J. Biochem. **136**, 293–300 (2004)
DOI: 10.1093/jb/mvh129

Novel Prolyl Tri/Tetra-Peptidyl Aminopeptidase from *Streptomyces mobaraensis*: Substrate Specificity and Enzyme Gene Cloning

Yukiko Umezawa[1,*], Keiichi Yokoyama[1], Yoshimi Kikuchi[1], Masayo Date[1], Kiyoshi Ito[2], Tadashi Yoshimoto[2] and Hiroshi Matsui[1,3]

[1]Institute of Life Sciences, Ajinomoto Co., Inc., Kawasaki 210-8681; [2]School of Pharmaceutical Sciences, Nagasaki University, Nagasaki 852-8521; and [3]Graduate School of Agriculture, Kyoto Prefectural University, Kyoto 606-8522

Received May 19, 2004; accepted June 2, 2004

The prolyl peptidase that removes the tetra-peptide of pro-transglutaminase was purified from *Streptomyces mobaraensis* mycelia. The substrate specificity of the enzyme using synthetic peptide substrates showed proline-specific activity with not only tripeptidyl peptidase activity, but also tetrapeptidyl peptidase activity. However, the enzyme had no other exo- and endo-activities. This substrate specificity is different from proline specific peptidases so far reported. The enzyme gene was cloned, based on the direct N-terminal amino acid sequence of the purified enzyme, and the entire nucleotide sequence of the coding region was determined. The deduced amino acid sequence revealed an N-terminal signal peptide sequence (33 amino acids) followed by the mature protein comprising 444 amino acid residues. This enzyme shows no remarkable homology with enzymes belonging to the prolyl oligopeptidase family, but has about 65% identity with three tripeptidyl peptidases from *Streptomyces lividans*, *Streptomyces coelicolor*, and *Streptomyces avermitilis*. Based on its substrate specificity, a new name, "prolyl tri/tetra-peptidyl aminopeptidase," is proposed for the enzyme.

Key words: gene cloning, processing enzyme, prolyl tri/ tetra-peptidyl aminopeptidase, *Streptomyces mobaraensis*, transglutaminase.

Abbreviations: AcOH, acetic acid; BNA, β-naphtylamide; DMSO, dimethylsulfoxide; pNA, *p*-nitroanilide; PTP-SM, prolyl tri/tetra-peptidyl aminopeptidase from *Streptomyces mobaraensis*; PVDF, poly vinylidene fluoride; SAM-P45, subtilisin-like serine protease from *Streptomyces albogriseolus*; SGMP II, *Streptomyces griseus* metallo-endoprotease; TGase, transglutaminase.

Transglutaminases (TGases) are widely distributed among mammals, plants, invertebrates, amphibians, fish, birds, and microorganisms. TGases are a family of enzymes that catalyze acyl transfer reaction between a γ-carboxyamide group of a glutamine residue in a peptide chain and a ε-amino group of a lysine residue, resulting in the formation of an ε-(γ-glutamyl) lysine cross-link (*1*, *2*).

Ando *et al*. (*3*) first found that *Streptomyces mobaraensis* (formerly classified as *Streptoverticillium mobaraense*) secretes TGase. The enzyme is now produced in a large scale using *S. mobaraensis* and is commercially available. It is used in the food industry for protein modification (*4*, *5*) and in the binding of meat or fish and gelled food products such as jelly, yogurt and cheese. Moreover, it has great potential for use in manufacturing materials for cosmetics, thermostable microcapsules, leather products, and carriers for immobilized enzymes. The total amino acid sequence of TGase from *S. mobaraensis* has been determined, and the gene has been cloned from the *S. mobaraensis* chromosome and its nucleotide sequence determined (*6*–*8*). Recently, the crystal structure has been determined as well (*9*).

TGase from *S. mobaraensis* is excreted as an inactive zymogen with a pro-domain consisting of 45 amino acid residues, and after secretion, it is processed (*8*). The pro-domain is considered to have important roles, such as increasing the thermostability and the efficiency factor of secretion and extracellular folding of the protein (*10*). In order to activate TGase, it is necessary to cleave the pro-domain, and the processing protease may have important roles in regulating the activity of TGase. The activation of TGase was investigated recently and a metalloprotease was found in *S. mobaraensis* (*11*), but after the processing of pro-TGase, an additional extra tetrapeptide remained in N-terminal region of mature TGase. To remove the remaining N-terminal tetrapeptide, it is necessary to specifically cleave a Pro-X bond. Because of the unique cyclic structure of the proline residue, peptide bonds including proline residues are often resistant to peptidase activity, even to peptidases with broad specificity. A group of enzymes that can uniquely recognize the proline residue has been naturally evolved (*12*, *13*). More recently, Zotzel *et al.* (*14*) reported that the activated transglutaminase was further processed by a tripeptidyl aminopeptidase (SM-TAP) from *S. mobaraensis*. However, no clear evidence has been provided that the tetrapeptide is processed by the enzyme.

In this work we describe the purification and characterization of a peptidase related to the removal of the pro-domain from pro-TGase. By detailed studies of substrate specificity, we show that the enzyme has proline-specific tripeptidyl and tetrapeptidyl peptidase activities. Moreo-

*To whom correspondence should be addressed. Tel: +81-44-210-5892, Fax: +81-44-210-5897, E-mail: yukiko_umezawa@ajinomoto.com

ver, we show the cloning of the peptidase gene and the deduced amino acid sequence coded by the gene. This enzyme is a novel proline-specific peptidase, since clear homology with the other enzymes belonging to the prolyloligopeptidase family is not found. Thus we propose the new enzyme name "prolyl tri/ tetra-peptidyl aminopeptidase from *S. mobaraensis*" (PTP-SM).

MATERIALS AND METHODS

Enzymes—Natural TGase was purified form the culture medium of *S. mobaraensis* as described previously (*3*). Prolyl tripeptidylaminopeptidase from *Porphyromonas gingivalis*, and prolyl oligopeptidase (prolyl endopeptidase) from *Flavobacterium meningosepticum* were prepared as described previously (*15*). SGMP II was kindly provided by Dr. Takashi Kumazaki (Aomori University, Japan).

Reagents—Most *p*-nitroanilide (pNA)–derivative peptides were purchased from Bachem (Switzerland) or Peptide Institute (Japan). Prolinal and its derivatives, and β-naphtylamide (BNA)–derivative peptides were obtained as described previously (*16*).

Bacterial Strain and Growth Conditions—S. mobaraensis IFO 13819 was obtained from the Institute for Fermentation, Osaka, Japan. The strain was routinely grown in ISP-2 broth (4 g/liter yeast extract, 10 g/liter malt extract, 4 g/liter glucose, pH 7.2) at 30°C. For purification purposes, the organism was grown in 0.8-liter batch cultures of ISP-2 broth at 30°C with agitation at 120 rpm for 3 d.

Enzyme Activity Assays—The peptidase activity was determined throughout the purification and characterization work using H-alanine-alanine-proline-*p*-nitroanilide (H-Ala-Ala-Pro-pNA, 3 mM in DMSO) as a substrate. The reaction mixture consisting of 0.5 ml of 20 mM sodium phosphate buffer, pH 6.5, containing 50 μl of 3mM H-Ala-Ala-Pro-pNA was preincubated at 30°C for 3 min. The reaction was initiated by adding 50 μl of enzyme solution. After 10 min incubation at 30°C, the reaction was stopped by adding 0.4 ml of 50% AcOH, and then the absorbance was measured at 410 nm using a spectrophotometer (UV-1200; Shimadzu Co., Japan). One unit of enzyme activity was defined as the amount of enzyme that released 1 μmol of *p*-nitroaniline per min under the conditions used. The specific activity was defined as the enzyme activity in units per mg protein. Other peptide substrates were assayed in the same manner.

Next, the activity to cleave BNA-derivative peptides was assayed by measuring the amount of BNA liberated from the peptide-BNA (*15*, *16*). To 0.8 ml of 20 mM Tris-HCl buffer, pH 7.0, was added 0.1 ml of enzyme solution, and the mixture was preincubated at 37°C for 3 min. The reaction was initiated by adding 0.1 ml of 5 mM peptide-BNA solution (in 40% dioxan). After 10 min incubation at 37°C, 0.5 ml of Fast Garnet GBC salt (1 mg/ml containing 10 % Triton-X100 in 1 M acetate buffer, pH 4.0) was added, and the absorbance was measured at 550 nm. One unit of enzyme activity was defined as the amount of enzyme releasing 1 μmol of BNA per min.

Determination of Protein Concentration—Protein concentration was determined by the Coomassie Brilliant Blue G-250 staining method using a Protein Assay kit (Nacalai Tesque, Japan).

Purification of PTP-SM—All purification steps were performed at room temperature. Cells were harvested by centrifugation (9000 rpm, 30 min), washed twice with 20 mM Tris-HCl buffer, 30 mM sodium chloride, pH 7.5, resuspended in 0.1 M sodium phosphate buffer, pH 7.0, and shaken for 30 min at 4°C. The purification procedure consisted of the following steps.

Hydrophobic interaction chromatography (1) : The extract containing protein was applied to a Butyl Sepharose 4 Fast Flow column (1.6 cm i.d. × 10 cm; Amersham Biosciences) equilibrated with 50 mM sodium phosphate buffer, pH 7.0, containing 1.5 M ammonium sulfate. The retained proteins were eluted at 2 ml/min using an ammonium sulfate gradient from 1.5 to 0 M ammonium sulfate (180 ml), and a final isocratic step at 0 M ammonium sulfate in 50 mM sodium phosphate buffer, pH 7.0 (40 ml). The eluant was collected in 45.6 ml fractions.

Hydrophobic interaction chromatography (2): The active sample from the previous chromatographic step was applied to a Phenyl Sepharose HP column (1 ml; Amersham Biosciences) equilibrated with 50 mM sodium phosphate (pH 7.0) containing 1.5 M ammonium sulfate. Proteins were eluted at 2 ml/min, using a linear gradient from 1.5 to 0 M ammonium sulfate in the same buffer (15 ml). The eluant was collected in 5.8 ml fractions.

Chromatographic separations were carried out in a fast protein liquid chromatography system (Amersham Biosciences). Desalting and buffer exchange of active fractions were carried out by gel filtration on a PD-10 column (Amersham Biosciences).

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)—Purification was monitored by SDS-PAGE using the method of Laemmli (*17*). In this case, 4–20 % polyacrylamide gels were used. The molecular mass of the denatured enzyme was estimated using a broad-range molecular weight protein standard (Amersham Biosciences). Proteins were stained with Coomassie Brilliant Blue R-250.

Isoelectric Focusing—The isoelectric point (pI) was determined by isoelectric focusing using a PhastSystem (Amersham Biosciences). In this case, a pI 3–9 wide range gel was used.

pH and Temperature Profiles—The pH dependence was determined in the range of 4.0 to 8.5 using the following buffers: 20 mM sodium acetate, pH 4.3 to 5.6; 20 mM sodium phosphate, pH 5.3 to 7.8; and Tris-HCl, pH 6.7 to 8.3. The temperature dependence was determined in the range of 15 to 65°C in 20 mM sodium phosphate buffer, pH 6.5. In every case, activity was expressed as the percentage of the activity obtained at either the optimum pH or temperature.

pH and Thermal Stability—To measure the pH stability, the enzyme solution was incubated at different pH in 0.15 M GTA (3,3-dimethylglutaric acid:Tris:2-amino-2-methyl-1,3-propanediol = 1:1:1) buffer at 4°C overnight in the range from pH 3.0 to 10.0, and the remaining activity was measured. For thermal stability, the enzyme solution was incubated at each temperature for 10 min in 20 mM sodium phosphate buffer, pH 6.5, in the range of 4 to 60°C, and the remaining activity was measured.

Table 1. **Purification of PTP-SM from *S. mobaraensis*.**

Purification step	Volume (ml)	Protein (mg)	Total activity (units)[a]	Specific activity (units/mg)	Purification (fold)	Yield (%)
Sodium phosphate extract after centrifugation	550	385	308	0.8	1	100
Butyl-Sepharose FF	45.6	9	213	23.7	30.0	69.2
Phenyl-Sepharose HP	5.8	3.8	136	35.5	44.4	44.2

[a]Based on the enzymatic activity using H-Ala-Ala-Pro-pNA as the substrate, where one unit of activity equals 1.0 μmol of *p*-nitroaniline released per min.

Analysis of Potential Enzymatic Inhibitors—For inhibition studies, the enzyme solution was incubated with the inhibitor for 30 min at room temperature, and the residual activity was recorded. All agents except for *Streptomyces* subtilisin inhibitor–like protein were assayed at 1 to 10 mM; *Streptomyces* subtilisin inhibitor–like protein purified from the *S. mobaraensis* culture (*11*) was assayed at 100 μg/ml.

Sequence Analysis—Purified PTP-SM was blotted on a PVDF membrane using a Prosorb™ blotting kit (Applied Biosystems), and the sequence was analyzed on a Shimadzu model PPSQ-10 Protein Sequencer (Shimadzu Co., Japan).

DNA Manipulations—DNA manipulations were carried out using the methods described by Sambrook *et al*. (*18*). Nucleotide sequences were determined using a BigDye terminator cycle sequencing FS ready reaction kit and a model 377 DNA sequencer (both from Applied Biosystems).

Cloning and Sequencing of the PTP-SM Gene—A region having less degeneracy, as deduced from the determined N-terminal amino acid sequence of PTP-SM, Lys-Ile-Pro-Gly-Met-Lys-Phe-Val-Glu-Glu-Lys, was selected, and a synthetic oligonucleotide (5′-AAGATCCCCGGGATGAA-GTTCGTCGAGGAGAAG-3′) was generated. The chromosomal DNA of *S. mobaraensis* IFO13819, prepared according to the conventional method, was digested with various restriction enzymes recognizing 6-nucleotide sequences and then analyzed by the Southern blot hybridization method using the synthetic oligonucleotide as a probe to generate a single band of about 6 kb as detected by *Sac*I cleavage. Accordingly, the chromosomal DNA of *S. mobaraensis* IFO13819 was digested with *Sac*I and the fragment of about 6 kb was recovered by agarose gel electrophoresis with EASYTRAP Ver. 2 (Takara Shuzo Co. Ltd., Japan). The recovered fragment was inserted into the *Sac*I site of pUC18, which was introduced into a competent *Escherichia coli* JM109 cell (Takara Shuzo Co. Ltd., Japan), thereby producing a library. The library generated in this way was screened for the strain harboring the plasmid. This was done by screening the library through colony hybridization using the above ^{32}P-labelled synthetic oligonucleotide as a probe to obtain the intended gene. The plasmid recovered from this strain was designated as pUMP1. The nucleotide sequence of the fragment cloned pUMP1 was determined.

Purification of pro-TGase—pro-TGase was purified from the culture medium of *S.mobaraensis*. The cell-free culture medium extract containing pro-TGase was applied to a weak ion-exchange chromatography column, CM Sepharose Fast Flow (1.6 cm i.d. × 10 cm; Amersham Biosciences) equilibrated with 20 mM sodium acetate buffer, pH 5.0. The retained proteins were eluted at 2 ml/min, using a sodium chloride gradient from 0 to 0.5 M sodium chloride and a final isocratic step at 0.5 M sodium chloride in 20 mM sodium acetate buffer, pH 5.0.

Activation of pro-TGase—Purified pro-TGase (final concentration; 0.5 mg/ml) was incubated with enzyme solution (S/E = 100) in 0.1 M sodium phosphate buffer, pH 7.0, at 30°C overnight. The activity of the activated TGase was analyzed by the colorimetric hydroxamate procedure (*19*), SDS-PAGE and N-terminal amino acid sequence as outlined above.

RESULTS AND DISCUSSION

Enzyme Localization and Purification—The analysis of the ability to hydrolyze H-Ala-Ala-Pro-pNA in the cultivation supernatant and on the cell surface of *S. mobaraensis* IFO13819 clearly indicates that the enzyme is localized on the cell surface. The cell-associated enzyme was easily separated from the surface of mycelia by extraction with 0.1 M sodium phosphate buffer (pH 7.0). This indicates that PTP-SM is localized at the cell wall and is connected through ionic interactions. In contrast, SM-TAP was purified from the supernatant of 50-h-old cultures (*14*). We did not detect any PTP-SM activity in culture supernatants.

The enzyme was purified by two chromatographic steps. Results of the purification procedure are summarized in Table 1. Chromatographic separation on a hydrophobic interaction column (Butyl Sepharose 4 Fast Flow) allowed the isolation of PTP-SM activity, which eluted as a unique peak at 0.60–0.55 M $(NH_4)_2SO_4$. In this purification step, an important enrichment in specific activity

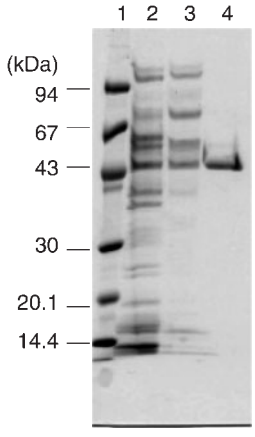

Fig. 1. **SDS-PAGE of PTP-SM active fractions obtained at different purification steps.** Lane 1, molecular mass markers; lane 2, cell extract; lane 3, Butyl Sepharose chromatography; lane 4, Phenyl Sepharose chromatography.

Table 2. **Effect of inhibitors on the activity of purified PTP-SM.**

Inhibitor	Concentration	Residual activity[a] (%)
None (control)	—	100.0
Phenylmethylsulfonyl fluoride	1 mM	0.0
Pefabloc SC	4 mM	59.9
N-Ethylmaleimide	1 mM	97.2
Iodoacetamide	5 mM	96.8
Leupeptin	1 mM	106.8
EDTA	10 mM	97.1
1,10-Orthophenanthroline	5 mM	67.2
Dithiothreitol	5 mM	94.1
Streptomyces subtilisin inhibitor–like protein	100 μg/ml	99.4
Z-Pro-Prolinal	1 mM	99.7
Z-Pro-(S)Prolinal	1 mM	105.8
Z-(S)Pro-(S)Prolinal	1 mM	111.2

[a]Results are for a 30-min incubation at room temperature in 20 mM sodium phosphate, pH 6.5, with H-Ala-Ala-Pro-pNA as the substrate.

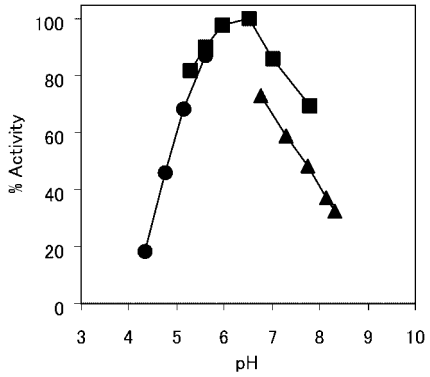

Fig. 2. **Effects of pH on PTP-SM activity using H-Ala-Ala-Pro-pNA as substrate.** The maximum activity at the optimum pH is given as 100%, which corresponds to 35.5 U/mg.

was obtained (Table 1). The enzyme eluted from the Phenyl Sepharose HP column at 0.60 M $(NH_4)_2SO_4$. This chromatographic step resulted in one major purified protein band eluted among the active fractions. The complete purification procedure yielded 44.2% of the total activity, with a 44.4-fold increase in purification.

Molecular Mass and Isoelectric Point—The molecular mass of the enzyme was estimated to be approximately 50 kDa by SDS-PAGE (Fig. 1). The isoelectric point was approximately 10.2. This value is rather high compared with other proline specific peptidases, except for prolyl oligopeptidase from *F. meningosepticum* (pI 9.6) (*15*).

pH and Temperature Profiles—In the amidolytic activity assay with H-Ala-Ala-Pro-pNA, the purified PTP-SM was active in a broad pH range from 4.3 to 8.3. The enzyme showed a pH optimum from pH 6.0 to 6.5 (Fig. 2). The optimum pH of SM-TAP was from 7.0 to 7.5, slightly different from PTP-SM. The enzyme showed broad stability between pH 4 and pH 9 for at least 12 h at 4°C. The optimum temperature was 45°C. Enzyme activity decreased significantly over 50°C. In 0.1 M sodium phosphate buffer, PTP-SM was stable at 30°C, and the stability for 10 min decreased significantly over 40°C.

Inhibition Profile—The activity was not affected by class-specific inhibitors of cysteine proteinases or metalloproteinases. Reducing agents also had no significant effect on activity. Activity was reduced to almost 40% in the presence of Pefabloc® SC (4.0 mM), and completely abolished in the presence of phenylmethylsulfonyl fluoride (1.0 mM), both of which are specific inhibitors of serine proteinases. Prolinal and its derivatives, which are typical prolyloligopeptidase inhibitors (*20*, *21*), had no effect on activity. The presence of the *Streptomyces* subtilisin inhibitor-like protein purified from *S. mobaraensis*

Table 3. **Comparison of the activities of various proline-specific peptidases against various peptide-BNA derivative substrates.**

Substrate S5 S4 S3 S2 S1 S1′	*S. mobaraensis* PTP-SM			*P. gingivaris* PTP			*F. meningosepticum* POP		
	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})
Pro-BNA		N.D.			N.D.			N.D.	
Ala-Pro-BNA		N.D.			N.D.			N.D.	
Z-Gly-Pro-BNA		N.D.			N.D.		0.12	116	967
Z-Ala-Pro-BNA		N.D.			N.D.		0.09	75.1	834
Gly-Ala-Pro-BNA	0.072	115	1,597	0.42	364	866	0.29	83.5	288
Z-Gly-Ala-Pro-BNA		N.D.			N.D.		0.31	128	412
Z-Ala-Gly-Pro-BNA		N.D.			N.D.		0.25	180	720
Gly-Ala-Gly-Pro-BNA	0.26	43	165		N.D.		0.45	149	331
Z-Gly-Ala-Gly-Pro-BNA		N.D.			N.D.		0.62	192	310
Ala-Gly-Ala-Gly-Pro-BNA		N.D.			N.D.		0.54	32.1	59.4

N.D., not detectable.

```
 1    gctcctatgagcatcgacgccgccagcagcgatcggttcggtctgaccgtcgacgccgacggcgagcgcgtgtggctggacgagcccggt

91    cggcccgtgccgctcgtgcggccgtgaaaggcccgaaaagagcccaagccgtgtgaactgcgaggacaaagggtctggcgcaacgcatgt

181   caccccagataagttcgccgcgacctttgcgaacccaggggagggcgcATGCGCAAGGCTCTCAGATCGCTGCTGGCGGCGTCGATGCTC
                                                      M  R  K  A  L  R  S  L  L  A  A  S  M  L

271   ATAGGAGCGATCGGCGCCGGCAGCGCCACGGCGGAGGCGGCGTCGATCACCGCCCCGCAGGCCGACATCAAGGACCGCATCCTGAAGATT
      I  G  A  I  G  A  G  S  A  T  A  E  A  A  S  I  T  A  P  Q  A  D  I  K  D  R  I  L  K  I

361   CCCGGGATGAAGTTCGTCGAGGAGAAGCCCTACCAGGGCTACCGCTACCTCGTGATGACGTACCGGCAGCCGGTGGACCACCGCAATCCC
      P  G  M  K  F  V  E  E  K  P  Y  Q  G  Y  R  Y  L  V  M  T  Y  R  Q  P  V  D  H  R  N  P

451   GGCAAGGGGACCTTCGAGCAGCGCTTCACCCTGCTCCACAAGGACACCGACCGGCCGACCGTGTTCTTCACGTCCGGCTACAACGTCTCC
      G  K  G  T  F  E  Q  R  F  T  L  L  H  K  D  T  D  R  P  T  V  F  F  T  S  G  Y  N  V  S

541   ACCAACCCCAGCCGCAGCGAGCCCACGCGCATCGTGGACGGCAACCAGGTGTCGATGGAGTACCGGTTCTTCACGCCGTCCCGGCCGCAG
      T  N  P  S  R  S  E  P  T  R  I  V  D  G  N  Q  V  S  M  E  Y  R  F  F  T  P  S  R  P  Q

631   CCCGCCGACTGGTCCAAGCTGGACATCTGGCAGGCGGCGAGTGACCAGCACCGCCTGTACCAGGCGCTGAAGCCGGTCTACGGGAAGAAC
      P  A  D  W  S  K  L  D  I  W  Q  A  A  S  D  Q  H  R  L  Y  Q  A  L  K  P  V  Y  G  K  N

721   TGGCTGGCCACGGGCGGCAGCAAGGGCGGCATGACGGCCACCTACTTCCGCCGCTTCTACCCGAACGACATGAACGGCACGGTCGCCTAC
      W  L  A  T  G  G  S  K  G  G  M  T  A  T  Y  F  R  R  F  Y  P  N  D  M  N  G  T  V  A  Y

811   GTCGCGCCCAACGACGTGAACGACAAGGAAGACTCGGCGTACGACAAGTTCTTCCAGAACGTCGGCGACAAGGCGTGCCGCACGCAGCTC
      V  A  P  N  D  V  N  D  K  E  D  S  A  Y  D  K  F  F  Q  N  V  G  D  K  A  C  R  T  Q  L

901   AACTCGGTGCAGCGCGAGGCGCTCGTCCGCCGCGACGAGATCGTCGCCCGCTACGAGAAGTGGGCTAAGGAGAACGGCAAGACGTTCAAG
      N  S  V  Q  R  E  A  L  V  R  R  D  E  I  V  A  R  Y  E  K  W  A  K  E  N  G  K  T  F  K

991   GTCGTCGGCAGCGCCGACAAGGCGTACGAGAACGTCGTCCTCGACCTGGTCTGGTCCTTCTGGCAGTACCACCTGCAGAGCGACTGCGCC
      V  V  G  S  A  D  K  A  Y  E  N  V  V  L  D  L  V  W  S  F  W  Q  Y  H  L  Q  S  D  C  A

1081  TCCGTCCCCGCCACCAAGGCGTCCACCGACGAGCTGTACAAGTTCATCGACGACATCTCGGGCTTCGACGGCTACACCGACCAGGGCCTG
      S  V  P  A  T  K  A  S  T  D  E  L  Y  K  F  I  D  D  I  S  G  F  D  G  Y  T  D  Q  G  L

1171  GAGCGCTTCACCCCGTACTACTACCAGGCGGGCACCCAGCTCGGCGCCCCTACGGTGAAGAACCCGCACCTCAAGGGCGTGCTGCGGTAC
      E  R  F  T  P  Y  Y  Y  Q  A  G  T  Q  L  G  A  P  T  V  K  N  P  H  L  K  G  V  L  R  Y

1261  CCCGGCATCAACCAGCCGCGCTCGTACGTCCCCCGCGACATCCCGATGACCTTCCGCCCCCGGCGCGATGGCGGACGTCGACCGCTGGGTG
      P  G  I  N  Q  P  R  S  Y  V  P  R  D  I  P  M  T  F  R  P  G  A  M  A  D  V  D  R  W  V

1351  CGCGAGGACAGCCGGAACATGCTCTTCGTGTACGGGCAGAACGACCCGTGGAGCGGTGAACCGTTCCGCCTGGGCAAGGGCGCCGCCGCC
      R  E  D  S  R  N  M  L  F  V  Y  G  Q  N  D  P  W  S  G  E  P  F  R  L  G  K  G  A  A  A

1441  CGGCACGACTACCGCTTCTACGCCCCGGGCGGCAACCACGGTTCCAACATCGCCCAGTTGGTGGCCGACGAGCGGGCCAAGGCCACGGCC
      R  H  D  Y  R  F  Y  A  P  G  G  N  H  G  S  N  I  A  Q  L  V  A  D  E  R  A  K  A  T  A

1531  GAGGTCCTGAAGTGGGCCGGTGTGGCGCCGCAGGCCGTCCAGAAGGACGAGAAGGCCGCCAAGCCGCTCGCGCCGTTCGACGCCAAGCTC
      E  V  L  K  W  A  G  V  A  P  Q  A  V  Q  K  D  E  K  A  A  K  P  L  A  P  F  D  A  K  L

1621  GACCGCGTGAAGAACGACAAGCAGAGCGCGCTGCGTCCGTAGggacccagtgcgtaaggcggcgggcgctcccggcgaggggcgcccgcc
      D  R  V  K  N  D  K  Q  S  A  L  R  P  *

1711  gtcgcgttccggaaggccccgggtgccgccgccggtgcttc
```

Fig. 3. **Nucleotide sequence of the *S. mobaraensis* PTP-SM gene.** The peptide sequence obtained by PTP-SM N-terminal sequencing analysis is underlined.

had no significant effect on enzyme activity. SM-TAP was inhibited by EDTA (*14*), but EDTA had no effect on PTP-SM (Table 2).

Substrate Specificity—The specificity of the purified enzyme was essentially confined to several chromogenic substrates. At first, for the purpose of determining the number of peptides that can be cleaved by PTP-SM, the hydrolytic activities of purified PTP-SM against various peptide-Pro-BNA derivative substrates were determined (Table 3). Enzyme activities were obtained when proline was the third or fourth residue from the N-terminus, indicating a prolyl-specific tripeptidyl and tetrapeptidyl peptidase activity, although the latter activity was very low. It was also revealed that the enzyme did not cleave when the α-amino group of the N-terminus was not free. These data indicate that the preparation of PTP-SM was free of any contamination by prolyl aminopeptidase, prolyl dipeptidyl peptidase, or prolylendopeptidase activities.

A comparison of the activity of PTP-SM with those of some typical proline-specific peptidases against various peptide-BNA derivative substrates was also performed. Prolyl tripeptidyl peptidase (PTP) from *P. gingivaris* (*22*) showed specificity similar to that of PTP-SM, but had only a prolyl tripeptidyl peptidase activity. In contrast, the specificity of prolyloligopeptidase (POP) from *F. meningosepticum* (*15*) is that of an endopeptidase.

To further confirm this specificity, the activities of PTP-SM against various tripeptide-colorimetric derivative substrates were determined (Table 4). Among the colorimetric substrates, H-Ala-Ala-Pro-pNA and H-Gly-Ala-Pro-BNA were hydrolyzed at the highest rates. Hydrolysis of Ala-X bonds by the enzyme was negligible. Most proline-specific peptidases showed alanyl activity

Table 4. **Relative activities of purified PTP-SM against various tripeptide-colorimetric derivative (pNA and BNA) substrates.**

Substrate S3 S2 S1 S1′	Activity (μmol/min/mg)
Ala-Ala-Pro-pNA	35.5
Lys-Pro-Pro-pNA	9.0
Ala-Phe-Pro-pNA	8.6
Gly-Ala-Pro-BNA	11.3
Gly-Ala-Ala-BNA	0.10
Ala-Ala-Ala-pNA	0.74
Phe-Pro-Ala-pNA	0.30
Pro-Leu-Gly-pNA	0.12
Val-Leu-Lys-pNA	0.00
Ala-Ala-Phe-pNA	0.98

```
PTP-SM   1   MRKALRSLLAASMLIGAI-----GAGSATAEAASITAPQADIKDRILKIPGMKFVEEKPY
Av-TAP   1   MRKALRWLLALAVLIGTLSTAGAATAAQPEATGSASAADTDIKDRLLAIPGMSLIQEKPY
Co-TAP   1   MRKALRWLLALVVLIGTV-----STAGAATAAEP---KAVDIKDRLLSIPGMSLIEEKPY
Li-TAP   1   MRKALRWLLALVVLIGTV-----STAGAATAAEP---KAVDIKDRLLSIPGMSLIEEKPY

PTP-SM   56  QGYRYLVMTYRQPVDHRNPGKGTFEQRFTLLHKDTDRPTVFFTSGYNVSTNPSRSEPTRI
Av-TAP   61  TGYRFFVLNYTQPIDHRHPSKGTFQQRITVLHKDTSRPTVFYTGGYNVSTTPSRREPTQI
Co-TAP   53  TGYRFFVLNYTQPVDHRHPSKGTFQQRITVLHKDVNRPTVFYTGGYNVSTNPSRREPTQI
Li-TAP   53  TGYRFFVLNYAQPVDHRHPSKGTFQQRITVLHKDVNRPTVFYTGGYNVSTNPSRREPTQI
                                                                      *
PTP-SM   116 VDGNQVSMEYRFFTPSRPQPADWSKLDIWQAASDQHRLYQALKPVYGKNWLATGGSKGGM
Av-TAP   121 VDGNQVSMEYRFFNPSRPDPADWSKLDIWQAASDQHRIFKALKAVYGQKWLSTGGSKGGM
Co-TAP   113 VDGNQVSMEYRYFTPSRPAPADWSKLDIWQAASDQHRIFKALKPLYSKNWISTGGSKGGM
Li-TAP   113 VDGNQVSMEYRYFTPSRPAPADWSKLDIWQAASDQHRIFKALKPLYSRNWISTGGSKGGM

PTP-SM   176 TATYFRRFYPNDMNGTVAYVAPNDVNDKEDSAYDKFFQNVGDKACRTQLNSVQREALVRR
Av-TAP   181 TATYYERFYPRDMDGVVAYVAPNDVVNDEDSAYDRFFANVGTKECRDKLNAVQREALVRR
Co-TAP   173 TATYYERFYPRDMDGVVAYVAPNDVVNKEDSAYDRFFARVGTDECRDKLNGVQREALVRR
Li-TAP   173 TATYYERFYPRDMDGVVAYVAPNDVVNKEDSAYDRFFARVGTDECRDKLNGVQREALVRR

PTP-SM   236 DEIVARYEKWAKENGKTFKVVGSADKAYENVVLDLVWSFWQYHLQSDCASVP--A-TKAS
Av-TAP   241 EPLEKKYAAYAADNGYTFTTIGSLDKAYEAVVLDYVWGFWQYSLLSDCDAIPADA-AHAT
Co-TAP   233 APLEKKYAAYAAENGYTFDTIGSLDRAYEAVVLDYVWGFWQYSTLADCADIP--ADAKNA
Li-TAP   233 APLEKKYAAYAAENGYTFDTIGSLDRAYEAVVLDYVWGFWQYSTLADCADIP--ADAKNA

PTP-SM   293 TDE-LYKFIDDISGFDGYTDQGLERFTPYYYQAGTQLGAPTVKNPHL-KGVLRYPGINQP
Av-TAP   300 DDA-IWDSVDTISGFSAYADQGLETYTPYYYQAGTQLGAPTIHFPYIEKKLVRY-GYQPP
Co-TAP   292 TDDAIWGSVDAISGFSAYTDQGLETYTPYYYQAGTQLGAPTIHFPHIEKKYIRY-GYQPP
Li-TAP   292 TDDAIWGSVDAISGFSAYTDQGLETYTPYYYQAGTQLGAPTIHFPHIEKKYIRY-GYQPP
                                  *
PTP-SM   351 RSYVPRDIPMTFRPGAMADVDRWVREDSRNMLFVYGQNDPWSGEPFRLGKGAAARHDYRF
Av-TAP   358 RNFVPRSIPMKFQPGAMRDVDGWVRHHARHMLFVYGQNDPWGAERFRLGAG--ARDSYVF
Co-TAP   350 RNFVPRSIPMKFEPWAMRDVDTWVRHNARHMLFVYGENDPWGAERFRLGHG--ARDSYVL
Li-TAP   350 RNFVPRSIPMKFEPWAMRDVDTWVRHNARHMLFVYGENDPWGAERFRLGHG--ARDSYVL
                    *
PTP-SM   411 YAPGGNHGSNIAQLVADERAKATAEVLKWAGVAPQAVQKDEKAAKPLAPFDAKLDRVKND
Av-TAP   416 AAPGLNHGANVAGLVADEKALATARILEWAGVASTAVQADPSAAKPLAKYDAKLDRRDVD
Co-TAP   408 TAPGMNHGANVAGLVPDQKARATARILDWAGVAPAKVQENPSAARPLATFDARLDQRDVE
Li-TAP   408 TAPGMNHGANVAGLVPDQKARATARILDWAGVAPAKVQENPSAARPLATFDARLDQRDVE

PTP-SM   471 KQSALRP
Av-TAP   476 REPTLRP
Co-TAP   468 REPALRP
Li-TAP   468 REPALRP
```

Fig. 4. **Multiple sequence alignment of *S. mobaraensis* PTP-SM and its bacterial homologues.** The sequences of the putative tripeptidyl aminopeptidase from *S. avermitilis* MA-4680 (Av-TAP) (EMBL: AP005042), the putative secreted tripeptidyl aminopeptidase from *S. coelicolor* A3(2) (Co-TAP) (EMBL: AL939113), and the secreted tripeptidyl aminopeptidase from *S. lividans* (Li-TAP) (EMBL: L46588) were aligned using the GENETIX multiple sequence alignment tool. Residues comprising the presumed catalytic triad are marked with asterisks; the consensus sequence around the active site serine in serine-type proteases, Gly-Xaa-Ser-Xaa-Gly-Gly, is underlined; and the regions of significant homology are indicated in bold and correspond to identities of at least 2 of 2, 3 of 3 or 3 of 4 of the aligned sequences.

10 to 100 times lower than the prolyl activity (*13*, *15*, *16*, *23*). This result shows that PTP-SM recognizes the proline structure more strongly than other proline-specific peptidases. It will be interesting to know the details of molecular recognition mechanism of PTP-SM for proline residues.

Sequence Analysis of PTP-SM—The N-terminal sequence of purified PTP-SM was analyzed and a sequence of 20 amino acids was obtained (Fig. 3). The region having the least degeneracy, which is deduced from the determined N-terminal amino acid sequence of PTP-SM, Lys-Ile-Pro-Gly-Met-Lys-Phe-Val-Glu-Glu-Lys, was selected and a synthetic oligonucleotide was generated. The PTP-SM gene was cloned from the chromosomal DNA of *S. mobaraensis* IFO13819 using this synthetic oligonucleotide as the ^{32}P-labelled probe. The nucleotide sequence of the fragment was determined, and found to correspond to that of PTP-SM as shown in Fig. 3. The amino acid sequence (20 residues) based on the enzyme was found, and the primary amino acid sequence of mature PTP-SM is shown in Fig. 3. The entire primary amino acid sequence containing the putative signal sequence and the pro-structure part of PTP-SM was determined. The molecular mass estimated from the amino acid sequence of the mature enzyme is 50,547. PTP-SM has proline specificity, however, the enzyme shows no homology with any other proline-specific enzymes. A homology search performed using the protein BLAST programs of the NCBI and EMBL databases revealed three tripeptidyl peptidases in *S. lividans* (EMBL: L46588), *S. coelicolor* (EMBL: AL939113), and *S. avermitilis* (EMBL: AP005042) coding for proteins showing

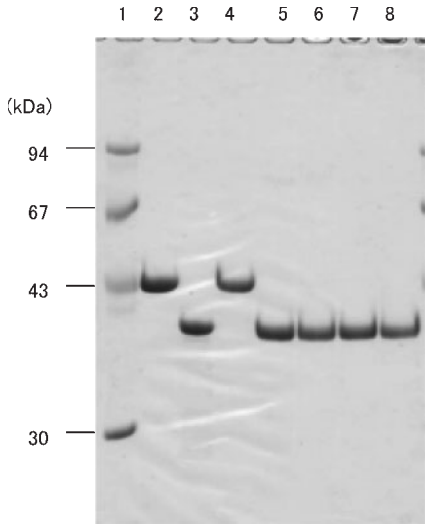

Fig. 5. **Electrophoretic analysis of activation of pro-TGase (pTG) by PTP-SM with and without proteases.** Purified pTG was incubated with enzyme solutions (pTG: enzyme = 100:1) at 37°C for 15 h. Lane 1, molecular mass markers; lane 2, purified pTG from *S. mobaraensis*; lane 3, purified TGase from *S. mobaraensis*; lane 4, pTG + PTP-SM; lane 5, pTG + SAM-P45; lane 6, pTG + SAM-P45 + PTP-M; lane 7, pTG + SGMP II; lane 8, pTG + SGMP II + PTP-SM.

about 65 % identity with PTP-SM (Fig. 4). According to the classification by Barret (*24*), tripeptidyl peptidases from *S. lividans* belong to the clan SC and the α/β hydrolase fold family. The results of this work suggested that PTP-SM is a member of a new family of proline-specific enzymes. Moreover, the catalytic triad in this enzyme as deduced by homology is Ser171, Asp371 and His417 (Fig. 4); the topology of these residues seems to be close to the catalytic triad of the prolyl oligopeptidase family (Ser-Asp-His), but different from those of the trypsin (His-Asp-Ser) and subtilisin (Asp-His-Ser) families of serine proteinases.

Processing of pro-Transglutaminase—The processing of pro-TGase has previously been reported. Dispase from *Bacillus polymixa* (*8*), SAM-P45 from *Streptomyces albogriseolus* (*10*, *25*), and TAMEP from *S. mobaraensis* (*11*) hydrolyze the peptide bond on the N-side of Phe (–4) in the pro-domain. Bovine chymotrypsin (*11*) hydrolyzes on the N-side of Arg (–3). Bovine trypsin (*8*) hydrolyzes on the N-side of Ala (–2) and converts the enzyme to an active form. However, to obtain the complete mature TGase, it is necessary to cleave on the C-side of Pro (–1); as the specificity of proline-specific enzymes is very narrow, it is expected that TGase must be processed in at least a two-step cleavage procedure (*11*). We used SAM-P45 and SGMP II, a metalloprotease from *Streptomyces griseus* (*26*, *27*) with and without purified PTP-SM to process pro-TGase. SDS-PAGE and activity measurement analysis showed that SAM-P45 and SGMP II convert pro-TGase to an active-form of TGase, but that PTP-SM alone could not cleave pro-TGase at all (Fig. 5). SM-TAP also gave the same result. To obtain information on the cleavage site, the N-terminal amino acid sequences of the products were analyzed. It was revealed that the cleavage site of PTP-SM with SAM-P45 or

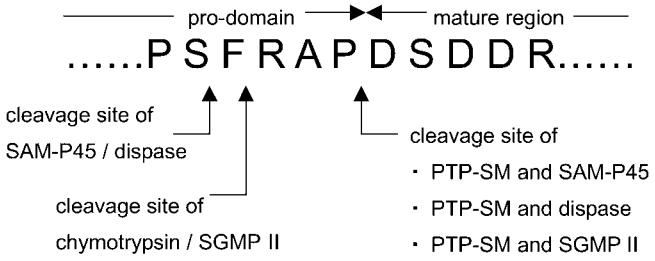

Fig. 6. **Cleavage site of TGase from *S. mobaraensis*.** The cleavage sites of SAM-P45, SGMP II, chymotrypsin, TAMEP and dispase with and without PTP-SM are indicated by arrows.

SGMP II is the C-side of Pro (–1), converting it into mature TGase with the same N-terminal amino acids as those of natural TGase. The result using dispase instead of SAM-P45 was the same. Moreover, the cleavage site of SGMP II is the same as that of chymotrypsin, the N-side of Arg (–3) (Fig. 6). Thus, it is shown that PTP-SM, strongly presumed to be related to the maturation of pro-TGase, cleaves the peptide bonds of Phe-Arg-Ala-Pro and Arg-Ala-Pro. This and the substrate specificity mean that PTP-SM has both tripeptidyl aminopeptidase activity and tetrapeptidyl aminopeptidase activity, but does not have endopeptidase activity.

This is the first report of the gene cloning of a prolyl tri/tetra-peptidyl aminopeptidase that acts in the maturation of TGase.

REFERENCES

1. Folk, J.E. and Finlayson, J.S. (1977) The epsilon-(gamma-glutamyl)lysine crosslink and the catalytic role of transglutaminases. *Adv. Protein Chem.* **31**, 1–133
2. Griffin, M., Casadio, R., and Bergamini C.M. (2002) Transglutaminases: nature's biological glues. *Biochem. J.* **368**, 377–396
3. Ando, H., Adachi, M., Umeda, K., Matsuura, A., Nonaka, M., Uchio, R., Tanaka, H., and Motoki, M. (1989) Purification and characterization of a novel transglutaminase derived from microorganisms. *Agric. Biol. Chem.* **53**, 2613–2617
4. Nielsen, P.M. (1995) Reactions and potential industrial applications of transglutaminases. *Food Biotechnol.* **9**, 119–156
5. Motoki, M. and Kumazawa, Y. (2000) Recent research trends in transglutaminase technology for food processing. *Food Sci. Technol. Res.* **6**, 151–160
6. Kanaji, T., Ozaki, H., Takao, T., Kawajiri, H., Ide, H., Motoki, M., and Shimonishi, Y (1993) Primary structure of microbial transglutaminase from *Streptoverticillium* sp. strain s-8112. *J. Biol. Chem.* **268**, 11565–11572
7. Washizu, K., Ando, K., Koikeda, S., Hirose, S., Matsuura, A., Takagi, H, Motoki, M., and Takeuchi, K. (1994) Molecular cloning of the gene for microbial transglutaminase from *Streptoverticillium* and its expression in *Streptomyces lividans*. *Biosci. Biotechnol. Biochem.* **58**, 82–87
8. Pasternack, R., Dorsch, S, Otterbach, J.T., Robenek, I.R., Wolf, S., and Fuchsbauer, H.-L. (1998) Bacterial pro-transglutaminase from *Streptoverticillium mobaraense*—purification, characterisation and sequence of the zymogen. *Eur. J. Biochem.* **257**, 570–576
9. Kashiwagi, T., Yokoyama, K., Ishikawa, K., Ono, K., Ejima, D., Matsui, H., Suzuki, E. (2002) Crystal structure of microbial transglutaminase from *Streptoverticillium mobaraense*. *J. Biol. Chem.* **277**, 44252–44260
10. Kikuchi, Y., Date, M., Yokoyama, K., Umezawa, Y., and Matsui, H. (2003) Secretion of active-form *Streptoverticillium*

mobaraense transglutaminase by *Corynebacterium glutamicum*: processing of the pro-transglutaminase by a cosecreted subtilisin-like protease from *Streptomyces albogriseolus*. *Appl. Environ. Microbiol.* **69**, 358–366
11. Zotzel, J., Keller, P., and Fuchsbauer, H.-L. (2003) Transglutaminase from *Streptomyces mobaraensis* is activated by an endogenous metalloprotease. *Eur. J. Biochem.* **270**, 3214–3222
12. Walter, R., Simmons, W., and Yoshimoto, T. (1980) Proline specific endo- and exopeptidases. *Mol. Cell. Biochem.* **30**, 111–127
13. Cunningham, D.F. and O'Connor, B. (1997) Proline specific peptidases. *Biochim. Biophys. Acta* **1343**, 160–186
14. Zotzel, J., Pasternack, R., Pelzer C., Ziegert, D., Mainusch M., and Fuchsbauer, H.-L. (2003) Activated transglutaminase from *Streptomyces mobaraensis* is processed by a tripeptidyl aminopeptidase in the final step. *Eur. J. Biochem.* **270**, 4149–4155
15. Yoshimoto, T., Walter, R., and Tsuru, D. (1980) Proline specific endopeptidase from *Flavobacterium*. *J. Biol. Chem.* **255**, 4786–4792
16. Yoshimoto, T., Fischl, M., Orlowski, R.C., and Walter. (1978) Post-proline cleaving enzyme and post-proline dipeptidyl aminopeptidase. *J. Biol. Chem.* **253**, 3708–3716
17. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacterio-phage T4. *Nature* **227**, 680–685
18. Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning. A Laboratory Manual* pp. 1339–1341, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
19. Folk, J.E. and Cole, P.W. (1966) Transglutaminase: mechanistic features of the active site as determined by kinetic and inhibitor studies. *Biochim. Biophys. Acta* **122**, 244–264
20. Yoshimoto, T., Kawahara, K., Matsubara, F., Kado, K., and Tsuru, D. (1985) Comparison of inhibitory effects of prolinal-containing peptide derivatives on prolyl endopeptidases from bovine brain and *Flavobacterium*. *J. Biochem.* **98**, 975–979
21. Wilk, S. and Orlowski, M (1983) Inhibition of rabbit brain prolyl endopeptidase by n-benzyloxycarbonyl-prolyl-prolinal, a transition state aldehyde inhibitor. *J. Neurochem.* **41**, 69–75
22. Banbula, A., Mak, P., Bugno, M., Silberring, J., Dubin A., Nelson, D., Travis, J., and Potempa, J. (1999) Prolyl tripeptidyl peptidase from *Porphyromonas gingivalis*. A novel enzyme with possible pathological implications for the development of periodontitis. *J. Biol. Chem.* **274**, 9246–9252
23. Inoue, T., Ito, K., Tozaka, T., Hatakeyama, S., Tanaka, N., Nakamura, K.T., and Yoshimoto, T. (2003) Novel inhibitor for prolyl aminopeptidase from *Serratia marcescens* and studies on the mechanism of substrate recognition of the enzyme using the inhibitor. *Arch. Biochem. Biophys.* **416**, 147–154
24. Barrett, A.J., Rawlings, N.D., and Woessner, J.F. (1998) *Handbook of Proteolytic Enzymes* pp. 369–372, Academic Press, Oval Road, London
25. Suzuki, M., Taguchi, S., Yamada, S., Kojima, S., Miura, K., and Momose, H. (1997) A novel member of the subtilisin-like protease family from *Streptomyces albogriseolus*. *J. Bacteriol.* **179**, 430–438
26. Kojima, S., Kumazaki, T., Ishii, S., and Miura, K. (1998) Primary structure of *Streptomyces griseus* metalloendopeptidase II. *Biosci. Biotechnol. Biochem.* **62**, 1392–1398
27. Tsuyuki, H., Kajiwara, K., Fujita, A., Kumazaki, T., and Isii, S. (1991) Purification and characterization of *Streptomyces griseus* metalloendopeptidases I and II. *J. Biochem.* **110**, 339–344