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Asp 280 residue is important in the activity of the *Escherichia coli* leader peptidase

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Abbreviations: SDS, sodium dodecyl sulfate; IPTG, isopropyl-1-thio-β-D-galactopyranoside; OmpA, outer membrane protein A; Lep, leader peptidase

Abstract

Leader peptidase is a novel serine protease in *Escherichia coli*, which catalyzes the cleavage of amino-terminal signal sequences from exported proteins. It is an integral membrane protein containing two transmembrane segments with its carboxy-terminal catalytic domain residing in the periplasmic space. Recently, the x-ray crystal structure of signal peptidase-inhibitor complex showed that Asp 280, a highly conserved consensus sequence of *E. coli* leader peptidase is the closest charged residue in the vicinity of two catalytic dyad, Ser 90 and Lys 145, and it is likely held in place by a salt bridge to Arg 282. Possible roles of Asp 280 and Arg 282 in the structure-catalytic function relationship were investigated by the site-directed mutagenesis of Asp 280 substituted with alanine, glutamic acid, glycine, or asparagine and of Arg 282 with methionine. All of mutants purified with nickel affinity chromatography were inactive using *in vitro* assay. It is surprising to find complete lose of activity by an extension of one carbon units in the mutant where Asp 280 is substi-tuted with glutamic acid. These results suggest that Asp 280 and Arg 282 are in a sequence which constitutes catalytic crevice of leader peptidase and are essential for maintaining the conformation of catalytic pocket.

Keywords: *Escherichia coli*, leader peptidase (signal peptidase) site-directed mutagenesis

Introduction

Leader(signal) peptidase is a member of novel class of serine proteinases which utilize serine and lysine residues for catalysis (Sung and Dalbey, 1992; Tschantz *et al*., 1993), and an essential protein in both prokaryotic and eukaryotic cells. It has been purified from *Escherichia coli* (Zwizinski *et al*., 1980), yeast (YaDeau and Blobel, 1989), chicken (Baker and Lively, 1987), and dog (Greenburg *et al*., 1989), and identified in *Salmonella typhimurium* (van Dijl *et al*., 1990), *Pseudomonas fluorescens* (Black *et al*., 1992) and *Bacillus subtilis* (van Dijl *et al*., 1992). Gene cloning and mutagenesis techniques have been employed to study the membrane biogenesis of leader peptidase and to determine its physiological role (Dalbey and Wickner, 1985 and 1987). The active site of the leader peptidase residues in the periplasmic domain, which is anchored to the membrane by two transmembrane segments (Figure 1A; Bilgin *et al*., 1990). It consists of a single polypeptide chain with a molecular weight of 35,988 Da (323 amino acid residues), contains one disulfide bond, and has an isoelectric point of 6.9 (Wolfe *et al*., 1983).

Recently, there has been a very important development in understanding the catalytic mechanism of leader peptidase. Paetzel *et al*. (1998) have solved the x-ray cystal structure of an *Escherichia coli* leader peptidase (Δ2-75, Mr 27,952 Da, 249 amino acids) which retains catalytic activity. The structure has been determined at 1.9 Å resolution with a 5S, 6S β-lactam inhibitor covalently bound as an acyl enzyme intermediate to Ser 90. In the structure of leader peptidase, aspartic acid 280 is the closest charged group in the vicinity of catalytic active sites, serine 90 and Lys 145, and it is likely held in place by a salt bridge to arginine 282 (Figure 1). In addition, there are four highly conserved regions of sequence which have been classified within the catalytic domain of signal peptidases commonly termed Box B, C, D, and E (Dalbey *et al*., 1997). Box E contains the highly conserved residues, Asp 280 and Arg 282 (Table 1).

The study of catalytic mechanism of leader peptidase has been specially emphasized in the antibacterial drug design since the location of the bacterial leader peptidase in either the periplasm or extracellular space makes accessible target for antibiotics. In order to identify a possible role of Asp 280 in the *E. coli* leader peptidase protein, we generated mutant proteins with specific substitutions using site-directed mutagenesis. The result of our study suggests that Asp 280 residue plays an important role in stabilizing *E. coli* leader peptidase structure, possibly, through salt bridges to Arg 282, however, it has not excluded the possibility of the catalytic role of Asp 280 in the leader peptidase protein.

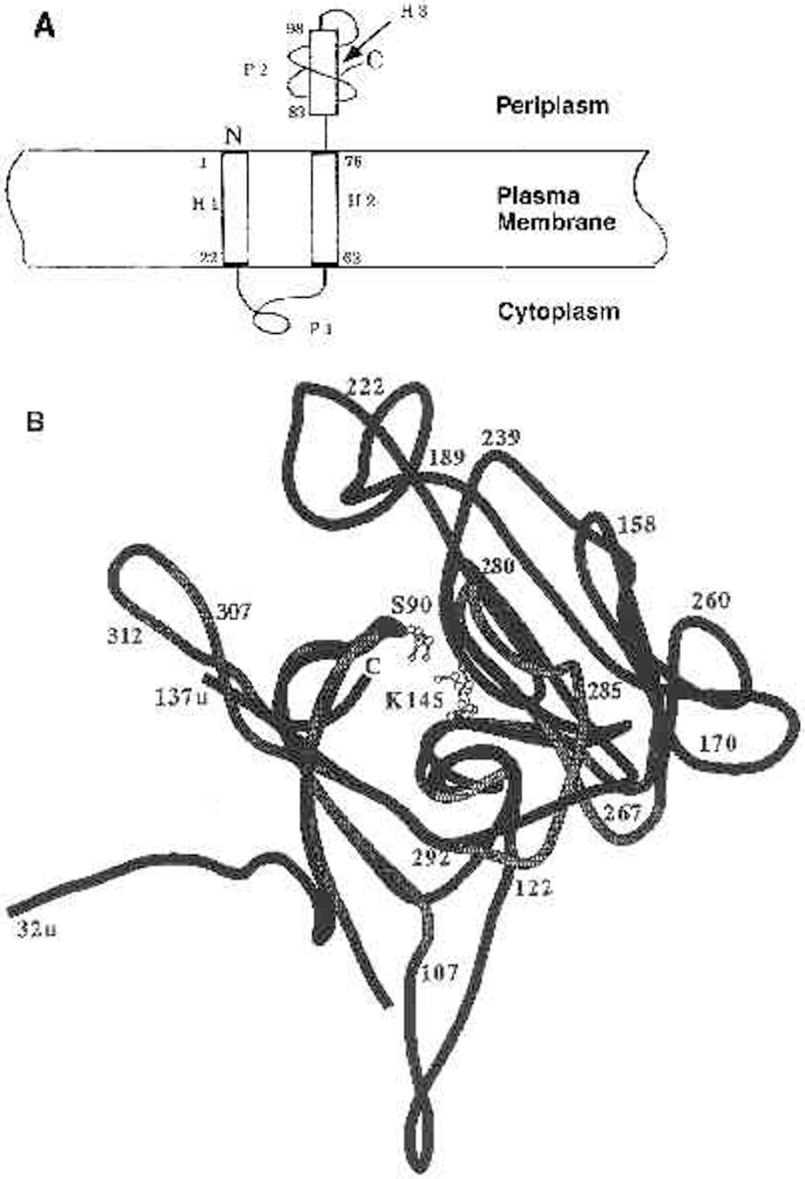

Figure 1. A. Membrane topology of *E. coli* leader peptidase. H1, H2, and H3 are hydrophobic domains, and P1 and P2 are polar domains. B. A ribbon diagram depicting the general fold of leader paptidase Δr 2-75. The Asp 280 is the nearest residue around the catalytic active sites, Ser 90 and Lys 145. The figure is reproduced from Paetzel *et al*., 1998.

Materials and Methods

Materials

Oligonucleotides were synthesized at the IDT(Integrated DNA Technologies, Inc., IA, U.S.A.). The Ni-NTA resin was purchased from Qiagen Inc.(CA, U.S.A.).

Bacterial strains and plasmids

E. coli strains, SB221 and MC1061 were from the Ohio State University. The cloning of the pro-OmpA nuclease A gene into the IPTG-inducible plasmid pONF1 (Takahara *et al*., 1985) and the overexpression of the protein were described by Chatterjee *et al*. (1995). Oligonucleotide-directed mutagenesis was used to engineer 6 consecutive histidine residues into the cytoplasmic domain of leader peptidase that can purify the mutants from the chromosome-expressed wild-type leader peptidase. Amino acid residues at the position 35-40 were substituted with histidine residues. The sequence of the oligonucleotide used is as follows: 5'-TTC GCA CCT AAA CGG CGG CGC GAA CGT CAT CAT CAT CAT CAT CAT GCT CGG GAC TCA CTG GAT AAA GCA- 3'.

Purification of the 6-His Tagged Leader Peptidase Proteins

The 6-His tag/nickel affinity chromatography was used to purify the overexpressed mutant leader peptidases away from the wild-type chromosome-expressed copies of leader peptidase. All leader peptidase proteins including the wild-type, contained the 6-His residues. *E. coli* MC1061 cells containing the pET plasmid encoding the mutant leader peptidase protein were grown in LB media (2L) containing 100 μg/ml ampicillin and 0.2% sucrose until an absorbance of 0.45 at 600 nm was reached. Expression was induced by the addition of arabinose to a final concentration of 0.2%, and the incubation of the cultures was continued for 4 h. The cells were pelleted and then resuspended in an equal weight of 50 mM Tris, pH 7.5, 10% sucrose. The cells were frozen by dropping them into liquid nitrogen and stored at -80˚C until required. Marble-sized nuggets of frozen cell suspension (30 g) were thawed by addition to 100 ml of buffer (10 mM Tris-Cl, pH 8.5, 5 mM EDTA, 20% sucrose) rapidly stirring at 23˚C. Lysozyme (20 mg) and DNase I (2 mg) were added and the suspension was stirred for 30 min at room temperature. It was then frozen in a bath of dry ice and ethanol, thawed in a bath of tap water, mixed with 1 ml of 1 M magnesium acetate, and stirred for 30 min at room temperature. The solution was then centrifuged at 18,000 rpm (4˚C, 30 min), and the pellet was resuspended in 30 ml buffer (10% glycerol, 10 mM triethanolamine chloride, pH 7.5) and centrifuged again. The membrane pellet was resuspended at 0˚C in binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl pH 8.0, 1% Triton X-100, 10 mM β-mercaptoethanol) with a Dounce homogenizer. Detergent-insoluble material was removed by centrifugation at 18,000 rpm. The Triton X-100 extract was applied at 4˚C to a 1 ml nickel column which was equilibrated with the binding buffer. The column was then washed with 20 ml of binding buffer followed by a second wash with wash buffer (60 mM imidazole, 0.5 M NaCl, 10 mM Tris, pH 8.0, 1 % Triton X-100, 10 mM β-mercapto-ethanol). The 6-His tagged leader peptidase was eluted by an imidazole gradient buffer (100, 200, 300, 400, and 500 mM imidazole each, 0.5 M NaCl, 20 mM Tris-HCl, pH 8.0). The eluted fraction was collected in 1 ml each concentration, assayed for protein by SDS-PAGE and visualized by Coomassie brilliant blue staining. Fractions containing leader peptidase activity were pooled and stored at -80˚C.

Purification of the pro-OmpA nuclease A

The *E. coli* strain SB221 bearing the plasmid pONF1 was used to overexpress the pro-OmpA nuclease A substrate, which is a hybrid protein of staphylococcal nuclease A fused to the signal peptide of the outer membrane protein (OmpA) (Takahara *et al*., 1985). The cells were grown to the late log phase in M9-casamino acids medium supple-mented with 5 μg/ml leucine and 5 μg/ml tryptophan. Pro-OmpA nuclease A was induced for 4 h by addition of 2 mM IPTG and purified as described by Chatterjee *et al*. (1995) and used as substrate for estimating the activity of leader peptidase.

Assay for the Processing of Pro-OmpA Nuclease A

Reaction mixture (13.5 μl) containing 1 μl of leader peptidase (final concentration 0.364 μM) was incubated at 37˚C with 9 μL of pro-OmpA nuclease A (6 μM) in 50 mM Tris, pH 8.0 in the presence of 1 % Triton X-100. The reaction was terminated by adding 5× dye supplemented with 100 mM $MgCl_2$, 5% SDS, 25% glycerol, 200 mM β-mercaptoethanol, 200 mM Tris (pH 6.8) and immediately freezing the mixture at -70˚C. The samples were analyzed on 17% SDS-polyacrylamide and the gel was stained with Coomassie Brilliant Blue. The concentration of Pro-OmpA nuclease was determined at 280 nm (E at 280 nm = 8.31).

Western blot analysis

0.5 μg of purified leader peptidase proteins was electrophoresed in a 17% SDS polyacrylamide gel and the proteins in the gel were then electroblotted to a nitrocellulose membrane. The membrane was treated with the anti-rabbit polyclonal leader peptidase antibody (1:10,000 dilution) and then with alkaline phosphatase (Pierce, IL, U.S.A.). The membrane was visualized using nitro blue tetazolium chrolide and 5-bromo-4-chloro-indoyl phosphate according to the manufacturer's protocol.

Results

Since it is likely that Asp 280 residue is critical to signal peptidase structure and function, each of mutants was constructed and purified to evaluate for a possible role of D280 using an *in vitro* assay. We have designated XNY (X, amino acids in the wild-type protein; N, the position of the amino acids; Y, the new substituted amino acids). The sequence of the mutagenic primers are shown in Table 2, and the sites of mutations can be seen in the working model of leader peptidase in Figure 1B. The genes encoding these mutant proteins were subcloned into the pET-23d vector, which is a T7 polymerase-dependent used in the expression of high levels of protein. The mutant proteins were then purified using a nickel affinity column as described in 'Materials and Methods'. The yield of each purified mutant was approximately the same. Typically, 4.6 mg of purified mutants was obtained from 1 L cell culture. Each of purified proteins, WT (lane 1), D280A (lane 2), D280E (lane 3), D280G (lane 4), D280N (lane 5), and R282M (lane 6) were shown in Figure 2A. The wild-type of leader peptidase was purified to its homogeneity but all of the mutant proteins were shown additional protein bands. In order to see antigenic

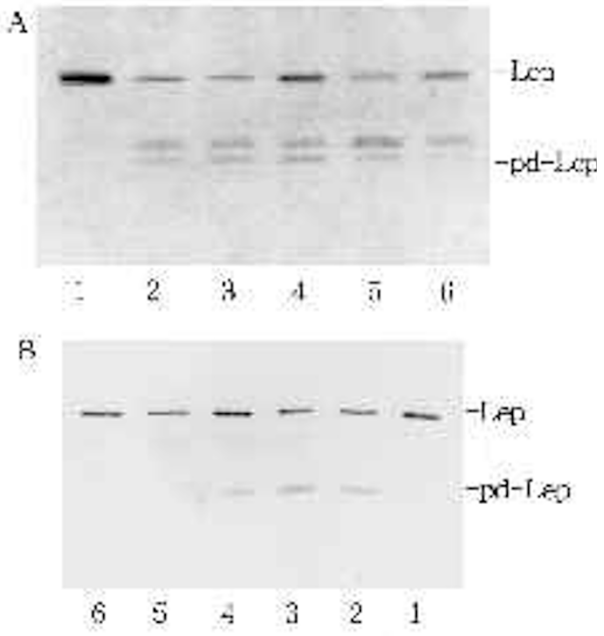

Figure 2. A. Purification of the mutant leader peptidases. WT (lane 1), D280A (lane 2), D280E (Lane 3), D280G (lane 4), D280N (lane 5), and R282M (lane 6), each of 0.5 μg, leader peptidase were applied to a SDS-PAGE (17%), and the gel was stained by Coomassie Brilliant Blue. B. Western blot analysis of the mutant leader peptidases. The proteins in the gel as indicated in panel A were reacted with the anti-rabbit polyclonal leader peptidase antibody and the color reaction was developed as described in the 'Materials and Methods'. Lep indicates the position of wild-type and mutant leader peptidases and pd-Lep indicates the position of partiallly degraded mutant leader peptidases.

Table 1. Conserved domain (E) of leader(signal) peptidases.

		* *
Lep (Eco)	272	GDNRDNSA**D**S**R**
Lep (Sty)	273	GDNRDNSA**D**S**R**
Lep (Pfl)	225	GDNRDNSN**D**S**R**
Lep (Hin)	297	GDHRDHSD**D**S**R**
Lep (Rca)	207	GDNRDNSE**D**S**R**
Sip (Bja)	195	GDNRDNSA**D**S**R**
Lep (Pla)	160	GDNRNNSY**D**SH
Sip (Mtu)	237	GDNRTHSA**D**S**R**
SpsB(Sau)	146	GDNREVSK**D**S**R**
SipS (Bsu)	145	GDNRRNSM**D**S**R**
SipT (Bsu)	154	GDNRLNSM**D**S**R**
SipU (Bsu)	148	GDNRLNSL**D**S**R**
SipP (pTA1015)	147	GDNRQESM**D**S**R**
SipP (pTA1040)	146	GDNRQNSM**D**S**R**
SipS (Bam)	146	GDNRRNSM**D**S**R**
SipT (Bam)	154	GDNRLNSM**D**S**R**
Sip (Bli)	147	GDNRQRSM**D**S**R**
Sip (Bca)	144	GDNRLSSW**D**S**R**
Imp1p	130	GDNLSHSL**D**S**R**
Imp2p	123	GDNYFHSI**D**SN
Sip (Mja)	138	GDNNPI H**D**PE
Sec11	102	GDNNAG-N**D**IS
Spc18	115	GDN--NAV**D**D**R**
Spc21	127	GDN--NEV**D**D**R**

Bam, *Bacillus amyloliquefaciens*; Bca, *Bacillus caldolyticus*; Bja, *Bradyrhizobium japonicum*; Bli, *Bacillus licheniformis*; Bsu, *Bacillus subtilis*; Eco, *Escherichia coli*; Hin, *Haemophilus influenzae*; Imp, Inner membrane protease; Lep, leader peptidase; Mja, *Methanococcus jannasxhii*; Mtu, *Mycobacterium tuberculosis*; Pfl, *Pseudomonas fluorescens*; Pla, *Phormidium laminosum*; Rca, *Rhodobacter capsulatus*; Sau, *Staphylococcus aureus*; Sip, signal peptidase; SPC, signal peptidase complex; Sty, *Salmonella typhimurium*.

reactivity of these purified mutant proteins, the western blot analysis was performed using anti-rabbit polyclonal leader peptidase antibody. The additional band (bottom band in Figure 2B) as well as the top band showing the whole structure of protein is also detected in the Asp 280 mutants, suggesting that it is the partially degraded *E. coli* leader peptidase protein. Since no additional band was seen in the wild-type and G272A mutant leader peptidase (data not shown), we believe that the utation of D280 impacts on the stable structure of leader peptidase protein and, therefore, the unstable protein is easily degraded during purification procedures.

Activity of mutant leader peptidases was assayed (Figure 3) using pro-OmpA nuclease A, an excellent substrate for the wild-type leader peptidase (Chatterjee *et al*., 1995). In comparison of the activity of wild-type leader peptidase with background activity, this assay system is 10 fold more sensitive than that of *in vitro* system that previously was used to show the serine 90 and lysine 145 are possible active site residues (Sung and Dalbey, 1992; Tschantz *et al*., 1993). An aliquot containing 0.364 μM (undiluted) of each purified leader peptidase proteins, after dilution (1:10, $1:10^2$, $1:10^3$, $1:10^4$, $1:10^5$) with a buffer (20 mM Tris-HCl, pH 8.0) was incubated with the pro-OmpA nuclease A substrate at 37˚C for 30 min and analyzed by SDS-PAGE and Coomassie Brilliant Blue staining. All of the mutant leader peptidases except D280G had $1/10^3$ the activity of wild-type protein corresponding to background enzymatic activity. It is interesting to note that the D280G mutant leader peptidase had a lower processing activity ($1/10^2$ to $1/10^3$ fold activity of wild-type protein). Since the residues, aspartic acid 280 and arginine 282 are possibly connected by a salt bridge, we believe that D280 and R282 residues are important in stabilizing the whole structure of leader peptidase protein. Three double leader peptidase mutants were made by remaining the charges (D280E, R282K), exchanging two residues (D280R, R282D), and engineering disulfide bond (D280C,

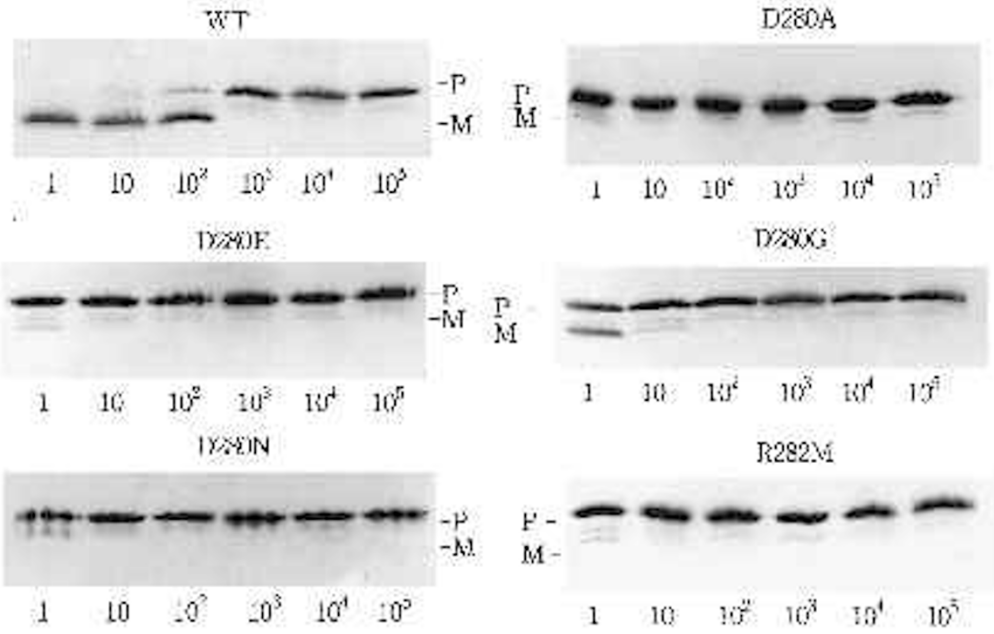

Figure 3. *In vitro* leader peptidase processing of the pro-OmpA protein. Leader peptidases, wild-type, D280A, D280E, D280G, D280N, and R282M were assayed for activity in the *in vitro* assay using pro-OmpA nuclease A as a substrate. The reaction was described in the 'Materials and Methods' and used either directly (without dilution) or after dilution (1:10, $1:10^2$, $1:10^3$, $1:10^4$, $1:10^5$) with a buffer (20 mM Tris-HCl, pH 8.0). The processing of pro-OmpA nuclease A (P) to nuclease A (M) was analyzed by SDS-PAGE using a 17 % polyacrylamide gel and stained by Coomassie brilliant blue staining.

Table 2. Sequence of leader peptidase mutants of the *Lep B* gene.

Sequence of oligonucleotides	Changes
GACAACAGCGCG<u>GCC</u>AGCCGTTACTGGGGC	D280A
GACAACAGCGCG<u>GAG</u>AGCCGTTACTGGGGC	D280E
GACAACAGCGCG<u>GGC</u>AGCCGTTACTGGGGC	D280G
GACAACAGCGCG<u>AAC</u>AGCCGTTACTGGGGC	D280N
GACAACAGCGCG<u>GAC</u>AGCCGTTACTGGGGC	D280M
GACAACAGCGCG<u>GTG</u>AGC<u>TGT</u>TACTGGGGC	D280C, R282C
GACAACAGCGCG<u>GAG</u>AGC<u>AAG</u>TACTGGGGC	D280E, R280K
GACAACAGCGCG<u>CGC</u>AGC<u>GAT</u>TACTGGGGC	D280R, R282D

The codons, which were changed to make the mutations, are underlined.

R282C) to mimic the bridge. All of three mutant leader peptidases were purified and assayed using pro-OmpA substrate. They also had a very low activity as the other single mutants (data not shown).

Discussion

Bacterial leader peptidases are not inhibited by any of the standard inhibitors to serine, cysteine, metallo-, or aspartic proteases (Zwizinski *et al*., 1981). Furthermore, extensive site-directed mutagenesis and chemical modification studies have revealed that bacterial leader peptidases have an essential Ser and Lys residue, but no essential His or Cys residues (Black MT, 1993; van Dijl *et al*., 1995). Based on these studies, it has been proposed that leader peptidases belong to a unique class of serine proteases which utilize a mechanism whereby the lysine acts as the general base to extract the proton from the hydroxyl side-chain of the serine.

Since Asp 280 and Arg 282 residues of *E. coli* leader peptidase are possibly connected by salt bridges around the catalytic active sites, Ser 90 and Lys 145, and they are the highly conserved residues in the Box E domain, it has been proposed that the residues possibly make essential contributions to the stability of active site or function of the protein. In this study, the single (D280A, D280E, D280G, D280N, and R282M) or double [(D280C, R282C), (D280E, R282K) and (D280R, R282D)] mutant leader peptidases are purified with a nickel affinity column and assayed using an *in vitro* assay, resulting in an inactive enzyme. This shows that the Asp 280 and Arg 282 residues in *E. coli* leader peptidase play a critical role in the activity of the protein. All of D280 mutant leader peptidases show additional two bands on Coomassie brilliant blue staining gel but one additional band on the western blot analysis. Since wild-type of purified protein shows homogeneous one band without an additional

band, we think that the D280 mutant produces additional bands by impacting the stable whole structure of protein. The purified R282M mutant also shows an additional band in the Coomassie brilliant blue staining gel like D280 mutants but not in the western blot analysis, suggesting that the additional band could be the product of impure leader peptidase protein rather than the partially degraded protein. When we performed CD spectroscopy on the purified K145A, the result of CD spectrum is similar to that of the wild-type protein (Tschantz *et al*., 1993). From the fact that there is no large conformational change even in K145A mutant and the x-ray structural aspect of Asp 280 with a salt bridge to R282, it is unlikely that Asp 280 mutant proteins are inactive as the result of global conformational changes. In order to show that there were no conformational changes within the mutant leader peptidases, a competition study between inactive D280 mutants and wild-type of leader peptidase for substrate binding will be taken as one of additional studies. It is also unlikely that Asp 280 play a role as a catalytic triad observed in the classic serine proteinases since it would require a significant substrate induced conformational change to make the Lys145 - Asp 280 interaction (Paetzel *et al*., 1998). In this stage, we speculate that Asp 280 provides primarily a structural role in maintaining the integrity of the active site residues.

In summary, there are unanswered questions regarding the exact mechanism of the leader peptidase. It is clear from *in-vitro* study that Asp 280 and Arg 282 are critical residues for the activity of leader peptidase protein, and it does not yet definitely provide the evidence as a proposed structure role. The forthcoming x-ray crystal structure of the soluble fragment of *E. coli* leader peptidase may contribute to answer this question regarding the detail catalytic mechanism of leader peptidase.

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Retraction

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The above article was retracted by the authors because of irreproducible *in vitro* assay data.