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Makoto Akioka,[a] Hiroaki Nakano,[b] Aya Horikiri,[a] Yoshiyuki Tsujimoto,[a] Hiroshi Matsui,[a] Tetsuya Shimizu,[b] Toru Nakatsu,[b] Hiroaki Kato[b] and Kunihiko Watanabe[a]*

[a]Department of Applied Biochemistry, Kyoto Prefectural University, Shimogamo, Sakyo, Kyoto 606-8522, Japan, and [b]Department of Structural Biology, Graduate School of Pharmaceutical Sciences, Kyoto University, 46-29 Yoshida Shimoadachi-cho, Sakyo, Kyoto 606-8501, Japan

Correspondence e-mail: kwatanab@kpu.ac.jp

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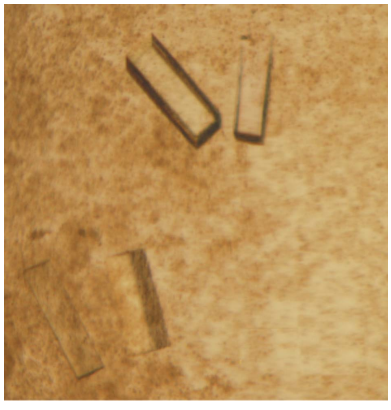

Overexpression, purification, crystallization and preliminary X-ray crystallographic studies of a proline-specific aminopeptidase from *Aneurinibacillus* sp. strain AM-1

To elucidate the structure and molecular mechanism of a characteristic proline-specific aminopeptidase produced by the thermophile *Aneurinibacillus* sp. strain AM-1, its gene was cloned and the recombinant protein was overexpressed in *Escherichia coli*, purified and crystallized using the hanging-drop vapour-diffusion method. X-ray diffraction data were collected to 1.8 Å resolution from the recombinant aminopeptidase crystal. The crystals belong to the orthorhombic space group $P2_12_12$, with unit-cell parameters a = 93.62, b = 68.20, c = 76.84 Å. A complete data set was also obtained from crystals of SeMet-substituted aminopeptidase. Data in the resolution range 20–2.1 Å from the MAD data set from the SeMet-substituted crystal were used for phase determination.

1. Introduction

A characteristic proline-specific aminopeptidase was found in the thermophilic *Aneurinibacillus* sp. strain AM-1 isolated from Arima Hot Spring, Kobe, Japan (Murai *et al.*, 2004). The enzyme has an exceptionally strong reaction to prolyl-*p*-nitroanilide (Pro-*p*NA) despite its many similarities to known leucyl aminopeptidases (Murai *et al.*, 2004; Arima *et al.*, 2005). In addition, a remarkable characteristic of the strain AM-1 aminopeptidase is that it contributes to the degradation of collagen, in which an extremely high level of proline residues (>20%) occurs, when used in combination with another collagenolytic protease. This enzyme differs essentially from typical proline iminopeptidases since it contains a metal, which is most likely to be zinc, for use in hydrolysis, in contrast to other proline iminopeptidases, which belong to a family of serine proteases (Kitazono *et al.*, 1994). Therefore, the aminopeptidase from strain AM-1 is expected to be a new type of aminopeptidase that has a preference for the hydrolysis of prolyl peptides. Therefore, we are attempting to reveal the structure of the strain AM-1 aminopeptidase and to elucidate its mechanism, which will explain its specificity for prolyl peptides. Here, after gene cloning of the proline-specific aminopeptidase from strain AM-1, the recombinant enzyme was overexpressed in *Escherichia coli*, purified and crystallized. A preliminary X-ray crystallographic study was then carried out for further structural analysis of the proline-specific aminopeptidase from strain AM-1.

2. Material and methods

2.1. Gene cloning, sequencing and plasmid construction for overexpression

The chromosomal DNA of strain AM-1 was isolated as described previously (Itoi *et al.*, 2006). A genomic library was constructed with partially *Sau*3AI-digested chromosomal DNA, the vector plasmid pUC119 and the host strain *E. coli* MV1184 (Yanisch-Perron *et al.*, 1985). *E. coli* clones producing the strain AM-1 aminopeptidase were screened by Western blotting analysis using a polyclonal antibody against the enzyme (Murai *et al.*, 2004). DNA sequencing was performed for both strands of the cloned fragment using PRISM dye primer-sequencing kits on a 310NT DNA sequencer (Applied Biosystems). The nucleotide-sequence data appear in the DDBJ/EMBL/

GenBank nucleotide-sequence database with accession No. AB271126. For construction of an overexpression plasmid, a DNA fragment containing from Ala28 to the termination codon, its 3′-flanking region (83 bp) and an artificial initiation codon ATG was synthesized by PCR with two primers (TGCTGCGCATATGGC-CCCGATCCAAC and CGCGGATCCGATGGAGATACCTGAG). After the DNA fragment had been digested with *Nde*I and *Bam*HI and ligated into the same sites of the vector plasmid pET-11a, the resulting hybrid plasmid for overexpression (pETAP-1) was used to transform *E. coli* BL21 (DE3) (Novagen).

2.2. Overexpression of the recombinant and selenomethionine-substituted (SeMet) aminopeptidases

All purification operations were performed at 277 K unless stated otherwise.

2.2.1. Recombinant aminopeptidase. After the cells had been grown for 4 h, the recombinant aminopeptidase was overexpressed in the transformant harbouring pETAP-1 at 310 K by the addition of isopropyl β-D-galactopyranoside (IPTG, final concentration 0.1 m*M*) to Luria broth [1%(*w*/*v*) peptone, 0.5%(*w*/*v*) yeast extract, 0.5%(*w*/*v*) NaCl pH 7.2] containing 50 µg ml^{-1} ampicillin and 10%(*w*/*v*) glucose for 3 h. Cells harvested from the culture (total 4 l) were resuspended in 100 ml buffer 1 (50 m*M* Tris–HCl pH 8.0) after washing with saline and then disintegrated by sonication. The cell extract obtained by centrifugation (15 000*g*, 20 min) was kept at 328 K for 30 min and denatured material was removed by centrifugation (10 000*g*, 25 min). The resulting supernatant was applied onto a DEAE-cellulose column equilibrated with buffer 1. Active flowthrough fractions were pooled, concentrated by ultrafiltration through an Amicon PM-30 membrane (Millipore) and dialyzed against buffer 2 [10 m*M* potassium phosphate buffer (KPB) pH 7.0]. The dialyzate was applied onto a hydroxyapatite column equilibrated with buffer 2. Elution was performed with a linear gradient of 10–500 m*M* KPB. The active fractions were pooled, concentrated by ultrafiltration, dialyzed against buffer 1 and concentrated using an Amicon Ultra-15.

2.2.2. SeMet aminopeptidase. After primary cultivation for 6.75 h, SeMet aminopeptidase was overexpressed in the transformant of *E. coli* B834 (DE3) (Novagen) harbouring pETAP-1 by the addition of IPTG (final concentration 0.1 m*M*) to M9 minimum medium supplemented with 0.3 mol ml^{-1} SeMet and 50 µg ml^{-1} ampicillin for 15 h. The cell extract was obtained and applied to consecutive DEAE-cellulose and hydroxyapatite column chromatography as described above for the recombinant enzyme. Active fractions eluted from the hydroxyapatite column were pooled, concentrated, dialyzed against buffer 1 and concentrated using an Amicon Ultra-15. The purity of the proteins was checked by SDS–PAGE.

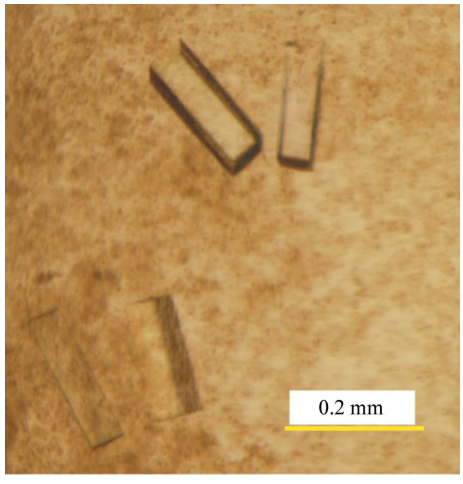

Figure 1
A crystal of the recombinant aminopeptidase obtained from 100 m*M* MES–NaOH pH 5.8, 0.2 *M* zinc acetate and 13%(*w*/*v*) PEG 6000. The crystal dimensions are about 0.2 × 0.08 × 0.08 mm.

2.3. Crystallization and data collection

The recombinant aminopeptidase was concentrated to 19 mg ml^{-1} in 50 m*M* Tris–HCl pH 8.0. Crystallization of the recombinant protein and its SeMet form were carried out using the hanging-drop vapour-diffusion method. Initial crystallization screening for the recombinant protein was carried out using Crystal Screens 1 and 2 (Hampton Research). 1 µl protein solution was mixed in a 1:1 ratio with each of the crystallization solutions and the resultant 2 µl drops were incubated at 299 K. Crystals of recombinant and SeMet aminopeptidase were flash-frozen using a solution comprising 100 m*M* MES–NaOH pH 5.8, 0.2 *M* zinc acetate, 18%(*w*/*v*) PEG 6000 and 20%(*v*/*v*) 2-propanol as a cryoprotectant. X-ray diffraction data were collected from recombinant protein crystals using Cu $K\alpha$ radiation on an R-AXIS VII imaging-plate system attached to a Rigaku rotating-anode generator (FR-E). For SeMet protein crystals, X-ray diffraction data were collected at BL44B2, SPring-8 (Hyogo, Japan). Data for MAD analysis were collected from a recombinant crystal and a SeMet-substituted crystal to 1.8 and 2.1 Å resolution, respectively. These diffraction data were integrated using *MOSFLM* (Leslie, 1992) and scaled using *SCALA* from the *CCP*4 program suite (Collaborative Computational Project, Number 4, 1994). For the SeMet protein crystals, three wavelengths, 0.9788 Å (peak), 0.9792 Å (edge) and 0.9639 Å (remote), were selected based on an X-ray absorption spectrum at the Se *K* edge. The CCD detector was placed at a distance of 180 mm and the exposure time of 5 s allowed us to obtain a 180° data set (0.5° frames).

3. Results and discussion

3.1. Gene cloning, overexpression and purification of the strain AM-1 aminopeptidase

The gene for the strain AM-1 aminopeptidase was cloned within a 4.1 kbp insert DNA fragment contained in a hybrid plasmid (pAH-1). The insert DNA fragment was subcloned into 2.5 kbp and the DNA sequences of the DNA fragment were determined. They revealed that the gene consisted of an open reading frame of 1368 bp capable of coding for a protein of 456 amino acids. However, the N-terminal amino-acid sequence (ten residues) determined using native protein purified from the culture supernatant of strain AM-1 (Murai *et al.*, 2004) starts at amino-acid position 28 (Ala28); therefore, the N-terminal region from amino-acid positions 1–27 functions as a signal sequence for secretion and these 27 N-terminal amino acids are missing from the native aminopeptidase purified from the culture supernatant. Therefore, the recombinant protein was modified by deleting the 27 N-terminal amino acids and by adding another Met at the N-terminus as an initiator for cytoplasmic overexpression (pETAP-1). As a result, fractions containing aminopeptidase activity were observed in the cell-free extract derived from *E. coli* cells harbouring pETAP-1. The recombinant aminopeptidase (71.1 mg) was purified 5.4-fold over the cell extract, with the yield being 34.2%, while the SeMet aminopeptidase (7.67 mg) was 11.1-fold purified over the cell extract, with the yield being 62.1%. The specific activity of the final sample of recombinant aminopeptidase (5.52 µmol min^{-1} per milligram of protein) was comparable with that of the native

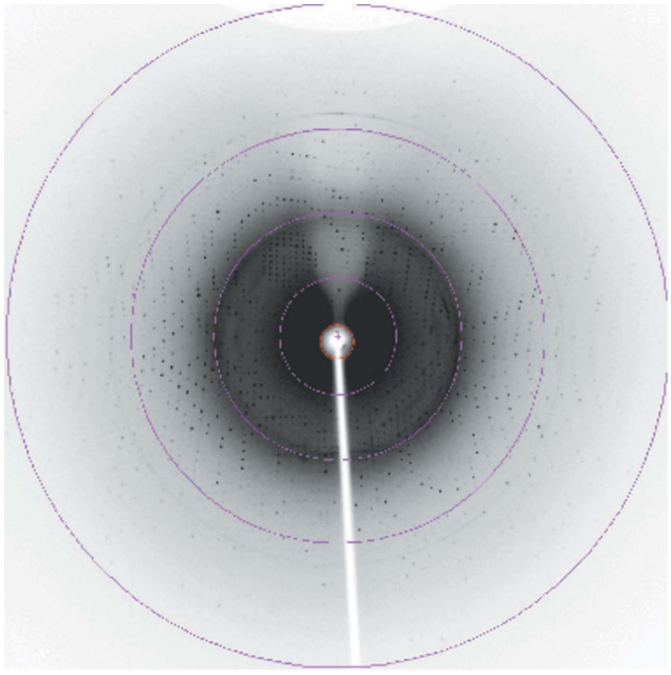

Figure 2
An X-ray diffraction image from a recombinant aminopeptidase crystal. Circles correspond to 1.8, 2.4, 3.6 and 7.1 Å resolution.

enzyme (8.83 µmol min^{-1} per milligram of protein), indicating that the function of the recombinant protein is nearly identical to that of the native protein.

3.2. Crystallization and X-ray crystallographic data

In the preliminary screening for crystallization conditions, rod-shaped microcrystals were found in a drop containing 100 m*M* sodium cacodylate pH 6.5 with 0.2 *M* magnesium acetate and 20%(*w*/*v*) PEG 8000 (Hampton Crystal Screen condition I-18) in 8 d. Subsequent refinement of the conditions gave the best crystallization conditions, which consisted of 100 m*M* MES–NaOH pH 5.8 with 0.2 *M* zinc acetate and 13%(*w*/*v*) PEG 6000. Crystals grew to dimensions of 0.2 × 0.08 × 0.08 mm at 299 K within 5 d (Fig. 1). The SeMet aminopeptidase was crystallized under the same conditions as those optimized for the recombinant aminopeptidase.

X-ray diffraction data were collected from the crystal of the recombinant aminopeptidase to 1.8 Å resolution (Fig. 2). The crystal belongs to the orthorhombic space group $P2_12_12$, with unit-cell parameters $a = 93.62$, $b = 68.20$, $c = 76.84$ Å. Assuming the presence of one molecule per asymmetric unit, the value of the Matthews coefficient V_M and the solvent content were calculated to be 2.73 Å^3 Da^{-1} and 54.9%, respectively (Matthews, 1968). Data statistics for the recombinant and SeMet aminopeptidase crystals are summarized in Table 1. Data in the resolution range 20–2.1 Å from the MAD data set were used for phase determination. Five selenium sites were found using the *SOLVE* program (Terwilliger & Berendzen, 1999). The initial phase was modified by solvent flattening using the *RESOLVE* program (Terwilliger, 2000) and the phases were extended to 1.8 Å. The initial model was constructed automatically using the *ARP*/*wARP* program (Perrakis *et al.*, 1999). On this occasion, the native data set at 1.8 Å resolution was combined with the phase output from *RESOLVE* and 5% of the reflections were selected for cross-validation. Refinement of the model structure of aminopeptidase is currently in progress.

Table 1
Statistics of X-ray diffraction data.

Values in parentheses are for the outermost resolution shell.

		SeMet enzyme		
	Recombinant enzyme	Edge	Peak	Remote
X-ray source	FR-E	BL44B2 (SPring-8)	BL44B2 (SPring-8)	BL44B2 (SPring-8)
Space group	$P2_12_12$			
Wavelength (Å)	1.5418	0.9792	0.9788	0.9639
Unique reflections	45919	29623	29574	29582
Completeness (%)	98.7 (97.1)	100.0 (100.0)	100.0 (100.0)	100.0 (100.0)
Resolution range (Å)	19.9–1.8 (1.9–1.8)	20.0–2.1 (2.21–2.1)	20.0–2.1 (2.2–2.1)	20.0–2. 1 (2.21–2.1)
R_{merge} (%)	5.8 (33.6)	9.3 (27.7)	9.3 (27.3)	9.5 (25.3)
Multiplicity	10.6 (10.5)	3.7 (3.6)	3.7 (3.6)	3.7 (3.6)
$I/\sigma(I)$	31.2 (6.1)	21.2 (5.5)	21.3 (5.5)	21.5 (5.8)

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