Acta Crystallographica Section F
Structural Biology and Crystallization Communications
ISSN 1744-3091

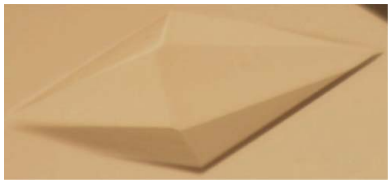
Crystallization and preliminary X-ray crystallographic analysis of Pz peptidase B from *Geobacillus collagenovorans* MO-1

Hiroaki Nakano,[a] Allin Hosokawa,[b] Ryuji Tagawa,[b] Koji Inaka,[c] Kazunori Ohta,[d] Toru Nakatsu,[e] Hiroaki Kato[e] and Kunihiko Watanabe[b]*

[a]Department of Pharmacy, Hyogo University of Health Sciences, 1-3-6 Minatojima, Chuo, Kobe 650-8530, Japan, [b]Division of Applied Life Sciences, Graduate School of Life and Environmental Sciences, Kyoto Prefectural University, Shimogamo, Sakyo, Kyoto 606-8522, Japan, [c]Maruwa Foods and Biosciences Inc., 170-1 Tsutsui-cho, Yamatokoriyama, Nara 639-1123, Japan, [d]Space Environment Utilization Center, Japan Aerospace Exploration Agency (JAXA), 2-1-1 Sengen, Tsukuba, Ibaraki 305-8505, Japan, and [e]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

Received 5 March 2012
Accepted 27 April 2012



Pz peptidase B is an intracellular M3 metallopeptidase that is found together with Pz peptidase A in the thermophile *Geobacillus collagenovorans* MO-1 and recognizes collagen-specific tripeptide units (-Gly-Pro-*X*-). These peptidases have low homology in their primary structures; however, their cleavage patterns towards peptide substrates are similar. In this work, Pz peptidase B was crystallized using the counter-diffusion method. Data were collected to a resolution of 1.6 Å at 100 K from a crystal obtained in the Japanese Experiment Module (JEM; also known as 'Kibo') at the International Space Station (ISS). The crystal belonged to the trigonal space group $P3_121$, with unit-cell parameters $a = b = 87.64$, $c = 210.5$ Å. A complete data set was also obtained from crystals of selenomethionine-substituted protein.

1. Introduction

The thermophile *Geobacillus collagenovorans* MO-1, which can degrade collagens, was isolated from soil. Several characteristic enzymes related to collagen degradation were found in this strain and have been extensively studied (Okamoto *et al.*, 2001; Miyake *et al.*, 2005; Itoi *et al.*, 2006). Two intracellular M3 metallopeptidases, Pz peptidases A and B, are critical in hydrolyzing collagen-derived peptides by recognizing a collagen-specific tripeptide unit, -Gly-Pro-*X*-, in this strain. Although the two enzymes show very low identity in their primary structure (22%), Pz peptidases A and B exhibit similar substrate specificity (Miyake *et al.*, 2005). Very recently, the crystal structure of Pz peptidase A was determined at 1.80 Å resolution; one of the striking features that was revealed was a tunnel that functions as a gateway for substrate and reactant molecules, with the active site located in the centre of the globular protein molecule (Kawasaki *et al.*, 2010). We are specifically interested in comparing the molecular structures of the two Pz peptidases and determining the catalytic mechanism of Pz peptidase B using X-ray crystallographic analysis. In contrast, the enzyme thimet oligopeptidase (TOP), a mammalian enzyme, has a similar substrate specificity to the Pz peptidases but shows a much lower identity (14% at most). In addition, there is only a small difference between the catalytic parameters of the Pz peptidases and of TOP (Sugihara *et al.*, 2007; Dando *et al.*, 1993). The structure of TOP has been reported not to contain a tunnel for substrates and reactants as in Pz peptidase A; rather, it contains a channel that runs the length of the entire protein molecule (Brown *et al.*, 2001; Ray *et al.*, 2004). Therefore, we were interested in identifying the differences in structure between Pz peptidase B, Pz peptidase A and TOP. Here, we report the overexpression, purification, crystallization and X-ray analysis of native and selenomethionyl (SeMet) Pz peptidase B.

2. Materials and methods

2.1. Protein expression and purification of Pz peptidase B

The gene for Pz peptidase B contained in a pUC119 plasmid was subcloned into a pET11b expression vector for overexpression (Miyake *et al.*, 2005). The plasmid, named pOPB-1, was introduced into *Escherichia coli* strain BL21 (DE3). The transformant was grown

Table 1
Data-collection statistics for SeMet-substituted and native crystals.

Values in parentheses are for the highest resolution shell.

	SeMet			Native	
Crystal	Peak	Edge	Remote	Grown on earth	Grown in space
Space group	$P3_121$			$P3_121$	$P3_121$
Wavelength (Å)	0.97919	0.97900	0.96396	0.90000	0.90000
Unit-cell parameters (Å)	$a = b = 87.99$, $c = 209.7$			$a = b = 88.15$, $c = 210.8$	$a = b = 87.64$, $c = 210.5$
Observed reflections	256545	227873	202190	420046	964525
Unique reflections	92600	81376	71728	47527	146530
Resolution (Å)	50.0–2.20 (2.24–2.20)	50.0–2.30 (2.34–2.30)	50.0–2.40 (2.44–2.40)	50.0–2.20 (2.24–2.20)	40.0–1.60 (1.62–1.60)
R_{merge} (%)	6.2 (36.5)	5.7 (34.6)	4.2 (23.5)	10.8 (25.8)	6.0 (41.1)
$\langle I/\sigma(I)\rangle$	29.7 (3.4)	30.8 (4.0)	32.0 (4.4)	62.1 (15.1)	31.9 (2.7)
Multiplicity	2.8 (2.6)	2.8 (2.8)	2.9 (2.8)	8.9 (8.2)	6.6 (4.1)
Completeness (%)	98.9 (98.4)	99.1 (99.9)	99.1 (99.9)	96.4 (99.7)	97.0 (96.1)

in LB medium containing 50 µg ml^{-1} ampicillin at 310 K. The purification method for Pz peptidase B has been described previously (Sugihara *et al.*, 2007). Briefly, cells harvested from the culture were washed with saline and resuspended in 100 ml buffer I (50 m*M* Tris–HCl pH 7.5) after disintegration by sonication. The supernatant was collected after centrifuging the homogenate at 6000*g* for 10 min. The supernatant was kept at 333 K for 30 min and denatured particulates were removed by centrifugation at 10 000*g* for 10 min. The resulting supernatant was applied onto a DEAE-cellulose column (GE Healthcare) equilibrated with buffer I. Elution was performed with a linear gradient of buffer I containing 0–0.5 *M* NaCl. The eluted fractions containing Pz peptidase B were concentrated using an Amicon PM-10 membrane (Millipore) and the concentrate was further purified using a Sephacryl S-300 column (GE Healthcare) equilibrated with buffer I. The active flowthrough was pooled and concentrated. The product homogeneity was determined by SDS–PAGE and functional assessment of Pz peptidase B. A standard assay of Pz peptidase B activity in purification was performed using Pz-PLGPR (Bachem, Bubendorf, Switzerland) as reported previously (Miyake *et al.*, 2005; Sugihara *et al.*, 2007). One unit of enzyme activity was defined as the amount of enzyme that hydrolyzed 1 µmol Pz-PLGPR in 1 min at 333 K.

2.2. Crystallization and data collection

The purified Pz peptidase B was concentrated to 20 mg ml^{-1} in 50 m*M* Tris–HCl pH 7.5. Initial crystallization screening was carried out with Crystal Screen, Crystal Screen 2 (Hampton Research) and Wizard 1, 2 and 3 (Emerald BioSystems) using both the hanging-drop and sitting-drop vapour-diffusion methods. 1 µl protein solution was mixed in a 1:1 ratio with each of the crystallization solutions and the resultant drops were incubated at 293 K. Dodecahedron-shaped crystals grew in drops containing 0.1 *M* bis-Tris–HCl pH 5.9, 2 *M* ammonium sulfate after 3 d. Subsequent refinement of the conditions gave optimal crystallization conditions consisting of 0.1 *M* bis-Tris–HCl pH 5.9, 2.3 *M* ammonium sulfate. Crystals obtained from drops prepared by mixing 2 µl protein solution and 2 µl reservoir solution grew to dimensions of 0.5 × 0.2 × 0.1 mm at 297 K within 5 d (Fig. 1). SeMet-substituted Pz peptidase B was crystallized using the same conditions as those optimized for native Pz peptidase B. Crystals of native and SeMet-substituted Pz peptidase B were flash-cooled using a solution consisting of 0.1 *M* bis-Tris–HCl pH 5.9, 2.3 *M* ammonium sulfate with 22%(*v*/*v*) 2-propanol as a cryoprotectant. X-ray diffraction data were collected from native and SeMet-substituted protein crystals at 93 K using an ADSC Quantum 210r CCD detector on BL-5A at the Photon Factory, High Energy Acceleration Research Organization, Tsukuba, Japan. The CCD detector was placed at a distance of 200 mm; an exposure time of 3 s allowed a 180° data set to be obtained (0.5° oscillation angle). Data were collected from a native crystal to a resolution of 2.2 Å and MAD data were collected from an SeMet-substituted crystal to resolutions of 2.2 Å (peak) to 2.4 Å (remote). Crystals suitable for high-resolution X-ray crystallographic analysis were obtained using a combination of the microseeding and counter-diffusion methods at the International Space Station (ISS). Seed crystals were obtained on Earth under the conditions described above and were transferred into 100 m*M* bis-Tris pH 5.9, 2.3 *M* ammonium sulfate. Space-grown crystals were obtained during the course of the Fourth Session of JAXA's Microgravity Protein Crystallization Experiment (JAXA-PCG), which was performed using the Protein Crystallization Research Facility (PCRF) in the Japanese Experiment Module (JEM; also known as 'Kibo') at the ISS. The crystal was transferred to cryoprotectant consisting of 0.1 *M* bis-Tris–HCl pH 5.9, 2.3 *M* ammonium sulfate, 30%(*v*/*v*) glycerol. Several minutes later, it was scooped up in a cryoloop and flash-cooled in liquid nitrogen. It was then mounted on a goniometer in a nitrogen stream at 100 K. The X-ray diffraction data were collected using a Rayonix MX-225HE CCD detector on BL44XU at SPring-8, Hyogo, Japan. A total of 360 images were collected at a crystal-to-detector distance of 160 mm with an oscillation angle of 0.5° and an exposure time of 5 s per image. Data were collected to a resolution of 1.6 Å. All data sets were indexed, integrated and scaled with the *HKL*-2000 program suite (Otwinowski & Minor, 1997).

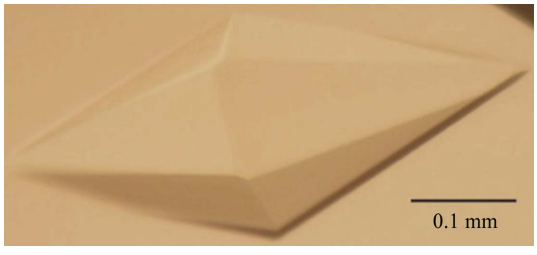


Figure 1
A crystal of recombinant Pz peptidase B obtained using 0.1 *M* bis-Tris–HCl pH 5.9, 2.3 *M* ammonium sulfate on earth. The crystal dimensions are about 0.5 × 0.2 × 0.1 mm. The scale bar is 0.1 mm in length.

3. Results and discussion

In the initial screening, cubic crystals with dimensions of approximately 50 × 50 × 50 µm were obtained within 3 d using the condition 50 m*M* Tris–HCl pH 9.0, 25 m*M* trimethylamine *N*-oxide, 12%(*v*/*v*) PEG 1000. However, further optimization of crystal growth under

similar conditions yielded no crystals that diffracted to beyond 5.0 Å resolution. In contrast, X-ray diffraction data were collected from dodecahedron-shaped crystals of native Pz peptidase B grown on Earth and at the ISS to resolutions of 2.2 and 1.6 Å, respectively. The crystal grown at the ISS belonged to the trigonal space group $P3_121$ or $P3_221$, with unit-cell parameters $a = b = 87.64$, $c = 210.5$ Å. Assuming the presence of one molecule per asymmetric unit, the calculated Matthews coefficient (V_M) was 3.4 Å^3 Da^{-1} (Matthews, 1968); the solvent content of the crystal was calculated to be 63.8%. The high-resolution data set collected from crystals obtained in the JAXA-PCG project was used as a reference for scaling all native data sets and the combined data were used for phase determination and refinement. Crystals obtained in Kibo at the ISS were dodecahedron-shaped with dimensions of approximately 0.5 × 0.3 × 0.2 mm. The crystals obtained in space were of better quality (1.6 Å resolution) than those obtained on Earth (2.2 Å resolution) and are suitable for more precise structure analyses. The difference in resolution indicates that the microgravity in space yields crystals of high quality. Since gravity is one of the parameters controlling the quality of protein crystals, and buoyancy-driven convection and sedimentation are greatly reduced in space, the environment in space has been posited to be ideal for crystallization of Pz peptidase B. As a result, parameters other than gravity dominate the crystal-growth mechanism in space and hence provide a unique opportunity to study the crystal-growth process. SeMet-substituted Pz peptidase B crystals diffracted to beyond 2.4 Å resolution on BL-5A at the Photon Factory. In order to determine the structure using the MAD method, three wavelengths for data collection, 0.97919 Å (peak), 0.97900 Å (edge) and 0.96396 Å (remote), were selected on the basis of the X-ray absorption spectrum at the Se *K* edge. Data-collection statistics for SeMet-substituted and native crystals are summarized in Table 1.

MAD data in the resolution range 50.0–2.4 Å were used for phase determination. Nine selenium sites were found using the *SOLVE* program (Terwilliger & Berendzen, 1999). The space group was determined to be $P3_121$ from an electron-density map using the initial phases. The initial phases were modified by solvent flattening using the *RESOLVE* program (Terwilliger, 2000) and were extended to 2.2 Å. The initial model was constructed automatically using the *ARP*/*wARP* program (Langer *et al.*, 2008). On this occasion, a native data set at 1.6 Å resolution was combined with the phase output from *RESOLVE* and 5% of the reflections were selected for cross-validation. Data were collected to 1.6 Å resolution on BL44XU at SPring-8 because the long *c* axis of the crystals ($c = 210.5$ Å) prevented the collection of high-resolution data. Refinement of the model structure of Pz peptidase B is currently in progress. In addition, crystallization conditions for complexes of Pz peptidase B with the inhibitors PPI-1 and PPI-2 [PPI-1, benzyloxycarbonyl-(L,D)-Phe-(PO_2CH_2)-(L,D)-Ala-Lys-Ser; PPI-2, Gly-Pro-Phe-(PO_2CH_2)-Gly-Pro-Nle], which also inhibit Pz peptidase A (Kawasaki *et al.*, 2007), are now being analyzed.

This study was supported by the JAXA-PCG project High Quality Protein Crystal Growth Experiment Onboard 'Kibo' conducted by JAXA. We thank the staff of beamline BL-5A, Photon Factory, Japan and of beamline BL44XU, SPring-8, Japan. This work was supported in part by the Hyogo University of Health Sciences Research grant for 2102 (to HN).

References

Brown, C. K., Madauss, K., Lian, W., Beck, M. R., Tolbert, W. D. & Rodgers, D. W. (2001). *Proc. Natl Acad. Sci. USA*, **98**, 3127–3132.
Dando, P. M., Brown, M. A. & Barrett, A. J. (1993). *Biochem. J.* **294**, 451–457.
Itoi, Y., Horinaka, M., Tsujimoto, Y., Matsui, H. & Watanabe, K. (2006). *J. Bacteriol.* **188**, 6572–6579.
Kawasaki, A., Nakano, H., Hosokawa, A., Nakatsu, T., Kato, H. & Watanabe, K. (2010). *J. Biol. Chem.* **285**, 34972–34980.
Kawasaki, A., Nakano, H., Tsujimoto, Y., Matsui, H., Shimizu, T., Nakatsu, T., Kato, H. & Watanabe, K. (2007). *Acta Cryst.* F**63**, 142–144.
Langer, G., Cohen, S. X., Lamzin, V. S. & Perrakis, A. (2008). *Nature Protoc.* **3**, 1171–1179.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
Miyake, R., Shigeri, Y., Tatsu, Y., Yumoto, N., Umekawa, M., Tsujimoto, Y., Matsui, H. & Watanabe, K. (2005). *J. Bacteriol.* **187**, 4140–4148.
Okamoto, M., Yonejima, Y., Tsujimoto, Y., Suzuki, Y. & Watanabe, K. (2001). *Appl. Microbiol. Biotechnol.* **57**, 103–108.
Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
Ray, K., Hines, C. S., Coll-Rodriguez, J. & Rodgers, D. W. (2004). *J. Biol. Chem.* **279**, 20480–20489.
Sugihara, Y., Kawasaki, A., Tsujimoto, Y., Matsui, H. & Watanabe, K. (2007). *Biosci. Biotechnol. Biochem.* **71**, 594–597.
Terwilliger, T. C. (2000). *Acta Cryst.* D**56**, 965–972.
Terwilliger, T. C. & Berendzen, J. (1999). *Acta Cryst.* D**55**, 849–861.