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A New Aminopeptidase from the Keratin-Degrading Strain *Streptomyces fradiae* var. k11

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Abstract An aminopeptidase gene fragment was isolated from a keratin-degrading strain, *Streptomyces fradiae* var. k11, by PCR amplification using a degenerate primer set designed based on the partial amino acid sequence of the native enzyme. The gene, designated *sfap*, encoded a polypeptide of 461 amino acids comprised of three domains: a signal peptide, a mature region, and a C-terminal propeptide. The aminopeptidase, SFAP, had highest amino acid sequence identity (79%) with a putative aminopeptidase from *Streptomyces griseus* subsp. griseus NBRC 13350. The gene with and without C-terminal propeptide was successfully overexpressed in *Escherichia coli* BL21 (DE3), and the gene without C-terminal propeptide encoded a functional enzyme. Purified recombinant SFAP exhibited optimal activity at pH 8.0 and 60 °C, and retained >60% peak activity over a broad range of temperature. The enzyme was thermal and pH stable, and showed metalloprotease characteristics, which was inhibited by EDTA but activated by Ca^{2+} and Co^{2+}. This is the first study to report the gene cloning and expression of a leucine aminopeptidase from *S. fradiae*.

Keywords Aminopeptidase · *Streptomyces fradiae* var. k11 · C-terminal propeptide

Introduction

Aminopeptidases (APs) are exopeptidases that catalyze the hydrolysis of amino acid residues from the N terminus of peptides and proteins [1, 2]. These enzymes are associated

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with many biological functions. Some of them play a role in the catabolism of exogenously supplied peptides and are necessary for the final steps of protein turnover. In addition, they are involved in some specific functions, such as protein maturation, protein degradation, hormone level regulation, and cell cycle control [3–5].

Streptomyces are gram-positive bacteria and have been the source of commercially important bioactive molecules, including enzyme inhibitors, antibiotics, and many valuable enzymes such as proteases [6, 7]. The APs from *Streptomyces* are of particular interest for use in biochemical and biomedical applications [8]. APs can be classified into families and clans based on their sequence homology. Many known *Streptomyces* APs, such as those from *Streptomyces griseus*, *Streptomyces septatus*, and *Streptomyces exfoliatus*, are metallopeptidases and belong to clan M28. Because of their strict enantioselectivity, high thermal stability, and ability to function as amidases and esterases, M28 APs are considered to be available for industrial applications [9–11].

So far, only one leucine AP from *Streptomyces fradiae* has been purified and characterized [12] and no leucine AP-coding gene has been cloned. In this study, an extracellular AP secreted from *S. fradiae* var. k11 during feather degradation was purified and the coding region was subjected to cloning, sequencing, and expression in *Escherichia coli*. To understand the enzymatic properties of the isolated *Streptomyces* AP, we performed a preliminary analysis of the recombinant enzyme.

Materials and Methods

Strains, Plasmids, and Chemicals

S. fradiae var. k11 [13] was from our laboratory stock. *E. coli* JM109 and BL21 (DE3) (Invitrogen, USA) were used for gene library construction, isolation of plasmids, transformation, and gene expression. Plasmids pUC19 and pGEM-T Easy (Promega, USA), and pET-22b(+) (Novagen, USA) were used for subcloning. *LA Taq* DNA polymerase (TaKaRa, Japan) and Phusion High-Fidelity DNA polymerase (New England Biolabs, UK) were used for polymerase chain reactions (PCR). L-Leu *p*-nitroanilide (Leu-pNA) (Sigma, USA) was used as the substrate for the aminopeptidase. All other chemicals are of analytical grade and commercially available.

Medium and Culture Conditions

S. fradiae var. k11 was grown in Gause's synthetic medium (0.1% KNO_3, 2% soluble starch, 0.05% K_2HPO_4, 0.05% $MgSO_4$, 0.05% NaCl, 0.001% $FeSO_4$) at 30 °C for 48 h. To induce AP production, the high-density culture of *S. fradiae* var. k11 was transferred to the feather medium (0.3% chicken feathers, 0.1% carbamide, 0.1% KH_2PO_4, 0.1% $(NH_4)_2SO_4$) for growth at 30 °C for 48~60 h. Luria-Bertani (LB) medium (0.5% yeast extract, 1% tryptone, and 1% NaCl) containing 100 μg ml^{-1} ampicillin was used to cultivate the recombinant *E. coli.*

PCR Amplification, Cloning, and Sequencing

The native enzyme secreted by *S. fradiae* var. k11 into feather medium was purified to electrophoretic homogeneity by ammonium sulfate precipitation. Then the precipitate was

suspended in 20 mM Tris–HCl (pH 8.0), dialyzed against the same buffer, and concentrated using polyethylene glycol 8000. The clear supernatant was loaded onto a HiTrap Q Sepharose XL column (GE Healthcare, Sweden) equilibrated with the same buffer. Proteins were eluted using a linear gradient of NaCl (0–1.0 M) in the same buffer. Fractions having enzyme activity were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis. Protein elution only showed one band, which was then excised from the gel and subjected to liquid chromatography–electrospray ionization–tandem mass spectrometry (LC-ESI-MS/MS) analysis. Three internal peptides were identified: APDI PLANVK, WGAEELGLIGSK, and VGVPVGGLFTG. Based on these partial amino acid sequences, a pair of degenerate primers (P1, 5′-GACATCCCSCTSGCSAACGTSAAG-3′ and P2, 5′-GTGAASAGGCCGCCSACSGGSAC-3′, S stand for C or G) was designed for PCR amplification of part of the gene using genomic DNA from *S. fradiae* var. k11 as a template. PCR reactions were carried out as follows: 94 °C for 4 min, followed by 32 cycles at 98 °C for 60 s, 72 °C for 2 min 30 s, with final extension at 72 °C for 7 min. The amplified fragment was purified and ligated into pGEM-T Easy for sequencing and basic local alignment search tool (BLAST) analysis.

A genomic library was constructed as described [13]. Briefly, a colony PCR amplification method was used for target gene isolation using the primers F1 (5′-ACCTCACGCAGTTCCAGTCG-3′) and F2 (5′- CCGATCATGTCGAAGTTCAG-3′). The positive AP clone was isolated and sequenced.

Sequence Analysis

Sequence assemble was performed using programs from the Vector NTI Suite 10.0 software. DNA and protein sequence similarity searches, conserved domain analysis, and mature peptide predictions were carried out using the BLAST server (http://www.ncbi.nlm.nih.gov/BLAST/). The AP signal peptide was predicted using SignalP (http://www.cbs.dtu.dk/services/SignalP/). Protein classification was determined by reference with the Merops peptidase database (http://merops.sanger.ac.uk). Multiple sequence alignment was performed using the clustalW program (http://www.ebi.ac.uk/clustalw/).

Expression of *sfap* in *E. coli*

Two DNA fragments without the signal peptide sequence, one encoding the propeptide plus the mature enzyme (*sfappro*), the other encoding only the mature enzyme (*sfap*), were amplified by PCR from the genomic DNA of *S. fradiae* var. k11. For *sfappro* amplification, primers P3, 5′-AAA*GAATTC*GGCACCCTCCGCGGCCC-3′ (*Eco*R I site italicized) and P4, 5′-ACC*AAGCTT*TCAGAAGGTCAGCGAGAAGC-3′ (*Hin*d III site italicized) were used, and for *sfap* amplification, primers P5, 5′-AAA*GAATTC*GGCACCCTCCGCGGCCC-3′ (*Eco*R I site italicized) and P6, 5′-ACC*AAGCTT*TTAGGCCGGCGGCTCGG-3′ (*Hin*d III site italicized) were used. PCR amplification reactions were carried out as follows: 98 °C for 30 s, followed by 30 cycles at 98 °C for 10 s, 68 °C for 50 s, with final extension at 72 °C for 7 min. The PCR products were purified by agarose gel electrophoresis, digested with *Eco*R I and *Hin*d III, and then cloned into the corresponding sites of pET-22b (+). pET-22b-*sfappro* and pET-22b-*sfap* were verified by sequencing and were transformed into *E. coli* BL21 (DE3) competent cells for protein expression.

Positive transformants were grown at 37 °C overnight in LB medium supplemented with ampicillin (100 μg ml^{-1}). Cultures were diluted 1:100 into fresh medium containing

ampicillin and grown at 37 °C under aerobic conditions until the absorbance at 600 nm reached 0.6. Isopropyl-β-D-1-thiogalactopyranoside (IPTG) was then added to a final concentration of 0.8 mM and the cells were induced at 28 °C for 12 h with agitation at 220 rpm.

Purification of the Recombinant AP Enzyme

To purify the recombinant enzyme, cultures of the positive transformants were centrifuged at 12,000×*g* for 10 min at 4 °C and the cell-free supernatants were dialyzed by ultrafiltration using Vivaflow 200 (Sartorius Stedim Biotech, Germany). Following ultrafiltration, 2 ml of the concentrated dialysate was applied to a HiTrap Q Sepharose XL FPLC column (GE Healthcare) equilibrated with 20 mM Tris–HCl buffer (pH 8.0). Proteins were eluted using a linear gradient of NaCl (0–1.0 M NaCl) in the same buffer. Fractions having enzyme activity were subjected to SDS-PAGE using a 5% polyacrylamide stacking gel above a 12% separating gel followed by staining with 0.2% Coomassie brilliant blue R-250. The concentration of the purified enzyme was determined by the Bradford assay, using bovine serum albumin as a standard [14].

Enzyme Assay

Aminopeptidase activity assays were performed by incubating purified enzyme in 50 mM Tris–HCl (pH 8.0) containing 3.2 mM Leu-pNA substrate at 37 °C and measuring absorbance at 405 nm to determine *p*-nitroaniline released. Initial reaction rates were determined from the linear portion of the optical density profile. One unit of enzyme activity was defined as the amount of enzyme that liberated 1 μmol of *p*-nitroaniline (ε= 10,600 M^{-1} cm^{-1} at 405 nm) per minute [15].

Enzymatic Characterization

The optimal pH for purified recombinant enzyme activity was determined at 37 °C using the following buffers: McIlvaine buffer (0.2 M Na_2HPO_4, 0.1 M citric acid) for pH 3.0 to 8.0, 0.1 M Tris–HCl for pH 8.0 to 9.0, or 0.1 M glycine–NaOH for pH 9.0 to 11.0. The effect of pH on enzyme stability was determined by incubation of the purified recombinant enzyme at 37 °C for 1 h at pH 2.0–11.0 using the indicated buffers and then measuring enzyme activity under standard conditions.

The temperature for optimal purified recombinant enzyme activity was determined at the optimum pH as determined above at temperatures ranging from 30 °C to 90 °C. The thermostability of the purified recombinant enzyme was monitored by pre-incubating the enzyme in 0.1 M Tris–HCl buffer (pH 8.0) at 60, 70, or 80 °C for 2, 5, 10, 20, 30, and 60 min without substrate. The residual enzyme activity in each case was then assayed under standard assay conditions.

The effects of different metal ions on purified recombinant enzyme activity were determined at 37 °C in 0.1 M Tris–HCl buffer (pH 8.0) supplemented with 0.1 mM or 1 mM $CaCl_2$, $CoCl_2$, $NiSO_4$, $CuSO_4$, $MgSO_4$, $MnSO_4$, $ZnSO_4$, $Pb(CH_3CO_2)_2$, $HgCl_2$, and metal chelator ethylenediaminetetraacetic acid (EDTA).

The K_m and V_{max} values for the purified recombinant enzyme were determined by Lineweaver–Burk analysis. The enzyme activity was measured at 37 °C in Tris–HCl buffer (pH 8.0) containing 0.3 to 3.0 mM Leu-pNA as substrate.

Results

Gene Cloning

A 653-bp DNA fragment was amplified by PCR using degenerate primers (P1 and P2) designed based on the above partial amino acid sequences of the purified aminopeptidase from *S. fradiae* var. k11. Based on the partially identified sequence, primers F1 and F2 were synthesized and used to screen the constructed *S. fradiae* var. k11 genomic library. Approximately 6,200 recombinants were screened and one clone containing the gene *sfap* was identified and sequenced. The resulting nucleotide sequence of the gene, designated *sfap*, was deposited in GenBank under accession no. AJ781828.

Sequence Analysis

Based on sequence analysis, the open reading frame of *sfap* was comprised of 1,386 bp encoding 461 residues of the putative aminopeptidase, named SFAP. A putative Shine-Dalgarno sequence (ribosome-binding site), GGAG, was identified 7-bp upstream of the ATG start codon of *sfap*. SignalP indicated the presence of a signal peptide at residues 1–42. The boundary between the mature enzyme and the proenzyme was predicted to be

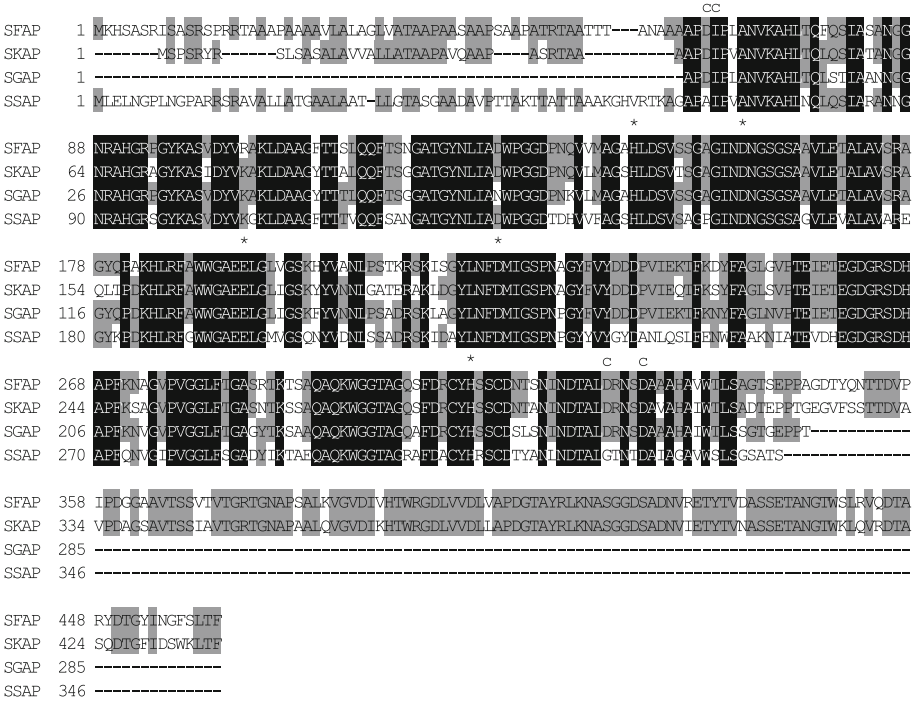

Fig. 1 Primary structure comparison among *Streptomyces* aminopeptidases (APs). SFAP, AP from *Streptomyces fradiae*; SKAP, AP from *Streptomyces* sp. KK565; SGAP, AP from *Streptomyces griseus*; SSAP, AP from *Streptomyces septatus* TH-2. All sequences reflect the full-length enzyme. Residues that are conserved among all sequences are highlighted in *black*. Residues potentially involved in zinc coordination are marked (*) and are fully conserved in all of the four APs. Residues regulated APs by calcium are marked (*C*)

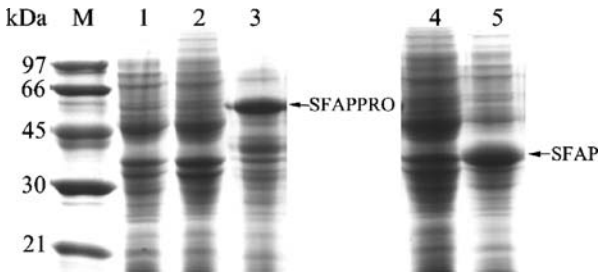

Fig. 2 SDS-PAGE analysis of recombinant SFAPPRO and SFAP expression in *E. coli* BL21 (DE3) cells. *M* lower protein molecular mass markers (kDa), lane *1* cell lysate from IPTG induction of empty pET-22b(+) vector, lane *2* cell lysate from pET-22b-*sfappro* transformant prior to IPTG induction, lane *3* cell lysate from IPTG induction of pET-22b-*sfappro*, lane *4* cell lysate from pET-22b-*sfap* transformant prior to IPTG induction, lane *5* cell lysate from IPTG induction of pET-22b-*sfap*

between Ala-346 and Gly-347, indicating that SFAP contains a C-terminal propeptide that is likely cleaved following secretion. Three SFAP homologs were identified in GenBank using BLAST and an alignment was generated. The deduced amino acid sequence exhibited highest identity (79%) to a putative aminopeptidase from *S. griseus* subsp. griseus NBRC 13350, and exhibited 71% identity to leupeptin-inactivating enzyme 1 precursor (LIE1) from *S. exfoliatus*, an enzyme with experimentally verified function [11]. The predicted mature peptide contained 304 residues with a calculated molecular weight of 31.5 kDa. Five conserved residues, H147, D159, E194, D222, and H309, were found in SFAP, implicating in the coordination of two zinc atoms (see asterisks in Fig. 1) as previously identified *Streptomyces* APs.

Expression of *sfappro* and *sfap* in *E. coli*

The recombinant plasmids pET-22b-*sfappro* and pET-22b-*sfap* were constructed and transformed into *E. coli*. After IPTG induction, cell cultures were analyzed for enzyme activity. The cell lysates and culture supernatants of the pET-22b-*sfap*-positive transformants displayed aminopeptidase activity, whereas the pET-22b-*sfappro*-positive transformants and the negative control containing empty pET-22b(+) vector had no enzyme activity. SDS-PAGE analysis indicated expression of SFAPPRO and SFAP following induction (Fig. 2). The observed apparent molecular weight of SFAPPRO and SFAP was about 50 kDa and 36 kD, respectively, suggesting that the C-terminal propeptide was not cleaved in the *E. coli* host cells.

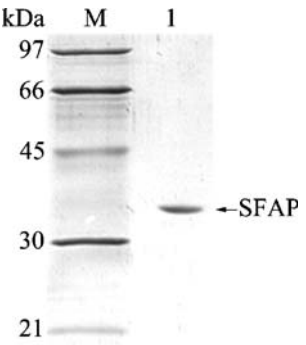

Fig. 3 SDS-PAGE analysis of purified recombinant SFAP. *M* protein molecular mass markers (kDa), lane *1* SFAP following anion exchange chromatography

Purification of Recombinant SFAP

Recombinant SFAP was purified from LB medium by sequential ultrafiltration and ion-exchange column chromatography. The specific activity of the purified recombinant SFAP was 198 U mg^{-1} after 18.6-fold purification, and the final activity yield of the enzyme was 20.6%. The purified enzyme migrated as a single band with an apparent molecular weight of about 36 kDa on a 12% SDS-polyacrylamide gel (Fig. 3), the same as that for the native *S. fradiae* var. k11 enzyme isolated from feather medium (data not shown), but higher than the calculated molecular weight (31.5 kDa). Similar results have been obtained for other APs, such as SGAP from *S. griseus* and SSAP from *S. septatus* TH-2 [8].

Properties of Recombinant SFAP

Purified recombinant SFAP was active at alkaline pH, with optimum activity at pH 8.0 (Fig. 4a). It was stable over a wide pH range, retaining >60% activity from pH 5.0–11.0 (Fig. 4b). The optimal temperature for enzyme activity was 60 °C at pH 8.0 (Fig. 4c) and the enzyme retained over 55% of the maximum activity when assayed at 40–80 °C. The purified recombinant SFAP was thermostable, retaining >80% of the initial activity after pre-incubation at 60 °C or 70 °C for 1 h and at 80 °C for 5 min (Fig. 4d).

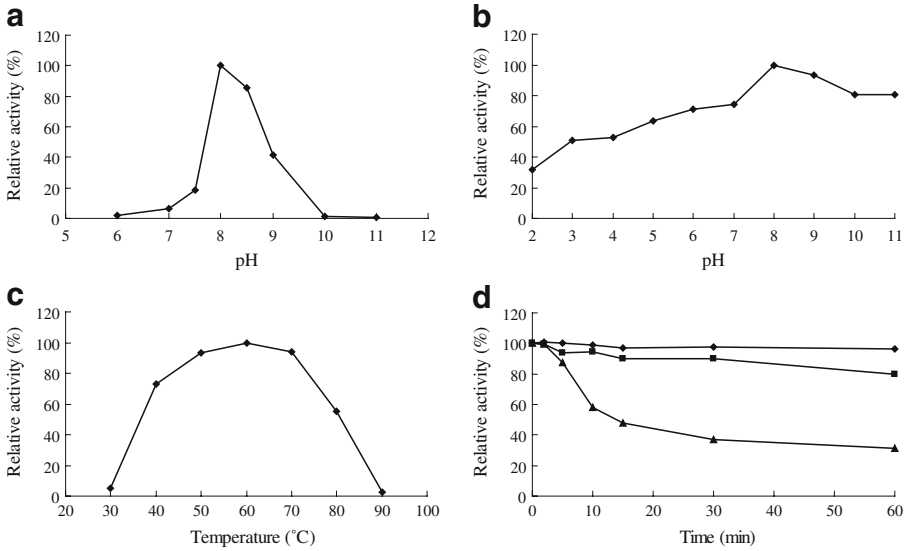

Fig. 4 Characterization of purified recombinant SFAP. **a** Effects of pH on SFAP activity. Enzyme activity was measured in 0.1 M McIlvaine buffer (pH 3.0–8.0), 0.1 M Tris–HCl (pH 8.0–9.0), or 0.1 M glycine-NaOH (pH 9.0–11.0) at 37 °C using L-Leu *p*-nitroanilide (Leu-pNA) as substrate. The maximum activity of recombinant SFAP was defined as 100%. **b** Effects of pH on SFAP stability. The effect of pH on enzyme stability was estimated by measuring the AP activity under standard conditions after incubation in the same buffer system at pH 2.0–11.0 at 37 °C for 1 h. The activity of untreated recombinant SFAP was defined as 100%. **c** Effects of temperature on SFAP activity. The AP activity was determined at temperatures over the range of 30–90 °C at the optimum pH. **d** SFAP thermostability. The thermostability of the purified recombinant SFAP was determined by measuring the residual enzyme activity under standard assay conditions after pre-incubating the enzyme in 0.1 M Tris–HCl (pH 8.0) at 60 °C (*filled diamond*), 70 °C (*filled square*), or 80 °C (*filled triangle*) without substrate for 2, 5, 10, 20, 30, or 60 min. The activity of untreated recombinant SFAP was defined as 100%. Each value in the panel represents mean of triplicates

Table 1 Effects of various divalent metal ions on the aminopeptidase activity of purified recombinant SFAP.

Chemicals	Relative activity (%)	
	0.1 mM	1 mM
CK[a]	100	100
$CaCl_2$	302	316
$CoCl_2$	188	176
$MgSO_4$	91	86
$MnSO_4$	69	61
$CuSO_4$	45	18
$HgCl_2$	44	18
$NiSO_4$	43	41
$ZnSO_4$	23	3
$Pb(CH_3CO_2)_2$	5	0

[a] No divalent metal ions were added to the reaction system

The enzyme was significantly inhibited by the metalloprotease inhibitor, EDTA, and lost 43% and 66% of initial activity in the presence of 0.1 mM and 1.0 mM EDTA, respectively. The effect of various metal ions on the enzyme activity is shown in Table 1. Enzyme activity was inhibited in the presence of heavy metal ions except for Co^{2+}, which substantially activated the enzyme. Approximately, threefold activation of SFAP by Ca^{2+} was observed.

The K_m and V_{max} values of SFAP using Leu-pNA as a substrate were 0.63 mM and 243 μmol min^{-1} mg^{-1}, respectively.

Discussion

APs have been proposed to be involved in post-translational modification and maturation of proteins, removing signal peptides from proteins following membrane transport, and in transformation of inactive proenzymes into their biologically active forms [4, 5]. During casein degradation, APs also play an important role in the uptake of nutrients [16]. *S. fradiae* var. k11 exhibits robust keratin hydrolysis ability and several proteases have been isolated from this strain [13]. Previous studies have indicated that the serine proteases from *S. fradiae* var. k11, SFP1 and SFP2, have some keratinolytic activity [17, 18]. Thus, we assume that SFAP plays a role in the processing of proenzymes. However, further analysis of interactions among *S. fradiae* var. k11 proteases and APs is required to identify the mechanism of keratin hydrolysis.

In this study, we determined that SFAP has a C-terminal propeptide domain. The mature SFAP enzyme exhibited markedly activity, but no AP activity was detected for the SFAP proenzyme (SFAPPRO). SDS-PAGE analysis indicated that the C-terminal propeptide of SFAP was not cleaved in the *E. coli* expression system. Propeptides are typical structural components of many extracellular proteases [19]. Extracellular proteases are synthesized as inactive precursors generally comprised of three or four domains including a signal sequence, a proteolytic domain, an N-terminal propeptide, and a C-terminal propeptide. The N-terminal propeptide has been assigned the function of chaperone and is thought to facilitate correct folding of the mature enzyme. The C-terminal propeptide has been demonstrated to participate in the secretion of the protein [20–22]. Serine proteases SFP1 and SFP2 contain N-terminal propeptide domains and the SFP2 proenzyme has been shown to exhibit markedly higher activity than the mature SFP2 enzyme [13, 17]. However,

further analysis will be required to determine the function of the C-terminal propeptide of SFAP.

Characterization of SFAP indicated that it was highly active over a broad range of temperature (40–80 °C) and exhibited good thermal stability (80 °C for 30 min). SFAP also showed pH stability over a wide pH range, retaining over 50% of the initial activity after pre-incubation at pH 3.0–11.0. These superior properties make *S. fradiae* AP more potential in research and industrial processes [23]. Moreover, the enzymatic properties of SFAP might be improved by protein engineering.

A property shared by most APs from *Streptomyces* spp. is their dependence on divalent metal ions for activity. SFAP was significantly activated by Ca^{2+}, as is the *S. griseus* AP (SGAP), which belongs to the same family as SFAP [24, 25]. The Ca^{2+}-binding site in SGAP has been recently identified [26]. Four residues (Asp-3, Asp-173, Asp-174, and Asp-262) were shown to be involved in SGAP modulation by cooperation with Glu-196 and calcium to increase the catalytic activity [27]. Based on the alignment of several *Streptomyces* APs, these five residues were also found to be conserved in the sequence of SFAP, implying that SFAP is also a Ca^{2+}-modulated AP. The addition of Hg^{2+}, Ni^{2+}, Cu^{2+}, and Zn^{2+} inhibited the activity of SFAP significantly, suggesting that SFAP is a thiol-sensitive metalloprotease because these heavy metal ions bind free mercapto groups (-SH) in cysteine residues. In this study, we also found that Co^{2+} substantially enhanced SFAP activity; Co^{2+} only enhanced SGAP activity slightly [8]. The Co^{2+}-binding site has been reported to be a dinuclear site [28], but the effects of Co^{2+} on SFAP remains to be determined in detail.

In conclusion, SFAP has low sequence similarity to other known APs. Furthermore, no AP genes from *S. fradiae* have previously been cloned, expressed, or characterized. Thus, the present study delineating the sequence and functional properties of an AP from *S. fradiae* will further our understanding of bacterial APs and advance comparative analyses. In addition, this study will likely contribute to understanding the relationship between bacterial keratinolytic enzyme systems and the mechanism of keratin hydrolysis.

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