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Crystallization and preliminary X-ray analysis of ervatamin B and C, two thiol proteases from *Ervatamia coronaria*

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Two highly stable cysteine proteases, ervatamin B (ERV-B) and ervatamin C (ERV-C), purified from the latex of the medicinal plant *E. coronaria* have been crystallized at room temperature. Crystals of ERV-B and ERV-C diffract to 2.5 and 2.6 Å, respectively. The space group is $P2_12_12_1$ for the crystals of both proteases with unit-cell parameters $a = 47.5$, $b = 58.8$ and $c = 68.8$ Å, and $a = 43.8$, $b = 82.6$ and $c = 133.1$ Å, respectively. A self-rotation function for ERV-C indicates a twofold non-crystallographic symmetry relating the two molecules in the asymmetric unit.

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1. Introduction

Ervatamia coronaria is a flowering plant indigenous to India and has various medicinal and other applications (Council of Scientific and Industrial Research, 1952). A search for useful biochemical constituents in the latex of the plant yielded multiple cysteine proteases with novel properties. Two proteases, designated ervatamin B (ERV-B) and ervatamin C (ERV-C), have been purified to homogeneity and characterized (Kundu *et al.*, 1998; Sundd *et al.*, 1998). Conserved N-terminal amino-acid residues typical of plant cysteine proteases and considerable N-terminal sequence similarity to papain and stem bromelain indicate that they belong to the papain superfamily, with which they have many properties in common. Their estimated molecular masses (26 kDa for ERV-B and 25 kDa for ERV-C), activity towards natural substrates, maximal activation by various reducing and chelating reagents, inhibition by thiol-specific inhibitors, autolytic properties, pH optimum (7.5–8.0), temperature optimum (323–328 K), stability in organic solvents such as methanol and ethanol *etc.* are typical of papain (Glazer & Smith, 1971) and other members of this superfamily. However, they also exhibit some novel properties and are distinct from each other and from papain in many respects (Kundu *et al.*, 1998; Sundd *et al.*, 1998). A striking property of these two ervatamin proteases is their remarkably high stability. The enzymes retained both activity and structure (Kundu *et al.*, 1998) even after prolonged exposure to extreme pH range (2.0–12.0), strong denaturants (8 *M* urea, 4 *M* GuMU, SDS), high temperature (338–343 K for 15 min) or organic solvents, which are known to inactivate most enzymes, and these properties are unique among the reported plant cysteine proteases. Spectroscopic studies on their conformational transitions (Kundu *et al.*, 1998) also reveal novel observations for the proteases. A study of their folding pattern compared with other plant cysteine proteases might be of particular interest, as the sequential unfolding of domains in papain (Edwin & Jagannadham, 1998) is absent in these proteases under similar conditions.

Thus, ervatamin B and ervatamin C seem to be endopeptidases with interesting properties and may have useful applications in food industry and medicine, as do papain and stem bromelain (Kaneda *et al.*, 1997). For a better insight into the underlying structural basis of their unique physicochemical properties, probable applications and structure–function relationship, a knowledge of the three-dimensional structures of the two proteases is important.

2. Methods and results

Small needle-shaped crystals of ERV-B were obtained in a dialysis bag during protein purification, when fractions eluted from an SP-Sepharose ion-exchange column at pH 5.0 were pooled, precipitated by 80% ammonium sulfate and dialysed at pH 7.0. A sparse-matrix screen (Jancarik & Kim, 1991) was employed using a Hampton Research screen kit (Hampton Research, California, USA) for crystallizing ERV-C. In a successful experiment, the protein solution (3 µl, 13.0 mg ml^{-1}) was mixed with an equal volume of reservoir solution containing 0.05 *M* monobasic potassium phosphate as salt and 20%(*w*/*v*) PEG 8000 as precipitant. Needle-shaped crystals (Fig. 1) were obtained in 6 d. The same crystals were also obtained from 0.2 *M* ammonium sulfate and 30%(*w*/*v*) PEG 4000.

Diffraction intensities were recorded on a 30 cm MAR Research imaging-plate detector placed on a Rigaku RU-200 rotating-anode

Table 1
Crystallographic characteristics and summary of data collection.

	ERV-B	ERV-C
Crystal size (mm)	0.05 × 0.05 × 0.05	0.4 × 0.15 × 0.05
Precipitant	$(NH_4)_2SO_4$	PEG 8000
Space group	$P2_12_12_1$	$P2_12_12_1$
Unit-cell dimensions (Å)		
a	47.5	43.8
b	58.8	82.6
c	68.8	133.1
Z	4	8
Unit-cell volume (10^6 Å^3)	0.192	0.482
Solvent content (%)	34	50
V_m† (Å^3 Da^{-1})	1.85	2.41
Maximum resolution (Å)	2.8	3.0
Number of measured reflections	13035	58825
Number of unique reflections	5103	10357
$I/\sigma(I)$ overall	5.2	8.9
Completeness overall	79.2	86.6
R_{merge}‡ overall	0.136	0.098
Resolution range of last shell	2.86–2.8	3.07–3.0
Completeness in last shell	84.0	92.2
R_{merge}‡ in last shell	0.287	0.327

† V_m is the crystal packing parameter (Matthews, 1968). ‡ $R_{merge} = \sum_i |I_i - \langle I \rangle| / \sum \langle I \rangle$, where I is an individual intensity measurement and $\langle I \rangle$ is the average intensity for this reflection with summation over all data.

generator running at 50 kV and 100 mA, using a copper target. The crystal-to-detector distance was set to 150 mm for both crystals. ERV-B and ERV-C crystals diffract to 2.5 and 2.6 Å, respectively. The images were collected at a series of 1° oscillations with exposures of 600 and 720 s, respectively, at room temperature. Data at 2.8 Å from ERV-B and at 3.0 Å from ERV-C were autoindexed and integrated, scaled and reduced using the programs *DENZO* and *SCALEPACK* (Otwinowski, 1993) implemented on a Silicon Graphics INDY system.

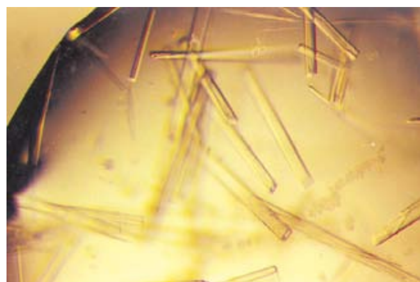

Figure 1
Crystals of ERV-C.

Crystal data and data-collection characteristics for both are listed in Table 1.

The solvent content for ERV-B using a molecular mass of 26 kDa is 33.7%, with the volume-to-mass ratio V_m = 1.85, assuming one molecule in the asymmetric unit. This value falls within the normal range (1.7–3.5 Å^3 Da^{-1}) given by Matthews (1968) for globular protein crystals, but is rather low, indicating a close packing of the molecules in the unit cell. For ERV-C (25 kDa), assuming one molecule per asymmetric unit, the V_m value is 4.83 Å^3 Da^{-1}, which is outside the observed range. On the other hand, with two molecules per asymmetric unit, the V_m value is 2.41 Å^3 Da^{-1}, which falls within the normal range, giving a solvent content of 50.0%.

A self-rotation function for ERV-C was calculated using the program *POLARRFN* in the *CCP*4 package (Collaborative Computational Project, Number 4, 1994), with a Patterson radius of integration of 35 Å and data in the 15–4 Å resolution range. The highest non-origin peak in the function appeared at ψ = 90, φ = 315 and κ = 180°, with a height 45% that of the origin peak, indicating that the two molecules in the asymmetric unit are related by a non-crystallographic twofold axis. Molecular-replacement calculations have been performed for both ERV-B and ERV-C. Structure solution and refinement are in progress.

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