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

Preliminary X-ray crystallographic analysis of the catalytic domain of prophenoloxidase activating factor-I

Shunfu Piao, Ji Young Jung, Ji Won Park, Jaewon Lee, Bok Leul Lee and Nam-Chul Ha*

College of Pharmacy and Research Institute for Drug Development, Pusan National University, Jangjeon-dong, Geumjeong-gu, Busan 609-735, South Korea

Correspondence e-mail: hnc@pusan.ac.kr

Received 30 March 2006
Accepted 4 July 2006

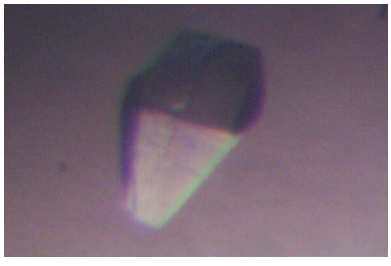
Clip-domain serine proteases (SPs) have been identified in invertebrates as crucial enzymes that are involved in diverse extracellular signalling pathways. Prophenoloxidase (proPO) activating factor-I (PPAF-I), a catalytically active clip-domain SP, cleaves proPO. To date, no crystal structures of a catalytically active clip-domain SP have been determined. Here, the results of crystallization and preliminary X-ray analysis of the SP domain of PPAF-I are reported. The crystal of the PPAF-I SP domain was obtained using the hanging-drop vapour-diffusion method in a precipitant solution containing 0.15 *M* lithium sulfate, 30% polyethylene glycol 4000 and 0.1 *M* Tris–HCl pH 8.0. The crystal diffracts X-rays to 1.7 Å resolution using a synchrotron-radiation source. The crystal belongs to space group $P2_12_12_1$, with one molecule in the asymmetric unit and unit-cell parameters $a = 38.3$, $b = 53.3$, $c = 116.6$ Å, $\alpha = \beta = \gamma = 90^\circ$. A molecular-replacement solution has been found using kallikrein as a starting model, resulting in an interpretable electron-density map.

1. Introduction

Both vertebrates and invertebrates employ serine protease (SP) cascades to mediate rapid and local reactions in response to diverse physiological or pathological stimuli in the extracellular region. Clip-domain SPs, which contain one or two clip domain(s) at the N-terminus of the SP domain, have been identified in the immune response and embryonic development of invertebrates. Easter from *Drosophila melanogaster* is a well characterized clip-domain SP and is expressed and secreted during oogenesis as an inactive zymogen (Anderson, 1998; Morisato & Anderson, 1995).

Clip-domain SPs can be divided into catalytic and non-catalytic groups according to their proteolytic activity. The non-catalytic group members do not exhibit any proteolytic activity owing to replacement of the serine residue at the active site by glycine. The overall structure of the SP domain of the non-catalytic group is similar to those of chymotrypsin-like SPs (Piao *et al.*, 2005). In contrast, the SP domain of the catalytic group is distinct from those of chymotrypsin-like SPs. One feature of the SP domain of the catalytic group is the replacement of two cysteine residues (Cys137 and Cys202 in the chymotrypsin numbering) which form a disulfide-bond linkage by other amino acids (Fig. 1). Another distinctive feature is a short insertion between the His and Asp residues of the catalytic triad (Fig. 1). The short insertion contains two cysteine residues which are expected to form a disulfide-bond linkage. Moreover, the short insertion has been predicted to contain a calcium-binding site (Rose *et al.*, 2003). The clip-domain SPs containing the short insertion are referred to as easter-type SPs because *Drosophila* easter exhibits the features mentioned above. However, no crystal structures of a catalytically active clip-domain SP have been determined.

Two easter-type SPs have been cloned and characterized from the large beetle *Holotrichia diomphalia* (Kwon *et al.*, 2000; Lee *et al.*, 1998). ProPO activating factors-I (PPAF-I) and III (PPAF-III) cleave proPO and PPAF-II, a non-catalytic group member, respectively (Kwon *et al.*, 2000; Lee *et al.*, 1998). Here, we report the crystallization and preliminary X-ray crystallographic analysis of the SP domain of PPAF-I from *H. diomphalia*.



2. Materials and methods

2.1. DNA construction and protein purification

Overexpression and purification of the SP domain of PPAF-I (residues 88–365) have been described previously (Piao *et al.*, 2005). The purified protein was concentrated to 5–10 mg ml^{-1} for crystallization using an ultrafiltration device (Millipore).

2.2. Crystallization, data collection and X-ray analysis

The crystals of the SP domain of PPAF-I were obtained by the vapour-diffusion technique at 287 K. Initial crystallization screening was performed by the sitting-drop method with Crystal Screen HT, a high-throughput sparse-matrix screening kit (Hampton Research, USA), in a 96-well plate using 0.5 µl protein solution (10 mg ml^{-1}) mixed with 0.5 µl of each precipitant solution. Crystals of various shapes were obtained in a few days in 12 of the 96 conditions. One condition (0.2 *M* lithium sulfate, 30% polyethylene glycol 4000, 0.1 *M* Tris–HCl pH 8.0), in which many tiny diamond-shaped crystals grew, was chosen for optimization (Fig. 2). The crystallization conditions were optimized to produce high-quality single crystals (0.2 mm in diameter; Fig. 2) in droplets containing 1 µl protein solution (5 mg ml^{-1}) and 1 µl precipitant solution consisting of 0.2 *M* lithium sulfate, 30% polyethylene glycol 4000, 0.1 *M* Tris–HCl pH 8.5. The droplets were equilibrated by the hanging-drop vapour-diffusion method against 1 ml of the same precipitant solution at 287 K for one week.

2.3. Crystallographic data collection

For X-ray data collection, a single crystal was briefly immersed into precipitation solution containing 10% glycerol as a cryoprotective agent. The crystal was flash-frozen in a stream of nitrogen gas at 100 K. Diffraction data were collected from a single crystal on beamline 4A of Pohang Accelerator Laboratory (Korea) at a wavelength of 0.9794 Å using an ADSC Q210 CCD detector with an exposure time of 2 s, a rotation angle of 1° and a crystal-to-detector distance of 130 mm. Diffraction was observed to a maximum resolution of 1.6 Å; however, data beyond 1.7 Å were weak and were not included in the processing. A complete data set was indexed, processed and scaled with *DENZO* and *SCALEPACK* from the *HKL*-2000 program suite (Otwinowski & Minor, 1997).

3. Results and discussion

Crystallization of the full-length PPAF-I was unsuccessful despite extensive screening of crystallization conditions. We suspected that free motion between the two domains of PPAF-I prevents the crystallization of full-length PPAF-I. In order to obtain crystals, we separately expressed the clip and SP domains of PPAF-I according to the boundary obtained from the proteolysis experiment reported previously (Piao *et al.*, 2005). The SP domain of PPAF-I was successfully overexpressed for crystallization screening. However, crystallization was not successful for the clip domain of PPAF-I.

Preliminary analysis of the X-ray diffraction data using the autoindexing routine in *DENZO* (Otwinowski & Minor, 1997) indicated that the crystal belongs to the primitive system, point group 222, with

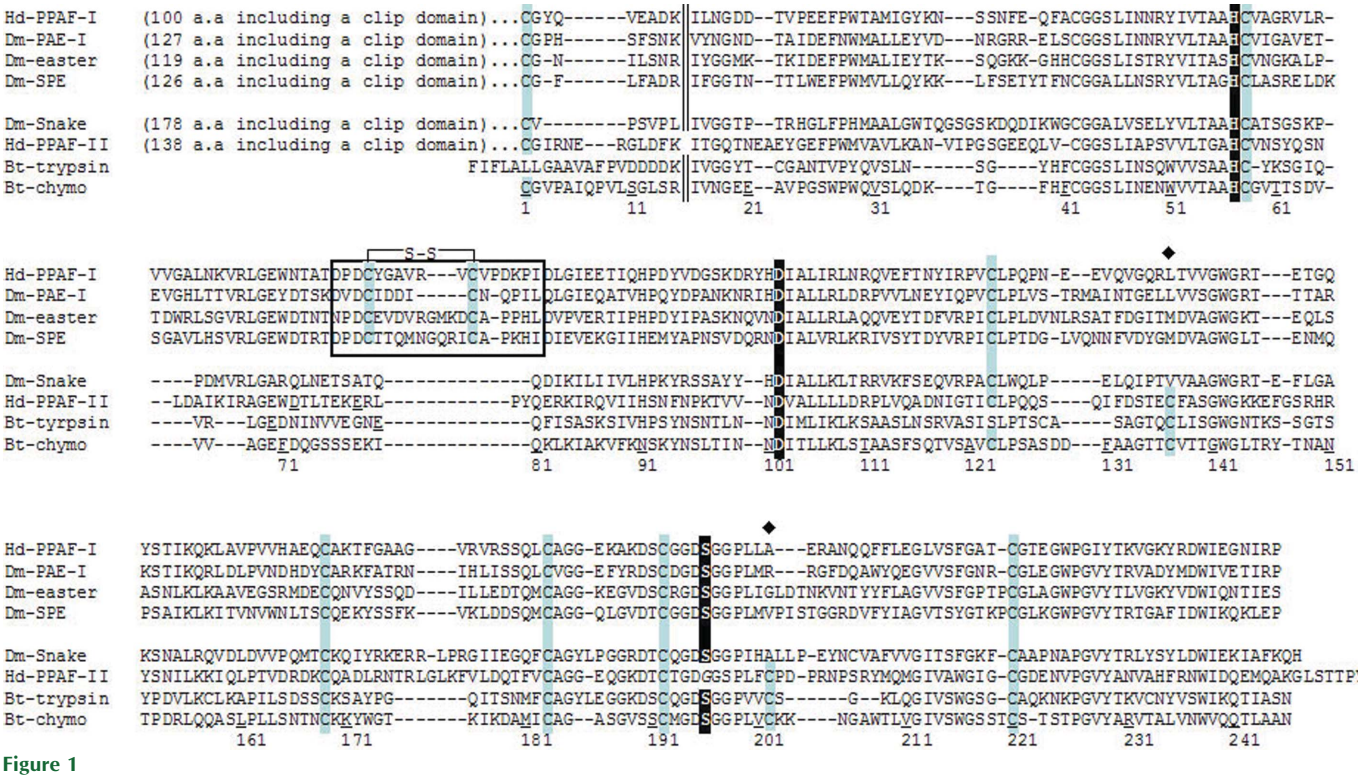


Figure 1
Sequence alignment of the SP domains of clip-domain SPs with trypsin and chymotrypsin. Four easter-type SPs, PPAF-II and bovine trypsin and chymotrypsin (from top to bottom; Hd, *H. diomphalia*; Dm, *Drosophila melanogaster*; Bt, *Bos taurus*; chymo, chymotrypsin) are shown. The alignment was performed using *ClustalW* (Thompson *et al.*, 1997) and then adjusted based on the conserved cysteine residues (shaded in cyan). The cleavage sites for zymogen activation are indicated by double lines and the catalytic triads (His, Asp, Ser) at the active sites are highlighted. The calcium-coordinating residues of PPAF-II and trypsin are underlined. The easter-type SP-specific insertion, indicated by a rectangle, contains two cysteine residues forming a putative disulfide bond. An invariant disulfide-bond linkage of SPs (Cys137 and Cys202 of chymotrypsin) is absent from all the catalycally active clip-domain SPs and is marked with diamonds. Every ten amino acids of chymotrypsin are underlined with a residue number.

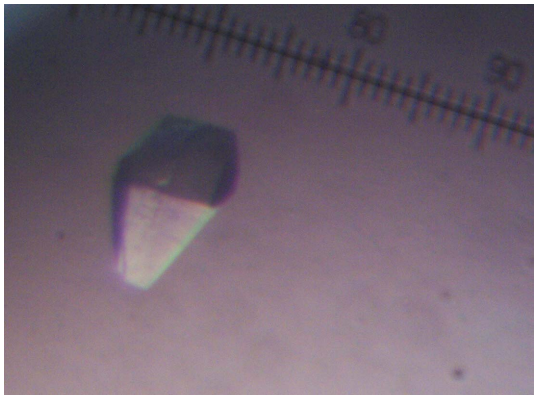


Figure 2
A crystal of the SP domain of PPAF-I. Approximate dimensions are 0.2 × 0.1 × 0.1 mm. Eight divisions on the scale represent 0.1 mm.

unit-cell parameters $a = 38.3$, $b = 53.3$, $c = 116.6$ Å, $\alpha = \beta = \gamma = 90^\circ$. Analysis of the X-ray diffraction pattern showed that along the h, k and l axes reflections were only present if h, k and $l = 2n$, identifying the space group as $P2_12_12_1$. The data set was 98.4% complete in the resolution range 100–1.70 Å, with $R_{\rm merge} = 6.7\%$. The crystals contain one molecule in the asymmetric unit, corresponding to a Matthews coefficient ($V_{\rm M}$) of 1.9 Å^3 Da^{-1} and a solvent content of 34.4% (Matthews, 1968). The crystallographic parameters and data-collection statistics are summarized in Table 1.

To date, many crystal structures of serine proteases have been deposited in the PDB. Kallikrein, a plasma serine protease (PDB code 2any; Tang *et al.*, 2005), was chosen as a search model for molecular replacement (MR) because it shares the highest sequence identity (34%) with the SP domain of PPAF-I. The MR procedure was performed with the program *AMoRe* (Navaza, 2001). The rotation with the highest correlation coefficient was applied to the search model and was used in the subsequent translation-function calculation. The calculation of the translation function gave one peak with a correlation coefficient of 24.8%, while the next solution exhibited a correlation of 20.5%. Rigid-body refinement with the best solution yielded a correlation coefficient of 35.5% and an R factor of 51.5% in the resolution range 10–3.5 Å. The solution produced an interpretable electron-density map, although it gave a relatively high R factor. The atomic model was refined using the program *CNS* to an $R_{\rm free}$ of below 30%. From the present model, the unique short insertion containing two cysteine residues is visible in the electron-density map, which will provide a clue as to how the insertion is involved in the function of easter-type SPs.

In conclusion, we obtained a high-quality crystal of the SP domain of PPAF-I and we are refining the structure of the SP domain of PPAF-I using the diffraction data set from the crystal. The crystal structure of PPAF-I will serve as a representative model of easter-type SPs to elucidate the molecular mechanism by which the clip-domain SPs recognize and catalyze the substrates in various biological processes.

Table 1
Data-collection and processing statistics.

Values in parentheses are for the highest resolution shell.

X-ray source	Beamline 4A, Pohang Accelerator Laboratory
Wavelength (Å)	0.9794
Temperature (K)	100
Unit-cell parameters (Å, °)	$a = 38.3$, $b = 53.3$, $c = 116.6$, $\alpha = \beta = \gamma = 90$
Space group	$P2_12_12_1$
$V_{\rm M}$ (Å^3 Da^{-1})	1.9
Solvent content (%)	34.4
Resolution (Å)	100–1.70 (1.76–1.70)
Unique reflections	27064
Redundancy	7.7 (4.3)
Completeness (%)	98.4 (96.0)
$R_{\rm merge}$ (%)	6.7 (25.8)
Average $I/\sigma(I)$	34.0 (3.0)

We thank the staff members at beamline 4A of Pohang Accelerator Laboratory (Korea) for the data collection. This project was supported by Programs of the National Research Laboratory (M10400000028-04J0000-02) grants to BLL and N-CH from the Korea Ministry of Science and Technology. This research was partly supported by Korea Research Foundation Grant (KRF-2004-041-C00247) to N-CH and Pusan National University Research Grant 2004 to N-CH.

References

Anderson, K. V. (1998). *Cell*, **95**, 439–442.
Kwon, T. H., Kim, M. S., Choi, H. W., Joo, C. H., Cho, M. Y. & Lee, B. L. (2000). *Eur. J. Biochem.* **267**, 6188–6196.
Lee, S. Y., Cho, M. Y., Hyun, J. H., Lee, K. M., Homma, K. I., Natori, S., Kawabata, S. I., Iwanaga, S. & Lee, B. L. (1998). *Eur. J. Biochem.* **257**, 615–621.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
Morisato, D. & Anderson, K. V. (1995). *Annu. Rev. Genet.* **29**, 371–399.
Navaza, J. (2001). *Acta Cryst.* D**57**, 1367–1372.
Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
Piao, S., Song, Y. L., Kim, J. H., Park, S. Y., Park, J. W., Lee, B. L., Oh, B.-H. & Ha, N.-C. (2005). *EMBO J.* **24**, 4404–4414.
Rose, T., LeMosy, E. K., Cantwell, A. M., Banerjee-Roy, D., Skeath, J. B. & Di Cera, E. (2003). *J. Biol. Chem.* **278**, 11320–11330.
Tang, J., Yu, C. L., Williams, S. R., Springman, E., Jeffery, D., Sprengeler, P. A., Estevez, A., Sampang, J., Shrader, W., Spencer, J., Young, W., McGrath, M. & Katz, B. A. (2005). *J. Biol. Chem.* **280**, 41077–41089.
Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997). *Nucleic Acids Res.* **25**, 4876–4882.