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Crystallization and preliminary X-ray crystallographic studies of the plant aspartic proteinase cardosin A

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Abstract

The plant aspartic proteinase cardosin A was crystallized using vapour diffusion. Crystals belong to the monoclinic space group $C2$, cell dimensions a = 116.9 (2), b = 87.2 (8), c = 81.3 (1) Å, β = 104.4 (4) , and contain two molecules in the asymmetric unit related by a non-crystallographic twofold axis. Diffraction data were collected at room temperature with radiation from a synchrotron source up to 2.85 Å resolution. When the crystals were flash cooled to 110 K in a nitrogen stream the same resolution limit could also be obtained on a rotating-anode source. Recently, synchrotron radiation together with flash cooling led to an improvement of the diffraction data to 1.72 Å resolution.

1. Introduction

Aspartic proteinases (AP) constitute a group of enzymes widely distributed in nature. They are found in vertebrates, plants, fungi, yeast, bacteria and retroviruses. In general they have an acidic pH optimum for activity and are inhibited by pepstatin (Umezawa *et al.*, 1970). There are two aspartic residues in the catalytic site, which preferentially cleave peptide bonds between hydrophobic residues within extended peptide substrates (Fruton, 1970, 1976; Hofmann & Hodges, 1982; Kay & Dunn, 1992). Amino-acid sequences of plant AP's obtained to date (Runeberg-Roos *et al.*, 1991; Cordeiro *et al.*, 1994; Asakura *et al.*, 1995) differ from those of other members of the family by an inserted segment of about 100 amino-acid residues, which has been suggested to be involved in the vacuolar targeting of these enzymes (Guruprasad *et al.*, 1994).

Structural models of many AP's have been determined by X-ray crystallography from a wide diversity of sources which include mammalian, fungal, yeast and retroviral AP's (see Protein Data Bank, Abola *et al.*, 1987). However, no plant AP has been structurally characterized by X-ray diffraction to date, as it has proved very difficult to obtain good quality diffracting crystals.

Cardosin A is the most abundant of the two plant AP's (the other, a less specific proteinase from a related but different gene, has been named cardosin B) isolated from the stigmas of *Cynara cardunculus*. L. (Veríssimo *et al.*, 1996). It is a 45 kDa vacuolar protein, glycosylated in both of its two subunits (30 and 15 kDa), with a sugar content of 9.6% of its native molecular weight (Costa *et al.*, 1997). It is expressed with the characteristic plant insertion of 100 residues (Faro *et al.*, 1995). It is found in large quantities in protein storage vacuoles of the stigmatic papillae and, much less abundantly in vacuoles of epidermal cells of the style (Ramalho-Santos *et al.*, 1997). Although its physiological role remains unclear, it has been suggested to have, as primary function, interaction with pathogens or/and pollen, and possibly posseses a secondary function in the annual senescence of the flower (Ramalho-Santos *et al.*, 1997). Additionally it has been shown that, like chymosin, cardosin A initiates milk clotting by cleaving the peptide bond between Phe105 and Met106 in κ-casein (Veríssimo *et al.*, 1996). Indeed, an enzymatic preparation containing this coagulating agent is traditionally used in Portugal in cheese manufacture. Both its physiological properties, such as its strict location, its specificity properties and its high expression, as well as its applications in cheese production, make cardosin A an interesting enzyme for structural studies (Bento *et al.*, 1996). Here we report the crystallization and preliminary diffraction analysis of plant AP cardosin A.

2. Materials and methods

Native cardosin A was isolated and purified following Faro *et al.* (1987). Extensive crystallization trials were possible after the procedure was improved (Veríssimo *et al.*, 1996), which in addition increased the degree of purity while maintaining the specificity and the kinetic properties.

Crystals of cardosin A were obtained using the Crystal Screen™ from Hampton Research, based on the sparse-matrix sampling method of Jancarik & Kim (1991). Needles appeared in three of the 50 test solutions, and were selected for further crystal optimization. Drops of 2 µl of protein solution, obtained by dissolving the lyophilized protein in deionized water to 8–20 mg ml^{-1}, and supplemented by an aliquot of precipitating solution, were deposited on polystyrene microbridges (from DROP, Devis Realisation Outillage Precision, Grenoble), located inside the reservoirs of Linbro boxes (Flow Laboratories, Inc., McLean, VA), and equilibrated against 500 µl of each tested precipitant solution.

Three diffraction data sets were successively obtained. The first was collected at room temperature on the BW7A wiggler beamline (λ = 0.901 Å) at the EMBL Outstation, DESY, Hamburg. The crystal was mounted in a sealed thin-walled glass capillary tube. The second was collected with in-house equipment, under cryo conditions with an Oxford Cryosystems Cryostream, using an Enraf–Nonius FR570 rotating anode operating at 4.5 kW, a Huber graphite monochromator (Cu $K\alpha$, λ = 1.5418 Å). The cryoprotectant, PEG 4K, was an integral component of the crystallization. The third set was obtained under cryo conditions with a synchrotron source, at the beamline X11 (λ = 0.9091 Å) at the EMBL Hamburg Outstation, DESY. All three sets were recorded on Mar Research imaging plates.

The diffraction images were integrated with *DENZO* and the intensities were scaled and merged with *SCALEPACK*

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Table 1. *Cardosin A crystals diffraction-data statistics*

	Crystal *A*	Crystal *B*	Crystal *C*
Experimental conditions	Synchrotron BW7A, room temperature	Cu $K\alpha$ radiation, 110 K	Synchrotron X11, 110 K
Cell dimensions			
a (Å)	118.9 (1)	117.6 (3)	116.9 (2)
b (Å)	88.3 (2)	87.4 (1)	87.20 (8)
c (Å)	82.5 (1)	82.0 (3)	81.3 (1)
β (°)	104.49 (4)	104.5 (2)	104.44 (4)
Resolution limits (Å)	15.0–2.85	30.0–2.85	35.0–1.72
Mosaicity (°)	0.15–0.25	0.60	0.95
Measured reflections	63522	164684	950405
Independent reflections	18976	18778	84399
Completeness (%) (outer shell)†	98.8 (99.8)	97.5 (95.0)	96.9 (99.3)
$I>3\sigma(I)$ (%) (outer shell)†	85.1 (66.0)	79.6 (51.9)	70.5 (31.1)
$I/\sigma(I)$ (%) (outer shell)†	14.8 (5.8)	10.6 (3.3)	15.9 (2.5)
$R_{\rm merge}(I)$ (%) (outer shell)†	7.6 (23.6)	10.3 (28.9)	6.2 (41.3)

† Outer shell: 2.90–2.85 Å crystal *A*; 2.90–2.85 Å crystal *B*; 1.75–1.72 Å crystal *C*.

(Otwinowski & Minor, 1997). The *CCP*4 package (Collaborative Computational Project, Number 4, 1994) was used to calculate observed structure factors (*TRUNCATE*), to normalize them (*ECALC*) and to calculate the self-rotation function (*POLARRFN*).

3. Results and discussion

Cardosin A crystals were difficult to obtain. Protein isolated from flowers of different sources, showing similar biochemical characteristics, reacted differently under identical crystallization conditions. The relatively high sugar content (*ca* 10%) of the protein, with associated carbohydrate branching variations and structure flexibility (Costa *et al.*, 1997), makes the crystalline order of the molecules difficult to achieve. Cardosin A crystals needed very long nucleation times, taking about three months to appear, as agglomerates of thin plates (Fig. 1). They grew optimally in sitting drops with a protein concentration of 12 mg ml^{-1} equilibrated against a precipitant solution of PEG 4 K 40%, of sodium citrate buffer 0.1 *M* (pH 5.5) and of ammonium acetate 0.2 *M*. Macroseeding was used to improve the quality: the agglomerates of plates were broken down and individual plates were transferred to new drops, previously equilibrated against the same crystallization conditions. The crystals were then allowed to grow for two further months. Several trials were necessary, with success rate of about 30%, to obtain good diffracting crystals (Fig. 2).

Three sets of X-ray data were collected from three different crystals using different radiation sources and different instrumental conditions. In the first set, using synchrotron radiation, the crystal did not diffract beyond 2.85 Å resolution (Table 1, crystal *A*) and was prone to radiation damage. However, a crystal measured with in-house equipment (Table 1, crystal *B*), proved that cryogenic conditions improved the resolution limits of diffraction and minimized such damage. A third crystal diffracted to 1.72 Å when exposed to synchrotron radiation under cryogenic conditions (Table 1, crystal *C*).

Cardosin A crystals contain two molecules in the asymmetric unit with a calculated solvent content of *ca* 47% (Matthews, 1968). The self-rotation function (Fig. 3) revealed a non-crystallographic twofold axis deviating *ca* 13° from axis *a*.

Future work will be devoted to solving the structure of cardosin A by molecular replacement. The crystallization of

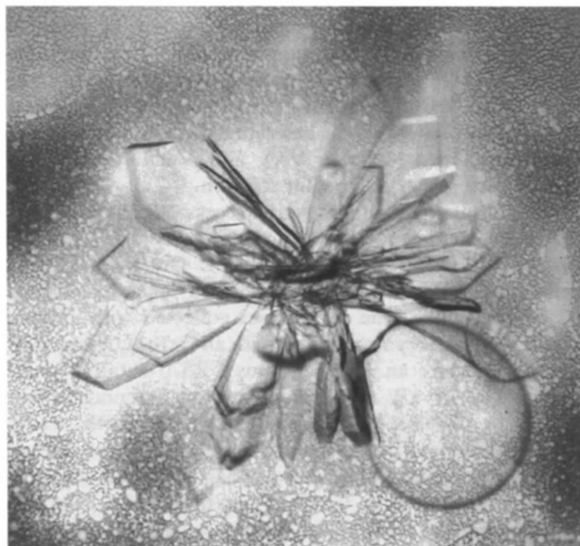

Fig. 1. Initial crystals of cardosin A. Agglomerates of thin plates that were used for macroseeding.

Fig. 2. Example of a cardosin A seeded crystal (largest dimension about 1 mm) used for data collection.

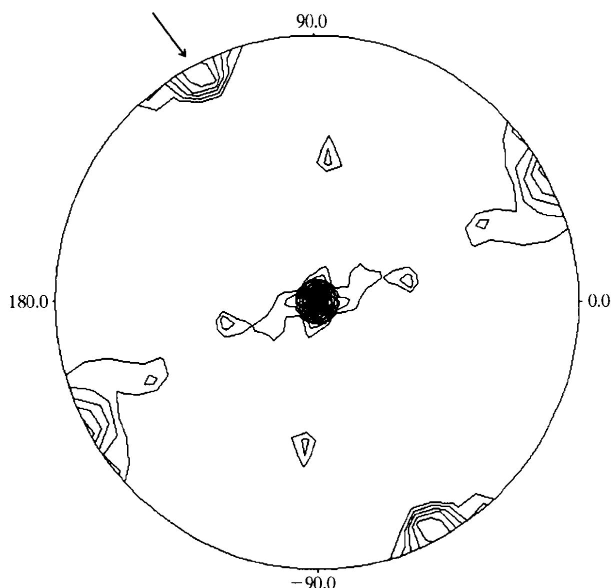

Fig. 3. Section $\kappa = 180^\circ$ of the self-rotation function. The marked peak ($\omega = 88$, $\varphi = 118$, $\kappa = 180^\circ$) with a height of 5.1 map r.m.s. (34% of the origin peak) corresponds to a non-crystallographic twofold axis which lies at *ca* 13° from axis *a*. The self-rotation function was calculated using normalized structure factors and a Patterson radius of 15 Å for data between 15.0 and 2.85 Å resolution.

protein complexes with different inhibitors will be undertaken in order to examine the enzyme specificity.

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