J. Biochem. 121, 1088-1095 (1997)

Identification of Reactive Site of a Proteinaceous Metalloproteinase Inhibitor from *Streptomyces nigrescens* TK-23

Sailaja S. Seeram,* Kazumi Hiraga,* Atsushi Saji,* Misao Tashiro,† and Kohei Oda*,[1]

*Department of Applied Biology, Faculty of Textile Science, Kyoto Institute of Technology, Sakyo-ku, Kyoto 606; and †Department of Food Science and Nutrition, Kyoto Prefectural University, Kyoto 606

Received for publication, January 8, 1997

Streptomyces metalloproteinase inhibitor (SMPI), isolated from _Streptomyces nigrescens_ TK-23, is a small proteinaceous metalloproteinase inhibitor consisting of 102 amino acid residues and two disulfide bridges. SMPI specifically inhibits metalloproteinases such as thermolysin. After prolonged incubation with a catalytic amount of thermolysin, it is cleaved at Cys64-Val65 [Murai, H., Hara, S., Ikenaka, T., Oda, K., and Murao, S. (1985) _J. Biochem._ 97, 173-180]. Hence, for identification of the reactive site, mutants were constructed by substituting Val65 with various amino acid residues (Leu, Ile, Phe, Tyr, Gly, Ser, Lys, and Glu). The mutants were analyzed for inhibitory activity. Among them, V65I, V65L, V65F, and V65Y retained strong inhibitory activity, whereas V65S, V65G, V65K, and V65E showed very weak inhibitory activity against thermolysin. The K_i values were found to be of the order of 10^{-10} M by using a fluorogenic substrate, MOCAc-Pro-Leu-Gly-Leu-A_2pr(Dnp)-Ala-Arg-NH_2. In addition, susceptibility to enzyme degradation was analyzed by means of limited proteolysis with thermolysin. Mutants which retained strong inhibitory activity were cleaved by thermolysin only at the reactive site, in the same way as native SMPI. The mutants which showed weak inhibitory activity underwent rapid degradation. These results were consistent with the substrate specificity of thermolysin. Based on these results, the reactive site of SMPI was identified as Cys64-Val65.

Key words: K_i value, metalloproteinase, proteinaceous inhibitor, reactive site, _Streptomyces_.

Metalloproteinases are widely distributed in plants, animals, and microorganisms. So far about 30 families of metalloproteinases have been recognized (*1*). However, in contrast to serine proteinase inhibitors, very few proteinaceous metalloproteinase inhibitors have been identified. *Streptomyces* metalloproteinase inhibitor (SMPI) (*2, 3*), isolated from *Streptomyces* nigrescens TK-23, was the first proteinaceous microbial metalloproteinase inhibitor to be identified. Subsequently several other proteinaceous metalloproteinase inhibitors have been isolated from various microorganisms: a periplasmic metalloproteinase inhibitor from *Erwinia crysanthemi* (*4*), an inhibitor from *Pseudomonas aeruginosa* (*5*), and two metalloproteinase inhibitors from *Serratia marcescens* SM6 (*6*) and *Serratia marcescens* ATCC 21074 (SmaPI) (*7*). Three anticoagulant proteins (serine proteinase inhibitors) were isolated from *Streptoverticillium cinnamoneum* subsp. *cinnamoneum* (SAC I, II, III), and SAC I inhibited thermolysin (*8, 9*). Some metalloproteinase inhibitors have also been isolated from animals, *e.g.*, a family of tissue inhibitors of matrix metalloproteinases (TIMPs), snake venom metalloproteinase inhibitors (*10*), and antihemorrhagic factors (*11*). However SMPI shows no sequence similarity to any of these metalloproteinase inhibitors, or to the serine proteinase inhibitors.

SMPI inhibits a wide range of bacterial metalloproteinases. It is a small protein with 102 amino acid residues, and 2 disulfide bridges (Fig. 1), and it reacts with thermolysin in the stoichiometric ratio of 1:1. When incubated with a catalytic amount of thermolysin, it is gradually cleaved at Cys64-Val65 (*3*).

Finkenstadt and Laskowski Jr. (*12*) and Ozawa and Laskowski Jr. (*13*) proposed that the reactive site of serine proteinase inhibitors is cleaved by the target enzymes. This approach has been successfully applied for the identification of the reactive site of many serine proteinase inhibitors. On the other hand, for metalloproteinases, in spite of their ubiquitous occurrence, very few proteinaceous inhibitors are identified and there is no clearly defined reactive site for metalloproteinase inhibitors. In our previous work the reactive site of SMPI was assigned tentatively as Cys64-Val65, as this bond is cleaved by thermolysin (*3*). However, this observation is insufficient for definitive identification of the reactive site. In order to determine the reactive site of SMPI unambiguously, we have carried out mutational analysis.

In this paper we describe the construction and expression

[1] To whom correspondence should be addressed: Tel.: +81-75-724-7763, Fax: +81-75-724-7760, E-mail:bika@ipc.kit.ac.jp

Abbreviations: A_2pr(Dnp), N^3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl; FAGLA, 3-(2-furylacryloyl)glycyl-L-leucine amide; MES, 2-(*N*-morpholino)ethanesulfonic acid; MOCAc, (7-methoxycoumarin-4-yl)acetyl; SMPI, *Streptomyces* metalloproteinase inhibitor. Amino acid residues are represented by one-letter or three-letter abbreviations. Mutants are identified by giving the wild-type residue followed by the residue number and the substituted residue.

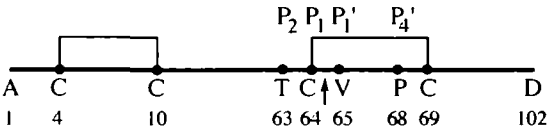

Fig. 1. **Topological structure of SMPI.** The arrow indicates the putative reactive site. The solid circles indicate amino acid residues, and the letters P_1, P_2, P_1', and P_4', are according to the nomenclature of Schechter and Berger (*39*). The abbreviated single letters and numbers represent corresponding amino acid residues and their positions.

of wild-type and mutant SMPI genes in *E. coli*, and identification of the reactive site based on the inhibitory activities and susceptibilities to enzyme degradation of the mutant proteins.

EXPERIMENTAL PROCEDURES

Materials—Plasmid pMPI321 containing the SMPI gene, cloned from *Streptomyces nigrescens* TK-23, was a generous gift from Prof. H. Takahashi, Tokyo University. All the mutant primers were kindly synthesized by Prof. Kainosho and his colleagues at Tokyo Metropolitan University. PCR kit was purchased from Perkin Elmer, Chiba. All the restriction enzymes, ligation kit, gene-clean kit, *etc.* used were commercial products. Thermolysin and native SMPI were kindly donated by Daiwa Kasei, Osaka. Glutathione Sepharose 4B and CM-Sepharose CL-6B were purchased from Pharmacia Biotech AB, Uppsala, Sweden. Reduced glutathione was purchased from Wako Pure Chemical Industries, Osaka. Phosphoramidon and thrombin were purchased from Sigma Chemical. FAGLA and MOCAc-Pro-Leu-Gly-Leu-A_2pr(Dnp)-Ala-Arg-NH_2 were purchased from Peptide Institute, Osaka.

Construction of Wild-Type SMPI (wSMPI) and Mutant SMPIs—Wild-type SMPI and mutant SMPIs were constructed as described in Fig. 2. (a) The signal sequence of SMPI was deleted by polymerase chain reaction (PCR) as shown in Fig. 2a. The plasmid pMPI 321, containing the 1.2 kb SMPI gene (*14, 15*), was digested with *Acc*I. The resulting fragment (600-700 bp) was used as the template for PCR. A *Bam*HI site was introduced at the 5′ terminal of the SMPI gene corresponding to the mature inhibitor by using the oligonucleotide primers 400BA(+) 5′-GCTCCGGCGGGATCCGCCCCGAG-3′ and 101SM(−) 5′-GAGGACGCCCCGGGTCGGCGCTC-3′. PCR was performed for 25 cycles (97°C, 1 min; 50°C, 1 min; 72°C, 1 min). (b) The amplified DNA fragment (350-400 bp) was subcloned into the *Sma*I site of pBF19 (*Bam*HI site-disrupted pUC19) plasmid as shown in Fig. 2b. The cloned gene was confirmed by restriction enzyme digestion of the newly created *Bam*HI site and dideoxy DNA sequencing. Mutant SMPIs were constructed according to the recombinant PCR method (*16*) using the *Eco*RI/*Sal*I-digested 400 bp fragment of pBF19-SMPI as a template. The mutagenic inside primers used were (newly created restriction sites are shown in brackets): V65G, 200NO(+) (*Not*I) 5′-GGTGACCTGCGGCCGCTTCCCGTG-3′; V65S, 211PS(+) (*Pst*I) 5′-AGGTGACCTGCAGCCGCTTCCCGTG-3′; V65I, 600ET(+) (*Eco*T22I) 5′-GGAAGGTGACATGCATCCGCTTCCCG-3′; V65L, VL65(+) 5′-GAAGGTGACCTGCCTCCGCTTCCCG-3′; V65F, VF65(+) 5′-GAAGGTGACCTGCTTCCGCTTCCCGT-3′; V65K, 800AO(+) (*Aor*51HI) 5′-AAGGTGACCTGCAAGCGCTTCCCGTG-3′; V65E, 611AO(+) (*Aor*51HI) 5′-AGGTGACCTGCGAGCGCTTCCCGTG-3′. As shown in Fig. 2c, the PCR-amplified fragments were subcloned into the *Bam*HI-*Sma*I sites of pGEX 4T-1 expression plasmid, transformed into *E. coli* BL21 strain, and confirmed by restriction enzyme digestion of newly created sites and/or DNA sequencing. DNA sequencing was carried out using a Taq Dye Deoxy™ Terminator Cycle Sequencing kit (Perkin Elmer) with a Perkin Elmer model 373A DNA sequencer.

Expression and Purification of Recombinant SMPIs—Cells containing the required plasmid were cultured overnight in 100 ml of 2×YT (1.6% polypeptone, 1.0% yeast extract, 0.5% NaCl, pH 7.2) medium. The 2×YT medium (2 liters) was inoculated with overnight preculture (1% v/v). Cells were grown at 37°C until the A_{600} reached 0.8-1.0, then glutathione S-transferase-SMPI (GST-SMPI) fusion protein production was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.4 mM. After 2 h induction the cells were collected by centrifugation (7,000 rpm, 20 min), suspended in 200 ml (1 : 10 volume) of 1×PBS (phosphate-buffered saline, 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3) supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF), and disrupted by sonication (Astrason XL model XL 2020). Triton X-100 was added to a final concentration of 1% and incubated for 30 min on ice to aid in solubilization of the fusion protein. All the purification steps were monitored by 1-chloro-2,4-dinitrobenzene (CDNB) assay (*17*) or by assay of inhibitory activity towards thermolysin and SDS-PAGE (*18*).

(a) Affinity column chromatography: The bacterial sonicate after centrifugation, *i.e.*, the supernatant containing fusion protein, was applied to a GST affinity matrix *i.e.*, a Glutathione Sepharose 4B column, previously equilibrated with 1×PBS (pH 7.3) supplemented with 1% Triton X-100 at 4°C. The column was washed with 200 ml of 1× PBS and eluted with 50 ml of elution buffer (50 mM Tris-HCl, pH 9.0, 10 mM GSH) at room temperature. The fractions containing inhibitor were pooled, and SMPI protein was separated from the fusion protein by thrombin digestion. For this purpose, 2.0 μg thrombin/1 mg fusion protein was added and the mixture was incubated for 16 h at 25°C, then applied to a CM-Sepharose column.

(b) CM-Sepharose column chromatography: In CM-Sepharose column chromatography, buffer B (50 mM H_3BO_3, 50 mM KCl, 50 mM Na_2CO_3, pH 8.2) was used, except for the V65E mutant. For V65E, phosphate buffer (50 mM KH_2PO_4/Na_2HPO_4, pH 6.0) was used. The thrombin digests were diluted in 5 volumes of buffer and applied to a CM-Sepharose CL-6B column, previously equilibrated with the same buffer. After washing of the column, proteins were eluted with a linear gradient of 0-0.5 M NaCl. Fractions containing SMPI protein were pooled and dialyzed against distilled water at 4°C, then lyophilized.

Production of Anti-SMPI Polyclonal Antibodies and Western Hybridization—Anti-SMPI polyclonal antibodies were raised by conventional methods. Mice were immunized by subcutaneous injections using native SMPI (15 μg/mouse). Freund's complete adjuvant was used in 1 : 1 (v/v) sterile PBS buffer (2.7 mM KCl, 137 mM NaCl, 1.5 mM KH_2PO_4, 8.1 mM $Na_2HPO_4 \cdot 7H_2O$, pH 7.4). Sera were

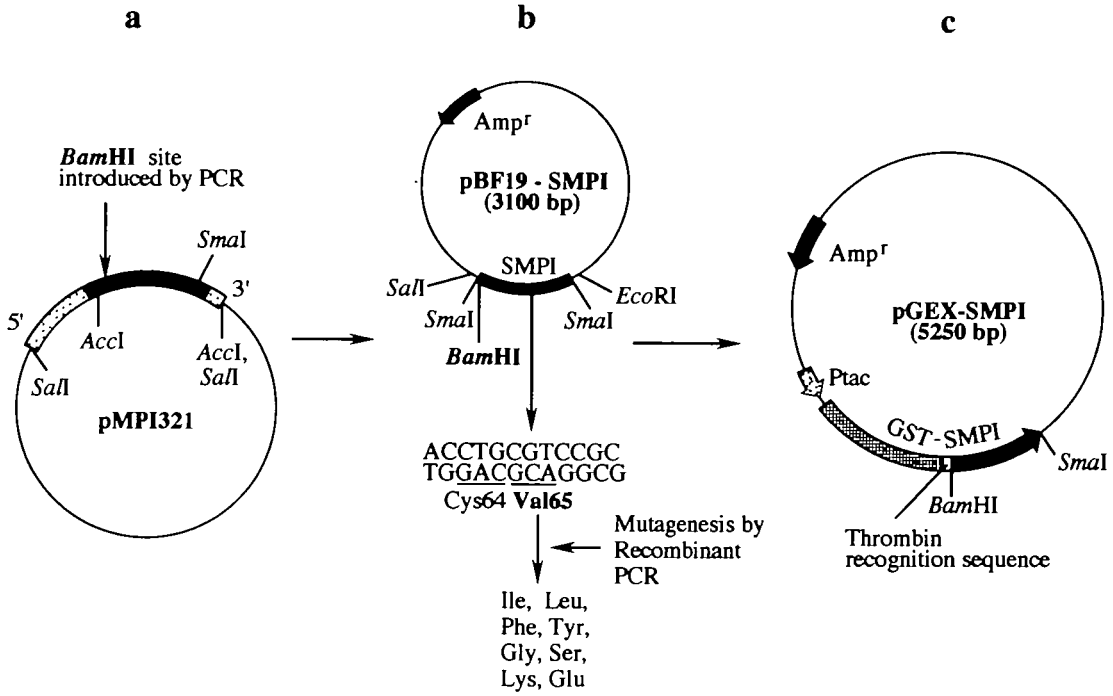

Fig. 2. **Schematic representation of construction and expression of wild-type and mutant SMPIs.** (a) The pMPI321 plasmid, containing *SalI-SalI* 1.2 kb genomic SMPI sequence, was digested with *AccI*. The resulting fragment was used as the template for the construction of wild-type SMPI. The *Bam*HI site was introduced by PCR to delete the signal sequence. (b) PCR-amplified fragments were subcloned into pBF19 vector. Mutant SMPIs were constructed according to the recombinant PCR method by using the *Eco*RI/*SalI*-digested 400 bp fragment of pBF19-wSMPI. (c) Wild-type and mutant genes were expressed by using pGEX 4T-1 expression plasmid. In the pGEX expression plasmid, inhibitor genes were inserted between the *Bam*HI and *Sma*I sites downstream of the GST gene, just after the thrombin recognition sequence.

collected at 10-day intervals and titer was monitored by Western blotting. After obtaining satisfactory titer the whole blood was collected and incubated at 37°C for 30 min, and sera were obtained by centrifugation. Sodium azide was added (0.02%) as a preservative, and the solution was stored at −70°C.

Western hybridization of expressed proteins was carried out according to the method of Towbin *et al.* (*19*), by using mouse anti-SMPI antibodies and peroxidase-conjugated mouse IgG antibodies.

Amino Acid Sequencing—(a) N-Terminal amino acid sequencing of wild-type SMPI: Wild-type SMPI (0.5 nmol/lane) was electrophoresed by Tricine-SDS-PAGE (*20*) and the protein was electrotransferred onto polyvinylidene difluoride (PVDF) membrane, stained with Coomassie Brilliant Blue R-250 and destained with 50% methanol. The protein band was sequenced by an Applied Biosystems 476A protein sequenator according to the method of Matsudaira (*21*).

(b) N-terminal amino acid sequencing of scissile bond region: Since metalloproteinases cleave the amino side of a scissile bond, the substituted residue can be confirmed. A mutant protein (2 nmol) was incubated with a catalytic amount of thermolysin at 25°C for 36 h, electrophoresed by Tricine-SDS-PAGE, electrotransferred onto PVDF membrane, and sequenced as described above.

Circular Dichroism (CD) Spectra—The CD spectra were measured on a Jasco J-720 model CD spectropolarimeter with 0.5-1.0 mg/ml protein in Milli Q water at 25°C. The data were collected from the far UV region (190 nm; quartz cell of 0.02 mm path length) to the near UV region (350 nm; a 1 cm path length cell).

Assay for Inhibitory Activity—Inhibitory activity towards thermolysin was analyzed using casein substrate as described previously (*2*).

Kinetic Analyses—Since there are no active site determinants for metalloproteinases, active enzyme concentration was determined by active site titration with a stoichiometric inhibitor, phosphoramidon. Concentrations of thermolysin and phosphoramidon were estimated from UV absorption using $\varepsilon_{278nm} = 663{,}000\ M^{-1}\cdot cm^{-1}$, and $\varepsilon_{280nm} = 5{,}550\ M^{-1}\cdot cm^{-1}$ (*22*), respectively. Thermolysin (0.18 μM) was preincubated with various concentrations of phosphoramidon (0.02-0.2 μM) for 5 min at 25°C. Preincubated FAGLA (1 mM) was added and the decrease in absorbance was measured at 345 nm on a Beckman DU-7000 spectrophotometer. Residual enzyme activity (%) was plotted against concentration of inhibitor. The concentration of inhibitor required to give complete inhibition (*i.e.*, equal to that of the active enzyme) was determined from the intercept on the abscissa. SMPI inhibitor concentrations were determined from $E^{1\%}_{280nm} = 5.56$ (*2*), and amino acid analysis. Molecular weights were considered to be 10,300 for native SMPI, and 10,500 for *E. coli*-expressed recombinant proteins, since they possess 2 extra N-terminal amino acid residues (Gly and Ser) corresponding to the *Bam*HI site of the expression vector.

All the reactions were carried out in 50 mM MES (pH 6.5), 10 mM $CaCl_2$, 0.005% Triton X-100 (v/v) buffer. Fluorimetric analyses were conducted by means of inter-

nally quenched fluorescent peptide assays using MOCAc-Pro-Leu-Gly-Leu-A_2pr(Dnp)-Ala-Arg-NH_2 substrate (*23*). Hydrolysis of the substrate results in the release of intensely fluorescent methoxycoumarin peptide, MOCAc-Pro-Leu-Gly. Increase of fluorescence per unit time (ΔI/min) was observed at λ_{ex} 328 nm, and λ_{em} 393 nm with a Hitachi model F-3010 fluorescence spectrophotometer at 25°C.

Calculation of inhibition constants (K_i): Thermolysin (1.208 nM) was incubated at 25°C for 15 min with various concentrations of SMPI inhibitor (0.2-3.0 nM; in the case of the Tyr mutant, 2-30 nM) in 980 μl of buffer. Then 20 μl of substrate was added to give a final concentration of 8 μM. In the case of V65S mutant, enzyme (25 nM) and inhibitor (100 nM-5 μM) were preincubated in 40 μl of buffer and 960 μl of buffer containing the substrate (final concentration 1 μM) was added. ΔI/min was observed for 1 min and initial velocities were calculated. From the initial velocities, free enzyme concentrations were calculated. Non-linear least-squares analysis of the data was done using a computer program; the association constants (K_a) were determined by plotting free enzyme concentration [E] *versus* inhibitor concentration [I_o] according to the method of Tashiro *et al.* (*24*) and K_i was calculated as $K_i = 1/K_a$.

Limited Proteolysis—Limited proteolysis was done at 25°C in 50 mM Tris-HCl (pH 7.5), 10 mM $CaCl_2$, 100 mM NaCl buffer. Thermolysin (100 pmol) and inhibitor (10 nmol) were incubated at an enzyme-inhibitor ratio of 1:100 (mol/mol). Aliquots were withdrawn at intervals. The enzyme reaction was stopped by adding an inhibitor, talopeptin (*25*), and the products were analyzed by Tricine-SDS-PAGE.

RESULTS

To identify the reactive site of SMPI, various mutant SMPIs were constructed, taking into account the substrate specificity of thermolysin. Thermolysin cleaves predominantly the amino side peptide bond of amino acid residues with bulky hydrophobic side chains, such as Leu, Ile, Phe, and Tyr residues. Hence, for the mutational analysis 8 substitution mutants were constructed. Val65 was replaced with amino acid residues which have good affinity, Leu (V65L), Ile (V65I), Phe (V65F), and Tyr (V65Y), or a poor affinity, Ser (V65S), Gly (V65G), Lys (V65K), and Glu (V65E), for thermolysin.

Expression and Purification of Wild-Type SMPI and Mutant SMPIs—The recombinant wild-type SMPI gene was constructed by the PCR method using a genomic SMPI fragment as the template. Mutant SMPI genes were constructed by the recombinant PCR method using recombinant wild-type SMPI gene as a template. The wild-type and mutant SMPIs were expressed in *E. coli* by using the pGEX expression system (Fig. 2). The proteins were expressed as glutathione S-transferase fusion proteins and separated by thrombin digestion. Expressed proteins were purified by glutathione affinity column chromatography and CM-Sepharose column chromatography, and approximately 5-7 mg of protein was obtained from 2 liters of culture with a yield of 40-50%. The purity was confirmed by SDS-PAGE (Fig. 3). In the DNA sequencing of V65F, 2 clones were identified with nonspecific incorporation of a single nucleotide (T to A), which resulted in a new SMPI mutant V65Y. Since thermolysin cleaves the amino side of the amino acid residues, the reactive site residue, *i.e.*, the substituted residue, can be confirmed. In that way the tyrosine residue was confirmed by N-terminal amino acid sequencing, after limited proteolysis with thermolysin.

E. coli-expressed SMPI proteins contained 2 extra amino acid residues, Gly and Ser, at the N-terminus, corresponding to the *Bam*HI site of the expression vector. The amino-terminal amino acid sequencing of the wild-type SMPI revealed the sequences Gly-Ser-Ala-Pro-Ser- and Ser-Ala-Pro-Ser-, *i.e.*, partial degradation of the first amino acid residue had occurred during thrombin digestion. Western blotting was performed in fusion protein form, and all the proteins hybridized to anti-SMPI polyclonal antibodies (data not shown). The CD spectra (Fig. 4) were taken for native, wild-type, V65I, V65L, and V65G proteins. The spectral patterns of native and recombinant proteins were indistinguishable, which indicated that the structure and conformation of the native and recombinant proteins were similar.

Inhibitory Activity against Thermolysin—Thermolysin-inhibitory activity was initially analyzed by using casein as a substrate. Only V65I, V65L, V65F, and V65Y mutants retained inhibitory activity, whereas V65G, V65S, V65K, and V65E did not show any inhibitory activity. ID_{50} values were of pmol order. When 64 pmol of enzyme was used, the ID_{50} values were found to be as follows: native 45 pmol, wild-type 42 pmol, V65I 27 pmol, V65L 100 pmol, V65F 47 pmol, V65Y 341 pmol. The order of inhibitory potency was V65I > native, wild-type, V65F > V65L > V65Y.

Kinetic Analyses—Kinetic analysis was performed by using the fluorogenic substrate MOCAc-Pro-Leu-Gly-Leu-A_2pr(Dnp)-Ala-Arg-NH_2, which has been used for matrix metalloproteinases. The K_m, k_{cat}, and k_{cat}/K_m values towards thermolysin were determined to be 9.4 μM, 1.2 s^{-1}, and 1.3×10^5 $M^{-1} \cdot s^{-1}$, respectively. The inhibition curves obtained for various SMPIs are shown in Fig. 5. Native, wild-type, V65I, and V65F showed the strongest inhibition, followed by V65L, and V65Y (Fig. 5A), while V65S, V65G, V65K, and V65E (Fig. 5B) showed inhibitory activity only when 50-200 times more inhibitor than enzyme was used. Inhibition constants, K_i values, were calculated by non-linear least-squares analysis and were about 10^{-10}; 10^{-9};

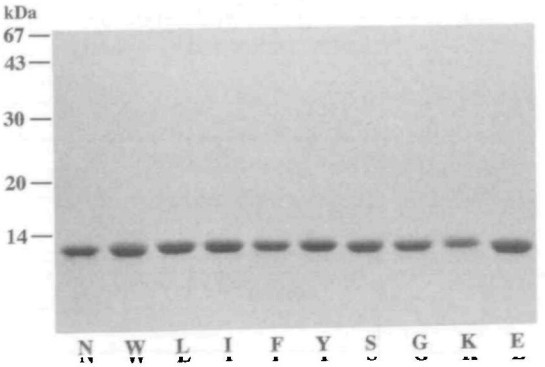

Fig. 3. **SDS-PAGE of various mutants after purification.** N, native; W, wild-type. Other letters represent one-letter abbreviations of corresponding substitution mutants. Wild-type and mutant proteins were purified as described in "EXPERIMENTAL PROCEDURES," and approximately 5 μg protein was electrophoresed on 16% SDS-PAGE gel under denaturing conditions.

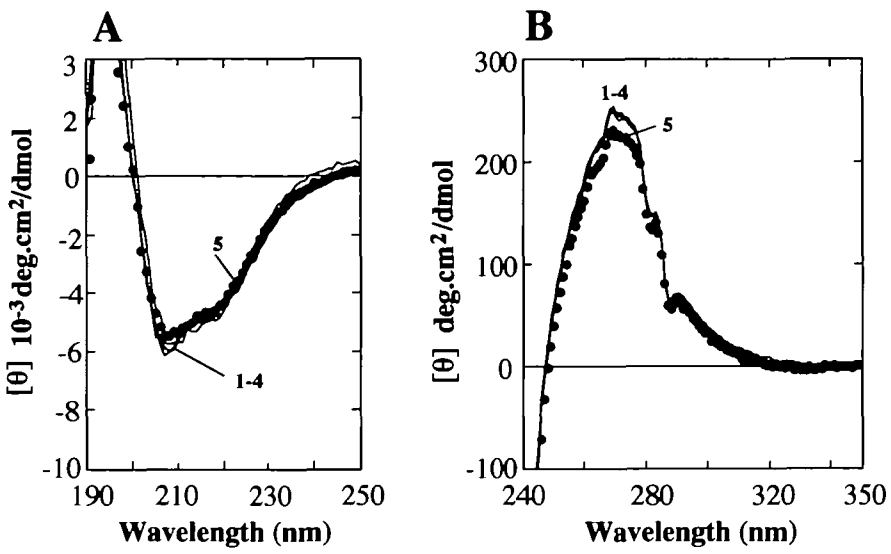

Fig. 4. **Circular dichroism (CD) spectral patterns of native and recombinant proteins.** 1-4, native, wild-type, V65I, and V65L; 5, V65G. CD spectra were taken with 0.5-1.0 mg/ml protein in Milli Q water at 25°C. Data were collected in the far (panel A) and near (panel B) UV regions.

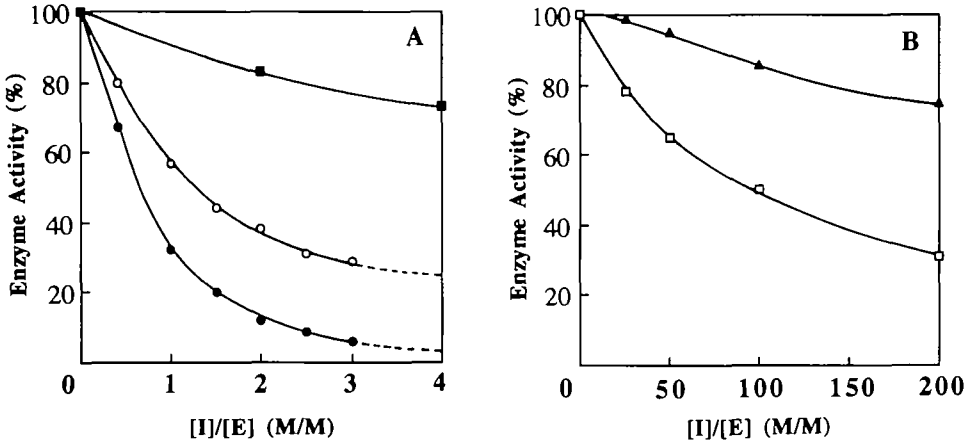

Fig. 5. **Inhibition curves obtained for various SMPIs.** Native, wild-type, V65I, and V65F showed similar patterns, represented by filled circles; open circles represent V65L; filled squares represent V65Y; open squares represent V65S; and V65G, V65K, and V65E also showed similar patterns, represented by filled triangles. Enzyme-inhibitor mixtures were incubated in 50 mM MES (pH 6.5), 10 mM $CaCl_2$, and 0.005% Triton X-100 (v/v) buffer for 15 min and the remaining enzyme activities were measured by using a fluorogenic substrate. For native, wild-type, V65I, V65F, V65L, and V65Y (panel A), 1.208 nM enzyme and various concentrations of inhibitor were incubated in a volume of 980 and 20 μl of buffer containing substrate was added before measuring the residual enzyme activity. For V65S, V65G, V65K, and V65E (panel B), 25 nM enzyme and various concentrations of inhibitor were incubated in 40 μl of buffer and 960 μl of buffer containing substrate was added before measuring the residual enzyme activity. Note that V65S, V65G, V65K, and V65E (panel B) showed inhibition at a 50-200-fold excess of inhibitor over enzyme

and 10^{-6} M for V65I, native, wild-type, V65F, V65L; for V65Y; and for V65S, respectively. Table I shows the K_i values determined for native, wild-type, and mutant inhibitors. V65I showed approximately 2 times stronger inhibition and V65L, V65Y, and V65S showed nearly 8, 60, and 35,000 times weaker inhibition than the native SMPI, respectively. For the calculation of K_i value, 8 μM (final concentration) substrate was used (about 0.85 K_m) so that free enzyme concentrations could be calculated accurately. We considered that dissociation influenced by the substrate is negligible within the 1 min assay time. Therefore the presented K_i values in Table I are the observed K_i values.

Limited Proteolysis with Thermolysin—To investigate the stability of proteins to degradation by thermolysin, limited proteolysis was carried out. The proteolysis patterns were analyzed by Tricine-SDS-PAGE. When cleavage occurs at the reactive site, two protein bands should be seen after reduction of the cystine bridge by mercaptoethanol, and electrophoresis under denaturing conditions. Two bands of molecular weight approximately 6,500 and 4,000,

TABLE I. **Calculated K_i values for various SMPIs against thermolysin.** K_i values were calculated as described under "EXPERIMENTAL PROCEDURES."

Inhibitor	K_i (M)
Native (Val)	1.01×10^{-10}
Wild-Type (Val)	1.14×10^{-10}
V65Leu	7.75×10^{-10}
V65Ile	5.46×10^{-11}
V65Phe	9.80×10^{-11}
V65Tyr	6.06×10^{-9}
V65Ser	3.54×10^{-6}
V65Gly	ND
V65Lys	ND
V65Glu	ND

ND, not determined.

corresponding to the N-terminal half and the C-terminal half were seen initially, but the smaller fragment gradually disappeared during destaining. When native, wild-type and mutant SMPIs were incubated with catalytic amounts of enzyme, 4 types of cleavage patterns were observed (Fig.

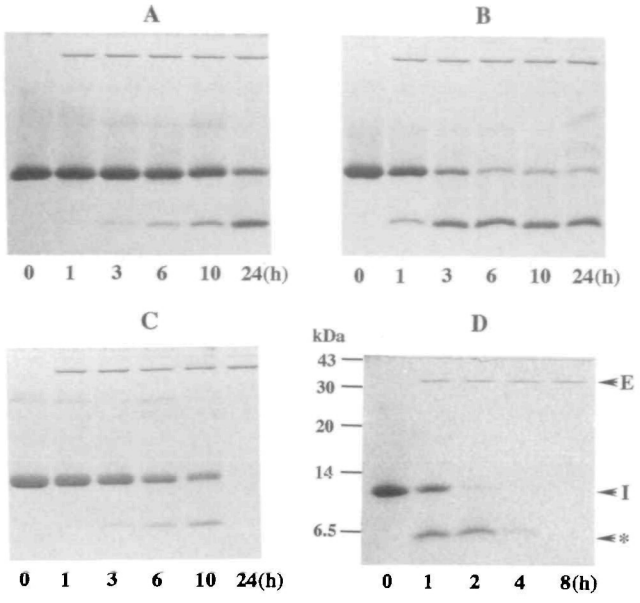

Fig. 6. **Tricine-SDS-PAGE patterns of limited proteolysis with thermolysin.** SMPIs (10 nmol) were incubated with 100 pmol of enzyme (approximately 1:100 enzyme-inhibitor ratio) in 50 mM Tris-HCl (pH 7.5), 10 mM $CaCl_2$, 100 mM NaCl buffer at 25°C, and aliquots were withdrawn periodically. Electrophoresis was carried out on Tricine-SDS-PAGE gels under denaturing conditions. The patterns were of 4 types. (A) N, WT, V65I, V65F, V65Y; (B) V65L; (C) V65S; (D) V65G, V65K, V65E, as explained in "RESULTS" and "DISCUSSION." E, enzyme; I, inhibitor. *, N-terminal half of the cleaved inhibitor.

6). Native, wild-type, V65I, V65F, and V65Y showed similar patterns, as shown in Fig. 6A: the cleaved peptide appeared at around 6 h incubation, and the proteins were stable for at least 24 h. As shown in Fig. 6B, V65L was also stable for at least 24 h but the cleavage was very fast, as the cleaved peptide can be seen at 1 h incubation. As illustrated in Fig. 6C, V65S showed a slow cleavage rate, but was degraded after 10 h incubation. V65G, V65K, and V65E were very unstable to the enzyme, and the cleaved peptide was visible at 1 h, as shown in Fig. 6D.

DISCUSSION

Even though several metalloproteinase inhibitors have been identified, little is known about their reactive sites. Tissue inhibitors of metalloproteinases (TIMPs) (*26*) and opossum serum inhibitor (oprin) (*10*) are not cleaved by the reacting enzymes. In contrast, *Streptomyces* metalloproteinase inhibitor (SMPI) is cleaved by thermolysin. As shown in Fig. 1 the scissile bond is present in a cystine bridge, the P_2 residue is threonine, and the P_4' residue is proline (Fig. 1). These features are commonly found in serine proteinase inhibitors. Laskowski Jr. and Kato described the inhibitory mechanism of serine proteinase inhibitors as the "standard mechanism" (*27, 28*). SMPI has many of the characteristics of standard mechanism inhibitors, and therefore, it may have an inhibitory mechanism similar to that of serine proteinase inhibitors. Hence for the identification of the reactive site, mutational analysis was carried out. As the standard mechanism inhibitors bind to the cognate enzymes in a substrate-like manner, SMPI mutants at the P_1' residue were constructed, based on the substrate specificity of thermolysin.

For the expression of SMPI in *E. coli* various methods were examined initially (data not shown), such as pIN III $OmpA_2$ (*29*), pIN III OmpA SSI (pOS1-t_2 plasmid) (*30*), pET 11b, and pBS (Bluescript) expression systems. However no system expressed SMPI successfully. The reason could be the instability of the SMPI-containing expression plasmids or the highly basic nature of the protein [pI value is 10.3 (*2*)]. Finally we expressed the wild-type and mutant genes as glutathione-S transferase fusion proteins by using the pGEX expression system. Expressed SMPI proteins were purified by using affinity column chromatography and ion exchange column chromatography. Wild-type SMPI, having two extra N-terminal amino acid residues, had similar properties to those of the native inhibitor. The proper folding of the proteins was confirmed by the CD spectra (Fig. 4); the native and recombinant molecules showed similar patterns.

In the present study mutational analysis was carried out from two viewpoints. One was the inhibitory properties. In the preliminary analysis with casein as a substrate, only V65I, V65L, V65F, and V65Y were found to retain inhibitory activity. Later, in the kinetic analysis with a highly sensitive fluorogenic substrate, the other mutants, V65S, V65G, V65K, and V65E, were also found to show weak inhibition (Fig. 5). As shown in Table I, V65L and V65Y mutants showed greater K_i values than native, wild-type, V65I, and V65F. As expected, V65S showed a very high K_i value. For the calculation of the K_i value of V65S, the enzyme and inhibitor were preincubated in a small volume of buffer (40 μl) and diluted to 1 ml for assay. Since V65S is a weak inhibitor, the dilution-influenced dissociation of the enzyme-inhibitor complex may have a considerable effect, and another factor is degradation by the enzyme (Fig. 6C). Hence, the observed K_i value of the V65S mutant might be far higher than its intrinsic value. The K_i values for V65G, V65K, and V65E could not be determined under the present experimental conditions because of rapid degradation of these molecules by the enzyme (Fig. 6D).

The second aspect of this study was limited proteolysis, by which the stability of the mutants to the enzyme was analyzed. All the mutants which retained strong inhibitory activity were specifically cleaved in a pattern similar to that of native SMPI (Fig. 6, A and B). In contrast, all the mutants which showed weak inhibitory activity underwent gradual degradation by thermolysin (Fig. 6, C and D). The metalloproteinase from *Bacillus subtilis* var. *amylosacchariticus*, which was weakly inhibited by native SMPI, cleaved it at many sites including the reactive site (*31*). It is assumed that in a similar way, the mutants which showed weak inhibitory activity might have served as substrates for thermolysin. It is possible that the first cleavage might have occurred at the reactive site and/or somewhere near the reactive site, followed by further degradation.

Thermolysin, a neutral thermostable metalloproteinase, was isolated from *Bacillus thermoproteolyticus* (*32*) by Endo in 1962. Since then, its substrate specificity has been extensively studied by using various kinds of substrates (*33-38*). Thermolysin hydrolyses peptide bonds involving Leu, Ile, Phe, Val, Tyr, and also many other residues. Morihara and Tsuzuki (*33*) analyzed the substrate specificity of thermolysin using Z-Gly-X-NH_2 and Z-Phe-X-Ala

substrates. In their study, based on the K_m and k_{cat} values, Leu (highest k_{cat}) and Phe (lowest K_m) were found to be the most sensitive residues at P_1', for thermolysin. In the present study the limited proteolysis patterns of V65L mutant (Fig. 6B) revealed faster hydrolysis (higher k_{cat}) than others, explaining the reason for its higher K_i value. Pozsgay *et al.* (*34*) determined K_i values for thermolysin using *N*-1-carboxy-2-phenylethyl derivatives of amino acid amides of *p*-aminobenzoate, and obtained greater K_i values for Leu and Tyr residues than Phe. It is noteworthy that the results obtained in the present study using mutant proteins, which have proper folding and large size, are in good agreement with the results obtained by other research groups using various kinds of synthetic substances which are relatively small and do not contain the appropriate conformational structure.

To conclude, in the present study, the K_i values and limited proteolysis patterns are in good agreement with the substrate specificity of thermolysin. Based on these results the Cys64-Val65 scissile bond was identified as the reactive site of *Streptomyces* metalloproteinase inhibitor, SMPI.

None of the proteinaceous inhibitors so far reported inhibit thermolysin except for SMPI and SAC I. SMPI is the only proteinaceous metalloproteinase inhibitor available for the analysis of the enzyme mechanisms of thermolysin-like enzymes. There is no existing well studied proteinaceous inhibitor for metalloproteinases. Having a broad range of inhibitory specificity, monomeric nature, small size, and a distinct reactive site, SMPI protein exhibits the necessary characteristics to serve as an excellent model inhibitor for metalloproteinases. For the determination of its structure, NMR analysis is being carried out.

We are grateful to Dr. H. Takahashi of Tokyo University for generously providing pMPI321 plasmid containing the SMPI gene, and to Drs. M. Kainosho and A. Ono of Tokyo Metropolitan University for synthesizing mutant oligonucleotide primers. We would also like to thank researchers at Kyoto Institute of Technology, especially Dr. S. Takahashi, for useful advice on protein purification, Dr. K. Hayashi for kind help in amino acid analysis and Dr. N. Tanaka for the analysis of CD spectra.

REFERENCES

1. Rawlings, N.D. and Barrett, A.J. (1995) Evolutionary families of metallopeptidases in *Methods in Enzymology* (Barrett, A.J., ed.) Vol. 248, pp. 183-228, Academic Press, New York
2. Oda, K., Koyama, T., and Murao, S. (1979) Purification and properties of a proteinaceous metallo-proteinase inhibitor from *Streptomyces nigrescens* TK-23. *Biochim. Biophys. Acta* **571**, 147-156
3. Murai, H., Hara, S., Ikenaka, T., Oda, K., and Murao, S. (1985) Amino acid sequence of *Streptomyces* metallo-proteinase inhibitor from *Streptomyces nigrescens* TK-23. *J. Biochem.* **97**, 173-180
4. Létoffé, S., Delepelaire, P., and Wandersman, C. (1989) Characterization of a protein inhibitor of extracellular proteases produced by *Erwinia chrysanthemi*. *Mol. Microbiol.* **3**, 79-86
5. 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
6. Suh, Y. and Benedik, M.J. (1992) Production of active *Serratia marcescens* metalloprotease from *Escherichia coli* by α-hemolysin HlyB and HlyD. *J. Bacteriol.* **174**, 2361-2366
7. 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
8. Tanabe, M., Asano, T., Moriya, N., Sugino, H., and Kakinuma, A. (1994) Isolation and characterization of *Streptoverticillium* anticoagulant (SAC), a novel protein inhibitor of blood coagulation produced by *Streptoverticillium cinnamoneum* subsp. *cinnamoneum*. *J. Biochem.* **115**, 743-751
9. Tanabe, M., Kawahara, K., Asano, T., Kato, K., and Kakinuma, A. (1994) Primary structure and reactive site of *Streptoverticillium* anticoagulant (SAC), a novel protein inhibitor of blood coagulation produced by *Streptoverticillium cinnamoneum* subsp. *cinnamoneum*. *J. Biochem.* **115**, 752-761
10. Catanese, J.J. and Kress, L.F. (1992) Isolation from opossum serum of a metalloproteinase inhibitor homologous to human α1B-glycoprotein. *Biochemistry* **31**, 410-418
11. Yamakawa, Y. and Omori-Satoh, T. (1992) Primary structure of the antihemorrhagic factor in serum of the Japanese habu: a snake venom metalloproteinase inhibitor with a double-headed cystatin domain. *J. Biochem.* **112**, 583-589
12. Finkenstadt, W.R. and Laskowski, M., Jr. (1965) Peptide bond cleavage on trypsin-trypsin inhibitor complex formation. *J. Biol. Chem.* **240**, 962-963
13. Ozawa, K. and Laskowski, M., Jr. (1966) The reactive site of trypsin inhibitors. *J. Biol. Chem.* **241**, 3955-3961
14. Tanaka, K., Saito, H., Arai, M., Murao, S., and Takahashi, H. (1988) Cloning and expression of the metallo-proteinase inhibitor (S-MPI) gene from *Streptomyces nigrescens*. *Biochem. Biophys. Res. Commun.* **155**, 487-492
15. Tanaka, K., Aoki, H., Oda, K., Murao, S., Saito, H., and Takahashi, H. (1990) Nucleotide sequence of the gene for a metalloproteinase inhibitor of *Streptomyces nigrescens* (SMPI). *Nucleic Acids Res.* **18**, 6433
16. Higuchi, R. (1990) Recombinant PCR in *PCR Protocols: A Guide to Methods and Applications* (Innis, M.A., Gelfand, D.H., Sninsky, J.J., and White, T.J., eds.) pp. 177-183, Academic Press, New York
17. Habig, W.H., Pabst, M.J., and Jakoby, W.B. (1974) Glutathione S-transferases. *J. Biol. Chem.* **249**, 7130-7139
18. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685
19. Towbin, H., Staehelin, T., and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**, 4350-4354
20. Ploug, M., Jensen, A.L., and Barkholt, V. (1989) Determination of amino acid compositions and NH_2-terminal sequences of peptides electroblotted onto PVDF membranes from tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis: Application to peptide mapping of human complement component C3. *Anal. Biochem.* **181**, 33-39
21. Matsudaira, P. (1987) Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. *J. Biol. Chem.* **262**, 10035-10038
22. Kitagishi, K. and Hiromi, K. (1984) Binding between thermolysin and its specific inhibitor, phosphoramidon. *J. Biochem.* **95**, 529-534
23. Knight, C.G., Willenbrock, F., and Murphy, G. (1992) A novel coumarin-labelled peptide for sensitive continuous assays of the matrix metalloproteinases. *FEBS Lett.* **296**, 263-266
24. Tashiro, M., Kihira, Y., Katayama, Y., and Maki, Z. (1989) Purification and characterization of a major trypsin inhibitor, FMTI-II, from foxtail millet grain. *Agric. Biol. Chem.* **53**, 443-451
25. Murao, S., Katsura, M., Fukuhara, K., and Oda, K. (1980) New metalloproteinase inhibitor (MK-I) produced by *Streptomyces mozunensis* MK-23. *Agric. Biol. Chem.* **44**, 701-703
26. Murphy, G., Koklitis, P., and Carne, A.F. (1989) Dissociation of tissue inhibitor of metalloproteinases (TIMP) from enzyme complexes yields fully active inhibitor. *Biochem. J.* **261**, 1031-1034
27. Laskowski, M., Jr. and Kato, I. (1980) Protein inhibitors of

proteinases. *Annu. Rev. Biochem.* **49**, 593-626
28. Laskowski, M., Jr. (1980) An algorithmic approach to sequence →reactivity of proteins. *Biochem. Pharmacol.* **29**, 2089-2094
29. Ghrayeb, J., Kimura, H., Takahara, M., Hsiung, H., Masui, Y., and Inouye, M. (1984) Secretion cloning vectors in *Escherichia coli*. *EMBO J.* **3**, 2437-2442
30. Taguchi, S., Yoshida, Y., Matsumoto, K., and Momose, H. (1993) Improved leader and putative terminator sequences for high-level production of *Streptomyces* subtilisin inhibitor in *Escherichia coli*. *Appl. Microbiol. Biotechnol.* **39**, 732-737
31. Tsuru, D., Fujita, Y., Morikawa, S., Ito, K., and Yoshimoto, T. (1992) Degradation of *Streptomyces* metalloproteinase inhibitor (SMPI) by neutral protease from *Bacillus subtilis* var. *amylosaccharitícus*. *Biosci. Biotech. Biochem.* **56**, 1275-1278
32. Matsubara, H. (1970) Purification and assay of thermolysin in *Methods in Enzymology* (Perlmann, G.E. and Lorand, L., eds.) Vol. XIX, pp. 642-651, Academic Press, New York and London
33. Morihara, K. and Tsuzuki, H. (1970) Thermolysin: Kinetic study with oligopeptides. *Eur. J. Biochem.* **15**, 374-380
34. Pozsgay, M., Michaud, C., Liebman, M., and Orlowski, M. (1986) Substrate and inhibitor studies of thermolysin-like neutral metalloendopeptidase from kidney membrane fractions. Comparison with bacterial thermolysin. *Biochemistry* **25**, 1292-1299
35. Hersh, L.B. and Morihara, K. (1986) Comparison of the subsite specificity of the mammalian neutral endopeptidase 24.11 (enkephalinase) to the bacterial neutral endopeptidase thermolysin. *J. Biol. Chem.* **261**, 6433-6437
36. Matsubara, H., Sasaki, R., Singer, A., and Jukes, T.H. (1966) Specific nature of hydrolysis of insulin and tobacco mosaic virus protein by thermolysin. *Arch. Biochem. Biophys.* **115**, 324-331
37. Matsubara, H. (1966) Observations of the specificity of thermolysin with synthetic peptides. *Biochem. Biophys. Res. Commun.* **24**, 427-430
38. Matsubara, H., Singer, A., Sasaki, R., and Jukes, T.H. (1965) Observations on the specificity of a thermostable bacterial protease "Thermolysin." *Biochem. Biophys. Res. Commun.* **21**, 242-247
39. Schechter, I. and Berger, A. (1967) On the size of the active site in proteases. I. Papain. *Biochem. Biophys. Res. Commun.* **27**, 157-162