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Function of the signal peptide and N- and C-terminal propeptides in the leucine aminopeptidase from *Aeromonas proteolytica*

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Abstract

The leucine aminopeptidase from Aeromonas proteolytica (also known as Vibrio proteolyticus) (AAP) is a metalloenzyme with broad substrate specificity. The open reading frame (ORF) for AAP encodes a 54 kDa enzyme, however, the extracellular enzyme has a molecular weight of 43 kDa. This form of AAP is further processed to a mature, thermostable 32 kDa form but the exact nature of this process is unknown. Over-expression of different forms of AAP in Escherichia coli (with AAP's native leader sequence, with and without the N- and/or C-terminal propeptides, and as fusion protein) has allowed a model for the processing of wild-type AAP to be proposed. The role of the *A. proteolytica* signal peptide in protein secretion as well as comparison to other known signal peptides reveals a close resemblance of the *A. proteolytica* signal peptide to the outer membrane protein (OmpA) signal peptide. Over-expression of the full 54 kDa AAP enzyme provides an enzyme that is significantly less active, due to a cooperative inhibitory interaction between both propeptides. Over-expression of AAP lacking its C-terminal propeptide provided an enzyme with an identical k_{cat} value to wild-type AAP but exhibited a larger K_m value, suggesting competitive inhibition of AAP by the N-terminal propeptide (K_i~0.13 nM). The recombinant 32 kDa form of AAP was characterized by kinetic and spectroscopic methods and was shown to be identical to mature, wild-type AAP. Therefore, the ease of purification and processing of rAAP along with the fact that large quantities can be obtained now allow new detailed mechanistic studies to be performed on AAP through site-directed mutagenesis.

Keywords

Aeromonas proteolytica (*Vibrio proteolyticus*), Leucine aminopeptidase, Over-expression, Propeptide inhibition, Signal peptide, Zinc(II)

Introduction

Enzymes containing dinuclear metallo-active sites are central to numerous biological processes and, consequently, characterization of their structure and function is a problem of outstanding importance [1]. These enzymes play key roles in carcinogenesis, tissue repair, and protein degradation processes. In addition, some of the enzymes within this group catalyze the hydrolysis of phosphorus(V) compounds found in nerve gases and agricultural neurotoxins [1]. One of these, the leucine aminopeptidase from *Aeromonas proteolytica* (*Vibrio proteolyticus*) (AAP; EC 3.4.11.10)[1] is capable of removing nearly any hydrophobic N-terminal amino acid residue from a polypeptide chain [1]. Leucine aminopeptidases are widely distributed in nature and play important roles in many biological processes including protein catabolism, degradation of hormones, wound response in plants, polypeptide trimming prior to their presentation to MHC-I, regulation of migration and cell proliferation, as well as HIV infection and proliferation [2], [3], [4], [5], [6], [7], [8], [9]. Therefore, the determination of a detailed reaction mechanism for leucine aminopeptidases is a required pre-requisite for the design of highly potent, specific inhibitors that can function as potential pharmaceuticals.

The as-purified AAP enzyme is small (32 kDa), monomeric, and is fully activated by two equivalents of Zn(II). However, the open reading frame (ORF) of AAP encodes a 54,223 Da protein that consists of four domains—a signal peptide (21 amino acids), an N-terminal propeptide (85 amino acids), the mature protein (291 residues), and a C-terminal propeptide (107 amino acids) [10], [11]. Upon expression of AAP in *A. proteolytica* (*V. proteolyticus*), three major bands are present on SDS–PAGE with approximate molecular weights of 50, 44, and 32 kDa. The first band likely corresponds to the molecular weight of the full-length protein without its signal peptide, while the second lacks either part or all of the N- or C-terminal propeptide. The third band corresponds to the mature, wild-type enzyme. The higher molecular weight enzymes are thermally unstable and undergo processing at 70 °C to form a heat-stable 32 kDa protein; however, the nature of this process remains unknown [12]. AAP is partially digested by an unknown processing enzyme of which a potential candidate is a Zn(II)-dependent neutral protease co-expressed with AAP [13], [14]. Alternatively, AAP may undergo auto-processing, which would make it the only known co-catalytic metalloendopeptidase to undergo such a process.

The N-terminal propeptide of AAP has been shown to be an absolute requirement for proper folding of the enzyme [15]. In addition, this propeptide has been shown to display some inhibitory properties based on the observed increase in the K_m value towards l-leucine-*p*-nitroanilide (l-*p*NA) [16]. On the other hand, the role of the C-terminal propeptide remains unknown. It has been shown that the C-terminal propeptide exhibits high sequence identity (52.6%) and homology (63.9%) with 100 terminal amino acids of *Vibrio cholerae* hemagglutinin/proteinase and 47.9% identity and 59% homology with the last 100 amino acids of *Vibrio alginolyticus* serine exoproteinase A [10]. However, very little is known about the function of the C-termini of these enzymes, although similar to AAP, a truncation at the C-terminus of hemagglutinin/proteinase of *V. cholerae* resulted in an increase in the observed catalytic activity [17].

Herein, we report the over-expression of AAP in *Escherichia coli* in several different forms (i.e., with and without the native signal sequence, with and without the C-terminal propeptide, and as a fusion protein). The effect of the native signal peptide on the extracellular secretion of AAP from *E. coli* was evaluated and compared to those of other *E. coli* leader sequences. The inhibitory properties of the N- and C-terminal propeptides on the hydrolysis of l-*p*NA by AAP were also examined and, based on kinetic data, a role for the C-terminal propeptide is proposed. Combination of these data has allowed a model for AAP processing to be proposed. Finally, the kinetic and spectroscopic properties of the recombinant, 32 kDa form of AAP were examined and compared to those of mature AAP.

Materials and methods

All chemicals used were purchased from Sigma and Fisher and were of the highest quality available. All biochemicals were purchased from Promega unless stated otherwise. All DNA and bacterial manipulations were performed under sterile conditions in a Laminar Flow unit (Agnew Higgins, Garden Grove, CA).

Comparison of the signal peptide of AAP to other signal peptides

Analysis of the propeptide sequence was performed using a web-based service operated by the Swiss Institute of Bioinformatics, using program ProtScale (http://us.expasy.org/cgi-bin/protscale.pl). This service uses the method developed by Kyte and Doolittle [18] with a window size of seven amino acids. The figures were generated using IgorPro (Wavematrix, Lake Oswego, OR). The AAP sequence accession number in the EMBL Data Library is Z11993 [10]. Comparison to other signal peptides such as PelB, MalE, and OmpA was performed using identical parameters [19].

Preparation of the AAP gene for cloning

The vep-pap vector containing DNA encoding the ORF (54 kDa protein) was kindly provided by Fred A. Wagner of the University of Nebraska. The plasmid was purified from *E. coli* JM109 cells (*rec*A1 *end*A1 *gyr*A96 *thi hsd*R17 (r_K–, m_K+) *rel*A1 *sup*E44 Δ(*lac-pro*AB) [F′ *tra*D36 *pro*AB *lac*I^{q}ZΔM15]) using Promega's Wizard *Plus* Minipreps DNA Purification System (Promega, Madison, WI) according to the manufacturer's instructions. The plasmid (100 µg) was incubated with 5 µl *Hin*dIII (10 U/µl) (Promega, Madison, WI) for 2 h at 37 °C. The digest was then isolated from the agarose gel (0.8%) using the Geneclean II Kit (Bio 101, Vista, CA). The plasmid was stored in doubly distilled, sterile water at −20 °C.

PCRs were performed on the vep-pap digest using the following primers (restriction enzymes recognition sites are underlined): (1) for the pT7 vector: 54 kDa form: 5′-CTA GCATATGAA ATA TAC CAA AAC G-3′ and 5′-CTA GAAGCTTAG TGA CGT ATG TCA G-3′, (*Nde*I and *Hin*dIII); (2) for the pMAL-c2X vector: 5′-AGAATTCAT GCC GCC GAT TAC ACA G-3′ and 5′-CTAAGCTTC AGC CTG GTG TTG GTG T-3′, (*Eco*RI and *Hin*dIII); and (3) for the pET27b(+): (i) for the 43 kDa AAP (ΔC-AAP): 5′-CTCCATGGA TAT GAA ATA TAC CAAA-3′ and 5′-CTAAGCTTC AGC CTG GTG TTG GTGT-3′, (*Nco*I and *Hin*dIII); (ii) for the 32 kDa form of AAP: 5′-AGA TAT GCC GCC GAT TACA-3′ and 5′-CTCCATGGC AGC CTG GTG TTG GTG T-3′, (*Nco*I and *Hin*dIII). All oligomers were obtained from Operon Technologies,

Alameda, CA. PCR was carried out with Ready-To-Go PCR Beads (Amersham–Pharmacia Biotech, Piscataway, NJ) with the following cycling parameters: stage 1 (94 °C × 5 min) × 1; stage 2 (94 °C × 5 min, 55 °C × 30 s, and 72 °C × 30 s) × 25; and stage 3 (72 °C × 7 min) × 1; then cooled to 4 °C. The PCR product was then isolated from an 0.8% agarose gel.

Cloning of the AAP gene

Each of the isolated PCR products was ligated into pGEM-T Easy or pGEM-T vectors (AAP DNA containing EcoRI restriction enzyme site) (Promega, Madison, WI) and transformed into DH5α competent cells [F− ϕ80dlacZΔM15Δ(lacZYA-argF)U169 deoR recA1 endA1 hsdR17(rK−, mK+) phoA supE44 λ− thi-1 gyrA96 relA1] (Invitrogen, Carlsbad, CA). Each of the plasmids was subsequently isolated from the bacterial host as previously described. Each pGEM-AAP vector was subjected to digestion with their respective restriction enzymes (Promega, WI; Invitrogen, Carlsbad, CA; Fermentas, Vilnius, Lithuania) and the digests encoding AAP were isolated from an agarose gel. The pT7 vector (a gift from Dr. John W. Peters, Utah State University) was treated with NdeI and HindIII restriction enzymes while the pET-27b(+) vector (Novagen, Madison, WI) was digested with NcoI and HindIII and pMAL-c2X (New England Biolabs, Beverly, MA) was digested with EcoRI and HindIII restriction enzymes. All digests were isolated from agarose gels.

Each of the AAP genes and their respective plasmid digests were ligated using the LigaFast Rapid DNA Ligation System (Promega, Madison, WI) following Promega's protocol. The ligation products were transformed into DH5α cells as described above. The plasmids were then isolated and the presence of the DNA insert coding for AAP was confirmed by DNA sequencing (Biotechnology Center, USU). Four expression systems were obtained in this manner—pT7-54AAP (pt7-AAP), pET-27b(+)-43AAP (ΔC-AAP), pET-27b(+)-32AAP, and pMAL-c2X-32AAP. Plasmid pVSNMC (Δsp-AAP) encoding the 52 kDa form of AAP (lacking the first 23 amino acids) [15] was a gift from Prof. Kiyoshi Hayashi.

The DNA sequence of the AAP insert in the pET-27b(+)-43AAP vector revealed two point mutations: K140P and P294S. To reverse these point mutations, four oligomers were constructed: P140K: forward–5′ -GGC AAT GCT ACG TTT TGG TTG GAA GTT GTT CTC-3′ and reverse–5′ -GAG AAC AAC TTC CAA CCA AAA CGT AGC ATT GCC-3′ ; P294S: forward–5′ -GCA ACC GGT GAC ACA CCA ACA CCA GGC TGA AGC-3′ and reverse–5′ -GCT TCA GCC TGG TGT TGG TGT GTC ACC GGT TGC-3′ . The reaction was carried out using the Quick Change Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The reaction products were transformed into E. coli XL1-Blue competent cells (recA1 endA1 gyrA96 thi-1 hsdR17supE44 relA1 lac [F′ proAB lacIqZ Δ M15 Tn10 (Tetr)]) and grown on LB‑agarose plates with a kanamycin concentration of 50 μg/mL. A single colony from the plate was grown in 50 mL LB with 50 μg/mL kanamycin. Plasmids were isolated using Qiaprep Spin Miniprep kit (Qiagen, Valencia, CA). The resulting DNA sequence was identical to the published DNA sequence of AAP [10].

The following bacterial hosts were used for over-expression: BL21(DE3)pLysS cells (F− ompT hsdSB(rB−mB−) gal dcm (DE3) pLysS (CmR)); AD494(DE3)pLysS cells (Δara− leu7967 ΔlacX74 ΔphoAPvuII phoR ΔmalF3 F′ [lac+(lacIq)pro] trxB::kan (DE3) pLysS, (CmR)) (both strains from Novagen, Madison, WI); and BL21 Star (DE3) (F− ompT hsdSB (rB−mB−) gal dcm rne131 (DE3)) (Invitrogen, Carlsbad, CA). For each plasmid, the transformation was carried out according to the manufacturer's instructions. Upon transformation, each plasmid was grown in 25 mL of sterile LB medium with 100 μg/mL ampicillin (pT7 and pMAL-c2X-derived vectors) or 50 μg/mL kanamycin (pET-27 b(+) vectors). When OD600 values reached 0.6–0.8, 0.85 mL aliquots were removed and mixed with 0.15 mL glycerol. The stock cultures were then stored in liquid nitrogen until needed.

Starter cultures and cell growth

Colonies from stock cultures were obtained by scraping the surface of the stock culture with a sterile pipette tip followed by immediate inoculation of sterile LB medium (25 mL). The starter culture was grown in a water bath at 37 °C until the OD_{600} was between 0.6 and 0.8. The resulting starter culture was used to inoculate a 5 L

fermentor growth (New Brunswick, Edison, NJ). The bacteria were further grown at 37 °C with aeration until the OD_{600} reached 1.2. At this point, the medium was induced with 1.2 g IPTG and grown for an additional 3 h at 37 °C (pMAL-c2X-32AAP). Alternatively, the temperature was lowered to 25 °C before the addition of 1.2 g IPTG and the bacteria were allowed to grow for 12–16 h. The cells were then harvested by centrifugation in a Beckman Avanti J-25 centrifuge (8000 rpm, 15 min, 4 °C). The cells were collected and frozen at −20 °C while the supernatant was collected and stored at 4 °C.

Purification of the MBP–AAP fusion protein

Typically, 2.5–3.0 g of cell paste was resuspended in 10 times their volume of 20 mM Tris buffer (pH 8.0), 200 mM NaCl, and 1 mM EDTA at 4 °C. Cells were lysed by sonication (4 × 1 min) with 1 min intervals with the tube containing cells kept on ice (Sonicator Cell Disruptor W-375, Heat System-Ultrasonics, Farmingdale, NY). The debris was separated from the cell extract by centrifugation for 40 min at 18,000 rpm at 4 °C. Clear supernatant was diluted with twice the volume of the same buffer and applied to a 1.5 × 18 cm amylose column (New England Biolabs, Beverly, MA) at a flow rate of 0.2 mL/min at 4 °C. The column was subsequently washed with 12 column volumes of the same buffer to elute any unbound protein. The fusion protein was eluted from the column using 20 mM Tris buffer (pH 8.0), 200 mM NaCl, 1 mM EDTA, and 10 mM maltose. Fractions containing protein were pooled and concentrated in an Amicon stirred cell equipped with a YM10 ultrafiltration membrane (Millipore, Bedford, MA). Protein concentrations were determined using a Bio-Rad assay (Bio-Rad Laboratories, Hercules, CA). The fusion protein was cleaved by factor Xa at a 50:1 ratio (by weight) at 10–20 °C.

Purification of AAP located in the supernatant

The supernatant, after centrifugation (containing LB and the protein), was saturated with ammonium sulfate. The precipitate was collected by centrifugation at 10,000 rpm for 45 min at 4 °C. The pellet, containing AAP, was re-suspended in 30 mL of 10 mM Tricine buffer (pH 8.0) containing 0.1 mM Zn(II). This AAP solution was dialyzed overnight against 4 L of the same Tricine buffer. The protein solution was then centrifuged at 18,000 rpm for 10 min (4 °C) to remove any insoluble precipitate. The supernatant was concentrated in an Amicon stirred cell to 5–10 mL and NaCl was added to a final concentration of 1 M. The sample was then loaded onto a phenyl-Sepharose column (2.5 × 33 cm) (Amersham Biosciences, Piscataway, NJ) pre-equilibrated with 10 mM Tricine buffer, pH 8.0, 1 M NaCl, and 0.1 mM $ZnSO_4$ to remove a brown pigment arising from LB medium that binds to AAP. pt7-AAP and Δsp-AAP (both 54 kDa proteins based on amino acid sequence) were eluted with 10 mM Tricine buffer, pH 8.0, and 0.1 mM $ZnSO_4$. Active fractions were concentrated to 5 mL and loaded onto a Fast Flow Q-Sepharose column (5 × 23 cm) (Amersham Biosciences, Piscataway, NJ) that had been equilibrated with 10 mM Tricine buffer, pH 8.0, 0.1 mM $ZnSO_4$. The protein was further separated from the LB-derived pigment by elution with a 0–1 M NaCl gradient. Active fractions were pooled and concentrated. Removal of any remaining pigment required a second phenyl-Sepharose chromatography step. Purified AAP used for kinetic experiments underwent proteinase K and heat treatment at 70 °C.

Purification of periplasmic AAP

Periplasmic AAP was purified by suspending ~10 g of cell paste in 50–100 mL Tricine buffer (10 mM Tricine, pH 8.0, 20% sucrose, and 0.1 mM Zn(II)) or 50–100 mL Tris buffer (33 mM, pH 8.0, 40% sucrose) with and without 1.5 mM EDTA. The cells were stirred to resuspend the pellet, placed on ice for 10–20 min at 4 °C, and then centrifuged (8500 rpm, 15 min at 4 °C). The cells were then resuspended in 30–50 mL Tricine that had been cooled to 4 °C (10 mM Tricine, pH 8.0, 0.1 mM Zn(II)) or 30–50 mL of water (4 °C) containing 1 mM $MgCl_2$ with less than 0.1 mg DNase I. The sample was shaken vigorously to resuspend the pellet followed by incubation on ice for 20–30 min. The protein solution was separated from debris by centrifugation (18,000 rpm, 40 min, 4 °C) and concentrated to 5 mL. The resulting solution was applied to a Fast Flow Q-Sepharose column (5 × 23 cm) (Amersham Biosciences, Piscataway, NJ) pre-equilibrated with 10 mM Tricine buffer, pH 8.0, 0.1 mM $ZnSO_4$. AAP was eluted with a 0–1 M NaCl gradient and active fractions were collected and concentrated to 5 mL followed by loading on an octyl-Sepharose column (3 × 30 cm) (Amersham Biosciences, Piscataway, NJ) that had been pre-

equilibrated with 10 mM Tricine buffer, pH 8.0, 1 M NaCl, and 0.1 mM $ZnSO_4$. AAP was eluted from the octyl-Sepharose column with 10 mM Tricine buffer, pH 8.0, 0.1 mM $ZnSO_4$, concentrated, and sent for Mass Spec analysis which revealed several impurities in the 7000–12,000 Da range. To further purify this AAP sample, the protein solution was concentrated in a Centricon with a 30 kDa cut-off membrane (YM30, Amicon) and then washed with 10 mM Tricine, pH 8.0, 0.1 mM Zn(II).

Purification of proteinase K treated AAP

To process recombinant AAP to the 32 kDa form (equivalent to native enzyme), the crude supernatant containing the protein after dialysis was treated with proteinase K at 37 °C. The activity of AAP towards l-*p*NA was monitored every 5 min. When the activity did not change after two consecutive measurements, the enzyme solution was heated at 70 °C. Upon concentration in an Amicon stirred cell containing a YM10 membrane to 5–10 mL, the enzyme was mixed with NaCl to a final concentration of 1 M. The enzyme solution was then applied to an octyl-Sepharose column (3 × 30 cm) (Amersham Biosciences, Piscataway, NJ) that had been pre-equilibrated with 10 mM Tricine buffer, pH 8.0, 1 M NaCl, and 0.1 mM $ZnSO_4$. AAP was eluted with 10 mM Tricine buffer, pH 8.0, containing 0.1 mM $ZnSO_4$. The pooled fractions of AAP were concentrated in an Amicon stirred cell. To remove any additional pigment associated with the protein, derived from LB broth, a second purification step was employed that involved applying the AAP sample to a Fast Flow Q-Sepharose column (Amersham Biosciences, Piscataway, NJ) (3 × 30 cm) pre-equilibrated with 10 mM Tricine buffer, pH 8.0, 0.1 mM $ZnSO_4$. AAP was eluted with a 0–1 M NaCl gradient. All purification steps were carried out at 4 °C and the purity of each final AAP sample was confirmed by SDS–PAGE.

Enzymatic assay of AAP

AAP was assayed for catalytic activity using l-*p*NA as the substrate [12]. In this assay, the hydrolysis of l-*p*NA was measured spectrophotometrically by monitoring the formation of *p*-nitroaniline at 405 nm (ε=10,800 M^{-1} cm^{-1}) in a cell with a 1-cm light path. All assays were performed on a Shimadzu UV-3101PC spectrophotometer equipped with a constant-temperature cell holder and an Isotemp 2013D water bath (Fisher Scientific, Pittsburgh, PA). All assays were carried out at 25 °C in 10 mM Tricine, pH 8.0, containing 0.1 mM $ZnSO_4$ using substrate concentrations between 0.2 and 8 K_m.

Apo-enzyme preparation

The purified and concentrated 32 kDa form of rAAP (10–20 mg/mL) was incubated with 10–15 mM disodium EDTA at 4 °C until no activity was observed (usually 1–3 days). To remove EDTA, the protein was dialyzed extensively against 100 times the volume of chelexed 50 mM Hepes buffer, pH 7.5. The activity of the dialyzed protein was less than 1% of the activity of the protein reconstituted with two equivalents of Zn(II). All plasticware used for dialysis and the dialysis tubing itself were presoaked in a disodium-EDTA/bicarbonate solution to remove all traces of metals and were then extensively washed with 50 mM Hepes buffer, pH 7.5, that had been run through a Chelex column.

Spectroscopic measurements

Electronic absorption spectra were recorded on a Shimadzu UV-3101PC spectrophotometer. Apo-protein samples in metal-free 50 mM Hepes buffer, pH 7.5, were degassed prior to incubating with Co(II) ($CoCl_2$: ⩾99.999%, Acros Organics, Geel, Belgium). All electronic absorption spectra were recorded at 25 °C.

Results

Over-expression of the entire ORF of AAP

Over-expression of the entire ORF of AAP in BL21(DE3) cells provided an active enzyme with a molecular weight of approximately 54 kDa located in the supernatant (pt7-AAP). Expression in BL21(DE3) cells yielded ~10 mg of enzyme in the supernatant while ~3 mg is retained within the cell. However, expression in the BL21Star cell line yielded approximately 40 and 4 mg of protein in the supernatant and within the cell, respectively. Amino acid sequence analysis revealed that the over-expressed enzyme found in the supernatant lacked the native signal sequence and as a result, its molecular weight was 51.6 kDa (Table 1). This form of the enzyme was thermostable and did not undergo processing. However, upon treatment of the supernatant form of pt7-AAP with proteinase K and heating to 70 °C for 1–2 h, the 51.6 kDa enzyme was processed to a 32 kDa form of AAP which has the same molecular weight and N-terminal amino acid sequence as wild-type AAP (Table 1).

Table 1. N-terminal amino acid sequence and molecular weight of each form of AAP based on cellular localization

Expression system	Location	N-terminal amino acid sequence	Molecular weight (Da)
Aeromonas proteolytica	Processed extracellular protein[a]	MPPIT	32,148 (100%)[b]
(*Vibrio proteolyticus*)	Periplasm[c]	—	30,923 (30%)[b] —
pT7-54AAP	Extracellular protein	EDKVW	51,550 (100%)
	Processed extracellular protein[d]	MPPIT	32,099 (100%)
			64,060 (20%)
pET27b(+)-43 AAP	Extracellular (supernatant)	EDKVW	40,709 (100%)
			39,654 (20%)
	Heat-treated extracellular protein	ND	8029 (100%)
		MPTTL	32,795 (40%)
	Periplasm	MPTTL	32,415 (100%)
pVSNMC	Extracellular protein	EDKVW	51,092 (100%)
	Proteinase K-treated extracellular protein	ASFVM	42,664 (100%)
	Processed extracellular protein[d]	MPPIT	31,866 (100%)
			32,774 (70%)
	Periplasm	ND	11,360 (100%)
		ND	44,302 (50%)
		MPTTL[e]	43,214 (35%)
		EDKVL and EEHNR	46,331 (25%)

N-terminal amino acid sequence and molecular weight of each form of AAP based on cellular localization
Molecular weights were obtained by matrix-assisted laser desorption ionization-time of Xight (MALDI-TOF) spectrometric analysis. The inten- sity of the peak is given in parenthesis.

ND—not determined.

[a] Heat treated at 70 °C (native protein).

[b] Broad peaks (1000 Da).

[c] Very low activity detected.

[d] Treated with proteinase K (37 °C) followed by heat treatment (70 °C).
[e] The only sequence obtained after extensive wash of the periplasmic fraction in a Centricon YM30.

Over-expression of the AAP ORF in *E. coli* using the pVSNMC plasmid (Δsp-AAP) provided active AAP enzyme in the growth medium. Transformation into BL21 Star (DE3) cells resulted in a significant increase in expression (up to 60 mg/L purified enzyme vs. 15 mg/L in BL21(DE3) cells). The molecular weight of the Δsp-AAP enzyme was found to be 51,092 Da (Table 1), which is slightly lower than the molecular weight deduced from the DNA sequence (52,026 Da). Amino acid sequencing and mass spectrometry analyses revealed that the signal peptide is absent so the Δsp-AAP enzyme starts with the same sequence as the N-terminal propeptide of AAP (Table 1). Therefore, the lower molecular weight indicates that the Δsp-AAP enzyme is truncated at the C-terminus.

To determine the catalytic properties of the recombinant 51 kDa form of AAP, the kinetic parameters K_m and k_{cat} were obtained using l-*p*NA as the substrate. The supernatant forms of both pt7-AAP and Δsp-AAP exhibited K_m values of 455 and 438 µM, respectively, and k_{cat} values of 464 and 525 min^{-1}, respectively (Table 2). Interestingly, the observed kinetic parameters for the 51 kDa forms of AAP are significantly different from those of wild-type AAP (K_m=10.6µM and k_{cat}=4280 min^{-1}). Additionally, the 51 kDa enzymes exhibited a time dependence for substrate cleavage (Fig. 1). Such a behavior is typically observed in inhibition studies with slow binding inhibitors, where upon prolonged incubation of the enzyme–inhibitor complex, the reaction is initiated with the addition of a substrate. This results in an increase of the velocity of the reaction due to dilution of the enzyme–inhibitor complex with the substrate [20].

Table 2. Kinetic parameters for the different forms of rAAP derived from pVSNMC

	K_m (µM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} $µM^{-1}$
51 kDa pt7-AAP (plasmid pT7-54AAP)	455 ± 38	464 ± 14	1.02
51 kDa sp-AAP (pVSNMC plasmid)	438 ± 35	525 ± 18	1.20
43 kDa sp-AAPa (pVSNMC plasmid)	11.3 ± 0.4	3843 ± 44	340
32 kDa sp-AAPb (pVSNMC plasmid)	11.8 ± 0.4	4050 ± 50	360
WT AAP (mature from Vibrio proteolyticus)	10.6 ± 0.3[c]	4280 ± 42	404

a. Proteinase K-treated, lacking part of the N-terminal propeptide.
b. Proteinase K-treated (37°C) and heat treated (70°C).
c. Typical values of K_m found in the literature vary from 10 to 18µM [12], [37].

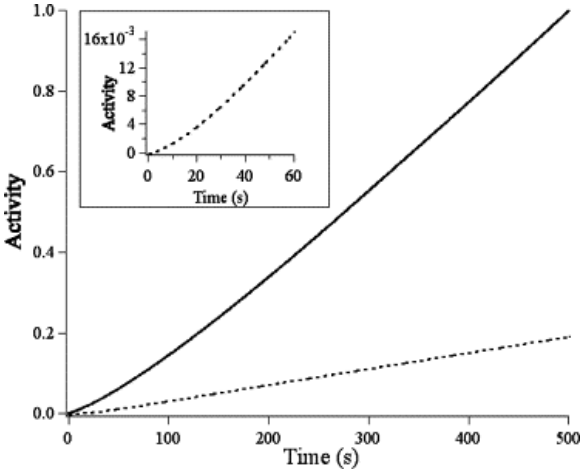


Fig. 1. Progress curve for the hydrolysis of l-*p*NA (990 µM) by ΔC-AAP (solid line) and Δsp-AAP (dashed line) at 25 °C in 10 mM Tricine buffer, pH 8.0. Inset: Expanded (0–60 s) region of l-*p*NA hydrolysis by Δsp-AAP.

Over-expression of a C-terminal truncated form of AAP

A 43 kDa form of AAP encoding the mature protein preceded by its N-terminal propeptide but lacking the C-terminal propeptide (ΔC-AAP) was over-expressed in a pET-27b(+) vector. Approximately 70 mg of pure enzyme was obtained from a 5 L culture using this expression vector and less than 1 mg of protein was retained within the cell. The predicted molecular weight of the pET-27 b(+)-43AAP (ΔC-AAP) enzyme is 43,235 Da, however, enzyme found in the supernatant had a mass of 40,709 Da based on Mass Spec data. N-terminal sequence analysis revealed that the observed mass difference of ~2.5 kDa is due to the loss of the first 21 amino acids of the N-terminal leader sequence of AAP, providing an enzyme with a molecular weight 40,880 Da (Table 1). Interestingly, Mass Spec analysis of the AAP enzyme retained within the cell revealed a truncated form that lacks most of the N-terminal propeptide sequence (Table 1).

The catalytic properties of the ΔC-AAP enzyme were also examined and ΔC-AAP exhibited a progress curve similar to those observed for pt7-AAP and Δsp-AAP (Fig. 1). The initial velocity was two times slower than the steady-state velocity. Kinetic parameters were obtained from the steady-state velocity at each substrate concentration by fitting these data to the Michaelis–Menten equation. Based on these fits K_m was found to be 142 μM, which is 10 times greater than that of wild-type AAP, however, the observed k_{cat} value was 4346 min^{-1}, which is identical (within error) to that observed for wild-type AAP (Table 3). Based on X-ray crystallography, the active site conformation of wild-type AAP does not change upon substrate binding [21], therefore, the N-terminal propeptide sequence must be responsible for the observed changes in kinetic parameters. Based on X-ray crystallographic analysis of AAP and with the help from a secondary structure prediction tool[2] [21] the first 13 amino acid residues of the N-terminal propeptide are likely disordered, suggesting that they can enter the active site of AAP and act as an inhibitor (Fig. 2). An estimate of the inhibitory properties of the N-terminal propeptide was obtained by fitting the experimental data to the Michaelis–Menten equation (1):

$$V=k_{cat}[E]_t[S]/(K_m+[S]), \quad (1)$$

where V is the velocity, k_{cat} is the reaction rate of the rate-limiting step, $k_{cat}[E]_t=V_{max}$ is the maximum velocity of the reaction, $[E]_t$ is the total enzyme concentration used in the reaction, [S] is the substrate concentration, and K_m is the Michaelis–Menten constant. Based on the fact that the k_{cat} value obtained for ΔC-AAP is identical (within error) to the k_{cat} value observed for wild-type AAP, the N-terminal propeptide is likely a competitive inhibitor that affects only K_m. Therefore, Eq. (2), which describes competitive inhibition, resembles Eq. (1) but includes the inhibition factor, K_i.

$$V=k_{cat}[E]_t[S]/(K_m(1+[I]/K_i)+[S]). \quad (2)$$

Since k_{cat} and V_{max} for ΔC-AAP and wild-type AAP are identical within error, and $[S]\rightarrow\infty, V\rightarrow V_{max}$:

$$K_{m1}=K_m(1+[I]/K_i), \quad (3)$$

where K_{m1} (142 μM) is the Michaelis constant of the enzyme inhibited by the N-terminal propeptide and K_m (11 μM) is the Michaelis constant of wild-type AAP. The K_i value obtained for the N-terminal propetide of ΔC-AAP was found to be 0.13 nM. This value is an estimate of the true K_i value and likely represents a high-end estimate since the propeptide is an integral part of AAP [22], [23], [24].

Table 3. Kinetic parameters of recombinant AAP obtained from the pET27b(+)-43AAP vector

pET27b(+)-43AAP	**K_m (μM)**	**k_{cat} (min^{-1})**	**k_{cat}/K_m (min^{-1} μM^{-1})**
Extracellular protein	142 ± 10	4346 ± 101	30.5

Heat-treated extracellular protein	16.6 ± 1.2	2211 ± 40	133
Treated extracellular protein[a]	10.4 ± 0.4	1519 ± 17	146
WT AAP (mature from *Vibrio proteolyticus*)	10.6 ± 0.3	4280 ± 42	404

a. Upon proteinase K and heat treatment at 70°C.

```
1         10         20         30         40         50
EDKVWISIGADANQTVMKSGAESILPNSVASSGQVWVGQVDVAQLAELSHNMHEE

cccceeeeccccchhhhhhccceecccccccccceeechhhhhhhhhhhhhhhhh

    60         70         80    85
HNRCGGYMVHPSAQSAMAASAMPTTLASFV

hhhhcceeeeechhhhhhhhccceeeeeec
```

Fig. 2. Prediction of the secondary structure of the N-terminal propeptide of AAP (c-random coil; e-extended strand; and h-alpha helix). This analysis was performed using a web-based program available through PBIL, Lyon, France at: http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_gw [21]. The first 13 amino acids (underlined) are largely unfolded and may enter the active site of AAP and inhibit the enzyme.

Over-expression of an N- and C-terminal truncated form of AAP

Over-expression of AAP lacking both the N-terminal and C-terminal propeptide sequences (plasmids pT7-32AAP and pET27b(+)-32AAP) resulted in the formation of inclusion bodies that could not be solubilized (Fig. 3). As a result, no enzyme activity was observed using these expression systems. The formation of inactive protein was hypothesized to be the result of the reducing environment in the cytosol, thus, preventing proper formation of the single disulfide bond in AAP. To test this hypothesis, *E. coli* strain AD494(DE3) pLysS was used, which is a thioredoxin reductase mutant strain, which has been shown to increase disulfide bond formation in the cytoplasm leading to the proper folding of proteins [25]. However, *E. coli* strain AD494(DE3) pLysS still provided only insoluble inclusion bodies of AAP.

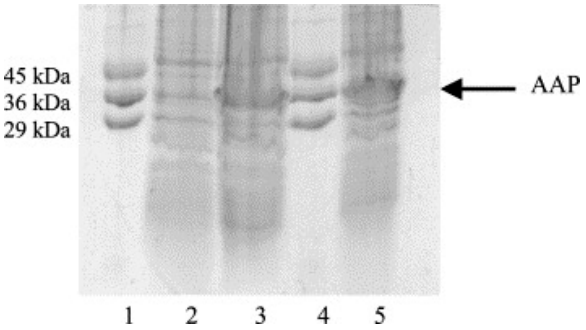


Fig. 3. SDS–PAGE for the over-expression of the 32 kDa form of AAP using pT7-32AAP expression vector (lanes: 1—protein marker; 2—cells before induction; 3—cells 3 h post-induction with IPTG; 4—protein marker; and 5—cell debris upon cell breaking).

To produce a soluble, active form of the 32 kDa AAP, a pMAL-c2X vector was used which contains a strong P_{tac} promoter [26], [27], [28]. This particular vector lacks the malE signal sequence and thus, the fusion protein is retained in the cytoplasm. In addition, a factor Xa cleavage site was designed into the vector directly preceding the cloning site of the AAP gene, to cleave the AAP enzyme from maltose-binding protein (MBP). The MBP–AAP fusion protein was over-expressed in both BL21 and AD494 cells and purified using an amylose column that selectively binds MBP. The molecular weight of the fusion protein was determined from SDS–PAGE to be ~70 kDa, which is in excellent agreement with the combined molecular weight of MBP and AAP (72 kDa). Cleavage of the fusion protein resulted in the disappearance of the 70 kDa band and the concomitant appearance of two new bands at 30 and 40 kDa, which correspond to AAP and MBP, respectively (Fig. 4). The band at approximately 30 kDa was less intense than the band corresponding to the MBP, suggesting that AAP was further processed by factor Xa. In addition, several bands were also observed in the SDS gel below 30 kDa consistent with additional processing of AAP by factor Xa.

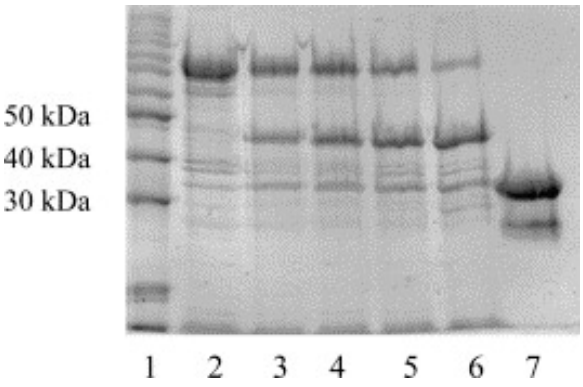


Fig. 4. SDS–PAGE showing the cleavage of the MBP–AAP fusion protein by factor Xa (lanes: 1—protein marker, 10 kDa spacing; 2—maltose-binding protein–AAP fusion protein; 3—digestion with factor Xa (2 h, 15 °C); 4—digestion with factor Xa (4 h); 5—digestion with factor Xa (8 h); 6—digestion with factor Xa (25 h); and 7—native AAP). Native 32 kDa AAP is often processed to a 28–29 kDa protein [38], [39].

Production of a soluble, active 32 kDa form of AAP

The recombinant 51 kDa form of AAP (pt7-AAP) was used to produce a heat stable, fully processed form of AAP that is identical to wild-type AAP. Since wild-type AAP has been suggested to be processed by a neutral protease, we treated pt7-AAP with proteinase K at 37 °C. Treatment of pt7-AAP with proteinase K was monitored by measuring AAP activity towards l-*p*NA, which revealed a continuous increase in initial velocity that eventually lapsed providing a straight line. These data suggest that AAP is no longer inhibited by its N-terminal propeptide, suggesting that proteinase K treatment provides a partially processed AAP enzyme with a molecular weight of 43 Da. N-terminal amino acid sequencing and Mass Spec data indicate that proteinase K treatment does in fact remove part of the N-terminal propeptide 81 amino acids (Table 1). The resulting 43 kDa enzyme exhibited K_m and k_{cat} values that are nearly identical to those of native AAP (K_m=10.8μM vs. 10.6 μM; k_{cat} 3800 vs. 4280 min^{-1} for processed pt7-AAP and WT AAP, respectively). Further processing did not occur under these conditions, even upon prolonged treatment of AAP with proteinase K.

Increasing the temperature of the N-terminal processed 43 kDa AAP enzyme to 70 °C in the presence of proteinase K resulted in additional processing of AAP. After heat treatment, a 32 kDa AAP enzyme was obtained that contained identical N- and C-terminal amino acid sequences to wild-type AAP (Table 1). Addition of a proteinase K inhibitor (PMSF) prior to heat treatment at 70 °C did not result in the formation of a 32 kDa form of AAP, suggesting that the 43 kDa form of AAP is not capable of auto-processing. The fully processed over-expressed 32 kDa form of AAP exhibited identical kinetic parameters to those of wild-type AAP (Table 2). Interestingly, processing of the ΔC-AAP enzyme with proteinase K and heat treatment at 70 °C provided an

enzyme with an identical K_m value as wild-type AAP (10.4 μM) but was much less active 1519 vs. 4346 min^{-1} for processed ΔC-AAP vs. wild-type, respectively (Table 3).

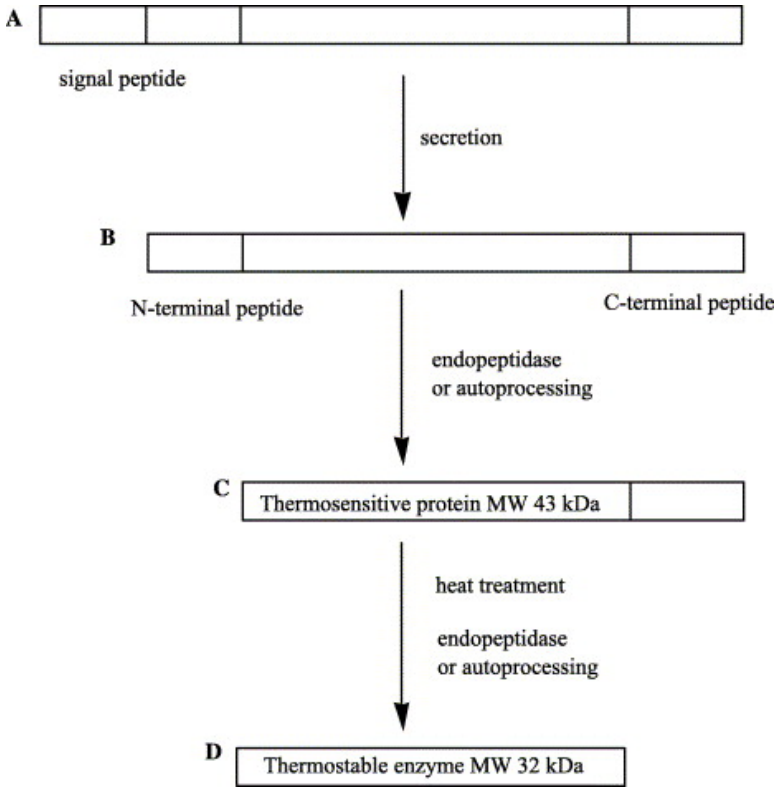
Spectroscopic characterization of the recombinant 32 kDa form of AAP

Since the proteinase K and heat treated 51 kDa form of AAP (pt7-AAP) displayed identical kinetic parameters and molecular weight as wild-type AAP (Table 1, Table 3), the structural integrity of the dinuclear Zn(II) active site was characterized by UV–Vis spectrometry. Reconstitution of the apo-32 kDa form of AAP (rAAP) with one and two equivalents of Co(II), provided electronic absorption spectra with extinction coefficients of ε_{525}=56 and 109 M^{-1} cm^{-1}, respectively. These data are in good agreement with the molar absorptivities and λ_{max} values observed for wild-type AAP in the presence of one and two equivalents of Co(II) (i.e., ε_{525}=45 and 97 M^{-1} cm^{-1}). UV–Vis titration of Co(II) into rAAP provided the number of divalent metal ions bound to the enzyme. Upon the addition of Co(II) to rAAP, the molar absorptivity at 525 nm increased gradually until two equivalents of Co(II) had been added. No significant change was observed upon the addition of excess Co(II), suggesting that rAAP binds only two divalent metal ions in the active site, identical to wild-type AAP.

Discussion

The gene encoding the leucine aminopeptidase from *A. proteolytica* (*V. proteolyticus*) (AAP) provides an enzyme with a molecular weight of 54 kDa. However, the "as-purified" wild-type enzyme from *A. proteolytica (V. proteolyticus)* is only 32 kDa. Very little is known about the nature of the cleavage process leading to mature enzyme, but expression of proproteins is a common feature among many proteases [23], [29], [30], [31], [32], [33]. Classic examples of protein activation through removal of a propeptide are the enzymes of the digestive tract expressed as inactive zymogens. In addition, several proteases, including metallo-enzymes such as subtisilin, thermolysin, carboxypeptidase A, and Npr from *Bacillus brevis* undergo processing upon secretion [23], [29], [30], [31], [32], [33]. Therefore, the development of a fundamental knowledge of maturation processes involving N-terminal leader sequences and N- or C-propeptides is an important outstanding problem.

The open-reading frame (ORF) of AAP contains four distinct domains: (i) a leader peptide, (ii) an N-terminal propeptide, (iii) the mature enzyme, and (iv) a C-terminal peptide (Scheme 1). Leader peptides typically direct a protein to a specific compartment within the cell, which in the case of AAP results in its transportation across the periplasmic space and the outer membrane so that it is secreted extracellularly. The AAP signal peptide appears to be very efficiently recognized by the *A. proteolytica* bacterial transport system since little to no AAP activity is found within the periplasmic space of the bacterium. Comparison of hydrophobicity plots of several signal sequences such as PelB, MalE, and OmpA suggests that the first 21 amino acids of the ORF of AAP constitute the signal peptide of AAP (Fig. 5). These data are in contrast to those reported by Guenet et al. [11] where they suggested that amino acids 73–106 constituted the signal peptide of AAP. Based on the hydrophobicity plots, the AAP signal peptide bears the closest resemblance to OmpA and shows distinct hydrophobicity, as opposed to the amino acids in positions 76–103 (Fig. 5, Fig. 6). This similarity to other signal peptides was hypothesized to lead to effective secretion of AAP from *E. coli* using the native AAP signal peptide. Secretion of AAP into the culture medium was observed with less than 10% of the total amount of AAP produced retained in the cell. A similar result was obtained when an *E.* coli leader sequence, PelB, replaced the native sequence in AAP. The observed hydrophobicity plots are also consistent with the prediction made by Van Heeke et al. [10] and are in excellent agreement with the (−3, −1) rule for signal sequences [34]. Conversely, the lack of initial 21 amino acids results in the accumulation of inactive protein in the bacterial cell (Fig. 3).



Scheme 1. Proposed processing of AAP during secretion and purification. The four different domains constituting AAP are: (1) the signal peptide (21 amino acids), (2) an N-terminal propeptide (89 amino acids), (3) the mature protein (291 amino acids), and (4) a C-terminal propeptide (107 amino acids). Upon secretion of AAP a thermostable, 32 kDa enzyme is formed through a 43 kDa protein intermediate.

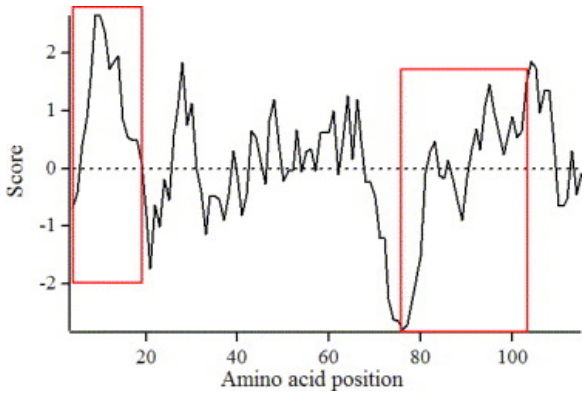


Fig. 5. Hydrophobicity plot of the propeptide sequence of AAP.

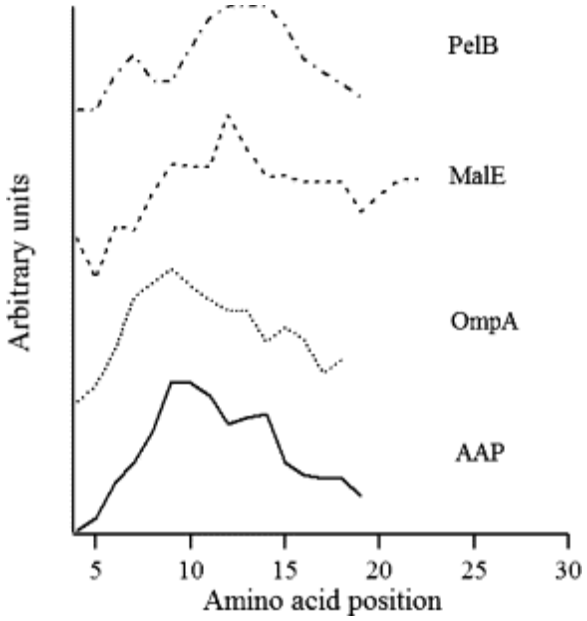


Fig. 6. Comparison of the hydrophobicity of known signal peptides.

The N-terminal propeptide was previously proposed to be required for proper folding of AAP [15]; however, its presence alone does not guarantee proper folding of the enzyme. If the signal peptide does not precede the N-terminal propeptide, AAP is not properly folded and is found in the insoluble cellular fraction [15]. The use of an *E. coli* thioredoxin reductase mutant strain, AD494, which in some cases helps in the formation of disulfide bonds in the cytoplasm [25], does not result in the formation of properly folded protein. There are six cysteines in the ORF of AAP—two in the N-terminal propeptide, C-terminal propeptide, and mature protein; the last two are not likely to be involved in protein folding as lack of the C-terminal propeptide results in the formation of active protein. These data suggest that proper protein folding may occur only in the periplasmic space or outside the cell where the environment is less reductive. Since thioredoxin usually acts on exposed disulfide bonds, the formation of the only disulfide bond within mature AAP (Cys223 and Cys227) may be critical for proper folding [35].

Several metalloenzymes have been shown to be inhibited by their respective N-terminal propeptides [23], [29], [30], [31], [32], [33]. Many of these propeptides are competitive inhibitors spanning a broad range of inhibition constants (0.1–2000 nM) [23], [36]. The N-terminal propeptide of AAP appears to be a competitive inhibitor of AAP with an approximate K_i value of 0.13 nM. This K_i value is very similar to that observed for N-terminal propetide inhibition of another metalloenzyme, carboxypeptidase A (K_i=2 nM) [24]. Upon cleavage of the N-terminal propeptide by proteinase K (ΔC-AAP), the inhibitory properties of AAP's propeptide are eliminated since the observed K_m value decreases from 142 to 11 μM, which is identical to wild-type AAP. Similarly, the proteinase K truncated form of the pVSNMC-encoded AAP enzyme exhibits a K_m value of 11 μM, a value that is also significantly lower than that for the unprocessed enzyme (438 μM) (Table 2, Table 3). In the latter case, upon proteinase K treatment the catalytic efficiency increases four times while a twofold decrease in k_{cat} is observed for ΔC-AAP. These data suggest that the C- and N-terminal propeptides of AAP interact with one another and that they likely inhibit the AAP enzyme in a cooperative fashion. Moreover, these data suggest that the C-terminal propeptide is involved in stabilizing AAP, particularly during the heat treatment to 70 °C since no decrease in activity is observed for AAP containing the C-terminal propeptide upon proteinase K and heat treatment. N- and C-terminal interaction is also consistent with the observed K_m values for the 51 kDa form of AAP (438 μM) vs. the C-terminal truncated form of AAP (142 μM). These data suggest that proteinase K closely resembles the native endopeptidase involved in processing AAP, since rAAP exhibits an identical molecular

weight, shares the same N-terminal sequence, exhibits identical kinetic properties, and is spectroscopically indistinguishable from wild-type AAP.

Based on these data, a mechanism for processing the 51 kDa form of AAP to its wild-type 32 kDa form can be proposed (Scheme 1). The ORF encodes a protein of a 54 kDa molecular weight. The protein is directed to the periplasm where it crosses the outer membrane; during this process the signal peptide is separated from the rest of the protein by a signal peptidase leaving a 51–52 kDa protein. This sequence of events is supported by the presence of an SDS–PAGE band for excreted AAP at approximately 50 kDa, corresponding to unprocessed AAP. In the following step, the N-terminal propeptide is removed by an endopeptidase. The current form of AAP still retains its C-terminal propeptide and the molecular weight is ~43 kDa but during heat treatment, the C-terminal propeptide is removed. Since inhibition of proteinase K with PMSF directly preceding the heat-treatment step inhibits the truncation at C-terminus, it is likely that an endopeptidase catalyzes this step. The final product is a 32 kDa, thermostable enzyme that is resistant to proteolysis by the endopeptidase responsible for AAP processing. The nature of this endopeptidase is unknown at this time, but a neutral protease that is co-produced with AAP in *A. proteolytica* (*V. proteolyticus*), is a likely candidate.

In conclusion, we have successfully over-expressed the leucine aminopeptidase from *A. proteolytica* (*V. proteolyticus*) in *E. coli* using its native signal peptide. The successful extracellular excretion of AAP from *E. coli* using its native signal peptide provides an additional signal peptide for the development of *E. coli* expression systems for proteins that do not fold properly in the cytosol. Characterization of the proteinase K-processed AAP enzymes allowed the first detailed model for the maturation of wild-type AAP to be proposed. A cooperative inhibitory interaction between the N- and C-terminal propeptides of AAP was observed and the kinetic characterization of the various forms of AAP revealed that the N-terminal propeptide is a strong inhibitor of AAP activity (K_i~0.13 nM). These data provide the basis for the design of a new class of leucine aminopeptidase inhibitors. In addition, a fully processed recombinant form of AAP was characterized by kinetic and spectroscopic methods and this enzyme was shown to be identical to the “as-purified” wild-type AAP enzymes. Therefore, the ease of purification and processing of rAAP along with the fact that large quantities can be obtained now allow new detailed mechanistic studies to be performed on AAP through site-directed mutagenesis.

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References

[1] J.M. Prescott, F.W. Wagner, B. Holmquist, B.L. Vallee Biochem. Biophys. Res. Commun, 114 (1983), pp. 646-652
[2] G. Pulido-Cejudo, B. Conway, P. Proulx, R. Brown, C.A. Izaguirre Antivir. Res, 36 (1997), pp. 167-177
[3] T. Hildmann, M. Ebnent, H. Pena-Cortes, J.J. Sanchez-Serrano, L. Willmitzer, S. Prat Plant Cell, 4 (1992), pp. 1157-1170
[4] K. Herbers, S. Prat, L. Willmitzer Planta, 194 (1994), pp. 230-240
[5] J. Beninga, K.L. Rock, A.L. Goldberg J. Biol. Chem, 273 (1998), pp. 18734-18742
[6] T. Saric, S.C. Chang, A. Hattori, I.A. York, S. Markant, K.L. Rock, M. Tsujimoto, A.L. Goldberg Nat. Immunol, 3 (2002), pp. 1169-1176
[7] I.A. York, S.C. Chang, T. Saric, J.A. Keys, J.M. Favreau, A.L. Goldberg, K.L. Rock Nat. Immunol, 3 (2002), pp. 1177-1184
[8] S. Mizutani, Y. Tomoda Am. J. Hypertens, 9 (1996), pp. 591-597
[9] T. Akada, Y. Tomoda, H. Miyashita, O. Niizeki, M. Abe, A. Sato, S. Satomi, Y. Sato J. Cell Physiol, 193 (2002), pp. 253-262

[10] G. Van Heeke, S. Denslow, J.R. Watkins, K.J. Wilson, F.W. Wagner Biochim. Biophys. Acta, 1131 (1992), pp. 337-340
[11] C. Guenet, P. Lepage, B.A. Harris J. Biol. Chem, 267 (1992), pp. 8390-8395
[12] J.M. Prescott, S.H. Wilkes Methods Enzymol. B, 45 (1976), pp. 530-543
[13] J.R. Merkel, E.D. Traganza, B.B. Mukherjee, T.B. Griffin, J.M. Prescott J. Bacteriol, 87 (1964), pp. 1227-1233
[14] M.E. Bayliss, S.H. Wilkes, J.M. Prescott Arch. Biochem. Biophys, 204 (1980), pp. 214-219
[15] Z.-Z. Zhang, S. Nirasawa, Y. Nakajima, M. Yoshida, K. Hayashi Biochem. J, 350 (2000), pp. 671-676
[16] J.H. Zhang, D.M. Kurtz, M.J. Maroney, J.P. Whitehead Inorg. Chem (1991), pp. 1359-1366
[17] A. Naka, K. Yamamoto, T. Miwatani, T. Honda FEMS Microbiol. Lett, 77 (1992), pp. 197-200
[18] J. Kyte, R.F. Doolittle J. Mol. Biol, 157 (1982), pp. 105-142
[19] J. Pratap, K.L. Dikshit Mol. Gen. Genet, 258 (1998), pp. 326-333
[20] R.A. Copeland **Enzymes: A Practical Introduction to Structure, Mechanism, and Data Analysis** (second ed.), Wiley-VCH, New York (2000)
[21] J. Garnier, J.-F. Gibrat, B. Robson Methods Enzymol, 266 (1996), pp. 540-553
[22] A. Serkina, T.F. Gorozhankina, A.B. Shevelev, G.G. Chestukhina FEBS Lett, 456 (1999), pp. 215-219
[23] Z. Hu, K. Haghjoo, F. Jordan J. Biol. Chem, 271 (1996), pp. 3375-3384
[24] B. San Segundo, M. Martinez, M. Vilanova, F. Aviles Biochim. Biophys. Acta, 707 (1982), pp. 74-80
[25] A.I. Derman, W.A. Prinz, D. Belin, J. Beckwith Science, 262 (1993), pp. 1744-1747
[26] C. di Guan, P. Li, P.D. Riggs, H. Inouye Gene, 67 (1988), pp. 21-30
[27] C.V. Maina, P.D. Riggs, A.G. Grandea 3rd, B.E. Slatko, L.S. Moran, J.A. Tagliamonte, L.A. McReynolds, C.D. Guan Gene, 74 (1988), pp. 365-373
[28] P. Riggs K. Struhl (Ed.), Current Protocols in Molecular Biology, Greene Publishing and Wiley Interscience, New York (1992), p. 16.6.1, 16614
[29] A. Markaryan, J. Lee, T. Sirakova, P. Kollattukudy J. Bacteriol, 178 (1996), pp. 2211-2215
[30] M. Taylor, K.C. Baker, G.S. Briggs, I.F. Connerion, N. Cummings, K.S. Pratt, D.F. Revell, R.B. Freedman, P.W. Goodenough Protein Eng, 15 (1995), pp. 59-62
[31] D. Baker, J. Silen, D. Agard Biochemistry, 31 (1992), pp. 12571-12576
[32] J.R. Winther, P. Sorensen, M. Kielland-Brandt J. Biol. Chem, 269 (1994), pp. 22007-22013
[33] T. Fox, E. de Miquel, J.S. Mort, A.C. Storer Biochemistry, 31 (1992), pp. 12571-12576
[34] G. Von Heijne Nucleic Acids Res, 14 (1986), pp. 4683-4690
[35] A. Holmgren J. Biol. Chem, 264 (1989)
[36] A.V. Serkina, T.F. Gorozhankina, A.B. Shevelev, G.G. Chestukhina FEBS Lett, 456 (1999), pp. 215-219
[37] C. Stamper, B. Bennett, T. Edwards, R.C. Holz, D. Ringe, G. Petsko Biochemistry, 40 (2001), pp. 7034-7046
[38] J.M. Prescott, S.H. Wilkes, F.W. Wagner, K.J. Wilson J. Biol. Chem, 246 (1971), pp. 1756-1764
[39] J.M. Prescott, S.H. Wilkes Methods Enzymol, 45 (1976), pp. 530-543

Notes

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[1] *Abbreviations used:* AAP, mature (32kDa) leucine aminopeptidase from *Aeromonas proteolytica* (*Vibrio proteolyticus*); rAAP, recombinant leucine aminopeptidase; pt7-AAP, recombinant leucine aminopeptidase over-expressed using pT7 vector (containing all domains of native AAP); Δsp-AAP, recombinant leucine aminopeptidase from pVSNMC expression vector (lacking the native leader sequence); ΔC-AAP, recombinant leucine aminopeptidase from pET27b(+)-43AAP expression system (lacking the C-terminal propeptide sequence); IPTG, isopropyl-β-d-thiogalactopyranoside; l-*p*NA, l-leucine-*p*-nitroanilide; Tricine, (*N*-tris[hydroxymethyl])methylglycine; malE, maltose-binding protein gene from *Escherichia coli*; MBP, maltose-binding protein; MHC-I, major histocompatibility complex-I; OmpA, outer membrane protein from *E. coli*; PelB, pectate lyase from *E. coli*; PMSF, phenylmethanesulfonyl fluoride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TOF-MS, time of flight mass spectroscopy; WT, wild-type. Numbers

preceding AAP, Δsp-AAP, and ΔC-AAP denote molecular weight of the respective form of the leucine aminopeptidase.

[2] Available at http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_gor4.html.