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Purification, crystallization and preliminary X-ray diffraction analysis of saxthrombin, a thrombin-like enzyme from *Gloydius saxatilis* venom

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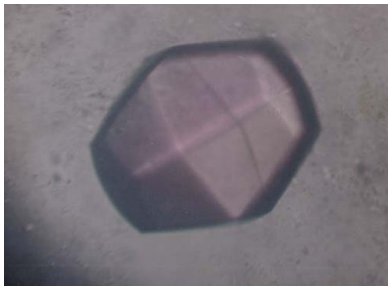
The snake-venom thrombin-like enzymes (SVTLEs) are a class of serine proteinases that show fibrinogen-clotting and esterolytic activities. Most TLEs convert fibrinogen to fibrin by releasing either fibrinopeptide A or fibrinopeptide B and cannot activate factor XIII. The enzymes hydrolyze fibrinogen to produce non-cross-linked fibrins, which are susceptible to the lytic action of plasmin. Because of these physiological properties, TLEs have important medical applications in myocardial infarction, ischaemic stroke and thrombotic diseases. Here, a three-step chromatography procedure was used to purify saxthrombin (AAP20638) from *Gloydius saxatilis* venom to homogeneity. Its molecular weight is about 30 kDa as estimated by SDS–PAGE. A saxthrombin crystal was obtained using the hanging-drop vapour-diffusion method and diffracted to a resolution limit of 1.43 Å. The crystal belongs to space group *C*2, with unit-cell parameters $a = 97.23$, $b = 52.21$, $c = 50.10$ Å, $\beta = 96.72^\circ$, and the Matthews coefficient (V_M) was calculated to be 2.13 Å^3 Da^{-1} with one molecule in the asymmetric unit.

1. Introduction

Snake venoms are mixtures of numerous proteins with enzymatic or toxic activities. The proteolytic enzymes contained in snake venoms affect the host coagulation process in various ways. These enzymes include the snake-venom thrombin-like enzymes (SVTLEs), which are distributed in several snake genera (Castro *et al.*, 2004). SVTLEs are serine proteinases and resemble thrombin in their ability to catalyze the last of the highly substrate-specific proteolytic reactions in the coagulation process.

Thrombin converts soluble fibrinogen to insoluble fibrin by proteolytic release of both fibrinopeptide A and fibrinopeptide B from the N-terminal disulfide knot of the fibrinogen A and B chains. Although TLEs also lead to clotting by hydrolysis of fibrinogen, there are many differences compared with thrombin. For instance, most SVTLEs only release one of the fibrinopeptides, mostly fibrinopeptide A, with the exception of a few TLEs such as jararacussin from *Bothrops jararacussu* venom (Castro *et al.*, 2004). In addition, TLEs cannot activate factor XIII, an important zymogen that is activated by thrombin. TLEs have some deletions in their primary structure in regions around the active-site cleft that allow the large substrate side chain to gain access to the base of the thrombin catalytic site. These changes may explain the differences in the function of TLEs compared with thrombin.

The unconventional polymers formed by TLEs cannot be identified by the coagulation cascade factor and these non-cross-linked fibrins are more susceptible to clearance by the lytic action of plasmin than the cross-linked fibrins formed by thrombin (Furukawa & Ishimaru, 1990). Because the non-cross-linked fibrins formed by TLEs are quickly degraded and cleaned away in the bloodstream, the thrombin-like enzymes have considerable clinical application in the treatment of various thrombo-embolic states and in the prevention of thrombosis (Huang *et al.*, 1999). Since the gene for batroxobin from *B. atrox moojeni* venom was isolated and analyzed (Itoh *et al.*, 1987), many other thrombin-like enzymes have been thoroughly studied and



several SVTLEs have been isolated and sequenced (Furukawa & Ishimaru, 1990).

Gloydius saxatilis is one of the most toxic snakes in the north of China and its venom affects platelet function and the blood-coagulation process in many ways. Sun *et al.* (2003) purified TLEs from *G. saxatilis* and used RT-PCR to amplify the cDNA of the gene in the venom gland of *G. saxatilis*. They determined a nucleotide sequence (AAP20638) that codes for a pre-zymogen protein of 258 amino acids with a predicted molecular weight of 29.8 kDa. Amino-acid sequence comparisons showed that it had a high identity to thrombin-like enzymes from other snakes (Sun *et al.*, 2003).

2. Materials and methods

2.1. Materials

Crude *G. saxatilis* venom was obtained from the Institute of Snakes and Snake Venoms of the Dalian Shedao Hospital (Dalian, People's Republic of China). DEAE-Sepharose, Phenyl-Sepharose and CM-Sepharose were purchased from Pharmacia (Sweden). The Crystal Screen kit was purchased from Hampton Research (USA). *p*-Toluenesulfonyl-L-arginine methyl ester (TAME) and bovine fibrinogen were purchased from Sigma (USA). Other reagents and chemicals of analytical grade were produced by Shanghai Biochemical Technology Co. (People's Republic of China).

2.2. Purification

Saxthrombin was purified from *G. saxatilis* venom by a three-step chromatography procedure as follows (see Fig. 1). Firstly, 1.4 g crude venom was dissolved in 15 ml loading buffer *A* (20 m*M* Tris–HCl pH 8.2) and centrifuged at 14 000 rev min^{-1} for 20 min. The supernatant was applied onto a DEAE-Sepharose Fast Flow column pre-equilibrated with loading buffer *A*. The column was washed with loading buffer *A* and then eluted with a 0.0–0.4 *M* linear NaCl gradient at a flow rate of 150 ml h^{-1}. All fractions were collected and individually analyzed for clotting activity using bovine fibrinogen. Peak 3, which had the greatest activity, was pooled. The peak 3 sample was dialyzed against loading buffer *B* (1.5 *M* ammonium sulfate, 50 m*M* Tris–HCl pH 7.5) and applied onto a Phenyl-Sepharose column, followed by elution with 400 ml of a linear ammonium sulfate gradient from 1.5 to 0 *M*. Peaks 3 and 4 were pooled and dialyzed against loading buffer *C* (20 m*M* sodium acetate/acetic acid pH 4.4). The pooled sample was concentrated and applied

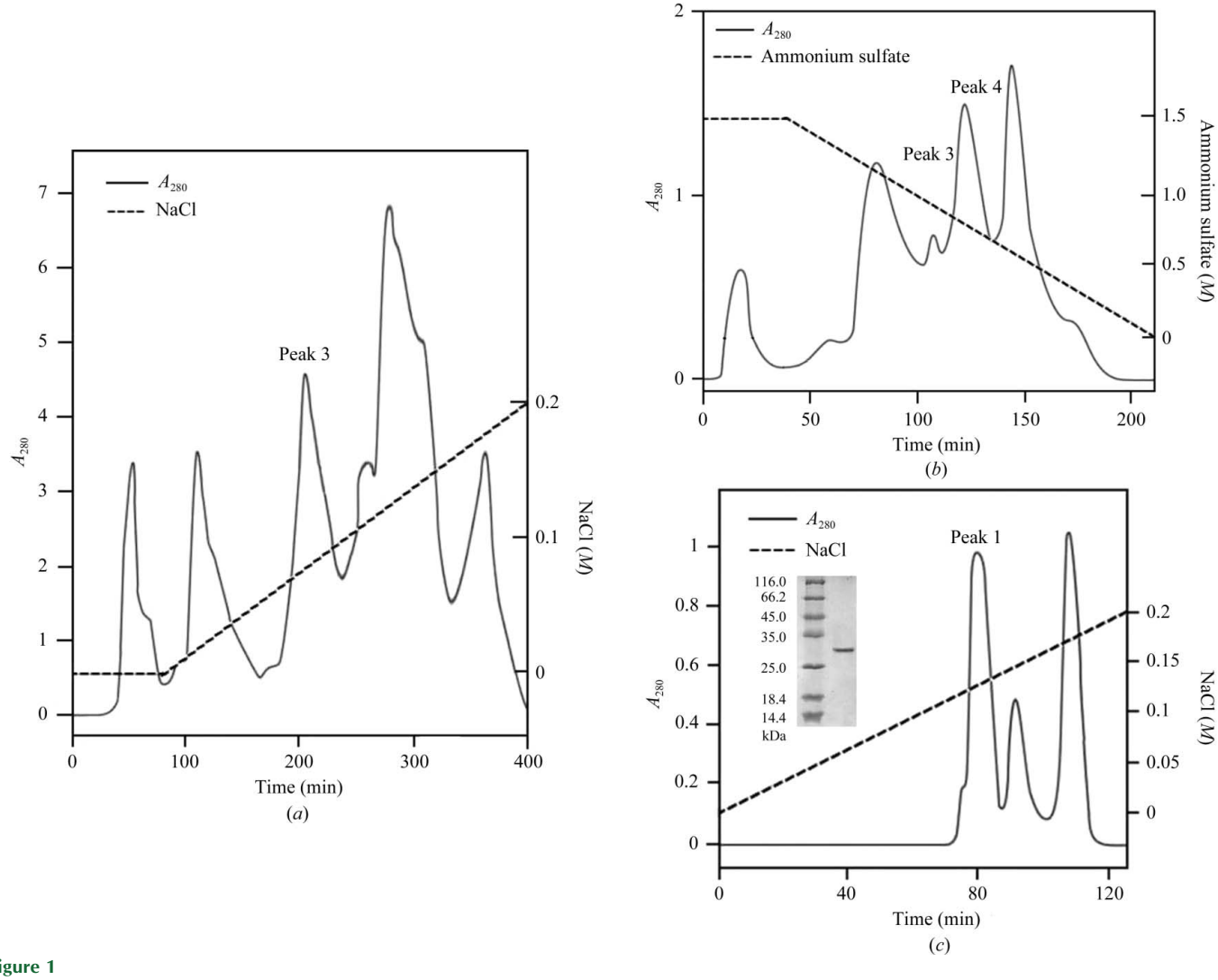


Figure 1
Preparation of saxthrombin using a three-step chromatography protocol. (*a*) DEAE-Sepharose anion-exchange chromatography. (*b*) Phenyl-Sepharose hydrophobic interaction chromatography. (*c*) CM-Sepharose cation-exchange chromatography.

onto a CM-Sepharose Fast Flow column pre-equilibrated with loading buffer *C*. The column was eluted with 300 ml of a linear NaCl gradient from 0.0 to 0.2 *M*. Peak 1 was pooled, concentrated and desalted. Saxthrombin was purified to high homogeneity and its molecular weight was approximately 30 kDa as estimated by SDS–PAGE, which is close to the predicted weight.

2.3. Enzyme assays

We used the method of Hahn *et al.* (1996) to measure the clotting activity of saxthrombin. The clotting activity was assayed at 310 K using 4 mg ml^{-1} bovine fibrinogen in 0.3 ml 50 m*M* Tris–HCl buffer pH 7.5 containing 0.9%(*w*/*v*) NaCl by recording the time required for the formation of clots after addition of the enzyme. The clots were observed at 310 K for several hours in order to investigate their stability.

The arginine esterase activity of saxthrombin was measured using the method of Nolan *et al.* (1976). The enzyme activity was assayed using TAME as a substrate. 0.5 µg purified enzyme was added to glass tubes containing 300 µl 0.5 m*M* TAME in 50 m*M* Tris–HCl pH 8.0 and 0.1% methanol. After thorough mixing, the increase in the absorbance at 247 nm was measured for 10 min at 293 K. One unit of TAME activity was defined as the amount of enzyme catalyzing the hydrolysis of 1 µmol of TAME per minute.

2.4. Crystallization

Crystallization experiments were performed at 293 K by the hanging-drop vapour-diffusion method, using Hampton Research Crystal Screens to screen for initial crystallization conditions. The protein was dissolved in 5 m*M* Tris–HCl pH 8.5 and its concentration was estimated to be 22 mg ml^{-1} using the Bradford assay. Microcrystals formed in conditions No. 46 and No. 95 of the Crystal Screen kit. Optimized crystals based on condition No. 46 were obtained by mixing 2 µl protein solution with 2 µl modified reservoir solution [containing 0.25 *M* calcium acetate, 0.1 *M* sodium cacodylate pH 6.5 and 16%(*w*/*v*) polyethylene glycol 8000] and then equilibrating against 300 µl reservoir solution. Two weeks later, a crystal of approximate dimensions 0.6 × 0.4 × 0.4 mm appeared (Fig. 2).

2.5. X-ray diffraction analysis and structure determination

All diffraction data were measured using a crystal cryoprotected in 0.20 *M* calcium acetate, 0.08 *M* sodium cacodylate pH 6.5, 12%(*w*/*v*) PEG 8000 and 20% glycerol. The crystal was directly flash-cooled in a stream of cold nitrogen gas at 105 K using an Oxford Cryosystems cooling device (Oxford Cryosystems Ltd, UK). X-ray diffraction data were collected using a MAR345 imaging-plate detector (MAR Research, Germany) with double-mirror focused Cu $K\alpha$ X-rays produced by a Rigaku RA-Micro007 rotating-anode generator. The oscillation angle was 1.0° and the exposure time was 10 min per frame. A total of 257 diffraction images were recorded from one crystal at a camera distance of 80 mm and the data were processed using *HKL*-2000 (Otwinowski & Minor, 1997). The data-collection and processing statistics are listed in Table 1.

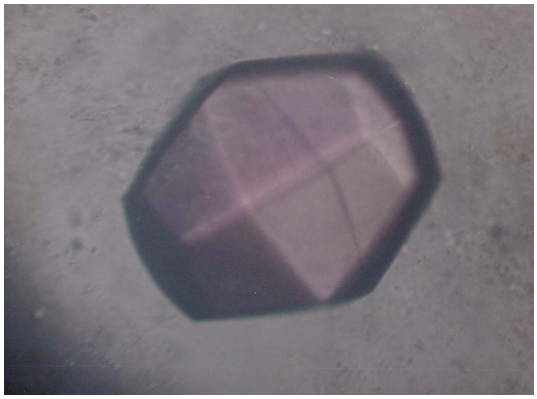


Figure 2
Crystal of saxthrombin.

Table 1
Data-collection and reduction statistics.

Values in parentheses are for the highest resolution shell.

Space group	*C*2
Unit-cell parameters (Å, °)	a = 97.23, b = 52.21, c = 50.10, β = 96.72
Molecules per ASU	1
Resolution limits (Å)	40–1.43 (1.48–1.43)
Observations	87989
Independent reflections	45350
Completeness (%)	98.2 (89.5)
$I/\sigma(I)$	7.7 (3.6)
$R_{\rm merge}$†	0.051 (0.291)

† $R_{\rm merge} = \sum_h \sum_j |I(h)_j - \langle I(h)\rangle| / \sum_h \sum_j I(h)_j$, where $I(h)_j$ is the observed intensity of a reflection and $\langle I(h)\rangle$ is the mean intensity of reflection h.

3. Results and discussion

Because the insoluble fibrin clots formed after intravenous injection of TLEs can rapidly be removed from the blood circulation, TLEs have been used in prevention of the formation of thrombi and in the reduction of blood viscosity. For these reasons, TLEs are purified on a large scale and are being commercially produced as clinical drugs. Here, we purified saxthrombin from *G. saxatilis* to homogeneity as evidenced by the single band on SDS–PAGE. The purified enzyme efficiently cleaves TAME with an arginine esterase activity of 2 554 U mg^{-1}.

Saxthrombin was crystallized in space group *C*2, with unit-cell parameters a = 97.23, b = 52.21, c = 50.10 Å, β = 96.72°. There is one molecule per asymmetric unit based on the Matthews coefficient ($V_{\rm M}$) of 2.13 Å^3 Da^{-1}, which corresponds to 42.4% solvent content. This TLE from *G. saxatilis* shares 86% amino-acid sequence identity with AaV-SP-I, a snake-venom serine proteinase from *Agkistrodon acutus* for which the structure has been solved (PDB code 1op0). Using this structure as a model, the phase problem was solved by molecular replacement. It is expected that our high-resolution structure will help us to understand the differences of this enzyme from thrombin and assist in thrombotic medicine research in the future.

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References

Castro, H. C., Zingali, R. B., Albuquerque, M. G., Pujol-Luz, M. & Rodrigues, C. R. (2004). *Cell. Mol. Life Sci.* **61**, 843–856.
Furukawa, K. & Ishimaru, S. (1990). *Medical Use of Snake Venom Proteins*, edited by K. F. Stocker, pp. 161–173. Boca Raton, FL, USA: CRC Press.

Hahn, B. S., Yang, K. Y., Park, E. M., Chang, I. M. & Kim, Y. S. (1996). *J. Biochem.* **119**, 835–843.
Huang, Q.-Q., Teng, M.-K. & Niu, L.-W. (1999). *Toxicon*, **37**, 999–1013.
Itoh, N., Tanaka, N., Mihashi, S. & Yamashina, I. (1987). *J. Biol. Chem.* **262**, 3132–3135.
Nolan, C., Hall, L. S. & Barlow, G. H. (1976). *Methods Enzymol.* **45**, 205–213.
Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
Sun, D. J., Yang, C. W., Yang, T. S., Yan, W. Q. & Wang, W. (2003). *Acta Zool. Sin.* **49**, 878–882.