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Crystallization and preliminary X-ray diffraction studies of the cysteine protease ervatamin A from *Ervatamia coronaria*

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The ervatamins are highly stable cysteine proteases that are present in the latex of the medicinal plant *Ervatamia coronaria* and belong to the papain family, members of which share similar amino-acid sequences and also a similar fold comprising two domains. Ervatamin A from this family, a highly active protease compared with others from the same source, has been purified to homogeneity by ion-exchange chromatography and crystallized by the vapour-diffusion method. Needle-shaped crystals of ervatamin A diffract to 2.1 Å resolution and belong to space group $C222_1$, with unit-cell parameters a = 31.10, b = 144.17, c = 108.61 Å. The solvent content using an ervatamin A molecular weight of 27.6 kDa is 43.9%, with a V_M value of 2.19 Å^3 Da^{-1} assuming one protein molecule in the asymmetric unit. A molecular-replacement solution has been found using the structure of ervatamin C as a search model.

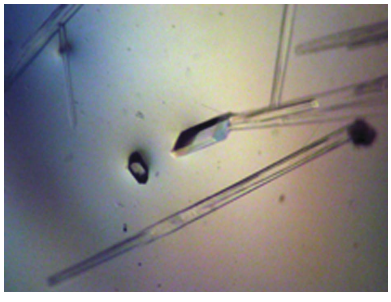
1. Introduction

Proteases are involved in all aspects of plant life cycles, such as enzyme regulation, mobilization of storage proteins during seed germination, programmed cell death, resistance against pathogens and insects and in most of the pathways for plant growth and development (Schaller, 2004). The plant proteases form the major catalytic classes serine, cysteine, aspartic and metalloproteases.

Plant cysteine proteases belong to a class which has been widely studied over the years. These enzymes are also used in industry owing to their high stability. Papain was the first plant cysteine protease discovered, in the latex of *Carica papaya* (Drenth *et al.*, 1968), and is the archetype of this class of enzymes involved in N-mobilization during seed germination, leaf senescence, a number of different cell deaths and plant defence mechanisms against insects and pathogens (Schaller, 2004).

Ervatamins are papain-like cysteine proteases found in the latex of the flowering plant *Ervatamia coronaria* (Sundd *et al.*, 1998). Three enzymes have been identified so far: ervatamins A, B and C of molecular weights 27.6, 23.2 and 22.5 kDa, respectively. Ervatamin A hydrolyzes denatured natural substrates such as casein and azo-albumin with high specific activities of 28 and 26.3 units mg^{-1}, respectively, compared with 9.0 and 7.5 units mg^{-1} for ervatamin B, and 11 and 4.5 units mg^{-1} for ervatamin C. It also shows high specific activity (14 units mg^{-1}) for synthetic substrates such as benzoyl-arginine *p*-nitroanilide (BAPA), while the other two ervatamins show negligible activity towards them (Nallamsetty *et al.*, 2003). The ervatamins also differ in stability between themselves: ervatamin C shows high stability and retains full activity up to 343 K (Sundd *et al.*, 1998), whereas ervatamin B and ervatamin A retain full activity up to 335 and 338 K, respectively (Kundu *et al.*, 2000; Nallamsetty *et al.*, 2003).

In order to elucidate the structural determinants for the underlying functional diversity of these multiple enzymes from the same latex, we have initiated a project to determine the three-dimensional structures of ervatamins by X-ray crystallography. Three-dimensional structures of ervatamins B and C have already been determined by our group (Chakrabarti *et al.*, 1999; Biswas *et al.*, 2003; Guha Thakurta *et al.*, 2004) and here we report the purification, crystallization and preliminary crystallographic analysis of ervatamin A.



2. Purification

Ervatamin A was purified from the latex of *E. coronaria* following a procedure which is a modification of the method described by Nallamsetty *et al.* (2003). Latex from young stems of the plant was collected in 10 m*M* sodium acetate buffer pH 5.0 containing 5 m*M* sodium tetrathionate and kept at 253 K for at least 24 h. It was thawed at room temperature and centrifuged at 17000*g* for 15 min to remove the gum. The clear supernatant, designated as the crude extract, was then applied onto a cation-exchange column of SP-Sephadex pre-equilibrated with 10 m*M* sodium acetate buffer pH 5.0 containing 5 m*M* sodium tetrathionate. Following the removal of the unadsorbed proteins, the column was eluted isocratically with 300 m*M* NaCl in the same buffer followed by a linear gradient of 300 m*M* to 1 *M* NaCl. The protein peak in the isocratic run was checked by 15% SDS–PAGE under reducing conditions, which showed essentially one band (Fig. 1). By changing the cation-exchange matrix from SP-Sepharose to SP-Sephadex and reducing the flow rate to 1 ml min^{-1} (instead of 6 ml min^{-1}), the second step of purification by gel filtration as described by Nallamsetty *et al.* (2003) could be avoided. The protein fraction in the isocratic run was checked for enzymatic activity and the molecular weight determined from 15% SDS–PAGE under reducing conditions confirmed this active peak to be ervatamin A. The enzyme was then dialyzed against 10 m*M* phosphate buffer pH 7.5 with 0.5 m*M* sodium tetrathionate. For crystallization, the tetrathionate-inactivated enzyme was concentrated to 16 mg ml^{-1} by ultrafiltration using an Amicon YM-10 (Millipore) membrane.

3. Crystallization

Crystallization experiments were performed both at room temperature and at 277 K. Initial crystals were obtained by the hanging-drop vapour-diffusion method using Hampton Research PEG/Ion screen solution No. 24 [0.2 *M* lithium acetate dihydrate, 20%(*w*/*v*) polyethylene glycol 3350] at both temperatures with 16 mg ml^{-1} tetrathionate-inactivated protein in 10 m*M* phosphate buffer pH 7.5. This condition was optimized and crystals suitable for X-ray diffraction studies were grown by the sitting-drop vapour-diffusion method with 6 µl protein solution and 6 µl reservoir solution at room temperature (Fig. 2). A solution of washed crystals was subjected to 15% SDS–PAGE under reducing conditions and stained with Coomassie Brilliant Blue, which revealed a single protein band corresponding to ervatamin A.

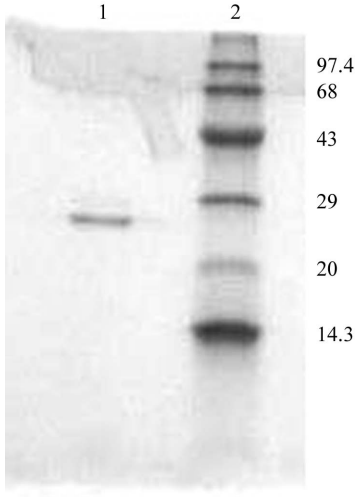


Figure 1
Coomassie-stained (15%) SDS–PAGE under reducing conditions. Lane 1, ervatamin A; lane 2, protein markers; molecular weights are marked in kDa.

Table 1
Crystallographic data of ervatamin A.

Values in parentheses refer to data in the highest resolution shell (2.14–2.10 Å).

Space group	$C222_1$
Unit-cell parameters (Å)	a = 31.10, b = 144.17, c = 108.61
Resolution (Å)	100–2.1
Rotation per exposure (°)	1
Exposure time (s)	600
Observed reflections	34453
Unique reflections	13803
Mosaicity (°)	0.39
Completeness (%)	93.1 (89.8)
Multiplicity	2.5
Mean $I/\sigma(I)$	3.9 (1.1)
$R_{\rm merge}$† (%)	6.78 (53.12)

† $R_{\rm merge} = [\sum(I - \langle I\rangle)^2/\sum(I^2)]^{1/2}$.

4. X-ray analysis

Diffraction data were collected in-house with a MAR300 imaging-plate system using Cu $K\alpha$ radiation generated by a Rigaku RU-200B rotating-anode generator operating at 50 kV and 100 mA equipped with the Osmic Confocal Max-Flux Optics system. Data were collected at room temperature using crystals of dimensions 0.2 × 0.1 × 0.1 mm with a crystal-to-detector distance of 140 mm and 1° oscillation per image. The crystals survived for up to 68 frames and then started degrading. Data were processed and scaled for these 68 frames using the *AUTOMAR* program suite (http://www.marresearch.com/automar/automar/run.htm). Ervatamin A crystals belong to the orthorhombic space group $C222_1$, with unit-cell parameters a = 31.10, b = 144.17, c = 108.61 Å. Data were processed to 2.1 Å and data statistics are summarized in Table 1. The Matthews coefficient ($V_{\rm M}$; Matthews, 1968) and the solvent content calculated using a molecular weight of 27.6 kDa for the protein are 2.19 Å^3 Da^{-1} and 43.9%, respectively.

5. Structure solution

Molecular-replacement calculations have been performed using the program *AMoRe* implemented in the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994). All-alanine models of papain (PDB code 9pap), actinidin (2act), ginger protease (1cqd), ervatamin B (1iwd) and ervatamin C (1o0e) were used as templates.

Figure 2
Crystals of ervatamin A.

The best results were obtained with ervatamin C as the model, yielding an R factor of 45.5% and a correlation factor of 40.76%, using data between 10 and 4 Å. Placement of the model obtained from molecular-replacement solution in the unit cell shows plausible packing interactions. Rigid-body refinement with this solution using *CNS* (Brünger *et al.*, 1998) gave $R = 44.9\%$ and $R_{\rm free} = 47.2\%$ with 5% data in the test set. Refinement is in progress.

References

Biswas, S., Chakrabarti, C., Kundu, S., Jagannadham, M. V. & Dattagupta, J. K. (2003). *Proteins Struct. Funct. Genet.* **51**, 489–497.

Brünger, A. T., Adams, P. D., Clore, G. M., DeLano, W. L., Gros, P., Grosse-Kunstleve, R. W., Jiang J.-S., Kuszewski, J., Nilges, M., Pannu, N. S., Read, R. J., Rice, L. M., Simonson, T. & Warren, G. L (1998). *Acta Cryst.* D**54**, 905–921.

Chakrabarti, C., Biswas, S., Kundu, S., Sundd, M., Jagannadham, M. V. & Dattagupta, J. K. (1999). *Acta Cryst.* D**55**, 1074–1075.

Collaborative Computational Project, Number 4 (1994). *Acta Cryst.* D**50**, 760–763.

Drenth, J., Jansonious, J. N., Koekoek, R., Swen, H. M. & Wolthers, B. G. (1968). *Nature (London)*, **218**, 929–932.

Guha Thakurta, P., Biswas, S., Chakrabarti, C., Sundd, M., Jagannadham, M. V. & Dattagupta, J. K. (2004). *Biochemistry*, **43**, 1532–1540.

Kundu, S., Sundd, M. & Jagannadham, M. V. (2000). *J. Agric. Food. Chem.* **48**, 171–179.

Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.

Nallamsetty, S., Kundu, S. & Jagannadham, M. V. (2003). *J. Protein Chem.* **22**, 1–13.

Schaller, A. (2004). *Planta*, **220**, 183–197.

Sundd, M., Kundu, S., Pal, G. & Jagannadham, M. V. (1998). *Biosci. Biotechnol. Biochem.* **63**, 1947–1955.