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Importance of Leu-5 and Pro-6 in the Inhibitory Activity of the *Serratia marcescens* Metalloprotease Inhibitor (SmaPI)

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The *Serratia marcescens* metalloprotease inhibitor (SmaPI) is a proteinase inhibitor toward *Serratia marcescens* metalloprotease (SMP). The three-dimensional structure of SmaPI was calculated by computer modeling using the structure complex between SMP and the *Erwinia chrysanthemi* inhibitor as a template. Based on this model structure, the substitution of the amino acid residues, Ala-4, Leu-5, Pro-6, and Thr-7, were located at the hinge region of the N-terminal segment by site-directed mutagenesis. Although the A4R and T7A mutant SmaPIs showed a nearly full inhibitory activity, the inhibitory activity of SmaPI decreased significantly when the Leu-5 was converted to Ala, Gly, Ile, or Val. Surprisingly, the L5I and L5V mutant SmaPIs showed less inhibitory activities than the L5A mutant. From these results, we suggested that the orientations and positions of respective aliphatic groups in the side-chain of position 5 mainly affected the inhibitory activity of SmaPI. The overall side-chain hydrophobicity was only slightly affected. The side-chain of the Leu-5 residue contributed approximately 0.79 kcal/mol out of 8.44 kcal/mol to the binding of SmaPI with SMP. The inhibitory activities of P6A and P6G were also severely decreased. The Pro-6 may have a critical role in maintaining the strict conformation of the N-terminal portion that may be important in the inhibitory activity of SmaPI. In conclusion, Leu-5 and Pro-6 have crucial roles in the inhibitory function of SmaPI toward SMP.

Keywords: Metalloprotease, Metalloprotease inhibitor, Site-directed mutagenesis, *Serratia marcescens*.

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Introduction

Metalloproteases comprise one of the four classes of proteolytic enzymes. While metalloproteases do not appear to be as abundant as serine proteinases, they play an important role in numerous physiological processes (Barrett *et al.*, 1986). Metalloproteases can be grouped into about 30 families (Rawlings and Barrett, 1995). Among them, the interstitial collagenase family (M10 family) is one of the largest families of metalloproteases and includes a variety of enzymes from organisms as diverse as bacteria, plants, and animals (Rawlings and Barrett, 1995). The interstitial collagenase families consist of two subfamilies, one containing eubacterial enzyme, conveniently termed the serralysin subfamily, and the other containing eucaryotic enzyme, termed the matrixin subfamily. Despite a low sequence homology between the two subfamilies, a very pronounced similarity was observed in the three-dimensional structures of the proteolytic domains of these proteases (Bode *et al.*, 1993; Stöcker *et al.*, 1995).

Members of the serralysin subfamily have been found in gram-negative bacteria. Serralysins are secreted as proproteins that require proteolytic activation. Enzymes of the serralysin subfamily are the alkaline proteinase from *Pseudomonas aeruginosa* and *Serratia marcescens*, as well as *Pseudomonas fragi*, *Proteus mirabilis* and *Escherichia freundii* (Decedue *et al.*, 1979; Wassif *et al.*, 1995). Serralysins can cause a wide range of pathogenic effects in infected hosts: (1) They enhance the vascular permeability by activating the tissue Hageman factor XII (Matsumoto *et al.*, 1984); (2) They activate the kinin-generating cascade (Maruo *et al.*, 1993); (3) They activate factor X and plasminogen, leading to the activation of influenza and paramyxovirus infectivity (Akaike *et al.*, 1989). These relations of serralysins in many pathogenic effects make structural studies of serralysins and their inhibitors interesting. However, the inhibitory and binding mechanism between serralysins and their inhibitors has not been extensively studied. Until now, inhibitors from

Erwinia chrysanthemi, *P. aeruginosa* and *P. fluorescens* were known as inhibitors toward the serralysin subfamily enzymes (Letoffe *et al.*, 1989; Duong *et al.*, 1992; Liao and McCallus, 1998).

We previously cloned the genes of SMP and *S. marcescens* metalloprotease inhibitor (SmaPI) from *S. marcescens* ATCC 27117, and the biochemical characterization of SmaPI was published (Kim *et al.*, 1995). Using a *Bacillus* expression system for the expression and secretion of SmaPI, the importance of the Leu-3 residue of SmaPI in inhibitory activity and binding with SMP was reported (Bae *et al.*, 1998). In a sequential deletion analysis of the N-terminal region of the SmaPI, SmaPI starting at the Ala-4 residue showed inhibitory activity toward SMP (Bae *et al.*, 1998). However, SmaPI starting at the Ala-8 residue had no detectable inhibitory activity (unpublished result).

Since SmaPI shows a very high SMP specificity, SmaPI is a model protein in order to understand the protein structure and function relationships that are necessary for the design of new protease inhibitors exhibiting high specificity. SmaPI is a monomeric protein and showed 30 to 40% identities in the amino acid sequence to other serralysin inhibitors (Leco *et al.*, 1994).

In the present work, we introduced several site-directed mutations at the region spanning from Ala-4 to Thr-7 for elucidation of the roles in the inhibitory activity of SmaPI on the basis of computer modeling of SmaPI using the *E. chrysanthemi* inhibitor as a template.

Materials and Methods

Media, enzymes and reagents The Luria-Bertani (LB) medium was made according to the protocol described by Sambrook *et al.* (1989). Bacto-tryptone, bacto-agar, and yeast extract were acquired from Difco Laboratories (Detroit, USA). Restriction endonucleases were obtained from New England Biolabs and used as recommended. Molecular weight standard proteins, protein assay dye reagents, and electrophoresis reagents were purchased from Bio-Rad (Richmond, USA). Metalloprotease (SMP) of *S. marcescens* ATCC 21074 was purified by the methods described in our previous article (Kim *et al.*, 1992; Yang and Kim, 1997). Unless indicated otherwise, all reagents were purchased in molecular biology grade.

Bacterial strains, plasmids, and growth conditions *Bacillus subtilis* DB431 and plasmid pZS124 were used as an expression host and expression vector, respectively, of the mutant SmaPI genes (Bae *et al.*, 1996). *B. subtilis* DB431 was cultivated in a LB medium at 37°C with vigorous shaking. When required, kanamycin was added at a concentration of 5 μg/ml to prevent loss of plasmid vectors (Park *et al.*, 1992).

Assays of SMP and SmaPI SMP activity was measured by monitoring the hydrolysis of azocasein as we previously described (Kim *et al.*, 1995). One Unit of SMP activity was defined as the amount of enzyme required to increase 0.01 of absorbance at 420 nm per min under experimental conditions. To measure SmaPI activity, purified SMP (3.33 U) was mixed with appropriately diluted SmaPI and measured by adding the substrate solution. One unit of SmaPI activity was defined as the amount of inhibitor that caused a 50% reduction of SMP activity under assay conditions.

Site-directed Mutagenesis of SmaPI Site-directed mutations were carried out using the PCR method (Nelson and Long, 1989; Moon *et al.*, 1996). The sequences of used primers: A4R, 5'-GGCAGTCTGCGGCTGCCGACCGCG-3'; L5A, 5'-GGCAGTCTGGCGGCGCCGACCGCG-3'; L5G, 5'-GGCAGTCTGGCGGGGCCGACCGCG-3'; L5I, 5'-GGCAGTCTGGCGATCCCGACCGCG-3'; L5V, 5'-GGCAGTCTGGCGGTGCCGACCGCG-3'; P6A, 5'-GGCAGTCTGGCGCTGGCGACCGCG-3'; P6G, 5' -GGCAGTCTGGCGCTGGGGACCGCG-3'; T7A, 5'-GGCAGTCTGGCGCTGCCGGCCGCGCAGTCG-3'. The mismatched codons are underlined. About 0.8 kb of the PCR products were eluted and digested with *Sal*I and then subcloned into the *Nae*I-*Sal*I digested pZS124 (Bae *et al.*, 1996). To confirm the mutagenesis, DNA sequencing was performed by the dideoxy chain-termination method.

Expression and Purification of SmaPI in *Bacillus subtilis* The wild-type and mutant SmaPIs were purified by the methods described in our previous article (Bae *et al.*, 1996).

Determination of Kinetic Parameters By using an artificial substrate, Nα-benzoyl-D,L-arginine-ρ-nitroanilide (BAPNA) (ε_{405} = 8,480/M min), kinetic parameters of SMP and SmaPI were determined. Enzyme assays were performed in 50 mM Tris-HCl (pH 7.0), 10 mM $CaCl_2$, and 10^{-5} M $ZnCl_2$ at 25°C. The K_i values were determined from slow-binding inhibition kinetics (Williams and Morrison, 1979). The enzyme concentration was 0.4 μM; this gives a measurable rate of substrate hydrolysis, as well as an observable rate of inhibitor binding over the steady-state time scale. Inhibitor concentrations varied between 4 μM and 8 μM. From the K_i value, Gibbs free energy (ΔG°_d) for dissociation of the SmaPI-SMP complexes was calculated according to the formula $\Delta G^\circ_d = -RT\ln K_i$.

Computer modeling of SmaPI Modeling by sequence homology was performed essentially following standard procedures (Greer, 1990; Ring and Cohen, 1993). Computer modeling was performed using the program HOMOLOGY (MS Inc., UK) running on a Silicon Graphic Crimson Workstation. The backbone conformation of SmaPI was modeled starting from appropriate segments of a *E. chrysanthemi* protease inhibitor (PDB code: 1SMP).

Results

Computer modeling of SmaPI The three-dimensional structure of SmaPI was predicted using the crystal structure of an inhibitor from *E. chrysanthemi* (PDB code, 1smp) as a template. When the primary sequences of SmaPI and the inhibitor from *E. chrysanthemi* were aligned, 41 amino acids of 102 amino acids were identical (approximately 40.2% identity). As indicated in Fig. 1, the two-loops region were modeled by a loop search. We applied 100 steps of a

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Erwinia  SSLRLPSAAELSGQWVLSGAEQHCDIRLN-T
SmaPI    GSLALPTAQSLAQQWEVADSERQCQIEFLAN
         .** **:* .*: ** ::.:*::*:*.:

Erwinia  DVLDGTTWKLAGDTACLQKLLPEAPVGWRPT
SmaPI    EQSETNGYQLVDRQRCLQSVFAAEVVAGAG-
         :  :     * .   ***  :    *..

Erwinia  PDGLTLTQADGSAVAFFSRNRDRYEHKLVDG
SmaPI    PDGIALLQADGSTLAFFSRDGDLYRNQLGAG
         ***::* *****::*****: * *.::*  *

Erwinia  SVRTLKKKA
SmaPI    DALTLKALA
           ..***  *
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Fig. 1. Sequence alignment of the serralysin inhibitors. Symbols: ★, exact matches; •, conservative substitutions. The start of the mature SmaPI is marked by an arrow. Erwinia, inhibitor from *E. chrysanthemi* (Letoffe *et al.*, 1989); P. aerug, inhibitor of *Pseudomonas aeruginosa* alkaline protease (Duong *et al.*, 1992).

molecular mechanics (MM) calculation only in the two-loop regions for optimization of the loop structure. Finally, we applied 100 steps of a MM calculation to remove the bad contacts in the whole inhibitor. These modeling studies, and the nature of the side-chain interaction, predicted that the serralysin-SmaPI binding would be stable although a difference in the binding contact were observed in loop 1 and loop 2. Like the *E. chrysanthemi* B374 inhibitor, SmaPI folds into a compact eight-stranded anti-parallel b-barrel with simple up-down topology (Fig. 2). The interior is tightly packed with hydrophobic amino acids. There are no hydrophilic side-chains in the interior of SmaPI.

Site-directed Mutagenesis of the Region Between Ala-4 and Thr-7 To elucidate their role in the inhibitory activity of SmaPI, we introduced eight site-directed mutations into the region between Ala-4 and Thr-7 of SmaPI: A4R, L5A, L5G, L5I, L5V, P6A, P6G, and T7A. All of these mutants were expressed in *Bacillus subtilis* as an extracellular soluble protein and purified from culture supernatant to a single band by tricine SDS-polyacylamide gel electrophoresis (data not shown). All of the mutants, except A4R SmaPI, showed an identical migration pattern on native gel electrophoresis to wild-type SmaPI. In addition, all of the mutants have similar thermal stability to the wild-type SmaPI. These facts suggest that amino acid replacements do not alter the overall conformation of SmaPI.

Specific Inhibitory Activity of The Mutant SmaPIs The specific inhibitory activity of the mutant proteins was measured (Fig. 3). The A4R mutant SmaPI has a nearly full inhibitory activity. The T7A mutant also showed a nearly equal activity to wild-type SmaPI. It is suggested that Ala-4

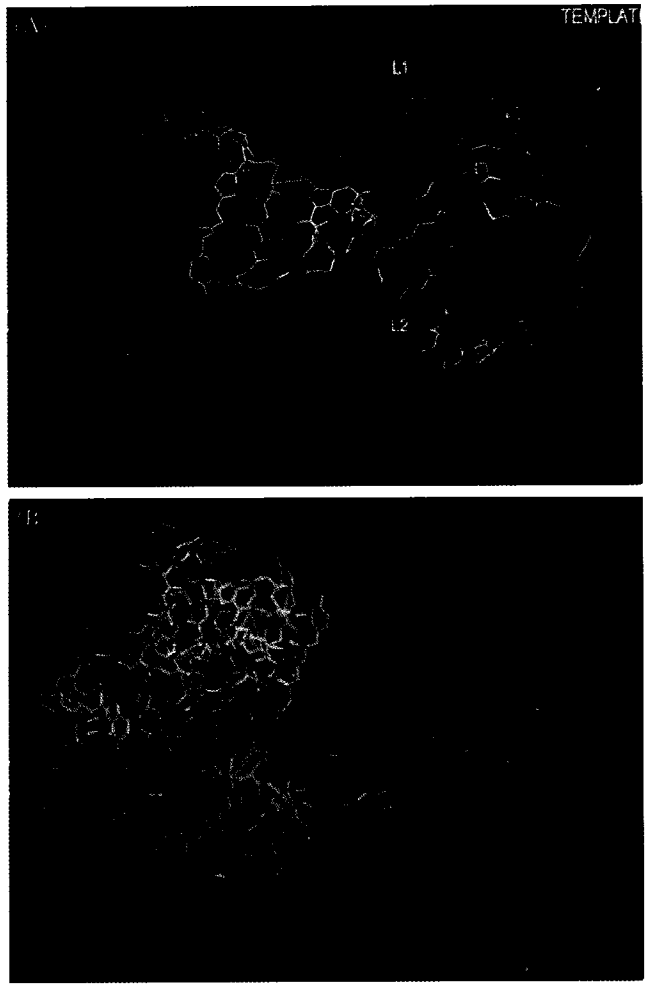

Fig. 2. Three-dimensional structure of SmaPI using computer modeling. (A) Comparison of structure among template, Model A, and Model B. (B) Structure of computer-simulated Model B SmaPI.

and Thr-7 are not critical in the inhibitory activity of SmaPI toward SMP.

To elucidate the role of the Leu-5 in the inhibitory activity of SmaPI, we replaced the Leu-5 with Ala, or Gly. The specific activities of L5A and the L5G mutant were decreased to about 35% and 16.8% of that of wild-type SmaPI, respectively. In order to elucidate the detailed role of the side-chain of the Leu-5 residue by changing its hydrophobicity, we constructed two more mutants, L5I and L5V. Surprisingly, the L5I and L5V mutants were less active than the L5A mutant SmaPI. This result may imply that the inhibitory activity of SmaPI toward SMP depends not on the overall side-chain hydrophobicity of residue 5 of SmaPI, but on the orientations and positions of respective hydrophobic methyl/methylene groups in the side chain.

To elucidate the role of the Pro-6 inhibitory activity of SmaPI, we replaced the Pro-6 with Ala or Gly. The P6A mutant showed severely decreased inhibitory activity (Fig. 3). The specific inhibitory activity of P6G also has reduced

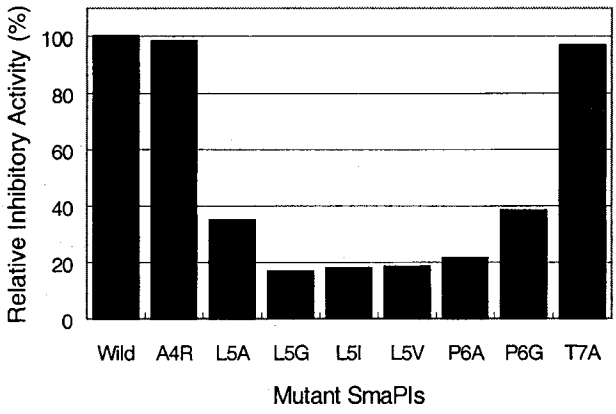

Fig. 3. Relative specific inhibitory activity of mutant SmaPIs.

inhibitory activity. However, specific inhibitory activity of the P6G mutant SmaPI retained was two times higher than that of the P6A mutant SmaPI.

Kinetic Analysis of The Mutant SmaPIs The equilibrium dissociation constant (K_i) of the mutant SmaPIs was determined using slow-binding inhibition kinetics (Table 1). The K_i value of A4R and T7A mutant SmaPI is slightly higher than that of the wild-type. However, the *K*i value of L5A and L5G mutant SmaPI is 2.5 and 3.6 times higher than that of the wild-type SmaPI, respectively. From the K_i value, ΔG°_d for dissociation of the SMP-SmaPI complexes was calculated. The ΔG°_d for the authentic SMP-SmaPI complex is 8.44 kcal/mol, whereas that for SMP-L5G SmaPI is 7.65 kcal/mol. By comparing the ΔG°_d of the SMP-SmaPI complex with that of the SMP-L5G complex, we estimated that the side-chain of the Leu-5 residue contributes approximately 0.79 kcal/mol to the binding of SmaPI with SMP. In short, about 10% of the free energy of the binding are attributable to the side-chain of the Leu-5 residue.

The K_i values of P6A and P6G mutant SmaPIs were also three and two times higher than that of wild-type SmaPI, respectively.

Discussion

The Serralysin subfamily belongs to the interstitial collagenase family, one of the largest families of metalloprotease, with a metzincin subfamily. Serralysins can cause a wide range of pathogenic effects in infected hosts. Therefore, it is not surprising that investigators focused on serralysin inhibitors as therapeutic agents. However, the mode of binding and the inhibition of serralysin inhibitors was not extensively studied.

Baumann *et al.* (1995) suggested that the N-terminal portion of the *E. chrysanthemi* inhibitor mainly interacted with the active site of SMP. In addition, we found that SmaPI (starting at the Ala-4 residue) had inhibitory activity toward SMP, but SmaPI (starting at the Ala-8 residue) had no detectable inhibitory activity (Bae *et al.*, 1998). These facts

Table 1. Inhibition constants (K_i) and Gibbs free energy values of Ala-4, Leu-5, Pro-6, and Thr-7 mutant SmaPIs.

Complex	Inhibitory constant, K_i (10^{-7} M)	ΔG°_d of SmaPI-SMP complexes (kcal/mol)
Wild-type SMP	7.13	8.44
A4R SMP	7.27	8.37
L5A SMP	18.1	7.88
L5G SMP	26.6	7.65
L5I SMP	26.1	7.66
L5V SMP	75.7	7.03
P6A SMP	20.3	7.81
P6G SMP	15.3	7.98
T7A SMP	7.98	8.33

The dissociation free energy of the SmaPI-SMP complexes was calculated according to the formula ΔG°_d= -RTln*K*i.

imply the possibility that the region between Ala-4 and Thr-7 may be important in the inhibitory activity of SmaPI.

Among the serralysin subfamily inhibitors, the Leu-5 residue is a highly conserved amino acid (Fig.1). In the modeled complex between the SmaPI and SMP, the Leu-5 makes hydrophobic contacts with the protease body on the edge of the active site cleft. In short, the side-chain of Leu-5 makes hydrophobic contacts with the aromatic ring of Tyr-169. L5A and L5G mutant SmaPIs showed severely decreased inhibitory activity. From the K_i value, we suggested that the side-chain of the Leu-5 residue contributes approximately 0.79 kcal mol^{-1} to the binding of SmaPI with SMP. Unexpectedly, L5I and L5V mutant SmaPIs have less inhibitory activity than the L5A mutant. It is suggested that the side-chain hydrophobic contribution of the residue in position 5 of SmaPI might be important in the inhibitory activity of SmaPI toward SMP, but the orientation and position of the aliphatic group in the side chain is also very important. In short, 1) one methylene group in position β of the residue 5 of SmaPI may be important, and 2) only one methyl/methylene group in the position γ may be necessary for inhibitory activity. Because Ile and Val have two methyl/methylene groups in position γ, L5I and L5V mutants seemed to have severely decreased inhibitory activities. The substitution with hydrophilic residues will be performed in spite of the very low possibility that the amino acid residues possessing hydrophilic side chains at the 5 position contribute to the interaction with SMP.

In the modeled SMP-SmaPI complex, it is the N-terminus of the inhibitor which contacts the protease active site, mainly through the S' sites. However, the N-terminal segment of the SmaPI seems to bind to SMP in a somewhat different way than one would expect for a substrate. In short, the N-terminal segment exhibits a strong twist that prevents the formation of a regular antiparallel β-strand. Baumann *et al.* (1995) suggested that Pro-6 of the *E. chrysanthemi* inhibitor might cause a strong twist of the N-terminal segment, but it is

unclear whether or not this twist is strictly required for inhibitory activity. To elucidate the role of the Pro-6 in the inhibitory activity of SmaPI, we replaced the Pro-6 with Ala or Gly. The P6A and P6G mutants showed severely decreased inhibitory activity. We suggest that the Pro-6 residue is important in maintaining the strong twist conformation of the N-terminal segment that is required for the inhibitory activity of SmaPI. In addition, Gly, rather than Ala in position 6, may be more tolerable in maintaining the twist conformation of the N-terminal segment.

In conclusion, Leu-5 and Pro-6 may have crucial roles in the inhibitory activity of SmaPI toward SMP. For elucidation of a more detailed role, crystallographic study on the SMP-mutant SmaPI complex is now in progress.

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References

Akaike, T., Molla, A., Ando, M., Akaiki, S. and Maeda, H. (1989) Molecular mechanism of complex infection by bacteria and virus analyzed by a model using serratial protease and influenza virus in mice. *J. Virol.* **63**, 2252-2259.

Bae, K. H., Lee, S. H., Kim, S. T., Lee, S. J., Shin, Y. C. and Byun, S. M. (1996) High-level expression and secretion of *Serratia marcescens* metalloprotease inhibitor in *Bacillus subtilis* by aid of subtilisin promoter and signal sequence. *Mol. Cells* **6**, 296-302.

Bae, K. H., Kim, I. C., Kim, K. S., Shin, Y. C. and Byun, S. M. (1998) The Leu-3 residue of *Serratia marcescens* metalloprotease inhibitor is important in inhibitory activity and binding with *Serratia marcescens* metalloprotease. *Arch. Biochem. Biophys.* **352**, 37-42.

Baumann, U., Bauer, M., Letoffe, S., Delepelaire, P. and Wandersman, C. (1995) Crystal structure of a complex between *Serratia marcescens* metallo-protease and an inhibitor from *Erwinia chrysanthemi*. *J. Mol. Biol.* **248**, 653-661.

Bode, W., Gomis-Rüth, F. and Stöker, W. (1993) Astacins, serralysins, snake venom and matrix metalloproteinases exhibit identical zinc-binding environments (HEXXHXXG XXH and Met-turn) and topologies and should be grouped into a common family, the 'metzincins'. *FEBS Lett.* **331**, 134-140.

Decedue, C. J., Broussand, E. A., Lansor, A. D. and Braymer, H. D. (1979) Purification and characterization of the extracellular proteinase of *Serratia marcescens*. *Biochim. Biophys. Acta* **569**, 293-301.

Duong, F., Lazdunski, A., Cami, B. and Murgier, M. (1992) Sequence of a cluster of genes controlling synthesis and secretion of alkaline protease in *Pseudomonas aeruginosa*: relationships to other secretory pathways. *Gene* **121**, 47-54.

Greer, J. (1990) Comparative modeling methods: application to the family of the mammalian serine protease. *Proteins* **7**, 317-334.

Kim, K. S., Lee, C. W., Lee, B. R. and Shin, Y. C. (1992) Autodigestion and stability of metalloprotease purified from Serratia marcescens ATCC 21074. *Kor. J. Microbiol.* **30**, 71-77.

Kim, K. S., Kim, T. U., Kim, I. J., Byun, S. M. and Shin, Y. C. (1995) Characterization of a metalloprotease inhibitor protein (SmaPI) of *Serratia marcescens*. *Appl. Environ. Microbiol.* **61**, 3035-3041.

Leco, K. J., Khokha, R., Pavloff, N., Hawkes, S. P. and Edwards, D. R. (1994) Tissue inhibitor of metalloproteases-3 (TIMP-3) is an extracellular matrix-associated protein with a distinctive pattern of expression in mouse cells and tissues. *J. Biol. Chem.* **269**, 9352-9360.

Letoffe, S., Delepelaire, P. and Wandersman, C. (1989) Characterization of a protein inhibitor of extracellular proteases produced by *Erwinia chrysanthemi*. *Mol. Microbiol.* **3**, 79-86.

Liao, C.-H. and McCallus, D. E. (1998) Biochemical and genetic characterization of an extracellular protease from *Pseudomonas fluorescens* CY091. *Appl. Env. Microbiol.* **64**, 914-921.

Maruo, K., Akaike, T., Inada, Y., Ohkubo, I., Ono, T. and Maeda, H. (1993) Effect of microbial and mite proteases on low and high molecular weight kininogens. Generation of kinin and inactivation of thiol protease inhibitory activity. *J. Biol. Chem.* **268**, 17711-17715.

Matsumoto, K., Yamamoto, T., Akaike, T., Miyoshi, S. and Maeda, H. (1984) Pathogenesis of serratial infection: activation of the Hageman factor-prekallikrein cascade by serratial protease. *J. Biochem.* **96**, 739-749.

Moon B. J., Vipond, I. B. and Halford, S. E. (1996) Site-directed mutagenesis studies with restriction endonuclease *Eco*RV to identify the role of Ile91 in recognition and catalysis. *J. Biochem. Mol. Biol.* **29**, 99-104.

Nelson, R. M. and Long, G. L. (1989) A general method of site-specific mutagenesis using a modification of the *Thermus aquaticus* polymerase chain reaction. *Anal. Biochem.* **180**, 147-151.

Park, J. H., Park, S. K., Jang, J. S., Lee, S. H. and Byun, S. M. (1992) Molecular cloning of the promoters derived from *Bacillus insolitus* ATCC23299. *Korean Biochem. J.* **25**, 403-408.

Rawlings, N. D. and Barrett, A. J. (1995) Evolutionary families of metallopeptidases. *Methods Enzymol.* **248**, 183-228.

Rings, C. S. and Cohen, F. E. (1993) Modeling protein structures: construction and their applications. *FASEB J.* **7**, 783-790.

Sambrook, J. E., Fritsch, E. F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Stöker, W., Grams, F., Baumann, U., Reinemer, P., Gomis-Rüth, F., Mckay, D. B. and Bode, W. (1995) The metzincins--topological and sequential relations between the astacins, adamalysins, serralysins, and matrixins (collagenases) define a superfamily of zinc-peptidases. *Protein Sci.* **4**, 823-840.

Wassif, C., Cheek, D. and Belas, R. (1995) Molecular analysis of a metalloprotease from *Proteus mirabilis*. *J. Bacteriol.* **177**, 5790-5798.

Williams, J. W. and Morrison, J. F. (1979) The kinetics of reversible tight-binding inhibition. *Methods Enzymol.* **63**, 437-467.

Yang, J. H. and Kim, S. S. (1997) Effect of pyrimidylsalicylate on the valine sensitive acetolactate synthase purified from *Serratia marcescens*. *J. Biochem. Mol. Biol.* **30**, 13-17.