

Crystallization of bothrombin, a fibrinogen-converting serine protease isolated from the venom of *Bothrops jararaca*

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Bothrombin, a snake-venom serine protease, specifically cleaves fibrinogen, releasing fibrinopeptide A to form non-crosslinked soft clots, aggregates platelets in the presence of exogenous fibrinogen and activates blood coagulation factor VIII. Bothrombin shares high sequence homology with other snake-venom proteases such as batroxobin (94% identity), but only 30 and 34% identity with human α -thrombin and trypsin, respectively. Single crystals of bothrombin have been obtained and X-ray diffraction data have been collected at the Laboratorio Nacional de Luz Síncrotron to a resolution of 2.8 Å. The crystals belong to the space group $P2_12_12_1$, with unit-cell parameters $a = 94.81$, $b = 115.68$, $c = 155.97$ Å.

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1. Introduction

Snake venoms are rich sources of metalloproteases and serine proteases that play key roles in haemorrhagia and blood-clotting disorders (Kornalik, 1990*a,b*). The metalloproteases often display haemorrhagic activity, demonstrate low substrate specificity and are inhibited by metal chelators such as EDTA. On the other hand, the serine proteases display higher substrate specificity, hydrolyze only specific bonds in peptides and are inhibited by DFP and PMSF.

Serine proteases have been isolated and characterized from a number of snake venoms and display a wide spectrum of activities such as cleavage of fibrinogen (Herzig *et al.*, 1970; Aragon-Ortiz & Gubensek, 1981; Iwasaki *et al.*, 1990; Aguiar *et al.*, 1996), release of bradykinin (Sato *et al.*, 1965), increase in capillarity and permeability (Sato *et al.*, 1965), β -fibrinogenase activity (Ouyang & Teng, 1976; Ouyang *et al.*, 1979), activation of factor V (Schiffman *et al.*, 1969; Rawala *et al.*, 1978), thrombin-like platelet activation (Marrakchi *et al.*, 1995) and induction of non-coagulant platelet aggregation (Niewiarowski *et al.*, 1979; Schmaier *et al.*, 1980).

The venom serine proteases are often referred to as thrombin-like proteases since they exhibit fibrinogen-clotting activity and have been the focus of many studies owing to their clinical and biochemical applications. These enzymes are divided into three basic classes depending on their ability to release fibrinopeptide A (class A), fibrinopeptide B (class B) or both fibrinopeptides A and B (class AB) from fibrinogen (Kornalik, 1990*a,b*). The thrombin-like enzymes usually belong to class A. The class B enzymes generally require long

periods of incubation with fibrinogen before the formation of fibrin is observed (Markland, 1983). The thrombin-like enzyme from *Bothrops insularis* (Selistre & Giglio, 1987) is capable of hydrolyzing fibrinogen and producing both fibrinopeptides A and B. It should be noted that unlike thrombin, these enzymes are incapable of activating factor XIII. Thus, they hydrolyze fibrinogen to produce a soft clot *i.e.* non-crosslinked fibrins, which are more susceptible to the lytic action of exogenous plasmin than the hard clots (crosslinked clots) formed by thrombin.

Intravenous injection of these thrombin-like enzymes causes rapid defibrinogenation arising from the proteolysis of fibrinogen, forming non-crosslinked fibrin monomers which are removed rapidly from circulation. Because of these physiological properties, thrombin-like enzymes act as procoagulants *in vitro*, converting fibrinogen to fibrin. On the other hand, they cause benign defibrination (Kornalik, 1990*a,b*). Batroxobin from the venom of *B. moojeni* and ancrod from the venom of *Agkistrodon rhodostoma* are produced commercially as clinical therapeutic drugs since they prevent thrombi formation and improve blood circulation by reducing blood viscosity. The serine protease reptilase is used clinically for the quantitative determination of fibrinogen in the plasma of patients undergoing heparin treatment.

Here, we present the results of the purification and crystallization of bothrombin, an enzyme from the venom of *B. jararaca* which releases fibrinopeptide A from fibrinogen, specifically cleaving Arg–Gly bonds to form a soft non-crosslinked clot, aggregates platelets in the presence of exogenous fibrinogen and

activates blood coagulation factor VIII. Bothrombin shares 94% homology with batroxobin (*B. moojeni*), which is used clinically to screen for disturbed fibrin formation and for the quantitative determination of plasma fibrinogen. The identities between bothrombin and the plasminogen activator isolated from the venom of *Trimeresurus stejnegeri* and trypsin are 58 and 34%, respectively.

2. Methods

2.1. Purification

The crude venom from *B. jararaca* was obtained as a lyophilized powder from a local serpentarium. 300 mg of this was dissolved in 1 ml of buffer (20 mM sodium phosphate pH 7.8) and centrifuged at 11 000 rev min⁻¹ for 10 min. The clear supernatant was applied to a Resource 15S (Pharmacia) column which had previously been equilibrated with the same buffer. The fraction which did not bind to this column was then applied onto a Sepharose-benzamidine CL-6B column and the unspecifically bound fractions were eluted by washing with 0.5 M NaCl. The serine protease bothrombin was eluted by changing the pH to 3.6 using 20 mM glycine buffer. The protein was tested for proteolytic activity and for its ability to release fibrinopeptide A.

The sample was pure as evidenced by silver stained SDS-PAGE gels and was monodisperse as demonstrated by dynamic light scattering (Dynapro 801).

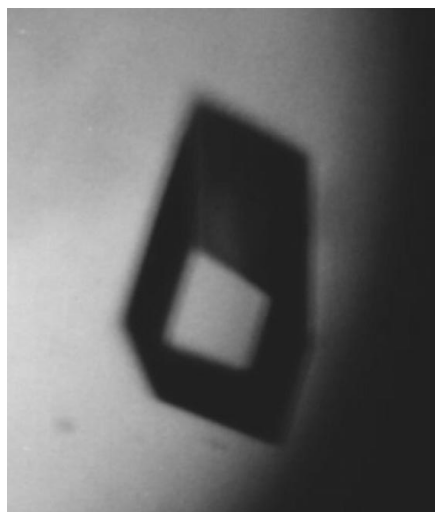


Figure 1
Photomicrograph of single crystals of *B. jararacam* serine proteinase (maximum dimensions 0.2 × 0.2 × 0.2 mm).

2.2. Crystallization

The protein was concentrated in a microconcentrator (Amicon) to a concentration of 5.0 mg ml⁻¹. Crystallization was performed at 1291 K by the hanging-drop vapour-diffusion method using 24-well tissue-culture plates. Initial trials were carried out using the screens produced by Hampton Research (Jancarik & Kim, 1991). Typically, 0.5 µl drops of protein solution were mixed with an equal amount of the screening solution. Single crystals with a minimum dimension of 0.2 mm were obtained when a 1 µl drop of the protein solution was mixed with an equal volume of a solution containing 100 mM MES pH 6.2, 10 mM zinc sulfate and 15% PEG MME 550 and equilibrated over a reservoir containing the same solution. Crystals were obtained after 6 d (Fig. 1).

2.3. X-ray diffraction data collection

For data collection, the crystal was transferred to a mother-liquor solution containing 20% glycerol and was flash-frozen. X-ray diffraction data were collected at the crystallographic beamline (Polikarpov *et al.*, 1988) at the Laboratório Nacional de Luz Síncrotron (LNLS, Campinas, Brazil). The synchrotron-radiation source at LNLS was set to a wavelength of 1.54 Å. Diffraction data were collected from 60 images using the oscillation method. Individual frames consisted of an oscillation of 1° with a crystal-to-detector distance of 260 mm. Diffraction intensity data were measured using a MAR 345 imaging-plate detector and were reduced and processed using the HKL program suite (Otwinowski & Minor, 1997).

3. Results

Examination of the systematic absences indicated that the crystals belong to the space group $P2_12_12_1$, with unit-cell parameters $a = 94.81$, $b = 115.68$, $c = 155.98$ Å. Calculation of the Matthews parameter (Matthews, 1968) resulted in a V_M of 2.79 Å³ Da⁻¹, assuming the existence of six molecules of bothrombin (molecular weight 25 591 Da) in the asymmetric unit. The crystals were highly mosaic; processing of the 153 850 observations to 2.8 Å resulted in an R_{merge} of 18.9% for 40 899 independent reflections. When only data to 3.2 Å was used, this resulted in an R_{merge} of 11.1% for 26 787 independent reflections. The relevant data collection and processing statistics are presented in Table 1.

Table 1

Data collection and processing statistics.

Space group	$P2_12_12_1$
Unit-cell parameters (Å)	$a = 94.81$, $b = 115.68$, $c = 155.98$
Maximum resolution (Å)	2.8
Resolution of data set (Å)	15–3.2
No. of unique reflections	26787
R_{merge}^\dagger 3.2 Å (2.8 Å) (%)	11.1 (15.2)
Completeness (%)	97.5
V_M (Å ³ Da ⁻¹)	2.79
No. of molecules per asymmetric unit	6
$I/\sigma(I)$ in outermost shell, 3.2–3.1 Å (2.86–2.8 Å)	3.5 (1.23)

$^\dagger R_{\text{merge}} = \sum \sum |I(h) - \langle I(h) \rangle| / \sum \sum I(h)$, where $I(h)$ is the observed intensity of the i th measurement of reflection h and $\langle I(h) \rangle$ is the mean intensity of reflection h calculated after scaling.

Bothrombin displays 58% identity and 71% homology with the plasminogen activator isolated from the venom of *T. stejnegeri*, whose structure has been refined at 2.5 Å to a crystallographic residual of 17.8% (Parry *et al.*, 1998). However, initial trials with the atomic coordinates of this protein failed to result in a molecular-replacement solution. We are currently generating a model based on this structure to be used as a probe for molecular replacement.

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