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D99 Type I Signal Peptidase Implicated Stabilizing the Protein Structure

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Type I signal peptidase is an integral membrane protein that functions to cleave signal peptides from secreted and membrane proteins. The enzyme serves as a potential target for the development of novel antibacterial agents due to its unique physiological properties. Despite being one of the best characterized enzymes, the catalysis of Type I signal peptidase still remains controversy over the catalytic serine/lysine dyad mechanism. It appears that the dyad proteases are generally less efficient than the prototypical serine/histidine/aspartic acid triad found in most enzymes, although Type I signal peptidase is an exception to this rule. In this paper, we have proposed that Type I signal peptidase may act as the serine/lysine/aspartic acid triad cataltytic mechanism. Therefore, the aspartic acid 99 residue in the *E. coli* signal peptidase was chosen and mutated to an alanine to see if there is any possible role of the aspartic acid in the catalytic function. Type I signal peptidase D99A protein was inactive *in vitro* assay using the procoat synthesized by *in vitro* transcription translation. However, the mutant was active using a highly sensitive *in vivo* assay. Pulse-chase experiments show that the replacement of aspartic acid 99 with alanine results in a very unstable signal peptidase molecule. Therefore, we conclude that it is unlikely that the residue is directly involved in catalysis, but rather plays an important role in stabilizing the protein structure.

Key words: Signal peptidase, site-directed mutagenesis

Proteases are wide spread in all types of bacteria, where they are involved in critical processes such as colonization and evasion of host immune defense, acquisition of nutrients for growth and proliferation, facilitation of dissemination, or tissue damage during infection [1]. Most of the proteases fall within four groups: the serine, cysteine, aspartic acid and metalloproteases.

The standard mechanism for the serine proteases involves, in addition to the serine residue that carries the nucleophilic attack, a proton donor/general base. It is called the catalytic triad. A histidine general base abstracts the proton from the side chain of serine, allowing the serine to act as a nucleophile and attack the carbonyl group of the amide bond within the protein substrate. The third player in the triad, an acidic residue, acts to orient the imidazolium ring of histidine residue (Fig. 1a). For other serine proteases, catalysis is achieved by a catalytic dyad in which a lysine residue plays the role of proton donor, and a third catalytic residue is not required (Fig. 1b) [2].

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E. coli signal peptidase is by far the most thoroughly characterized Type I signal peptidase. This enzyme serves as a potential target for the development of novel antibacterial agents because of its unique physiological properties. It is essential for the viability of *E. coli* and its active site is relatively accessible on the outer leaflet of the cytoplasmic membrane. It is widely distributed in the clinically relevant gram-positive bacteria as well as in *Chlamydia*. The enzyme is a unique protease insensitive to the classic protease inhibitors. Together, these features support the

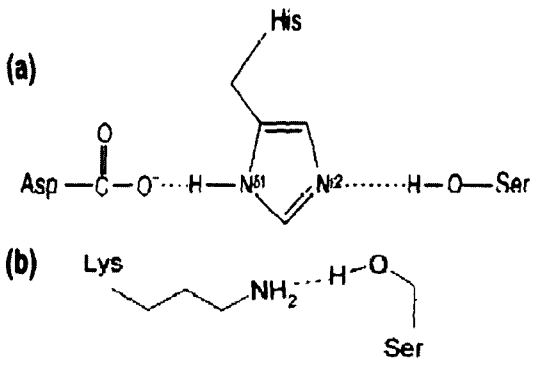

Fig. 1. Schematic representation of the serine protease. (a) The classical Ser/His/Asp catalytic triad. (b) The Ser/Lys cataytic dyad.

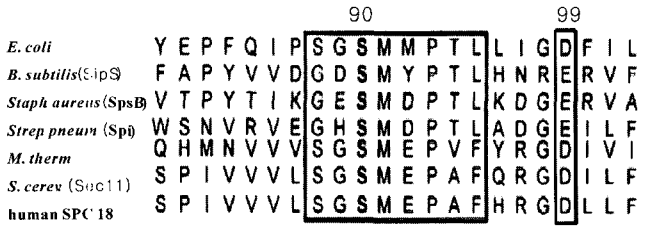

Fig. 2. Conserved domain of signal peptidases. The first big box corresponds to the conserved region of sequence in the Type I signal peptidase (SPase). The aspartic acid 99 in the *E. coli* signal peptidase are indicated. The representative signal peptidase are as follows: *Escherichia coli* SPase [5]; *Bacillus subtillis* SPase (SipS) [14]; *Staphylococcus aureus* SPase (SpsB) [15]; *Streptococcus pneumoniae* SPase (Spi) [16]; *Mycobacterium tuberculosis* SPase [17] ; *Saccharomyces cerevisiae* SPase (Sec11) [18]; Signal Peptidase Complex 18 [19].

choice of bacterial Type I signal peptidase as an ideal target for the developement of novel antibiotic therapies [3].

Type I signal peptidases have been classified into the evolutionary clan of serine proteases, which utilize a Ser/Lys catalytic dyad mechanism, whereby an -amino group of a lysine side chain acts as the general base to increase the nucleophilicity of the active serine. Among the many interesting unanswered questions about the function of Type I signal peptidases, perhaps the most critical one is how the lysine maintained in the unprotonated state so that it can function as the general base. Generally, it appears that the Ser/Lys dyad proteases are less efficient than the triad proteases except that *E. coli* signal peptidase which is very efficient against pre-protein substrate [4].

In this paper, we have proposed that Type I signal peptidase may act as the serine/lysine/aspartic acid triad cataltyt.c mechanism. So far, many aspartic acids of Type I signal peptidase located in the conserved domains have been studied and concluded that the residues are not related with the catalytic mechanism of signal peptidase I [5, 6]. In this paper, we have chosen aspartic acid 99 in the *E. coli* signal peptidase, locating in the non-conserved boxes based on the a structure-based multiple sequence alignment (Fig. 2). The results of this study suggest that aspartic acid 99 residue plays an important role in stabilizing *E. coli* signal peptidase structure. It is unlikely that this residue is directly involved in catalysis.

Materials and Methods

Bacterial strains and plasmids

E. coli strain SB221, BL21(DE3), IT41 and MC1061 were from the Ohio State University. The cloning of signal peptidase gene into pING plasmid, which contains signal peptidase under the *araB* promoter, was described in Dalbey and Wickner [7]. Oligonucleotide mutagenesis was performed using the Quick-change mutagenesis kit (Strategene, USA). The mutant D99A Type I signal peptidase (GAT mutated to GCC at position 99) was verified by plasmid isolation and sequencing using the sequenase 2.0 kit (USB, USA).

In vitro activity assay using the procoat substrate

Type I signal peptidase activity was measured by the post-translational conversion of procoat to coat protein and signal peptide. In this assay, procoat was synthesized *in vitro* according to the procedure in Zwinziski, *et al.* [8, 9]. 2 ml of *E. coli* MC1061 cultures expressing different signal peptidase were grown to an optimal density at 600 nm of 0.2, induced with arabinose for 2 hour, followed by centrifugation for 1 min to concentrate the cells. After resuspending in 0.3 ml of lysis buffer containing 20% sucrose, 10 mM Tris-HCl (pH 8.0), 10 mM EDTA, 1% Triton X-100, 1 mg/ml lysozome, 5 μg/ml deoxyribonuclease, 1 μg/ml ribonuclease, and 5 mM phenylmethylsulfonyl fluoride and incubating for 30 min at room temperature, cell extracts were used directly or diluted (1:10, 1:10, 1:50, 1:200, 1:400) and incubated with [^{35}S]-labeled procoat at 37°C for 30 min. The processing of procoat was analyzed on a 23% polyacrylamide gel.

In vivo activity assay of Type I signal peptidase

The activity of signal peptidase I *in vivo* was measured by examining the processing of outer membrane protein A precursor (pro-OmpA) in *E. coli* IT41, a temperature-sensitive signal peptidase strain [10, 11]. Briefly, *E. coli* IT41 was grown at 32°C in M9 medium [12] containing 0.5% fructose and 50 μg/ml each amino acid (except methionine). After reaching the early-log phase, cultures were shifted to 42°C for 1 hour to inactivate the temperature-sensitive signal peptidase. 0.2% Arabinose was added to the medium to induce synthesis of signal peptidase. Cells were labeled with [^{35}S]methionine for 15 seconds, and unlabeled methionine was added to a final concentration of 500 μg/ml. At the indicated times, samples were removed and quenched in 20% trichloroacetic acid. Samples were immunoprecipitated with antibody directed against outer membrane protein A and analyzed on a 12% SDS-poly-

acrylamide gel with a 5% stacking gel using a discontinuous buffer system. The gel were fixed and subjected to fluorography.

Pulse-chase assay

2 ml *E. coli* BL21 (DE3) cultures were grown in M9 media containing 0.5% fructose and 50 μg/ml each amino acids except methionine and induced with IPTG to a final concentration of 1 mM. After 1 hour induction, cells were labeled with 100 μCi of [^{35}S]methionine for 1 min and chased with an excess of nonradioactive methionine to a final concentration of 500 μg/ml. At the indicated times, 100 μl portions were removed and quenched to 20% ice-cold trichloroacetic acid. Samples were then immunoprecipitated [12] and analyzed by SDS-PAGE and fluorography [6].

Results

The standard mechanism for the serine proteases involves the Ser/His/Asp catalytic triad while *E. coli* signal peptidases are most likely regarded as a Ser/Lys dyad. In this work, the aspartic acid 99 residue in the *E. coli* signal peptidase was chosen to test if the residue has any possible role in the catalytic mechanism. As a first step, we have constructed the mutant D99A and *in vitro* activity of D99A was measured using radiochemically [^{35}S]labeled procoat. The Type I signal peptidase cleaves procoat to the coat protein. As can be seen in Fig. 3, a 50-fold diluted extract of *E. coli* MC1061 expressing wild-type *E. coli* signal peptidase catalyzes comparable cleavage of procoat, as does undiluted extract of *E. coli* MC1061 without plasmid. The pattern of cell extract containing D99A signal peptidase is similar to cell extract of MC1061 without plasmid, indicating that the mutant is a important residue in the *in vitro* activity.

In a second step, we evaluated the activity of D99A signal peptidase using a more sensitive *in vivo* assay, where Type I signal peptidase activity was measured in its native membrane environment. This assay examines whether the plasmid-encoded D99A signal peptidase can restore processing of pro-OmpA at the nonpermissive temperature of 42°C in the temperature-sensitive Type I signal peptidase strain, *E. coli* IT41 [13]. This strain bears a temperature sensitive signal peptidase encoded by the chromosomal *lepB* gene. The cell extracts containing wild-type or a plasmid encoding D99A mutant were pulse-labeled for 15 seconds with [^{35}S]methionine and chased with an excess of unlabeled methionine. Aliquots were removed at the indicated times and analyzed by immunoprecipitation to OmpA, SDS-polyacrylamide gel electrophoresis, and fluorography. While the processing $t_{1/2}$ of the *E. coli* IT41 wild-type signal peptidase is rapid within 10 seconds, it is slow in cell extract without plasmid ($t_{1/2}$ takes about 90 seconds). In Fig. 4, the D99A mutant has normal rapid processing as seen in the wild-type signal peptidase, indicating that the aspartic acid 99 residue is not required for the signal peptidase activity.

Next, immunoblot analysis has performed to identify the D99A signal peptidase protein. In contrast to the wild-type signal peptidase, the mutant signal peptidase was not detected by anti-rabbit polyclonal signal peptidase antibody (data not shown). We tested the stability of the D99A mutant with a pulse-chase experiment. As seen in the Fig. 5, D99A signal peptidase could not be detected after roughly 5 min, suggesting that the protein is unstable. This is compared to a general half-life of 60 min for wild-type signal peptidase. The additional band in the mutant could be the partially degraded *E. coli* signal peptidase protein.

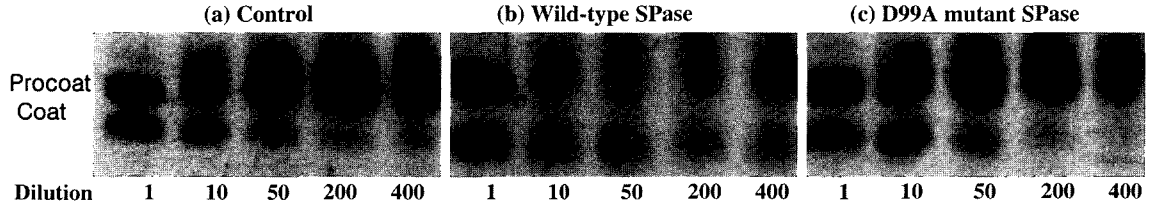

Fig. 3. *In vitro* activity of Type I signal peptidase using the M13 procoat protein. (a) *E. coli* MC 1061 extract as a control (b) *E. coli* MC 1061 extract expressing wild-type signal peptidase (c) *E. coli* MC 1061 extract expressing D99A mutant singal peptidase. *E. coli* MC 1061 extracts were tested for signal peptidase I activity by measuring the posttranslational processing of the M13 procoat at various dilutions of cell extract. Cell extracts were prepared as described in the "Materials and Methods". Undiluted or diluted (1:10, 1:50, 1:200 or 1:400) extracts were incubated for 30 min at 37°C with *in vitro* synthesized [^{35}S]procoat. The procoat was separated from the processed coat protein by using a 23% SDS-polyacrylamide gel.

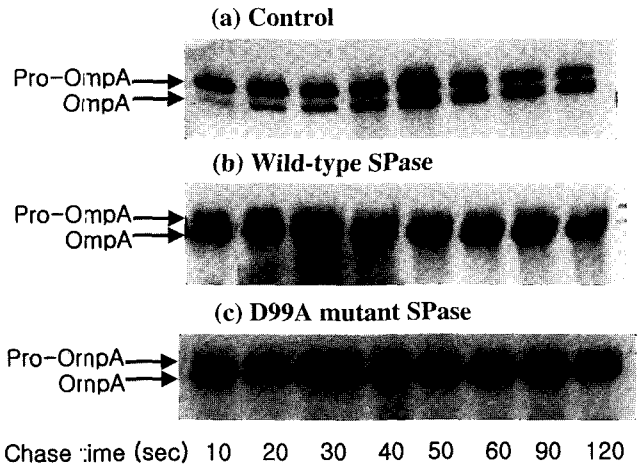

Fig. 4. *In vivo* processing of pro-OmpA for the activity of the Type I signal peptidase. (a) *E. coli* IT41 as a control (b) *E. coli* IT41 expressing wild-type signal peptidase (c) *E. coli* IT41 expressing D99A mutant singal peptidase. *E. coli* IT41 was grown at 30°C to the mid-log phase. After inducing signal peptidase I by the addition of arabinose (0.2%), cultures were shifted to 42°C for 1 h. Pro-OmpA processing was determined as described in the "Materials and Methods".

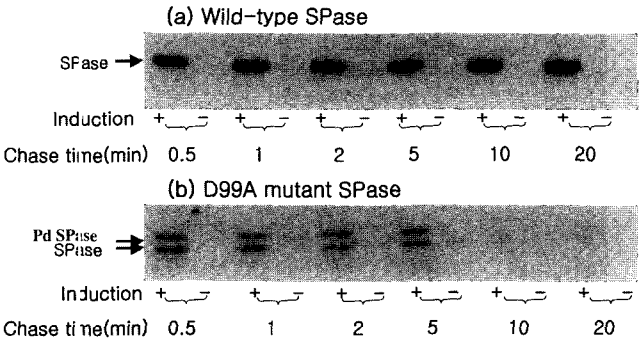

Fig. 5. *In vivo* stability of D99A mutant as analyzed by pulse-chase. (a) BL21(DE3) cells expressing wild-type signal peptidase (b) BL21(DE3) cells expressing D99A mutant singal peptidase. Two ml cultures of BL21(DE3) cells were labeled with 100 μCi of [^{35}S]methionine for 30s and then chased with 500 μg/ml of nonradioactive methionine. At the each chase point, 0.2 ml of the labeled culture was added to an equal volume of ice-cold trichloroacetic acid to quench the reaction. Each samples was immunoprecipitated with antiserum to signal peptidase and then analyzed by SDS-polyacrylamide gel electrophoresis and fluorography.

We believe that the mutation of D99A impacts on the stable structure of signal peptidase protein and, therefore, the unstable protein is easily degraded during purification procedure.

Discussion

Typically, the catalytic mechanism of serine proteases involves the catalytic triad, Ser/His/Asp [2] while Type I signal peptidase requires an essential Ser 90 [5] and Lys 145 [10]. Therefore, it has been proposed that Type I signal peptidase belongs to a novel serine protease which utilizes a mechanism whereby the lysine acts as the general base to extract the proton from the hydroxyl side-chain of the serine. The interesting question would be how lysine is maintained in the unprotonated state to function as the general base. In this paper, we try to look for an aspartic acid residue to act as a third player in the catalytic triad. Based on the consensus sequence of the signal peptidase family, Ser90 of *E. coli* enzyme is found in the second conserved domain (Box B) whereas the catalytic Lys 145 is in the fourth conserved domain (Box D).

There are 6 aspartic acids in the conserved domains, indicating that the residues are not important in the direct Type I signal peptidase catalytic mechanism. Now, the aspartic acid 99 in the Type I signal peptidase was chosen to see if the residue is important in the catalytic mechanism. Aspartic acid 99 residue is located outside conserved boxes but homologous to putative signal peptidase sequences presently available as seen in the Fig. 2. The result of *in vitro assay* shows that the aspartic acid 99 residue is critical for signal peptidase processing by the procoat substrate but the more sensitive *in vivo* assay demonstrates that the processing of pro-OmpA by *E. coli* signal peptidase encoding D99A and wild-type of pET23lep vector is rapid at 42 °C. Pulse-chase studies (Fig. 5) established that the replacement of aspartic acid 99 with alanine results in a very unstable signal peptidase molecule based on the fact that the enzyme could not detected after 5 min unlike the wild-type signal peptidase. In conclusion, the data seem to rule out possibility that aspartic acid 99 plays a direct role on catalysis. It is rather to stabilize the protein structure.

국문초록

Type I 신호펩디드 가수분해효소에 존재하는 D99 아미노산 잔기의 구조적 역할 가능성

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신호펩디드 가수분해는 모든 생물에 필수적인 단백질로 N-말단에 신호서열을 가진 단백질들의 신호서열을 잘라내는 효소로 serine 과 lysine을 활성부위로 하는 특이한 dyad serine 효소류로 알려져있다. 최근에 신호펩디드 가수분해효소의 특성으로 새로운 항생제 이용 가능성 때문에 그 활성 메카니즘의 연구가 중요시되고 있다. 본 연구는 *E. coli* 신호펩디드 효소가 serine/lysine/aspartic acid로 하는 전형적인 triad serine 효소류의 메카니즘을 갖는다는 가정하에 Type I 신호펩디드류의 하나인 *E. coli*에서 aspartic acid 99을 alanine으로 치환하여 효소의 enzymatic activity를 조사하였다. 그 결과 D99잔기는 *E. coli* Type I 신호펩디드 효소의 활성메카니즘에 직접적으로 관여한다기보다는 그 효소를 구조적으로 안정화하는데 중요한 역할을 할 가능성이 있는 것으로 암시되어진다.

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