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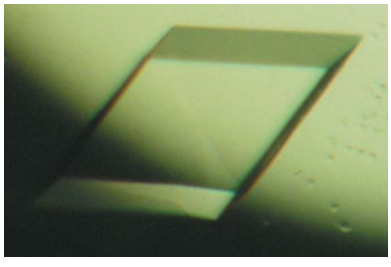
Cloning, expression, crystallization and preliminary X-ray crystallographic analysis of aspartyl aminopeptidase from the *apeB* gene of *Pseudomonas aeruginosa*

Aminopeptidases (APs) are a group of exopeptidases that catalyze the removal of amino acids from the N-termini of proteins and peptides. The APs are ubiquitous in nature and are of critical biological and medical importance because of their key role in protein degradation. *Pseudomonas aeruginosa* aspartyl aminopeptidase (PaAAP), which is encoded by the *apeB* gene, was expressed in *Escherichia coli*, purified and crystallized using the microbatch method. A preliminary structural study has been performed using the X-ray crystallographic method. The PaAAP crystal diffracted to 2.0 Å resolution and belonged to the rhombohedral space group *H*3, with unit-cell parameters $a = b = 133.6$, $c = 321.2$. The unit-cell volume of the crystal is compatible with the presence of four monomers in the asymmetric unit, with a corresponding Matthews coefficient V_M of 2.95 Å^3 Da^{-1} and a solvent content of 58.3%.

1. Introduction

The synthesis of new proteins and the degradation of unwanted or misfolded proteins are universal complementary processes that continuously occur in all living cells and are controlled by proteosomes. This catabolism of proteins is a key cellular function and must be under spatial and temporal control in order to avert damage to the cell. Aminopeptidases (APs) are a group of exopeptidases that cleave amino acids from the amino-termini of proteins or peptides. This proteolytic process is closely associated with many biological functions such as protein maturation, hormone-level regulation, protein degradation and cell-cycle control. These enzymes therefore play an important role in many pathological conditions, including cancer, cataracts, cystic fibrosis, leukaemia and HIV infection (Lazdunski, 1989; Pulido-Cejudo *et al.*, 1997). Proteosomes are the major protein-degradation machinery and digest proteins into short peptides containing 7–15 residues (Groll & Clausen, 2003). Many ATPases, such as Clps and PAN (energy-dependent proteases), recognize and unfold modified proteins, subsequently delivering the denatured polypeptides to the associated protease (Zwickl *et al.*, 1999). These proteolytic machines do not completely perform the proteolytic process or degrade proteins to single amino acids. They generate a pool of oligopeptides of different lengths which require further processing to generate free amino acids (Borissenko & Groll, 2005).

According to the MEROPS database (Rawlings *et al.*, 2000), APs can be classified into families and clans based on sequence homology. They are widely distributed in all organisms (bacteria, yeast and plant and animal tissues) and have critical biological roles and medical importance. The MH clan consists of metalloaminopeptidases that accommodate two Zn ions in the active site and are involved in catalytic hydrolysis processes (Auld, 2004). The structures of many metallopeptidases have been reported and mechanisms have been proposed based on their activities. The architectures of these biological molecules show various catalytically active oligomeric structures (monomers, dimers, trimers and tetramers). A dodecameric tetrahedral (TET) aspartyl aminopeptidase (AAP) from *Pseudomonas aeruginosa* (PaAAP), which is encoded by the *apeB* gene and belongs to the M18 family, has been reported. To date, TET AAP structures have been reported from various species: PDB entries 3l6s (*Homo sapiens*; Structural Genomics Consortium, unpublished work), 2glf (*Thermotoga maritima*; New York SGX



Research Center for Structural Genomics, unpublished work), 2glj (*Clostridium acetobutylicum*; New York SGX Research Center for Structural Genomics, unpublished work) and 1xfo (*Pyrococcus horikoshii*; Russo & Baumann, 2004). The AAP from *H. sapiens* (HsAAP; PDB entry 3l6s) shares 42.2% sequence similarity with PaAAP. PaAAP consists of 429 amino acids and has a molecular weight of about 46.7 kDa.

In this study, we studied the dodecameric TET AAP from *P. aeruginosa*, which is encoded by the *apeB* gene. This gene was cloned and expressed and the aspartyl aminopeptidase was purified and crystallized; a preliminary X-ray crystallographic study was carried out in order to understand its structural organization and unique catalytic reaction pathway.

2. Materials and methods

2.1. Cloning

The *apeB* gene was amplified *via* polymerase chain reaction (PCR) using the genomic DNA of *P. aeruginosa*. The sequences of the forward and reverse primers designed from the published genome sequence (Stover *et al.*, 2000) were 5′-TA**GGATCC**ATGCGCGCA-GAACTCAACC-3′ and 5′-AG**AAGCTT**TCAGGGCAGCTCGCT-GCTG-3′, respectively. The bases shown in bold indicate *Bam*HI and *Hin*dIII restriction sites. The PCR-amplified fragment was digested with *Bam*HI and *Hin*dIII and then cloned into the vector pET24a (Novagen), encoding a polypeptide with an N-terminal hexahistidine tag to facilitate protein purification.

2.2. Overexpression and purification

PaAAP was overexpressed in *Escherichia coli* BL21 (DE3) cells. The cells were grown at 310 K to an OD_{600} of 0.4–0.5 in Luria–Bertani medium containing 50 µg ml^{-1} ampicillin. Protein expression was induced by addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.5 m*M*. Cells were grown for 3 h at 310 K and were centrifuged at 6000 rev min^{-1} (Vision VS24-SMTi V5006A rotor) for 20 min at 277 K. The resulting cell pellet was resuspended in ice-cold lysis buffer (25 m*M* Tris pH 8.0, 100 m*M* NaCl, 1 m*M* EDTA, 0.1 m*M* PMSF) and homogenized with a sonicator (Sonomasher, S&T Science, Republic of Korea) for about 30 s. The crude cell extract was centrifuged at 20 000 rev min^{-1} (Vision VS24-SMTi V508A rotor) for 40 min at 277 K. All purification steps were performed at 277 K. The expressed PaAAP was purified using three steps. Firstly, the supernatant was purified on an Ni–NTA column (Qiagen). Unbound proteins were washed out extensively using buffer *A* (25 m*M* Tris pH 8.0 with 500 m*M* NaCl and 25 m*M* imidazole). PaAAP was eluted from the column using buffer *B* (25 m*M* Tris pH 8.0 containing 300 m*M* NaCl and 1 *M* imidazole) and the eluted

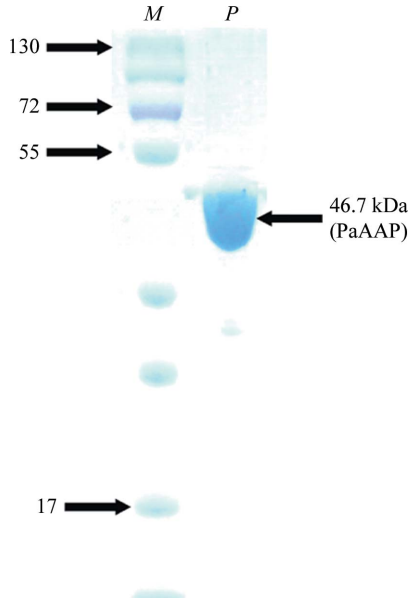


Figure 1
SDS–PAGE analysis of PaAAP during purification. Proteins were analyzed on 12% SDS–PAGE and stained with Coomassie Blue. Lane *M*, molecular-weight markers (labelled in kDa); lane *P*, purified PaAAP.

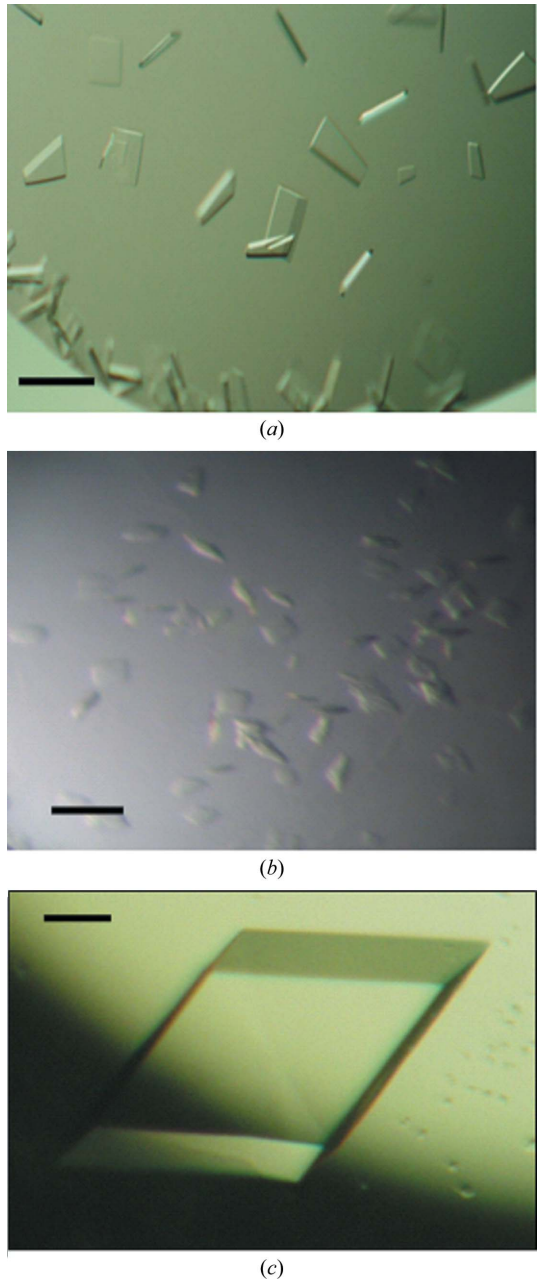


Figure 2
Crystals of PaAAP obtained using the sitting-drop method with (*a*) 0.01 *M* $NiCl_2{\cdot}6H_2O$, 0.1 *M* Tris–HCl pH 8.5, 1.0 *M* $LiSO_4{\cdot}H_2O$ and (*b*) 40%(*v*/*v*) PEG 300, 0.1 *M* HEPES pH 7.5, 0.2 *M* NaCl. (*c*) A crystal of PaAAP obtained using the microbatch method after 10 d using Crystal Screen Cryo condition No. 4 [40%(*v*/*v*) PEG 300, 0.1 *M* HEPES pH 7.5, 0.2 *M* NaCl]. The scale bar represents 0.1 mm.

Table 1
Data-collection statistics.

Values in parentheses are for the highest resolution shell.

Beamline	4A, PLS
Wavelength (Å)	0.97921
Resolution range (Å)	50–2.0
Space group	*H*3
Unit-cell parameters (Å)	$a = b = 133.6$, $c = 321.2$
Total No. of reflections	224910
No. of unique reflections	134321
Completeness (%)	97.9 (98.7)
Molecules per asymmetric unit	4
V_M (Å^3 Da^{-1})	2.95
Solvent content (%)	58.3
Average $I/\sigma(I)$	11.9 (3.3)
R_{merge}† (%)	6.8 (24.5)

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $\sum_{hkl}$ denotes the sum over all reflections and $\sum_i$ the sum over all equivalent reflections (Stout & Jensen, 1968).

protein fractions were dialyzed against buffer consisting of 20 m*M* Tris pH 8.0, 50 m*M* NaCl and 1 m*M* EDTA for the TEV cleavage reaction to remove the 6×His tag from the N-terminus. The resultant protein solution was then loaded onto a Q anion-exchange column and eluted with a 1 *M* linear NaCl gradient in 25 m*M* Tris pH 8.0. The resulting protein solution was applied onto a Superdex 200 column, which had previously been equilibrated with buffer consisting of 25 m*M* Tris–HCl pH 8.0, 50 m*M* NaCl, 1 m*M* dithiothreitol (DTT), for further purification. The homogeneity of the purified protein was assessed *via* SDS–PAGE (Fig. 1) and the protein was concentrated to 6 mg ml^{-1} in buffer *A* for crystallization screening.

2.3. Crystallization and X-ray data collection

Initial crystallization experiments were carried out with various screening kits (Crystal Screen, Crystal Screen 2, Crystal Screen Cryo, Crystal Screen 2 Cryo and Index from Hampton Research and Wizard from Emerald BioSystems) using a Hydra II e-drop automated pipetting system (Matrix). After 1 d, tiny plate-like crystals were observed in two different conditions (Figs. 2*a* and 2*b*). These initial hits were scaled up using the sitting-drop and microbatch (1 µl protein solution plus 1 µl reservoir solution) methods. Only one condition [Crystal Screen Cryo condition No. 4; 40%(*v*/*v*) PEG 300, 0.1 *M* HEPES pH 7.5, 0.2 *M* NaCl] produced significant-sized crystals after 10 d using the microbatch method at 295 K (Fig. 2*c*). A crystal was flash-cooled in liquid nitrogen with 5%(*v*/*v*) glycerol as a cryoprotectant. Data were collected using an ADSC Quantum 210 CCD detector on beamline 4A at Pohang Light Source (PLS), Republic of Korea. The crystal diffracted to 2.0 Å resolution and the data were integrated and scaled using *DENZO* and *SCALEPACK*, respectively (Otwinowski & Minor, 1997). Based on auto-indexing using the *DENZO* program, the PaAAP crystal belonged to the rhombohedral space group *H*3, with unit-cell parameters $a = b = 133.6$, $c = 321.2$ Å. The final data-collection statistics are summarized in Table 1.

3. Results and discussion

Initial high-throughput crystallization screening of PaAAP yielded diffraction-quality crystals (Fig. 2*c*) after 10 d from Crystal Screen Cryo condition No. 4 [40%(*v*/*v*) PEG 300, 0.1 *M* HEPES pH 7.5, 0.2 *M* NaCl]. A complete set of data (360 frames) was collected to 2.0 Å resolution with a crystal-to-detector distance of 180 mm and an oscillation angle of 0.5° per frame. The crystal belonged to space group *H*3. The volume of the PaAAP crystal is compatible with the presence of four monomers in the asymmetric unit, with a volume per

Figure 3
The $\chi = 180°$ section of the self-rotation function calculated with *MOLREP* (Vagin & Teplyakov, 2010) using data from 30 to 3 Å resolution.

unit molecular weight of the protein of 2.95 Å^3 Da^{-1} and a calculated solvent content of 58.3% (Matthews, 1968). Self-rotation functions were computed at $\chi = 60$, 90, 120 and 180° in the resolution range 30–3 Å to detect twofold, threefold, fourfold and sixfold rotation axes. Analysis of this self-rotation calculation (Fig. 3) confirmed that the PaAAP crystal had a multiple number of molecules in the asymmetric unit that were related by noncrystallographic twofold symmetry. A preliminary structure solution of PaAAP was obtained by molecular replacement (MR) using *MOLREP* (Vagin & Teplyakov, 2010; Winn *et al.*, 2011) with the crystal structure of HsAAP (PDB entry 3l6s), which shares 42.2% sequence identity, as a search model. The best results were obtained in space group *H*3, with a correlation coefficient of 53.7% and an *R* factor of 46.2%. Examination of the best MR solution structure showed good crystal packing, and no clashes were found between symmetry-related molecules. The model is currently being refined using *REFMAC*5 (Murshudov *et al.*, 2011). Structural information on PaAPP may provide insight into its enzymatic reaction mechanism and may help us to determine the characteristics of its unique catalytic reaction process.

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