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Active Site Aspartic Acid and Propeptide are Not Critical for the Activity and Secretion of *Serratia marcescens* Serine Protease in *Escherichia coli*

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A serine protease (SMS) from *Serratia marcescens* RH1, which is produced as a 112 kDa precursor composed of a typical NH_2-terminal signal peptide, a mature protease and a large COOH-terminal propeptide, was expressed by its own promoter and secreted extracellularly in *Escherichia coli*. Replacement of the SMS promoter and signal sequence by the ϕ10 promoter of bacteriophage T7 and OmpT signal sequence resulted in the secretion of active SMS in *E. coli* BL21(DE3). Deletion of the NH_2-terminal 54 amino acids containing Asp residue of the active site did not critically affect the activity and secretion of the protease. Furthermore, the deleted gene product lacking the entire propeptide was secreted as an active enzyme. Co-expressed mature protein and propeptide without covalent linkage were toxic to *E. coli* BL21(DE 3) but not to *E. coli* HB101.

KEY WORDS □ ***Serratia marcescens*, serine protease, secretion, OmpT signal peptide, aspartic acid, propeptide, toxicity**

Some of Gram-negative bacteria have been known to secrete extracellular proteases (4, 5, 10, 15, 22, 24). Among them, *Serratia marcescens*, belonging to the family Enterobacteriaceae, secretes four or more different proteases (12). The gene for the major metalloprotease (protease SM) has been cloned and sequenced (15). Protease SM had a propeptide of NH_2-terminal 16 amino acids and was secreted by a signal peptide-independent pathway requiring a specific transporter, analogous to the mechanisms of *Erwinia chrysanthemi* protease B and hemolysin secretion (11). Recently, the gene for the minor metalloprotease (Smp) of *S. marcescens* was also cloned and sequenced (5). The active Smp was expressed by its own promoter and secreted extracellularly in *E. coli*. Smp had high sequence homology (43%) to *E. carotovora* metalloprotease (7) and was composed of a signal peptide, a propeptide and a mature protein in contrast to protease SM, which indicates Smp is secreted by a signal peptide-dependent pathway.

S. marcescens RH1 produces a major 45 kDa metalloprotease and several minor serine proteases. The gene (*sms*) encoding one (SMS) of the serine proteases has been cloned and sequenced in *E. coli* (10). The cloned SMS was stably expressed by its own promoter and secreted extracellularly from *E. coli* host cells, and showed 88% homology to the serine protease (24) from *S. marcescens* IFO-3046. On the basis of the sequence homology (10) and NH_2-terminal amino acid sequencing, SMS seems to be produced as a 112 kDa precursor composed of a typical NH_2-terminal signal peptide (3.5 kDa), a mature protease (41 kDa) and a large COOH-terminal propeptide (67 kDa).

In this study, we report the secretion of SMS using OmpT signal sequence in *E. coli*. Deletion of the active site Asp or the entire propeptide did not critically affect the activity and secretion of SMS. Furthermore, co-expressed mature protein and propeptide without covalent linkage were toxic to *E. coli* BL21(DE3) but not to *E. coli* HB101.

MATERIALS AND METHODS

Bacterial strains and culture conditions

Serratia marcescens strain RH1 was previously described (10). *E. coli* HB101 (19) was used for amplification of plasmid DNA. *E. coli* BL21(DE3) and BL21(DE3)pLysS (23) were used for induction of the T7 ϕ10 promoter and secretion of SMS. Cells were grown in LB medium at 37°C with aeration. *E. coli* BL21(DE3)pLysS was grown in LB medium containing 25 μg/ml chloramphenicol.

Construction of the recombinant plasmids

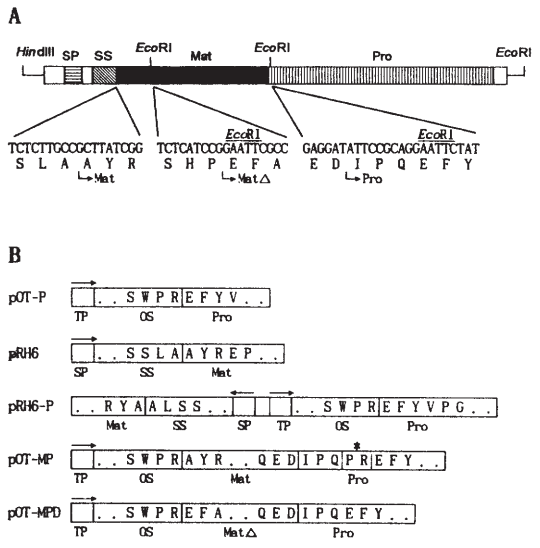

Fig. 1. *Structures of a 3.6 kb HindIII-EcoRI fragment of pRH4 (A) and SMS constructs (B).*
A. Only restriction sites that were pertinent are shown. The thin line represents vector DNA and the box insert DNA. The nucleotide and amino acid sequences of the junction regions of signal peptide, mature protease and propeptide are given below. Relevant features are shown as follows: ▥, promoter; ▧, signal peptide; ■, mature protease; ▤, propeptide.
B. The amino acid sequences at the junction between the genes are shown (in single letter code) for all plasmids. The asterisk indicates amino acid residues encoded by additional bases originating from *Bam*HI linker sequence. Mat represents SMS mature protease; Mat△, the mutant SMS mature protease lacking NH_2-terminal 54 amino acids; Pro, SMS propeptide; OS, OmpT signal peptide; SS, SMS signal peptide; SP, SMS promoter; TP, T7 ϕ10 promoter.

The plasmid pRH4 containing the promoter and entire structural gene of SMS in pUC19 was described before (10) (Fig. 1A). A 2.2 kb *Eco*RI fragment of pRH4 encoding propeptide was deleted and self-ligated to make pRH6 (Fig. 1B). To make pOT-P, the 2.2 kb *Eco*RI fragment of pRH4 was ligated with *Bam*HI linker (5′-CGGATCCG-3′) and inserted into the *Bam*HI site of the plasmid pET3 sec1-a which contains the bacteriophage T7 ϕ10 promoter, the OmpT signal sequence, a *Bam*HI cloning site and T7 transcription terminator, and directs proteins into the periplasmic space (16). The 2.8 kb *Bgl*II-*Cla*I fragment of pOT-P was subcloned into *Hin*dIII site of pRH6 to generate pRH6-P. To make pOT-MP, the gene encoding the entire mature SMS was amplified from pRH4 by polymerase chain reaction (PCR) using primers 1 and 2, and subcloned into pOT-P (Fig. 1B). The 3.2 kb *Eco*RI fragment of pRH4 was treated with Klenow fragment and ligated with *Bam*HI linker (5′-CGGATCCG-3′), and subcloned into the *Bam*HI site of pET3 sec1-a, resulting in pOT-MPD (Fig. 1B).

PCR and nucleotide sequencing

PCR reaction was performed as described by Saiki *et al.* (18). Oligonucleotides were synthesized to amplify the DNA fragment encoding the entire mature SMS. Primer 1 (5′-CGCTTATCGGGAACCAGGAC-3′) and primer 2 (5′-CCTGCGGAATATCCTCTTGGG-3′) correspond to the 5′ end and 3′ end of mature SMS, respectively. The sequencing reaction was done by the dideoxy chain termination method (20). Primers with the sequences 5′-GACATGCGTGCCATGCGTGCC-3′ and 5′-CCCAAGACCGGGGATATTCAC-3′, corresponding to the nucleotide 517 to 537 and 1462 to 1482 (10), respectively, were synthesized to determine the nucleotide sequences of the junction region of OmpT signal peptide-SMS mature protease and SMS mature protease-propeptide fusion proteins, respectively.

Protease assay

The protease activity was measured as described by Kwon *et al.* (6). The 2.5 m*l* of prewarmed 1% casein in 50 mM phosphate buffer (pH 8.0) and 0.5 m*l* of enzyme solution were mixed and incubated at 37° for 30 min. The reaction was stopped by adding stopping mixture containing 0.11 M trichloroacetic acid, 0.22 M acetic acid and 0.33 M sodium acetate, and centrifuged. The supernatant was subject to optical density determination at 280 nm. One unit of the protease activity was defined as an amount of enzyme which caused an increase in absorbance of 0.1 per 30 min.

Polyacrylamide gel electrophoresis (PAGE)

Native PAGE of SMS was done by the method of Laemmli (8), except sodium dodecyl sulfate (SDS) was omitted. Following electrophoresis in 6% continuous nondissociating polyacrylamide gels, the gels were placed on skim milk agar. Bands of clearing corresponding to skim milk hydrolysis appeared after 1 hr incubation at 37°C.

Cell fractionation and enzyme assay

Fractionation of extracellular, periplasmic and cytoplasmic enzyme was performed by the cold osmotic shock method (2). Cells were harvested and washed twice with 10 mM Tris-HCl (pH 7.5) containing 25% sucrose followed by shaking for 10 min at room temperature. After spinning at 9,000×*g* for 10 min, the pelleted cells were quickly and vigorously resuspended in ice-cold water followed by shaking for 10 min at 4°C and

centrifugation at 9,000×*g* for 10 min. The extracellular fraction is the sum of the culture supernatant and the two washes. The periplasmic fraction is the supernatant after treatment with ice-cold water. The precipitated cells were suspended in 10 mM Tris-HCl (pH 7.5) and disrupted with French Press (Amicon). The cytoplasmic fraction is the supernatant after disruption. The activities of β-lactamase and β-galactosidase were measured by the method of micro-iodometry (17) and as described by Miller (13), respectively.

RESULTS AND DISCUSSION

Secretion of SMS using OmpT signal sequence

To investigate the role of SMS signal peptide, SMS signal sequence was replaced by OmpT signal sequence using PCR to create pOT-MP encoding the mature peptide and propeptide of SMS following OmpT signal peptide under the control of T7 φ10 promoter (Fig. 1). *E. coli* BL21 (DE3) harboring pOT-MP produced the active protease extracellularly (Fig. 2). To show that the extracellular protease activity was not due to cell lysis, the β-lactamase and β-galactosidase activities were also assayed (Table 1). Most of the protease activity was found in the extracellular fraction of the cells, while most of the β-lactamase and β-galactosidase activities were localized in the periplasm and the cytoplasm, respectively. This result showed that the protease was truly excreted into the extracellular medium from *E. coli* cells. Furthermore, nondenaturing PAGE of the culture supernatant revealed the proteolytic band with the same mobility as that from pRH4 (Fig. 3). These results suggest that OmpT signal sequence, which directs proteins into the periplasmic space

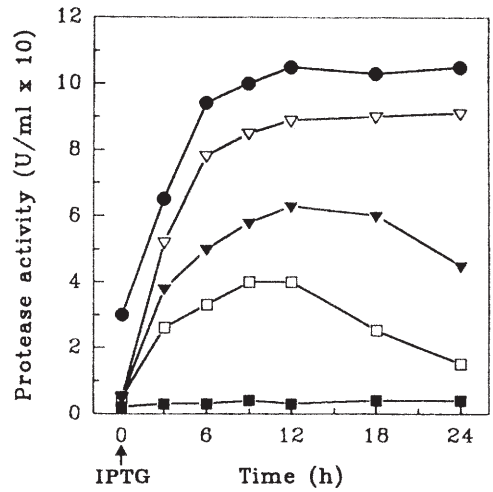

Fig. 2. *Time courses of production of SMS from E. coli strains.*
Each of overnight cultures (1 m*l*) of *E. coli* HB101 harboring pRH4 (●), and *E. coli* BL21 (DE-3) harboring pOT-MP (▽), pOT-MPD (▼), pRH6 (□) and pUC19 (■), was inoculated in 100 m*l* of LB medium containing 100 μg/m*l* of ampicillin. After aerobic cultivation at 37°C for 3 hrs, IPTG was added to *E. coli* BL21(DE-3) cells harboring pOT-MP, pOT-MPD and pRH-6 at a final concentration of 1 mM to induce the T7 φ10 promoter. Cells containing pRH4 and pUC19 were not induced by IPTG. The extracellular protease activities were assayed at intervals. Each point on the graph represent the mean of triplicate experiments. The data were adjusted with respect to cell mass.

Table 1. *Localization of the protease activity in E. coli cells[a] harboring SMS constructs.*

Enzymes	Plasmids	Enzymatic activity (U)		
		Extracellular	Periplasmic	Cytoplasmic
SMS	pRH4	105[b] (77± 5.3)[c]	11 (8± 1.6)	20 (15± 2.3)
	pOT-MP	89 (75± 5.5)	8 (7± 0.8)	10 (18± 1.8)
	pOT-MPD	63 (62± 3.9)	13 (13± 1.0)	25 (25± 1.9)
	pRH6	40 (59± 4.6)	12 (17± 2.9)	16 (24± 2.7)
	pUC19	1 (7± 1.1)	1 (7± 1.8)	13 (86± 5.9)
β-lactamase	pOT-MP[d]	87 (24± 2.3)	256 (70± 5.6)	22 (6± 1.1)
	pUC19	47 (6± 1.5)	730 (90± 6.2)	36 (4± 0.6)
β-galactosidase	pOT-MP[d]	0.066 (18± 3.4)	0.033 (9± 1.4)	0.269 (73± 4.8)
	pUC19	0.007 (2.3± 0.5)	0.004 (1.3± 0.2)	0.296 (96± 6.0)

[a] *E. coli* BL21(DE3) cells harboring pOT-MP, pOT-MPD and pRH6 were grown and induced by IPTG as described in Fig. 2. Cells were harvested at 12 hr after the addition of IPTG. Cells harboring pRH4 and pUC19 were not induced by IPTG.
[b] Enzyme activity represents the means of triplicate experiments.
[c] Values in parentheses indicate percent distribution of the enzymes, which is represented as mean standard deviation of the triplicate determinants.
[d] The enzymatic activities of the pOT-MPD and pRH6 samples were similar to those of the pOT-MP sample.

through the inner membrane, can replace SMS signal sequence for the extracellular secretion of SMS and that SMS signal sequence is required only for the export through the inner membrane, consistent with the idea of Shitaka *et al.* (21).

Deletion of propeptide

SMS has an exceptionally large COOH-terminal propeptide (67 kDa) (10). To investigate the effect of the deletion of this large propeptide on the activity and secretion of SMS, the entire propeptide was deleted from pRH4 to generate pRH6 which encodes the signal peptide and entire mature peptide under the control of its own promoter (Fig. 1). *E. coli* BL21(DE3) harboring pRH6 secreted the active SMS extracellularly, although total protease activity was somewhat lower than that from pRH4 (Fig. 2 and Table 1). Mature SMS with an apparent size of 60 kDa was found in the culture supernatants of *E. coli* BL21(DE3) harboring pRH6 on SDS-PAGE (data not shown), agreeing with the previous report (10). SMS from pRH6 showed the same mobility as that from pRH4 on nondenaturing-PAGE (Fig. 3). These results suggest that propeptide of SMS is not essential for the activity and secretion of SMS in *E. coli*, although it may play some role in the export of the mature protease through the outer membrane.

In contrast to our results, serine protease from *S. marcescens* IFO-3046 has shown that frame mutations in propeptide caused total loss of the protease activity (24) and that the mutated gene product lacking the entire propeptide was localized in the periplasmic space only as inactive form (14). On the basis of these observations, they suggested that SMS propeptide is essential for the extracellular production of the protease. Although the major difference between their protease constructs and ours is that we used OmpT signal sequence, whereas they used its own signal sequence, the exact reason for the discrepancy of the results is unclear. Studies involving site-directed mutation and immunological assay of the protease to identify the functional roles of propeptide are now in progress.

Deletion of the active site Asp residue

The plasmid pOT-MPD encodes OmpT signal peptide and the mutant SMS lacking the NH_2-terminal 54 amino acids containing the active site Asp residue under the control of T7 ϕ10 promoter (Fig. 1B). *E. coli* BL21(DE3) harboring pOT-MPD secreted the active SMS protease without loss of activity, although the total protease activity was somewhat lower than that from pRH4 (Fig. 2 and Table 1). Furthermore, the protease of the culture supernatant showed higher mobility on non-denaturing PAGE (Fig. 3), consistent with the fact that the mutant SMS made from pOT-MPD lacks the NH_2-terminal 54 amino acids. These results suggest that the active site Asp residue might not be essential for proteolytic activity even though it might contribute to maintenance of a favorable conformation of the active site.

The Asp residue is known to be essential in the proteolysis by forming charge-relay system. The crystallographic data also shows that the Asp residue is sufficiently close to the His to favour intramolecular interactions. But, Tonin from rat submaxillary gland, a serine protease with trypsin- and chymotrypsin-like activity, revealed the substitution of an Asp residue by a Leu residue without affecting the proteolytic activity of the enzyme (9). Furthermore, the finding that papain, a thiol-protease containing His and Cys in the active site, reacts in a similar fashion to serine proteases could indicate that the Asp residue might not be essential for proteolytic activity.

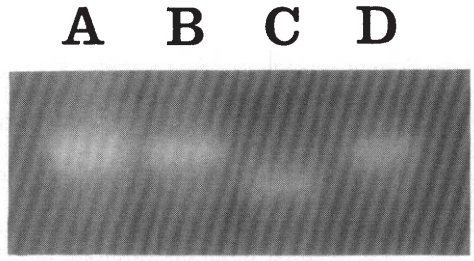

Fig. 3. *Nondenaturing PAGE of SMS.*
Cells were cultured and induced as described in Fig. 2, and were harvested at 12 hr after addition of IPTG. The supernatants were precipitated with ammonium sulfate (80% saturation) and applied to the nondenaturing PAGE as described in Materials and Methods. Lanes A to D represent the samples of *E. coli* HB101 harboring pRH4 (A) and *E. coli* BL21(DE-3) harboring pOT-MP (B), pOT-MPD (C) and pRH6 (D), respectively.

Table 2. *Transformation of E. coli strains by SMS constructs.*

Plasmids	Number of Transformants		
	BL21(DE3)	BL21(DE3)pLysS	HB101
pRH6-P	0	0	21700
pRH4	1280	1140	22300
pRH6	860	750	16900
pOT-MP	1250	1030	22100
pOT-MPD	1090	860	17400
pUC19	1440	1280	22200

E. coli strains were made competent by the standard calcium chloride procedure (19). The 300 nanograms of plasmids were used for each transformation. The values shown are the means of triplicate independent experiments.

Another explanation could be that the deletion of the NH_2-terminal region could induce a conformational change to bring another Asp close to the active site His and Ser. Final conclusions will have to rely on the three-dimensional structure of the enzyme.

Co-expressed pro- and protease domains are toxic to *E. coli* BL21(DE3)

To test the secretion of SMS by co-expressing the pro- and mature domains without covalent linkage, pRH6-P was constructed in *E. coli* HB101 (Fig. 1). The plasmid pRH6-P encodes the signal peptide and mature domain of SMS under the control of its own promoter, and the OmpT signal peptide and SMS propeptide under the control of ϕ10 promoter. However, transformation of pRH6-P into *E. coli* BL21(DE3) resulted in no transformants, whereas several plasmids used as controls transformed strains BL21(DE3) and HB 101 well (Table 2). Furthermore, transformation of pRH6-P into *E. coli* BL21(DE3)pLysS, which contains a chloramphenicol-resistant plasmid encoding T7 lysozyme inactivating T7 RNA polymerase in the absence of induction, yielded no transformants. Theses results strongly suggest that expression from pRH6-P is toxic to *E. coli* BL21(DE3) strains.

The synthesis of a membrane-bound MalE β-galactosidase hybrid protein, when induced by growth of *E. coli* on maltose, leaded to inhibition of cell division and eventually a reduced rate of mass increase (3). The hybrid protein was postulated to block some export site or cause the sequestration of some limiting factors involved in the export process. Furthermore, the transformation of the protease gene of human immunodeficiency virus (HIV) also yielded no transformants in BL21(DE3) (1). In contrast to our results, HIV protease was not toxic to *E. coli* BL21 (DE3)pLysS in the absence of induction. This result shows that the co-expression of SMS protease and propeptide by very low level of T7 polymerase may be sufficient to cause cell death. The toxicity of the SMS protease to *E. coli* cells may provide a method to isolate mutants of this protease. At present we are studying the mutagenesis and selection procedures which are of general utility for isolating mutants of proteases in *E. coli*.

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초 록: 대장균에서 *Serratia marcescens* 세린 단백질분해 효소의 활성과 분비에 대한 활성부위 Aspartic Acid와 Propeptide의 영향

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Serratia marcescens 균주 RH1 세린 단백질 분해효소(SMS)는 아미노 말단 signal peptide, 성숙형 효소 및 카복시 말단 propeptide로 이루어져 있으며, 대장균에서 자신의 promoter에 의해서 발현되고 세포외로 분비된다. SMS promoter와 signal sequence를 bacteriophage T7의 ϕ10 promoter와 OmpT signal sequence로 바꾸었을 때 대장균 BL21 (DE3)에서 활성 SMS가 분비되었다. 활성 Asp 잔기를 포함하는 아미노 말단 54 아미노산을 제거하였으나 SMS의 활성과 분비에 큰 영향을 미치지 않았다. 또한, 전체 propeptide가 제거되었을 때에도 활성 단백질 분해효소가 분비되었다. Covalent linkage 없이 성숙형 효소와 propeptide를 동시발현 시켰을 때, 대장균 BL21 (DE3)에 치사효과를 나타내었다.