forms are distinguished by the letters I and E.

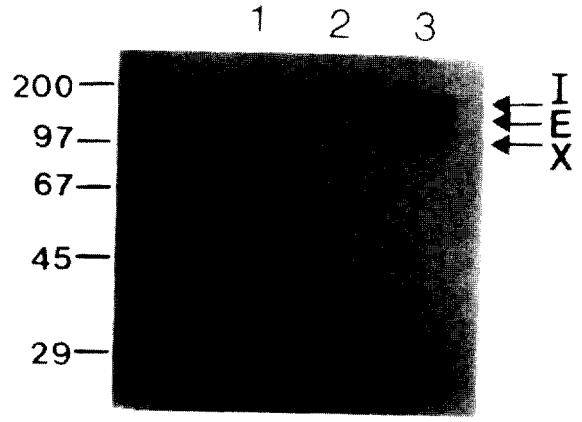

Fig. 6. Kinetics of PUL-E and PUL-X formation in *Bacillus* sp. S-1 by western blot of pullulanase extracts prepared. Cultures were sonicated with 20 mM PMSF to prevent further processing. Lane 1, cell extracts from 4 hr culture; lane 2, from 8 hr culture; lane 3, from 12 hr culture. Antibody used was 1:200 dilution of mouse anti-PUL-E. Blots were visualized using a secondary antibody linked to alkaline phosphatase. Large, intermediate, and small forms of the polypeptides are indicated by the letters I, E, and X, respectively. Intracellular and extracellular forms are distinguished by the letters I and E. Molecular markers used were prestained proteins ranging from 29 to 200 kDa.

Western blot of purified PUL-I differed in the apparent M_r of PUL-E. There was an apparent decrease in size of the polypeptide. The extracellular pullulanase was in a single enzyme form in the extracellular bacterial system. As described previously (8), only 140 kDa PUL-E form was extracellularly immunoreacted throughout the cultivation. With these results it was suggested that the AP protein is produced extracellularly as a single enzyme in alkaliphilic *Bacillus* sp. S-1; and thus concluded that when the 180 kDa M_r protein is present in cell extracts it can undergo processing during purification. The increase in AP activity has also been attributed to "folding" of the AP protein, thus increasing apparent enzyme activity, even though unfolded proteins are generally better substrates for proteolytic digestion than their folded counterparts (27). However, in alkaliphilic *Bacillus* sp. S-1 extracts the formation of a multiplicity of separable AP activities is an indication that limited proteolysis does not necessarily lead to loss of enzyme activity.

A number of questions containing the secretion of the AP remain to be answered. First, how can endogeneous proteases cause processing at the N-teminus? One possibility is that N-terminus of the intracellular pullulanase is accessible to proteases. Another possibility is that the 40 kDa peptide extension in PUL-I affect the overall conformation of

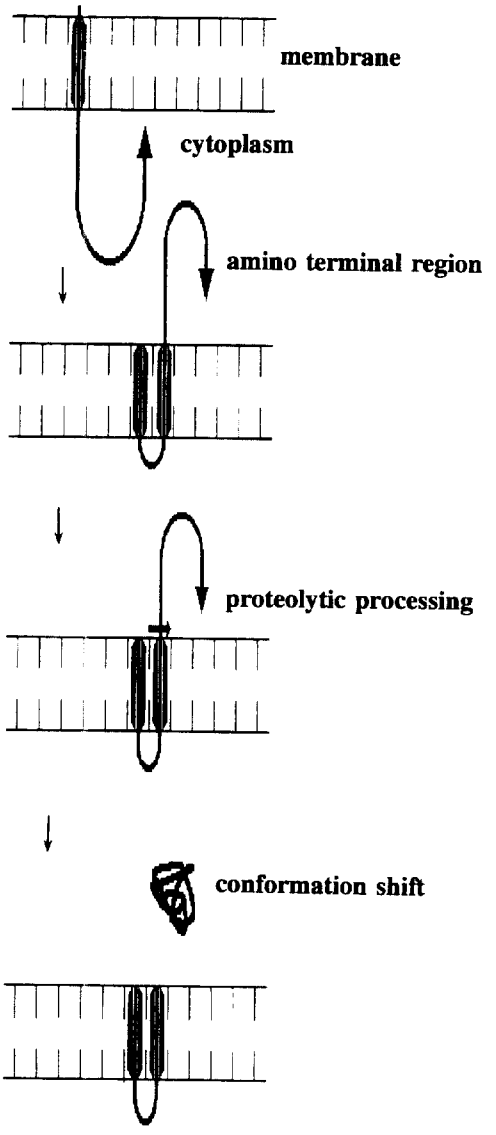

Fig. 7. Mode depicting the translocation of wild-type pullulanase in *Bacillus* sp. S-1.

the polypeptide in such a way as to expose its C-terminus to proteolytic attack. Second, the identity of the enzyme that processess the PUL-I is unknown. Processing may be carried out by nonspecific cytoplasmic or cytoplasmic membrane peptidases, since the length of processing is clearly different from that recognized by generally known peptidases (30).

In conclusion, pullulanase provides a simple and accessible experimental system for studying protein secretion in alkaliphilic *Bacillus* sp. S-1 as shown in Fig. 7. This protein is secreted by two-step mechanism which is well characterized both temporally and spatially. The first step involves the proteolytic cleavage of the N-terminal signal sequence. The second step, which is the rate-limitting step of the pathway, consists of the release

of the resulting, processed, membrane form to the extracellular medium.

References

1. **Ara, K., K. Igarashi, K. Saeki, S. Kawai, and S. Ito.** 1992. Purification and some properties of an alkaline pullulanase from alkalophilic *Bacillus* sp. KSM-1876. *Biosci. Biotech. Biochem.* **56**, 62-65.
2. **Bernfeld, P.** 1955. Amylases, α and β. *Methods Enzymol.* **1**, 149-158.
3. **Chambert, R. and M-F. Petit-Glatron.** 1988. Secretion mechanism of *Bacillus subtilis* levansucrase: characterization of the second step. *J. Gen. Microbiol.* **234**, 1205-1214.
4. **Davis, B.J.** 1964. Disk electrophoresis. II. Methods and application to human serum proteins. *Ann. N.Y. Acad. Sci.* **121**, 404-427.
5. **Gooderham, K.** 1983. *In* Techniques in Molecular Biology (Walkerand, J. M. and Gaastra, W., eds.), pp.49-87, Croom Helm Publishers, London.
6. **Kim, C.H.** 1994. Specific detection of pullulanase type I on polyacrylamide gels. *FEMS Microbiol. Lett.* **116**, 327-332.
7. **Kim, C.H., H.I. Choi, and D.S. Lee.** 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.
8. **Kim, C.H., H.I. Choi, and D.S. Lee.** 1993. Purification and biochemical properties of extracellular alkaline pullulanase from an alkalophilic *Bacillus* sp. S-1. *Biosci. Biotechnol. Biochem.* **57**, 1632-1638.
9. **Kim, Y.S. and C.H. Kim.** 1995. Purification and detailed characterization of a bifunctional amylase-pullulanase enzyme from *Bacillus circulans* having two different active sites on one polypeptide. *Eur. J. Biochem.* **227**, 687-693.
10. **Kornacker, M.G. and A.P. Pugsley.** 1989. Molecular characterization of *pulA* and its product, pullulanase, a secreted enzyme of *Klepsiella pneumoniae* UNF5023. *Molecular Microbiology* **4**, 73-85.
11. **Kuriki, T. and T. Imanaka.** 1989. Nucleotide sequence of the neopullulanase gene from *Bacillus stearothermophilus*. *J. Gen. Microbiol.* **135**, 1521-1528.
12. **Laemmli, U.K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4, *Nature* **227**, 680-685.
13. **Lee, M.J., B.S. Kang, D.S. Kim, Y.T. Kim, S.K. Kim, K.H. Chung, J.K. Kim, K.S. Nam, Y.C. Lee, and C.H. Kim.** 1997. Processing of an intracellular immature pullulanase to the mature form involves enzymatic activation and stabilization in alkalophilic *Bacillus* sp. S-1. *J. Biochem. Molec. Biol.* **30**, 46-54.
14. **Lee, M.J., Y.C. Lee, and C.H. Kim.** 1997. Intracellular and extracellular forms of alkaline pullulanase of an alkalophilic *Bacillus* sp. S-1. *Arch. Biochem. Biophys.* **337**, 308-316.
15. **Lowry, O.H., N.H. Rosenbrough, A.L. Farr, and A. Randall.** 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265-275.
16. **Melaswien, H., M. Paloheimo, and L. Hemio.** 1990. Nucleotide sequence of the α-amylase-pullulanase gene from *Clostridium thermohydrosulfurium*. *J. Gen. Microbiol.* **136**, 447-454.
17. **Nakamura, K., A. Nakamura, H. Takamatsu, H. Yoshikawa, and Yamane, K.** 1990. Cloning and characterization of a *Bacillus subtilis* gene homologous to *E. coli secY*. *J. Biochem.* **107**, 603-607.
18. **Nakamura, N., N. Sashihara, H. Nagayama, and K. Horikoshi.** 1989. Characterization of pullulanase and α-amylase activities of a *Thermus* sp. AMD33. *Starch/Starke* **41**, 112-117.
19. **Obido, F.J.C. and S.K.C. Obi.** 1988. Purification and characterization of a thermostable pullulanase from *Thermoactinomyces thalpophilus*. *J. Industrial Microbiol.* **3**, 343-350.
20. **Ohba, R. and S. Veda.** 1982. An inductive effector in production of extracellular pullulanase by *Aerobacter aerogenes*. *Agric. Biol. Chem.* **46**, 2425-2431.
21. **Ohba, R., H. Chaen, S. Hayashi, and S. Veda.** 1978. Immobilization od *Streptomyces flavochromogenes* pullulanase on tannic acid and TEAE-cellulose. *Biotechnol. Bioeng.* **20**, 665-676.
22. **Petit-Glatron, M.-F., F. Benyahia, and R. Chambert.** 1987. Secretion of *Bacillus subtilis* levansucrase: a possible two-step mechanism. *Eur. J. Biochem.* **163**, 379-387.
23. **Plant, A.R., H.W. Morgan, and R.M. Daniel.** 1986. A highly stable pullulanase from *Thermus aquaticus* YT-1. *Enz. Microb.Technol.* **8**, 668-672.
24. **Possot, O., d'Enfert, I., Reyss, and A.P. Pugsley.** 1992. Pullulanase secretion in *Escherichia coli* K-12 requires a cytoplasmic protein and a putative polytopic cytoplasmic membrane protein, *Molecular Microbiology* **6**, 95-105.
25. **Pugsley, A.P. and I. Reyss.** 1990. Five genes at the 3' end of the *Klepsiella pneumoniae pulC* operon are required for pullulanase secretion. *Molecular Microbiology* **4**, 365-379.
26. **Schulein, M. and B. Hojer-De.** 1984. Characterization of a new class of thermophilic pullulanases from *Bacillus acidopullulyticus*: comparison with *Aerobacter aerogense* pullulanase; compatibility with Aspergillus niger glucoamylase. *Ann. N.Y. Acad. Sci.* **434**, 271-274.
27. **Scotti, P.A., R. Chambert, and M.-F. Petit-Glatron.** 1995. Kinetics of the unfolding-folding transition of *Bacillus subtilis* levansucrase precursor. *FEBS Lett.* **360**, 307-309.
28. **Suzuki, Y., K. Hatagaki, and H. Oda.** 1991. A hyperthermostable pullulanase produced by an extreme thermophile, *Bacillus flavocaldarius* KP1228, and evidence for the proline theory of increasing protein thermostability. *Appl. Microbiol. Biotechnol.* **34**, 707-714.
29. **Yamashita, M., A. Nakagawa, N. Katsuragi, and Y. Murooka.** 1992. Role of lipid modification on a starch-debranching enzyme, *Klepsiella* pullulanase: comparison of properties of lipid-modified and unmodified pullulanases. *Molecular Microbiology* **6**, 389-394.
30. **Wolfe, P.B., P. Silver, and W. Wickner.** 1982. The isolation of homogeneous leader peptidase from a strain of *Escherichia coli* which overproduces the enzyme. *J. Biol. Chem.* **257**, 7898-7902.