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Purification and characterization of a novel tripeptidyl aminopeptidase from *Streptomyces lividans 66*

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Abstract An extracellular tripeptidyl aminopeptidase has been purified from *Streptomyces lividans 66* cell-free cultures. The enzyme is a major component of the secreted proteolytic activity. The protease removes only the N-terminal tripeptide from recombinant human GM-CSF and IL-3 but does not cleave recombinant human IL-6. The enzyme cleaves the synthetic tripeptide substrates APA-pNA and APM-pNA but does not cleave substrates with blocked amino terminals. Smaller substrates are not cleaved. The enzyme appears to be a serine protease of 55 kDa molecular weight. The pH optimum is between 7.5 and 8.5 but varies slightly with the substrate. The N-terminal sequence and amino acid composition have been determined.

Key words: Tripeptidyl aminopeptidase; Recombinant protein expression; Protein degradation; Serine proteinase; Fermentation; *Streptomyces lividans*

1. Introduction

Recombinant genetic expression systems employing *Streptomyces lividans* have been used to produce a variety of recombinant protein products [1–3]. Soluble biologically active proteins are secreted directly into the media thereby eliminating the difficulties associated with solubilizing and refolding proteins generated as inclusion bodies. These secreted proteins are vulnerable to degradation by extracellular proteases. Analysis of human recombinant granulocyte macrophage-colony stimulating factor (GM-CSF), interleukin-3 (IL-3), and erythropoietin (EPO) produced by *S. lividans 66* revealed the presence of degradation products missing the N-terminal tripeptide (−3 forms). While the production of extracellular proteases in *Streptomyces griseus* and several other species has been studied extensively, few enzymes have been identified in *S. lividans* [4–6]. A chymotrypsin-like activity has been reported in *S. lividans TK24* and a neutral protease has been reported in *S. lividans 66* [7–10]. We describe the isolation and characterization of a major extracellular protease from *S. lividans 66*. This enzyme is a tripeptidyl aminopeptidase (TAP) capable of removing the N-terminal tripeptide from both IL-3 and GM-CSF. Tripeptidyl peptidases have not been previously reported in *Streptomyces* or other eukaryotic species.

2. Materials and methods

2.1. Materials

GM-CSF and IL-3 were produced by Cangene via expression in *S. lividans 66*. N-AC-APA-pNA, Boc-AP, A-AMC, M-pNA, S-bNA, Boc-AAPA-pNA, and SASRIN resins were obtained from Bachem Biosciences. A-pNA, R-pNA, L-pNA, P-pNA, AA-pNA, GP-pNA, N-Bz-VGR-pNA, and N-Bz-R-pNA were obtained from Sigma. D-PFR-pNA was obtained from KabiVitrum (Stockholm, Sweden).

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Abbreviations: EPO, recombinant human erythropoeitin; GM-CSF, recombinant human granulocyte macrophage colony stimulating factor; IL-3, recombinant human interleukin-3; IL-6, recombinant human *N*-alanyl interleukin-6; PMSF, phenylmethanesulfonylfluoride; TAP, tripeptidyl aminopeptidase.

2.2. Purification of TAP

S. lividans 66 cells were removed from 11 litres of fermentation broth by ultrafiltration with a 0.45 μm filter (Pellicon System, Millipore). Proteins in the filtrate were concentrated 20-fold by ultrafiltration employing a membrane with a 10 kDa cutoff (Millipore). The protease activity was followed by assaying with APA-pNA and GM-CSF as described below. The protease was precipitated at 4°C by lowering the pH to 4.0 with 0.1M HCl. The precipitate was collected by centrifugation (Model J2-21, Beckman) at 10,000 × *g* at 4–10°C and was redissolved in 50 ml 10 mM Tris-HCl, pH 8.0. After dialysis against 4 litres of 10 mM Tris-HCl, pH 8.0 at 4°C, the protease was loaded at ambient temperature onto a 1.6 × 10 cm anion-exchange column (Q-Sepharose Fast Flow, Pharmacia) equilibrated with 10 mM Tris-HCl, pH 8.0. After washing with equilibration buffer, the bound protease was eluted with a 200 ml gradient from 0 to 500 mM NaCl at a flow rate of 2 ml/minute. The active fractions were pooled and made 2 M in ammonium sulfate. This material was loaded at ambient temperature onto a 1.6 × 10 cm hydrophobic interaction column (Phenyl-Sepharose Fast Flow, Pharmacia) equilibrated in 10 mM Tris-HCl, pH 8.0, 2 M ammonium sulfate. After washing with equilibration buffer, the column was eluted with a 200 ml gradient from to 2 to 0 M ammonium sulfate at a flow rate of 2 ml/min. The active fractions were assayed for purity by SDS-PAGE [11].

2.3. Assay of TAP with GM-CSF or IL-3

TAP was assayed by preparing 100-fold dilutions of column fractions with 20 mM Tris-HCl, pH 8.0. When GM-CSF was the substrate, 20 μl of the TAP dilution was added to 10 μl of GM-CSF (10 μg) and 20 μl 20 mM Tris-HCl, pH 8.0. The assays were incubated at 37°C for 2 h. 20 μl of 125 mM Tris-HCl, pH 6.8, 0.1% Bromophenol blue in 50% glycerol were added. Products were separated by native gel electrophoresis at constant current on a 17% polyacrylamide gel by a modification of the method of Davies [12] in which all buffers are pH-adjusted with H_2SO_4 (Fig. 1A). Product identity was initially established by amino acid sequencing.

When IL-3 was the substrate, 10 μl of TAP was added to 50 μl 20 mM Tris-HCl, pH 8.0 plus 40 μl IL-3 (2.5 μg/μl). The assays were incubated at 37°C. 25 μl aliquots were withdrawn at the desired time points and frozen on crushed dry ice. The products were separated by isoelectric focusing from pH 3–10 using a Multiphore II electrophoresis unit (LKB) and Pharmalyte 3–10 (Pharmacia) ampholytes. Intact IL-3 has a pI = 7.4. The −3 form demonstrates a pI = 7.1 (Fig. 1B). Products were initially identified by amino acid sequencing.

2.4. Assay of TAP with synthetic substrates

When APA-pNA was employed as substrate, the assay was conducted in a 96-well microtiter plate. 50 μl 100 mM Tris-HCl, pH 8.0, were added followed by 25 μl 3.2 mM APA-pNA. 25 μl of TAP were added to the wells and the absorbance was read at 405 nm. The assays

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were incubated at 37°C for 2 h. The absorbance was read at 405 nm at time points as needed. The activity (release of *p*-nitroaniline) was calculated from the change in absorbance.

Fluorescent substrates were assayed under the same conditions. β-Naphthylamine and 7-amino-4-methylcoumarin release were measured using the 400 nm/450 nm excitation/emission block in a Pandex Fluorescent Concentration Analyzer (Idexx).

2.5. Protease inhibition survey

For inhibitor studies, TAP stock was diluted 100-fold with 20 mM Tris-HCl, pH 8.0. The inhibitor was added to the enzyme solution and preincubated for 30 min at 22°C. When the preincubation was complete, the appropriate substrate was added and the assays were incubated at 37°C for the desired time period. Products were analyzed as described in the respective assays.

2.6. Protein sequencing

Purified TAP was adsorbed onto a polyvinylidenedifluoride (PVDF) membrane (Immobilon, Millipore) by the method of Matsudaira [13]. After visualization with Amido black, the sample was excised and used for amino acid analysis and automated Edman degradation for 15 cycles.

2.7. Synthesis of substrates

APA-pNA, APA-AMC, APM-pNA, and APS-bNA were synthesized by coupling Boc-AP with A-pNA, A-AMC, M-pNA, and S-bNA, respectively, by the mixed anhydride method followed by deblocking with trifluoroacetic acid. AAPA-pNA was prepared by deblocking Boc-AAPA-pNA with trifluoroacetic acid. *N*-Carbobenzoxy-APARSPA-pNA was synthesized by manual solid phase methods employing a SASRIN resin.

3. Results and discussion

Sequence analysis of degradation products found during the production of human recombinant GM-CSF, IL-3, and EPO revealed the proteolytic removal of the N-terminal tripeptide sequences (APA-, APM-, and APP- respectively). The synthetic substrate, APA *p*-nitroanilide (APA-pNA), was prepared as an analog of the GM-CSF amino terminal and was included in a survey of the proteolytic activities present in the cell-free fermentation broths.

As seen in Fig. 2, the APA-pNA cleaving activity was the major protease found in the survey. The presence of a two step cleavage via removal of AP followed by hydrolysis of A-pNA was eliminated by the low activities towards AP-pNA and A-pNA. The presence of an elastase-like endoproteinase was discounted by the lack of measurable cleavage of Boc-AAPA-pNA or N-Ac-APA-pNA in the filtrates [14–16].

The enzyme was purified as described above. Activity was monitored by assaying with both the spectrophotometric APA-pNA and the electrophoretic GM-CSF native gel assay (Fig. 1A). SDS-PAGE (Fig. 3) shows the purification obtained.

The enzyme demonstrates a molecular weight of 55,000 Da on SDS-PAGE. The first 15 residues of the N-terminal sequence obtained by automated Edman degradation gave the sequence Asp-Gly-His-Gly-His-Gly-Arg-Ser-Trp-Asp-Arg-Glu-Ala-Arg-Gly. The amino acid composition (excluding Cys and Trp) results are shown in Table 1.

The cleavage specificity of the pure enzyme was examined with both proteins and synthetic substrates. The enzyme readily removes the first N-terminal tripeptide from GM-CSF and IL-3 but fails to cleave IL-6 (possessing an additional N-terminal alanine residue). Attempts to force cleavage of IL-6 and further cleavage of GM-CSF and IL-3 by increasing the enzyme:substrate ratio by a factor of 1,000 while simultaneously extending the digestion time by a factor of 10 yielded no new products.

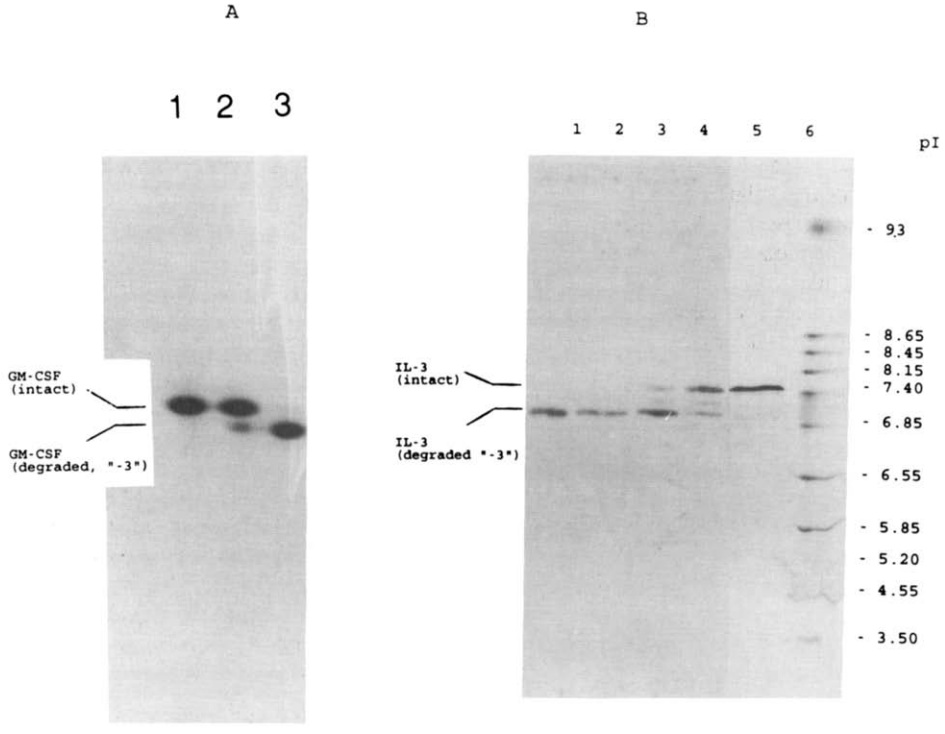

Fig. 1. Resolution of intact and (−3) degradation products from GM-CSF and IL-3. Proteins were digested and electrophoresed as described in section 2. (A) Separation of GM-CSF and GM-CSF(−3) form by native gel electrophoresis. Lane 1, intact GM-CSF; lane 2, mixture of GM-CSF and GM-CSF(−3); lane 3, purifed GM-CSF(−3). (B) Separation of IL-3 and IL-3(−3) form by isoelectric focusing. Lane 1, IL-3 + TAP, 4 h; lane 2, IL-3 + TAP, 2 h; lane 3, IL-3 + TAP, 1 h; lane 4, IL-3 + TAP, 0 h; lane 5, human erythrocyte carbonic anhydrase II (Sigma, pI = 7.4); lane 6, pI standards.

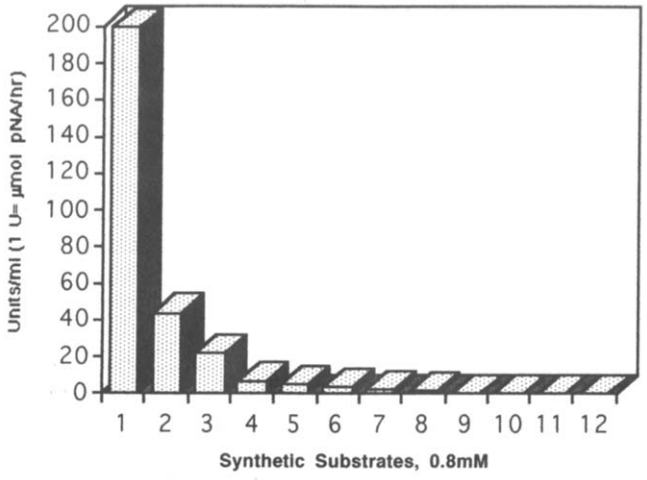

Fig. 2. Cleavage of synthetic substrates by *S. lividans* fermentation broth. The assays were conducted in 50 mM Tris-HCl, pH 8.0 with 0.8 mM substrate incubated at 37°C. The results are reported as micromoles of *p*-nitroaniline released in 1 h by 1.0 ml of fermentation broth. 1, APA- pNA; 2, D-PFR-pNA; 3, L-pNA; 4, R-pNA; 5, P-pNA; 6, AP-pNA; 7, A-pNA; 8, AA-pNA; 9, *N*-benzoyl-R-pNA; 10, Boc-AAPA-pNA; 11, *N*-acetyl-APA-pNA; 12, *N*-benzoyl-Y-pNA.

This resistance may be a function of the primary sequence or protection of the cleavage site by the folding of the target molecule. IL-3 undergoes a significant shift in pI from 7.4 to 7.1 upon removal of the APM tripeptide suggesting a structural rearrangement or the possible formation of a salt bridge by the new N-terminal.

Crystal structures show the first 14 residues of GM-CSF are solvent accessible and the GM-CSF(−3) form can be further degraded by enzymes attacking the N-terminal while NMR studies indicate that the N-terminal of IL-6 is also accessible [17,18]. It is also possible that the enzyme does not cleave tripeptides containing charged residues. EPO(−3) and GMCSF(−3) have arginine as their N-terminal residues and appear to resist further cleavage. The resistance of IL-6 may reside in the primary sequence, Ala-Pro-Val-Pro-Pro. The bulky valine side chain may prevent proper alignment in the active site. Alternatively, the secondary amide may be resistant to cleavage.

Table 1
Amino acid composition of isolated TAP

Amino acid	Mol percentage
Asp + Asn	13.6
Glu + Gln	10.9
Ser	4.7
Gly	10.0
His	2.2
Arg	7.4
Thr	6.3
Ala	14.3
Pro	7.2
Tyr	3.9
Val	6.4
Met	1.2
Ile	2.3
Leu	5.6
Phe	1.7
Lys	2.5

The protease is active against APA-pNA and the fluorogenic analog APA 7-amido-4-methylcoumarin (APA-AMC). The kinetic constants for cleavage of APA-pNA by TAP are K_m = 37 μM and V_{max} = 55 μmol · min^{-1} · mg^{-1} at pH 8.0. The enzyme cleaved APA-pNA 10 times as rapidly as the IL-3 analog, APM-pNA. This result agrees with those obtained when comparing the rates of GM-CSF and IL-3 cleavage. The tripeptide β-naphthylamide (bNA), APS-bNA, was also hydrolyzed.

The elastase substrate, N-Ac-APA-pNA, is completely resistant to cleavage by TAP. The N-blocked Boc-APS-bNA is not hydrolyzed by TAP. The extended N-blocked GM-CSF analogs, Boc-APARSPA-bNA and N-carbobenzoxy-APARSPA-pNA, were not affected by TAP but in a coupled assay the reporter groups were rapidly released by simultaneously incubating the molecules with TAP and TPCK-treated trypsin (Sigma). This clearly demonstrates the absolute requirement for an unblocked N-terminal group in the substrate. Additionally, TAP does not release the reporter group from Boc-AAPA-pNA, N-Bz-R-pNA, N-Bz-VGR-pNA, A-pNA, R-pNA, L-pNA, P-pNA, S-bNA, AA-pNA, GP-pNA, D-PFR-pNA, or AAPA-pNA. The enzyme has shown no abilitity to act on monoamino acid, dipeptide, or tetrapeptide substrates. The lack of activity towards D-PFR-pNA may result form the

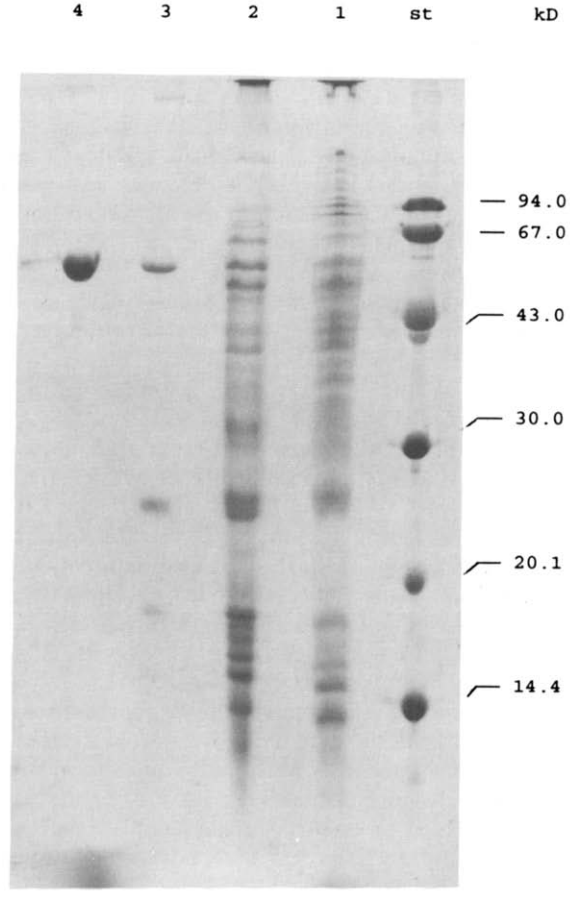

Fig. 3. SDS-PAGE demonstration of purification of TAP. Approximately 20 μg of protein were denatured under reducing conditions and analyzed by SDS-PAGE on 10% polyacrylamide gels. St, molecular weight standards; lane 1, 10 kDa ultrafiltration retentate; lane 2, redissolved pH 4.0 precipitate; lane 3, Q-Sepharose chromatography pool; lane 4, Phenyl-Sepharose chromatography pool.

Table 2
Inhibitor survey

Inhibitor	Percentage residual activity
None	100
PMSF, 1.6 mM	7
$HgCl_2$	
0.1 mM	99
1.0 mM	93
$CaCl_2$	
1.0 mM	96
10 mM	97
$CoCl_2$	
1.0 mM	98
10 mM	82
EDTA	
1.0 mM	95
10 mM	95
Iminodiacetic acid	
1.0 mM	82
10 mM	60
Dithiothreitol, 1 mM	86
Dithiothreitol (1 mM) + EDTA (10 mM)	97
Elastatinal, 0.1 mM	86
Chymostatin, 0.1 mM	75
Benzamidine, 10 mM	94
Ampicillin	
1.0 mM	93
10 mM	94

N-terminal D-isomer or the presence of the charged arginine as discussed above.

The results of an inhibitor survey are shown in Table 2. The enzyme was inhibited by phenylmethanesulfonylfluoride (PMSF) suggesting that it is a serine protease. TAP was not inhibited or stimulated by chelating agents, divalent cations, or sulfhydryl reagents thereby eliminating any relation to the subtilisins, thiol proteinases, or metalloproteinases. Pepstatin was also ineffective. The failure of the peptide aldehydes, elastatinal and chymostatin, may reflect TAP's lack of endoproteolytic activity.

The effect of pH on the cleavage of several substrates by TAP has been examined. The enzyme was active against APA-pNA from pH 5.0 to pH 9.5 with optimal activity at pH 8.0–9.0. Half maximal velocities were obtained at pH 7.1 and 9.8. The enzyme cleaved GM-CSF from pH 4.0 to pH 10.0 with a broad maximum ranging from pH 5.0 to pH 9.0. The broad maximum for GM-CSF probably reflects the extreme sensitivity of this substrate to TAP. As previously mentioned, the GM-CSF crystal structure shows that the N-terminal is highly accessible [17]. The enzyme also cleaved IL-3 at pH 5.0–9.0 with maximal activity at pH 7.0–8.5.

TAP appears to be a tripeptidyl aminopeptidase capable of cleaving substrates at the third amide bond from the N-terminal. While serine proteases with tripeptidyl aminopeptidase activity have been identified in mammals [19–21], enzymes of this type have not been reported in prokaryotes. The enzyme is similar in size to the lysosomal tripeptidyl aminopeptidases isolated from bovine anterior pituitary glands and porcine ovaries but does not exhibit the acidic pH optimum of those serine proteases [19,22]. The pH optimum more closely resembles that of the extracellular enzymes from human liver and erythrocytes but is considerably smaller than these 135,000 Da enzymes [20]. Examination of the TAP DNA sequence may provide some insight into evolutionary relationships between the prokaryotic and eukaryotic enzymes.

Bacterial secretion systems for heterologous protein expression can be adversely affected by secreted proteases. By isolating and characterizing a major protease involved in the degradation of biopharmaceutical products, the use of *Streptomyces* expression systems can be enhanced by the application of selective inhibitors or, ultimately, by the deletion of the protease gene from the host genome.

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