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Purification, characterization, crystallization and preliminary X-ray diffraction of acuthrombin-B, a thrombin-like enzyme from *Agkistrodon acutus* venom

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Acuthrombin-B, a thrombin-like enzyme from *Agkistrodon acutus* venom, has been isolated and purified to homogeneity by ion-exchange chromatography on DEAE-Sepharose, gel filtration on Sephacryl S-100 and fast performance liquid chromatography on DEAE-8HR. The protease is an acid protein (p*I* 6.0) consisting of two non-identical polypeptide chains (14.4 and 16 kDa) and there is no disulfide bond between the subunits. Its molecular weight is 27 kDa as estimated by gel filtration on Sephacryl S-100. The protease has arginine-esterase activity and hydrolyzes synthetic substrates such as *p*-toluenesulfonyl arginine methyl ester and α-*N*-benzoyl-L-arginine amide ethyl ester, and shows clotting activity with human fibrinogen, rabbit citrated plasma and human citrated plasma *in vitro*. The specific activity with human fibrinogen was estimated to be 230 NIH units mg^{-1}. The protease is considered as a serine-type protease and contains metal ion(s) to some extent, as indicated by the fact that its clotting and arginine-esterase activities could be completely inhibited by PMSF and partially inhibited by the chelating agent EDTA, while the thrombin inhibitor heparin had no effect on its clotting activity towards rabbit citrated plasma. Acuthrombin-B crystals with a resolution limit of 2.06 Å were obtained by conventional hanging-drop vapour diffusion. The crystals belong to space group $P2_1$ with unit-cell parameters a = 34.97, b = 53.58, c = 67.88 Å, β = 98.89° and contain one molecule per asymmetric unit.

1. Introduction

It is known that thrombin plays a crucial role in the central event of blood coagulation by converting soluble fibrinogen to insoluble fibrin through a proteolytic reaction which releases two special polypeptides (FPA and FPB) from the N-terminus disulfide (NDS) knot of the fibrinogen Aα and Bβ chains (Shier & Mebs, 1990). The resulting fibrin monomers aggregate to form the fibrin matrix meshwork, which is resistant to fibrinolytic degradation (Blomback *et al.*, 1978). Thrombin also has the ability to activate coagulation factor XIII. To understand the mechanism of formation of the insoluble fibrin clot by thrombin, a number of crystal structures of complexes of thrombin with fibrinopeptides or other synthetic peptides have been determined and analyzed (Chen *et al.*, 1995). On the other hand, in the investigation of toxic biological materials, many snake-venom enzymes have been found to possess the ability to destroy the fibrinogen structure, including the thrombin-like enzymes (TLEs) which result in the clotting of fibrinogen *in vitro* (Shier & Mebs, 1990). Commonly, these TLEs are glycoproteins with a single polypeptide chain. They exhibit fibrinogen-clotting activity without the help of clotting factors *in vitro* and also show esterolytic activity on synthetic substrates such as *p*-toluenesulfonyl arginine methyl ester (TAME) and α-*N*-benzoyl-L-arginine amide ethyl ester (BAEE); both activities can be inhibited by special inhibitors of serine-type proteases, such as diisopropyl fluorophosphate (DFP) and phenylmethylsulfonyl fluoride (PMSF), but cannot be inhibited by the thrombin inhibitor heparin (Shier & Mebs, 1990). The snake-venom TLEs reported can be divided into three classes: FP-A, FP-B and FP-AB, according to whether the fibrinogen is hydrolyzed to produce FPA, FPB or both. TLEs can be also classified into two types (S and M) according to the numbers of subunits involved. Most TLEs can be considered to be members of the S type consisting of a single polypeptide chain. M-type TLEs are made of identical or non-identical multi-subunits; to our knowledge, there are only few TLEs belonging to this type, such as the TLE from the venom of *Cerastes cerastes* (Nolan *et al.*, 1976).

Since most TLEs release only FPA from fibrinogen and have no ability to activate factor XIII, they hydrolyze fibrinogen to produce non-cross-linked fibrins which are more susceptible to the lytic action of the exogenous plasmin than the thrombin-induced clots (Turpie *et al.*, 1971). After intravenous injec-

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tion, TLEs cause a rapid defibrinogenation through the proteolytic effect on fibrinogen and form non-cross-linked fibrin monomers which can be removed from the circulatory system either by the fibrinolytic reaction or *via* the reticuloendothelial system (Mahir *et al.*, 1987; Silberman *et al.*, 1983). Therefore, unlike thrombins, most TLEs act *in vitro* as procoagulants by converting fibrinogen to fibrin, while *in vivo* they cause defibrinogenation (Chang & Huang, 1995). Owing to these physiological properties, some TLEs have been used to prevent the formation of thrombi on the foreign surfaces of prostheses and extracoporeal devices and to improve the blood circulation in various vascular disorders by reducing blood viscosity (Bell, 1974; Lattalo, 1983; Stöcker, 1988).

The discovery and characterization of new member(s) of the TLE family might provide promising drugs for the treatment of disorders of the blood-circulation system. Furthermore, studies on the structure–function relationships of new TLEs should contribute to deeper understanding of the molecular diversity in the TLE family and the differences between TLEs and thrombins. Encouraged by these ideas, we investigated the snake venom of Chinese mainland *Agkistrodon acutus*. As part of a series of studies on the structure–function relationships of snake-venom TLEs, the purpose here is to provide details of the purification, characterization, crystallization and preliminary X-ray diffraction analysis of acuthrombin-B, a new serine-type protease with fibrinogen-clotting and arginine-esterase activities.

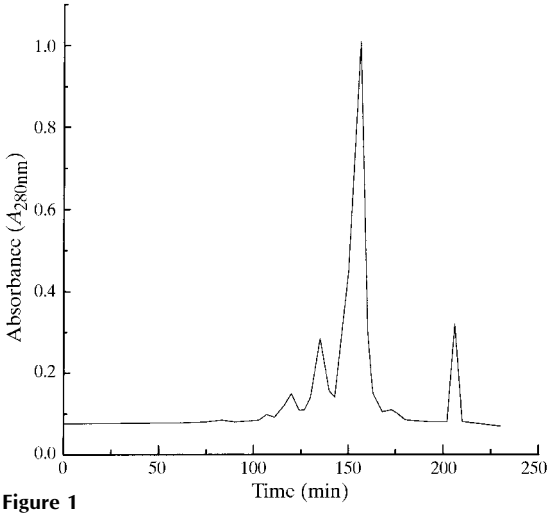

Figure 1
FPLC of acuthrombin-B. The column was equilibrated with 0.02 *M* Tris–HCl buffer (pH 8.0) and then eluted with 0.02 *M* Tris–HCl buffer (pH 8.0) with a 0–0.5 *M* linear concentration gradient of NaCl. The major peak which possessed the highest clotting activity was collected.

2. Experimental procedures

2.1. Materials

The dried crude venom of *Agkistrodon acutus* was obtained from the southern mountain region (Anhui, China). DEAE-Sepharose, CM-Sepharose, Sephacryl S-200, Sephacryl S-100 and ampholytes (pH 3–10) were produced by Pharmacia (Uppsala, Sweden). Waters 650E Advanced Protein Purification System and protein-Pak DEAE-8HR were purchased from Waters Co. (USA). Sodium dodecyl sulfate (SDS), acrylamide, *N*,*N*′-methylene bisacrylamide, *N*,*N*,*N*′,*N*′-tetramethyl ethylenediamine (TEMED) and PEG 4000 were purchased from Fluka Co. (Switzerland). Urea was produced by Promega (USA). Human thrombin and human fibrinogen were purchased from Tianjin Biochemical Technology Co. (Tianjin, China). Standard proteins for molecular weight estimation, BAEE, TAME, PMSF, benzoyl argininea-mide (BAA) and heparin were produced by Shanghai Dongfeng Biochemical Technology Co. (Shanghai, China). Other reagents and chemicals were of analytical grade from commercial sources.

2.2. Methods

2.2.1. Purification. Crude venom (2 g) was dissolved in 5 ml of starting buffer (0.02 *M* Tris–HCl pH 8.0), centrifuged to remove the insoluble materials at 4500*g*, extensively dialysed against the starting buffer and applied to a DEAE-Sepharose column (1.6 × 40 cm). The column was pre-equilibrated with starting buffer and eluted with 0.02 *M* Tris–HCl buffer containing a pH gradient from 8.0 to 6.5 and a 0–0.2 *M* NaCl gradient at a flow rate of 2 ml min^{-1}. The fraction possessing clotting activity was pooled, concentrated and applied to a Sephacryl S-200 column (1.6 × 80 cm). The column was pre-equilibrated with 0.15 *M* NaCl and eluted with the same solution. Similarly, the fraction from the gel filtration possessing the highest clotting activity was pooled, concentrated, desalted and applied to a protein-Pak DEAE-8HR column. The column was pre-equilibrated with 0.02 *M* Tris–HCl buffer (pH 8.0) and eluted with 0.02 *M* Tris–HCl buffer (pH 8.0, containing a 0–0.5 *M* NaCl gradient) at a flow rate of 1 ml min^{-1} (Fig. 1). The fraction with clotting activity was pooled, concentrated, desalted and freeze-dried for further characterization.

2.2.2. Molecular weight and isoelectric point estimation. The molecular weight was estimated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) with and without β-mercaptoethanol, and also by gel filtration on a Sephacryl S-100 column (0.9 × 100 cm) eluted with 0.15 *M* NaCl, the marker proteins used being RNAase (molecular weight 13 kDa), trypsin inhibitor (20.1 kDa), *Vitreoscilla* haemoglobin (31 kDa), egg albumin (45 kDa) and bovine albumin (68 kDa).

The isoelectric point was estimated by isoelectric focusing (IEF) on an Ampholine polyacrylamide slab gel using pH 3–10 ampholytes under the conditions specified by the manufacturer. After the focusing period, equal samples were cut from the gel and placed in 1 ml of distilled water and the pH gradient was measured potentiometrically after 24 h diffusion at 277 K. The slab gels were fixed and stained for proteins with Coomassie brilliant blue R250 (Vesterberg, 1973).

2.2.3. Carbohydrate assay. The neutral carbohydrate composition was determined by the method described by Dreywood (1946). 4 ml of anthrone reagent (0.2 g of anthrone dissolved in 100 ml of 98% H_2SO_4) was added to 1 ml of protease solution (1 mg ml^{-1}) on ice and incubated in boiling water for 15 min. After the reaction mixture was cooled to room temperature, the absorbance of the mixture was recorded at 640 nm. Glucose was used to obtained the standard curve.

2.2.4. Clotting activity. The clotting activity was assayed at 310 K with 0.5%(*w*/*v*) pre-heated human fibrinogen in 0.2 ml of 0.05 *M* Tris–HCl buffer pH 7.6 containing 0.15 *M* NaCl by recording the time required from the enzyme addition to the formation of clots (Quadar Pasha *et al.*, 1988). Human thrombin was used to obtain the standard clotting curve. One unit of clotting activity was defined as the amount of protein (in μM) necessary to induce the same response as the standard thrombin. The stability of the clot was investigated by keeping it under observation at 310 K for several hours. The rabbit citrated plasma and human citrated plasma were also used as substrates in the clotting assays.

2.2.5. Proteolytic activity. The caseinolytic activity was assayed by the method of Kunitz (1947). The protein sample (1 mg ml^{-1}) to be tested was incubated with

1%(*w*/*v*) casein in 2 ml of 0.1 *M* Tris–HCl buffer pH 7.6 for 30 min at 310 K before the addition of 3 ml of 5%(*v*/*v*) trichloroacetic acid. The mixture was then centrifuged at 4500*g* for 15 min. The absorbance of the supernatant was recorded at 280 nm. One unit of caseinolytic activity was defined as the amount of enzyme which induced a 0.001 absorbance unit increase in absorbance per minute.

2.2.6. Factor XIII activation activity. The factor XIII activation activity was determined according to Pirkle (1988). 0.2 ml of the protease solution (1 mg ml^{-1}) and 0.2 ml of 0.025 *M* $CaCl_2$ were added to the rabbit citrated plasma (0.2 ml). 30 min after the clotting of the reaction mixture, 1 ml of 5 *M* urea was added and the time required for the dissolution of the clot was recorded. Human thrombin acted as a control. The purified human fibrinogen was used instead of rabbit citrated plasma as a second control.

2.2.7. Arginine-esterase activity. The reaction mixture contained 0.5 ml of 10 m*M* BAEE, 10 m*M* BAA or 10 m*M* TAME, 0.5 ml of 0.1 *M* Na_2HPO_4/NaH_2PO_4 buffer (pH 7.6) and 0.2 ml of protease solution (1 mg ml^{-1}). The residual ester was determined with NH_2OH–$FeCl_3$ after incubation at 310 K for 30 min (Sato *et al.*, 1965).

2.2.8. Lethal activity. The lethal activity was assayed by intraperitoneal (ip) injection of 0.5 ml of protease solution (containing 0.15 *M* NaCl) into mice weighing 18–22 g. Five dose levels (0, 5, 10, 20 and 50 mg kg^{-1}) and six mice per dose level were used. The 50% lethal dose (LD_{50}) was determined according to the method of Litchfield & Wilcoxon (1949).

2.2.9. Haemorrhagic activity. The haemorrhagic activity was assayed by the method of Kondo *et al.* (1960). 0.1 ml of protease solution (0.5 mg ml^{-1}) was injected into depilated skin on the back of albino rabbits. In the controls, 0.1 ml of normal physiological saline was used instead of the protease solution. The animals were killed humanely 24 h later and the skins were removed. The minimum haemorrhagic dose (MHD) was defined as the amount of protease producing a haemorrhagic spot with a mean diameter of 10 mm.

2.2.10. Inhibition studies. The effects of different protease inhibitors and thrombin inhibitors on both clotting activity and arginine-esterase activity were examined. 2 ml of protease solution (1 mg ml^{-1}) was mixed with 0.2 ml of solution containing 500 m*M* EDTA, 0.5%(*w*/*v*) heparin or 100 m*M* PMSF. The mixture was then incubated at 310 K for 30 min and subjected to the fibrinogen assay, the citrated plasma-clotting assays or the arginine-ester hydrolysis assay.

2.2.11. Crystallization and preliminary X-ray diffraction analysis. The acuthrombin-B sample was checked for purity using SDS–PAGE and then concentrated to 30 mg ml^{-1}. The crystals were grown by conventional hanging-drop vapour diffusion using tissue-culture plates and siliconized glass cover slips (McPherson, 1982). For the first crystallization screen, 98 crystallization conditions were analyzed using the Hampton Crystal Screen based on the sparse-matrix sampling method of Jancarik & Kim (1991). The screen was carried out at room temperature (about 300 K) using 4 µl hanging drops. Crystals appeared in drops containing equal volumes of protein concentrate and a reservoir solution consisting of 0.1 *M* sodium HEPES buffer (pH 7.5), 10%(*v*/*v*) 2-propanol and 20%(*w*/*v*) PEG 4000. These crystals grew to approximate dimensions of 0.1 × 0.15 × 0.4 mm after two weeks, but were too small to be suitable for X-ray diffraction studies. To refine this crystallization condition, the pH and precipitant concentrations were optimized based on narrowing sets of conditions. Better quality crystals were obtained from drops containing equal volumes of protein concentrate and a reservoir solution consisting of 0.02 *M* Tris–HCl buffer (pH 7.2) and 20%(*w*/*v*) PEG 4000 after two weeks, but these crystals were still unsuitable for data collection. After many trial-and-error experiments, improved crystals could be obtained by the following method: 2.5 µl of protein concentrate was mixed with an equal volume of precipitant solution [0.02 *M* Tris–HCl pH 7.2, containing 20%(*w*/*v*) PEG 4000 and 0.01 *M* $MgCl_2$] and then equilibrated against 0.5 ml of the same precipitant solution. Single crystals of maximum dimensions 0.5 × 0.4 × 0.2 mm which were suitable for X-ray diffraction were obtained after two weeks (Fig. 2).

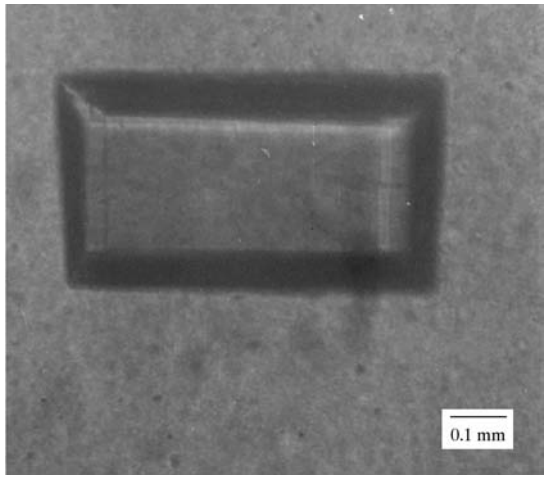

Figure 2
Crystal of acuthrombin-B.

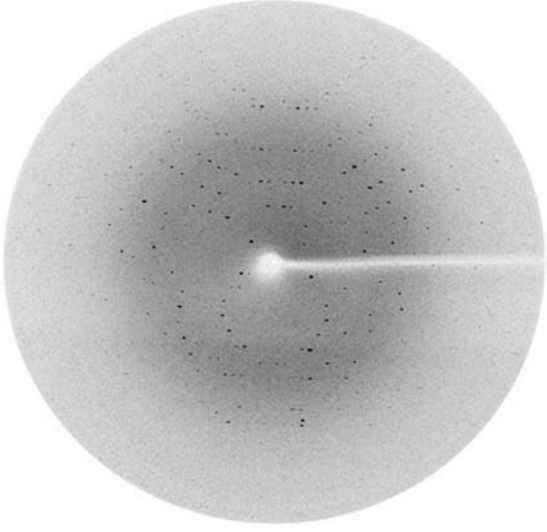

Figure 3
A diffraction pattern of the acuthrombin-B crystal.

Diffraction data were collected from one crystal at room temperature in our laboratory using a MAR Research imaging plate (diameter 300 mm) mounted on a MAR Research X-ray generator with a graphite monochromator and sealed copper-target tube. The working tube voltage and current were 40 kV and 50 mA, respectively. A total of 180 imaging frames were recorded at a 120 mm crystal-to-detector distance, 1° oscillation angle and 600 s exposure time per imaging frame (Fig. 3). The diffraction data were processed using *DENZO* and *SCALEPACK* (Otwinowski, 1993; Minor, 1993). The self-rotation function analysis was performed using *X-PLOR* (Brünger, 1992).

3. Results

3.1. Chemical characterization

On an SDS–PAGE gel under reducing or non-reducing conditions, the purified

Table 1
Comparison of enzyme K_m values for BAEE as substrate.

Source	Enzyme	K_m (M)	Reference
Bovine	Trypsin	5.0×10^{-5}	Penasse (1974)
Mammal	Thrombin	5.6×10^{-5}	Magnusson (1971)
Lachesis muta noct.	Arginine esterase	1.14×10^{-5}	Silva *et al.* (1985)
Lachesis muta muta	Fibrinogen-clotting enzyme	2.05×10^{-5}	Yarleque *et al.* (1989)
Crotalus adamanteus	Enzyme	2.05×10^{-4}	Markland & Damus (1971)
Cerastes vipera	Cerastobin	2.2×10^{-3}	Farid *et al.* (1989)
Cerastes cerastes	Proteinase RP34	3.0×10^{-4}	Laraba-Djebari *et al.* (1992)
Agkistrodon acutus	Acuthrombin-B	2.7×10^{-3}	See text

Table 2
Diffraction data collection and reduction statistics.

Space group	$P2_1$
Unit-cell parameters	
a (Å)	34.97
b (Å)	53.58
c (Å)	67.88
β (°)	98.89
Number of reflections	49684
Number of independent reflections	15616
Resolution limits (Å)	30.0–2.06
$R_{\rm merge}$† (%)	6.8 (28.4)‡
Completeness§ (%)	93.0 (90.8)‡

† $R_{\rm merge} = \sum\sum_i |\langle I(h)\rangle - I(h_i)_i| / \sum\sum_i I(h)_i$, where $I(h)_i$ is the intensity of the ith observation of reflection h and $\langle I(h)\rangle$ is the mean intensity of reflection h. § The completeness is the ratio of observed reflections to possible reflections. ‡ The values in parentheses are for the highest resolution shell (2.11–2.06 Å).

acuthrombin-B showed two protein bands corresponding to molecular weights of 14.4 and 16 kDa (Fig. 4), while its molecular weight was estimated to be 27 kDa by gel filtration on a Sephacryl S-100 column. The protease moved as a single band on a IEF gel and the isoelectric point was estimated to be 6.0. Moreover, the protease was found to be a glycoprotein as indicated by anthrone reagent, corresponding to about 1% neutral carbohydrates.

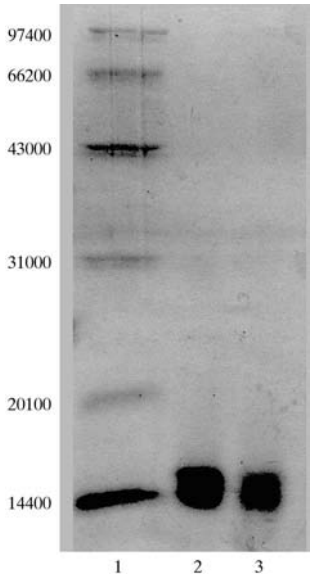

Figure 4
SDS–PAGE of acuthrombin-B. The samples were electrophoresed in 15% polyacrylamide and Tris–glycine buffer (pH 8.3). Lane 1, standard proteins; lane 2, acuthrombin-B under non-reducing conditions; lane 3, acuthrombin-B under reducing conditions.

3.2. Biological characterization

Acuthrombin-B was found to be able to clot human fibrinogen with a specific activity of about 230 NIH units mg^{-1}. The resulting clots were sensitive to gentle shaking by hand and were found to be unstable on standing after incubation at 310 K for several hours, indicating that the clots were unstable compared with those formed by thrombin treatment of fibrinogen. The arginine esters TAME and BAEE were found to be hydrolyzed by purified acuthrombin-B, while BAA was not. Using TAME and BAEE as substrates, the kinetic parameters K_m and $V_{\rm max}$ were 1.4×10^3 μM and 0.358 μM min^{-1} mg^{-1} and 2.7×10^3 μM and 0.474 μM min^{-1} mg^{-1}, respectively. For BAEE (Table 1), the apparent affinity of acuthrombin B is similar to that of cerastobin from *Cerastes vipera* venom. The dependence of the arginine-esterase activity on pH was examined in the pH range 3.0–11.0 with BAEE and TAME as substrates; the optimum point was found to be at about pH 7.5 in 0.02 M Tris–HCl buffer. Both the clotting and arginine-esterase activities could be noticeably inhibited by 10 mM PMSF or 50 mM EDTA but not by 0.05% (w/v) heparin. Furthermore, no clots were observed if the protease was incubated with 10 mM PMSF at 310 K for more than 30 min before the clotting-activity assay. The clotting time was prolonged by more than ten times if the protease was incubated with 50 mM EDTA at 310 K for more than 30 min before the clotting-activity assay, and the resulting clots were very sensitive to gentle shaking by hand. The clot formed by the action of acuthrombin-B on rabbit citrated plasma or human citrated plasma was found to dissolve 10 min after the addition of 5 M urea solution, indicated that the protease had no factor XIII activation activity. As a control, clots formed by the action of human thrombin on the same substrates did not dissolve even 24 h after the addition of 5 M urea solution. As a second control, clots formed by acuthrombin-B or human thrombin on purified human fibrinogen were found to dissolve in 5 M urea solution in 10 min.

Neither lethal activity nor haemorrhagic activity was observed, as shown by the facts that no mouse died 10 d after injection at the highest dose level (ip injection of 50 mg kg^{-1}) and that no haemorrhagic spot was produced after 100 mg of sample was injected into the depilated skin of the back of albino rabbits. Additionally, no caseinolytic activity was found.

3.3. Preliminary X-ray diffraction analysis

The crystals were found to belong to the monoclinic system, with unit-cell parameters a = 34.97, b = 53.58, c = 67.88 Å and β = 98.89°. The space group should be $P2_1$, since only the (0k0) reflections where $k = 2n + 1$ were absent. Based on the molecular mass and the unit-cell dimensions, only one acuthrombin-B molecule is present in each asymmetric unit, corresponding to a volume/mass ratio V_m of 2.513 $Å^3$ Da^{-1} (Matthews, 1968). The results of self-rotation function analysis also clearly indicated that there was no non-crystallographic symmetry in the asymmetric unit. Data collection and reduction statistics are summarized in Table 2.

4. Discussion

The serine-type protease acuthrombin-B was isolated and purified from the venom of *Agkistrodon acutus* by ion-exchange chromatography on DEAE-Sepharose, gel filtration on Sephacryl S-200 and FPLC on DEAE-8HR. The protease showed microhomogeneity, as indicated by its unique pI value, and a single protein band on SDS–PAGE. Crystals suitable for X-ray diffraction were obtained by conventional hanging-drop vapour diffusion. Similar to most TLEs purified from the snake venoms, acuthrombin-B possesses arginine-esterase activity, hydrolyzing synthetic substrates such as TAME and BAEE, and clotting activity with human fibrinogen, rabbit citrated plasma and human citrated plasma *in vitro*; both these activities can be thoroughly inhibited by PMSF but not by the thrombin inhibitor heparin. Furthermore, the protease has no ability to activate coagulation factor XIII. These experimental observations suggest that acuthrombin-B

might be a new member of the TLE family. However, the protease showed two protein bands corresponding to molecular weights of 16 kDa (α-subunit) and 14.4 kDa (β-subunit) on SDS–PAGE under reducing or non-reducing conditions, while only one peak appeared on the gel-filtration profile, corresponding to a molecular weight of 27 kDa. Only one molecule was observed to be present in each asymmetric unit of the crystal cell and the self-rotation function analysis clearly indicated that there was no non-crystallographic symmetry in the asymmetric unit. For these reasons, acuthrombin-B should be considered to consist of two non-identical subunits with the molecular formation $\alpha\beta$ and with no disulfide bonds between the subunits. To our knowledge, the TLEs from other snake venoms are of a single polypeptide chain type, with the exception of RP-34 from the venom of *Cerastes cerastes* (Bell, 1974) which consists of two identical subunits. Moreover, acuthrombin-B seems to contain metal ion(s), as its clotting and arginine-esterase activities could be significantly inhibited by EDTA. In contrast, to our knowledge, there are no metal ions in TLEs purified from other snake venoms, and their clotting and esterolytic activities are not affected by EDTA.

Acuthrombin-B might be useful as an anticoagulant drug since it has no lethal or haemorrhagic activities. In fact, many TLEs are being commercially produced as clinical theraputical drugs, such as Batroxobin from the venom of *Bothrops atrox* and its subspecies (Stöcker & Barlow, 1976) and Ancrod from the venom of *Agkistrodon rhodostoma* (Nolan *et al.*, 1976).

Owing to the differences in the important biological functions and molecular compositions between TLEs and thrombins and between the members of the TLE family, it is surely the case that significant differences must exist in their three-dimensional structures. As far as we are aware, no crystal structure in the TLE family has been reported. Therefore, the crystal structure determination of acuthrombin-B will not only reveal the structure–function relationship itself, but also deepen our knowledge of the molecular diversity of the clotting enzymes. It will also help us to understand the clotting mechanism through which fibrinogen is cleaved by TLEs and thrombins. This work is being carried out in our laboratory.

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