

Functional and structural analysis of two fibrinogen-activating enzymes isolated from the venoms of *Crotalus durissus terrificus* and *Crotalus durissus collilineatus*

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Fibrinogen-activating enzymes, widely distributed in Crotalidae and Viperidae venoms, are single-chain glycosylated serine proteases that display high macromolecular selectivity and are often referred to as thrombin-like enzymes (TLEs). TLEs serve as structural models to extend our understanding of the structure–function relationships of blood coagulation factors, have been clinically used for the treatment of thrombotic diseases, and are used as tools in clinical assays. The combination of gel filtration and ion-exchange chromatography proved to be successful in obtaining milligram quantities of pure samples of TLEs from the venoms of *Crotalus durissus terrificus* (white venom) and *Crotalus durissus collilineatus* (yellow venom). Functional characterization indicates that both enzymes preferentially degrade the B β chain of bovine fibrinogen and possess edema-inducing and coagulant activities. However, the TLE from *C. d. collilineatus* venom shows twofold higher coagulant activity with a minimum coagulant dose (MCD) of 0.6 $\mu\text{g}/\mu\text{l}$, whereas the enzyme isolated from *C. d. terrificus* indicated an MCD of 1.5 $\mu\text{g}/\mu\text{l}$. Molecular modeling of gyroxin and structural comparisons with other highly conserved snake venom serine proteases, underlines the key role played by the surface charge distribution and the double insertion in the 174-surface loop in macromolecular substrate recognition by TLEs.

Keywords *Crotalus durissus* sp; thrombin-like enzyme; functional characterization; crystallization; molecular modeling

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Introduction

Crotalidae venom serine and metalloproteases interfere in the regulation, control, and maintenance of the complex hemostatic system and hence; many of them have found uses in the diagnosis and treatment of hemostatic disorders such as protein C activator (PROTAC) (Pentapharm Ltd) and DEFIBRASE (American Diagnostica Inc.) [1]. The range of activities exhibited by these enzymes includes hemorrhagic, procoagulant, anticoagulant, platelet activation, and bradykinin-releasing effects [2]. Based on their functions, the procoagulant snake venom proteases are classified as factor I [thrombin-like enzymes (TLEs)], factor V, factor VII, factor X, groups C and D prothrombin activators [3].

Crotalidae and Viperidae venoms TLEs are single-chain glycosylated serine proteases that utilize the same structural scaffold and display high sequence similarities. Similar to the mammalian serine proteases, the aspartate residue is conserved in the S1 subsite at position 189 resulting in the preferential cleavage of substrate arginyl bonds. Although thrombin preferentially releases fibrinopeptide A from fibrinogen, TLEs release either fibrinopeptide A or B, rarely both with equal efficiency [4]. Since TLEs do not activate factor XIII, the resulting fibrin clot is not cross-linked hence, is easily degraded by the fibrinolytic system, and removed from circulation by the reticuloendothelial system causing acute defibrinogenating effects. Fibrinogen is an important component of the coagulation cascade and contributes to the maintenance of blood viscosity [5]. Epidemiological studies have demonstrated that fibrinogen is a major risk

factor for ischemic heart disease, stroke, and peripheral circulatory disturbances [6–8].

Since TLEs are clinically well tolerated with minimal secondary effects [4], they have been extensively tested in the treatment of several hemostatic disorders and serve as excellent structural models to understand structure–function–specificity relationships of blood coagulation factors.

In this work, two fibrinogen-activating enzymes from *Crotalus durissus terrificus* and *Crotalus durissus collilineatus* were purified and functionally characterized. In addition, structural studies with a member of TLE subgroup were carried out to shed light on the molecular basis for its mechanistic and macromolecular substrate recognition.

Materials and Methods

Purification

Desiccated crude venoms (100 mg) of *C. d. terrificus* (white) and *C. d. collilineatus* (yellow) venoms obtained from a local serpentarium were dissolved in 2 ml of 0.05 M ammonium formate buffer (pH 3.0) and centrifuged at 10,000 g to remove insoluble materials. Similar purification procedures were used for both the enzymes. The clear supernatant was applied to a Sephadex G-75 column, pre-equilibrated with the fore-mentioned buffer, linked to an FPLC (Pharmacia) system utilizing a flow rate of 30 ml/h. Protein elution was measured at 280 nm and esterase activity was monitored at 247 nm. Fractions with esterase and coagulant activities were pooled, dialysed in 0.05 M ammonium bicarbonate buffer (pH 8.0), and applied to an ES-502 N ion-exchange column (Shimadzu-HPLC) at a flow rate of 0.8 ml/min, and protein fractions were eluted using a linear 0–0.5 M ammonium bicarbonate gradient. The fractions showing esterase and coagulant activities were pooled, dialysed, lyophilized, and stored at 0°C. The homogeneity of the samples was confirmed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE).

Polyacrylamide gel electrophoresis

SDS–PAGE gels (12%; w/v) were prepared by the previously described method [9]. Samples were pretreated under reducing conditions (SDS plus β -mercaptoethanol) at a 100°C for 5 min. Gels were stained with 0.1% Coomassie brilliant blue R-250 in ethanol:acetic acid (5:1; v/v) for 15 min and destained in 10% acetic acid. The molecular masses were estimated by interpolation from a linear logarithmic plot of relative molecular mass

vs. distance of migration. Molecular weight markers used were phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovoalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa), and α -lactalbumin (14.4 kDa). Protein concentration was determined by the method of Bradford [10] using the lysozyme as the standard.

Enzyme assay

Esterase activity was determined using 0.01 M Na-P-tosyl-L-arginine-methyl ester (TAME) as the substrate in a solution containing 0.1 M Tris–HCl buffer, pH 7.8 and 37°C as described by Ehrempreis and Scheraga [11]. Enzyme solutions (50 μ l) were incubated in 2.5 ml of the TAME for 15 min at 37°C. The reactions were interrupted by the addition of 0.1 ml glacial acetic acid and the resulting products were measured at 247 nm. One unit of enzymatic activity was defined as the amount of enzyme capable of increasing the absorbance by 0.001 AU at 247 nm/min. Fibrinogen-clotting activity was carried out using human plasma incubated in 20 μ l of the fraction. The clotting time was determined as the time required for the first sudden appearance of the fibrin net. Results were expressed as the inverse percentage given to the following equation: $CT = 1/\Delta \times 100$, where CT is the clotting time and Δ the medium time in seconds.

Enzyme inhibitors and substrate specificity

Inhibition of the esterase activity was assayed after pre-incubation of 2 μ g of the enzyme in 0.1 M Tris–HCl buffer, pH 8.0 for 30 min at 37°C containing one of the following inhibitors: 20 mM phenylmethylsulfonyl fluoride (PMSF), 8 trypsin inhibitor unit (TIU)/ml aprotinin, 20 mM 1,10-phenanthroline, 20 mM leupeptin, 20 mM ethylenediaminetetraacetic acid (EDTA), 20 mM ethylene glycol-bis (β -amino ethyl ether) tetra acetic acid (EGTA), and 20 mM heparin, as previously described.

Enzyme (2 μ g) in 0.05 M Tris–HCl buffer, pH 7.6 containing 0.15 M NaCl was incubated with one of the following substrates: S-2251 substrate for plasmin and streptokinase-activated plasminogen or S-2238 chromogenic substrate for thrombin; for 10 min at 37°C. The chromogenic products were monitored at 405 nm.

Coagulant activity

Samples of 200 μ l of plasma were incubated with different amounts of the enzyme or crude venoms (0.5, 1, 2, 4, 6, 8, and 10 μ g) in 25 μ l phosphate-buffered saline (PBS) and the coagulation time was recorded.

The minimum coagulant dose (MCD) was defined as the minimum concentration of enzyme capable of coagulating human plasma in 1 min. As a control, a plasma aliquot was incubated with 25 μ l of a 0.25 mM solution of CaCl_2 and the coagulation time was determined as mentioned earlier. Inhibition of coagulant activity was assayed after pre-incubation of 20 μ g of the enzyme in 0.1 M Tris–HCl buffer, pH 8.0 for 30 min at 37°C containing either 2 mM PMSF or 2 mM benzamidine.

Fibrinogenolytic activity

The method described by Rodrigues *et al.* [12] was used with modifications to determine the fibrinogenolytic activity. Samples of 30 μ l of bovine fibrinogen (3 μ g/ μ l PBS, pH 8.0) were incubated with different amounts of enzyme (0.5–10 μ g) at 37°C for 2 h. The reaction was terminated by the addition of 30 μ l of 0.05 M Tris–HCl buffer, pH 8.8, containing 10% (v/v) 2-mercaptoethanol, 2% (v/v) SDS, and 0.05% (w/v) bromophenol blue and heating at 100°C for 5 min. The samples were then analysed by SDS–PAGE gels (13.5%; w/v).

Edema-inducing activity

PBS solutions (50 μ l) containing 5–100 μ g of the enzymes were injected in the subplantar regions of groups of five Swiss mice (18–22 g). Control animals were injected with 50 μ l of the PBS solution. After 0.5, 1, 2, 4, and 8 h, the progression of edema was evaluated with a low-pressure pachymeter, and expressed as the percentage of induced edema [13].

Crystallization

The CdtII-2 and CdcII-3 samples were concentrated to 16 mg/ml in microconcentrators (Amicon, Centricon) and stored in 0.02 M phosphate buffer (pH 7.8). Crystallization was carried out by the hanging-drop vapor diffusion method using ready-to-use protein crystallization kits commercialized by Hampton Research (Crystal Screen I and II) [14]. Typically, 1 μ l of protein solution was mixed with an equal volume of the screening solution and equilibrated over a reservoir containing 1 ml of the latter solution. Once initial crystallization conditions had been determined, they were optimized using grid screen method by varying the pH and precipitant concentration.

Molecular modeling and quality analysis

The atomic coordinates of protein C activator isolated from *Agkistrodon contortrix contortrix* venom [15,16] were used to obtain a structural model of the serine

proteases from *C. d. terrificus* venom, gyroXin based on restraint-based modeling as implemented in the MODELLER program [17]. The overall model was improved by enforcing the proper stereochemistry using spatial restraints and CHARMM energy terms, followed by conjugate gradient simulation based on the variable target function method [17]. The loops were optimized using ModLoop [18] based on the satisfaction of spatial restraints, without relying on a database of known protein structures. The overall and local quality analyses of the final model were assessed by VERIFY3D [19], PROSA [20], and VADAR [21]. Three-dimensional structures were displayed, analysed, and compared using the program COOT [22].

Results and Discussion

Purification of thrombin-like enzymes from *C. d. terrificus* and *C. d. collilineatus* venoms

Four peaks were obtained after crude venom fractionation on the Sephadex G-75 molecular exclusion column of both sources [Fig. 1(A) and (D)]. All fractions were tested for esterase and coagulant activities, and only peak 2 displayed both esterase and coagulant activities. The fractions corresponding to peak 2 were pooled and dialysed against 0.05 M ammonium bicarbonate buffer (pH 8.0) and submitted to an ES-502 N ion-exchange column. In both cases, the fractionation resulted in three major peaks [Fig. 1(B) and (E)]. The fractions were analysed by SDS–PAGE and tested for esterase and coagulant activities. On SDS–PAGE gels, under reduced conditions, the purified samples from both venoms (peak CdtII-2 from *C. d. terrificus* venom and peak CdcII-3 from *C. d. collilineatus* venom) migrated as a single protein band corresponding to a molecular weight of \sim 30 kDa [Fig. 1(C) and (F)].

Enzyme inhibitors and substrate specificity

As indicated in Table 1, both enzymes were completely inhibited by leupeptin, a strong inhibitor of serine proteases such as trypsin, plasmin, and kallikrein, in agreement with the tests with chromogenic substrates. PMSF, another canonical serine protease inhibitor, which covalently binds to histidine residues, also significantly inhibited both enzymes reducing activity to around 35% (Table 1). Inhibitors specific for metalloproteases did not affect the activity of either enzyme.

Both purified enzymes were assayed on synthetic chromogenic substrates for streptokinase-activated plasminogen (S2251) and thrombin (S2238). Both purified

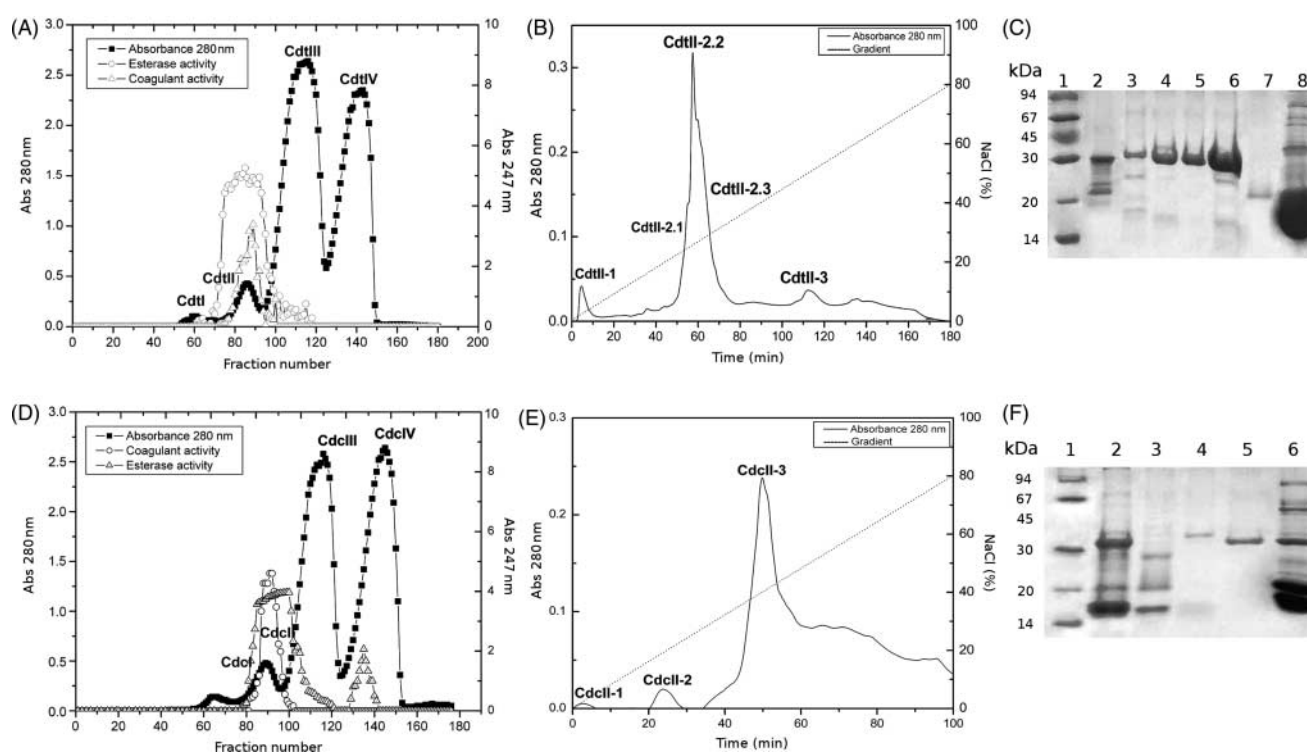


Fig. 1 Purification of thrombin-like enzymes from *Crotalus durissus terrificus* and *Crotalus durissus collilineatus* venoms (A) and (D) Gel filtration chromatograms of *Crotalus durissus terrificus* and *Crotalus durissus collilineatus* crude venom, respectively. (B) and (E) Anionic-exchange chromatograms of CdtII and CdcII peaks from gel filtration. (C) Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of *Crotalus durissus terrificus* fractions. Lane 1, low molecular weight markers; lane 2, CdtII fraction from gel filtration; lanes 3–7, fractions of CdtII-1, CdtII-2.1, CdtII-2.2, CdtII-2.3, and CdtII-3, respectively; lane 8, crude venom. (F) Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of *Crotalus durissus collilineatus* fractions. lane 1, low molecular weight markers; lane 2, CdcII fraction from gel filtration; lanes 3–5, fractions of CdcII-1, CdcII-2, and CdcII-3, respectively; lane 6, crude venom.

enzymes displayed significant activity on S2238 substrate, specific for thrombin, whereas no activity was observed for S2251 substrate. These results indicate that

these enzymes isolated from *Crotalus* sp (yellow and white venoms) are TLEs without detectable plasmin-like activity.

Table 1 Residual amidolytic activity of CdtII-2 and CdcII-3 in comparison with bovine trypsin in the presence of several inhibitors specific for serine proteases (phenylmethylsulfonyl fluoride, heparin, leupeptin, and aprotinin) and metalloproteases [ethylene glycol-bis (β-amino ethyl ether) tetra acetic acid, ethylenediaminetetraacetic acid, and 1,10-phenanthroline]

Inhibitor	Concentration	Residual activity (%)		
		Trypsin	CdtII-2	CdcII-3
Control	20 mM	100.0	100.0	100.0
PMSF	20 mM	65.0	31.7	43.6
EGTA	20 mM	91.2	100.0	100.0
EDTA	20 mM	100.0	91.6	92.6
Leupeptin	20 mM	5.4	0.0	0.0
Heparin	20 mM	97.2	100.0	100.0
1,10-Phenanthroline	20 mM	100.0	100.0	100.0
Aprotinin	8 TIU/ml	5.5	95.2	94.6

Activity monitored at 405 nm (n = 3).

Coagulant activity on human plasma

Both crude venoms of *C. d. terrificus* and *C. d. collilineatus* snakes showed a similar MCD ($\sim 8.2 \mu\text{g}/\mu\text{l}$) (Fig. 2). However, the purified enzyme from *C. d. collilineatus* venom displayed twofold higher coagulant activity with a minimum coagulant activity of $0.6 \mu\text{g}/\mu\text{l}$, whereas the enzyme isolated from *C. d. terrificus* indicated an MCD of $1.5 \mu\text{g}/\mu\text{l}$ (Fig. 2).

Fibrinolytic activity on purified bovine fibrinogen

Both purified TLEs from *C. d. terrificus* and *C. d. collilineatus* venoms degraded the $\text{A}\alpha$ and $\text{B}\beta$ chains of fibrinogen, in a dose–time-dependent manner (Fig. 3). The results showed that both enzymes preferentially degraded the $\text{B}\beta$ chain of fibrinogen and secondarily $\text{A}\alpha$ chain, at high enzyme dose ($>5 \mu\text{g}$) while the $\text{C}\gamma$ chain was unaffected (Fig. 3).

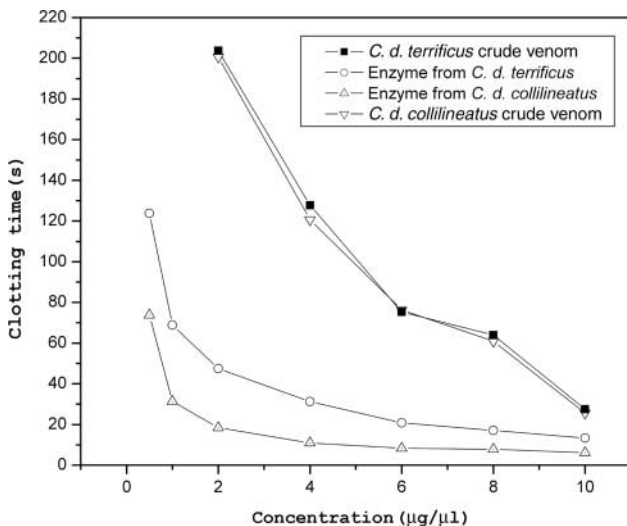


Fig. 2 Clotting time of coagulant activity of CdtII-2 and CdcII-3

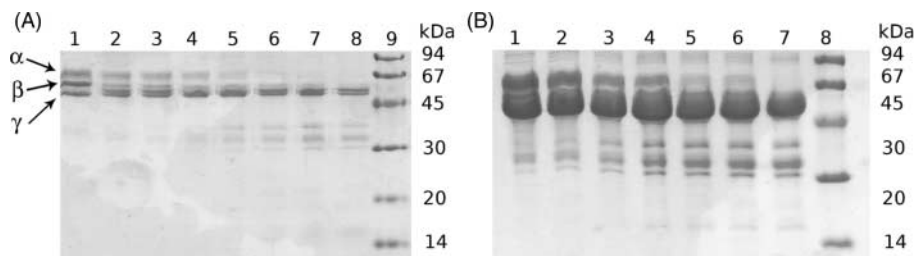


Fig. 3 Sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis of cleavage products of bovine fibrinogen by CdtII-2 and CdcII-3 Fibrinogen was prepared at $3 \mu\text{g}/\mu\text{l}$ and incubated with the enzyme at $0.5\text{--}10 \mu\text{g}$ for 2 h at 37°C . The fibrinogen incubated for 2 h was used as a control. (A) *Crotalus durissus terrificus*. Lane 1, control (fibrinogen); lane 2, $0.5 \mu\text{g}$; lane 3, $1.0 \mu\text{g}$; lane 4, $2.0 \mu\text{g}$; lane 5, $4.0 \mu\text{g}$; lane 6, $6.0 \mu\text{g}$; lane 7, $8.0 \mu\text{g}$; lane 8, $10.0 \mu\text{g}$; and lane 9, low molecular weight markers. (B) *Crotalus durissus collilineatus*. Lane 1, control (fibrinogen); lane 2, $0.5 \mu\text{g}$; lane 3, $1.0 \mu\text{g}$; lane 4, $2.0 \mu\text{g}$; lane 5, $4.0 \mu\text{g}$; lane 6, $6.0 \mu\text{g}$; lane 7, $8.0 \mu\text{g}$; and lane 8, low molecular weight markers.

Edema-inducing activity

Both enzymes, in a dose-independent manner, induced an edema of around 50% in the first 30 min with a subsequent decrease in a time-dependent manner (Fig. 4). The decrease was faster when the animal was treated with the enzyme isolated from *C. d. collilineatus* venom (Fig. 4).

Crystallization

Single crystals of the serine protease isolated from *C. d. collilineatus* venom (white venom) (CdcII-3) were obtained under several crystallization conditions. Single crystals were obtained when $2 \mu\text{l}$ protein droplet was mixed with an equal volume of reservoir solution consisting: (1) 0.1 M Tris–HCl, pH 8.0, 20% PEG 8000, and 0.2 M ammonium sulfate [Fig. 5(A)]; (2) 0.1 M sodium cacodylate, pH 6.5, 25% PEG 4000, and 0.2 M sodium chloride [Fig. 5(B)]; (3) 0.1 M HEPES, pH 7.5, 24% PEG 3350, and 0.2 M lithium chloride [Fig. 5(C)]; and (4) 0.1 M sodium cacodylate, pH 6.5, 18% PEG 8000, and 0.2 M magnesium chloride [Fig. 5(D)]. X-ray diffraction data of these crystals will be collected and their structures determined to elucidate the molecular basis for macromolecular recognition and activation of coagulation factors.

Molecular modeling of gyroxin

Gyroxin, a snake venom serine protease (SVSP) from *C. d. terrificus* venom, shares high sequence and structural similarity (up to 90%) with other SVSPs including a C-terminal extension uniquely observed in serine proteases isolated from snake venom glands (Fig. 6). Besides the high sequence and structural homology, each SVSP displays a different biological role, with high substrate selectivity, such as plasminogen activator [from *Trimeresurus stejnegeri*, (TSV-PA)], protein C activator

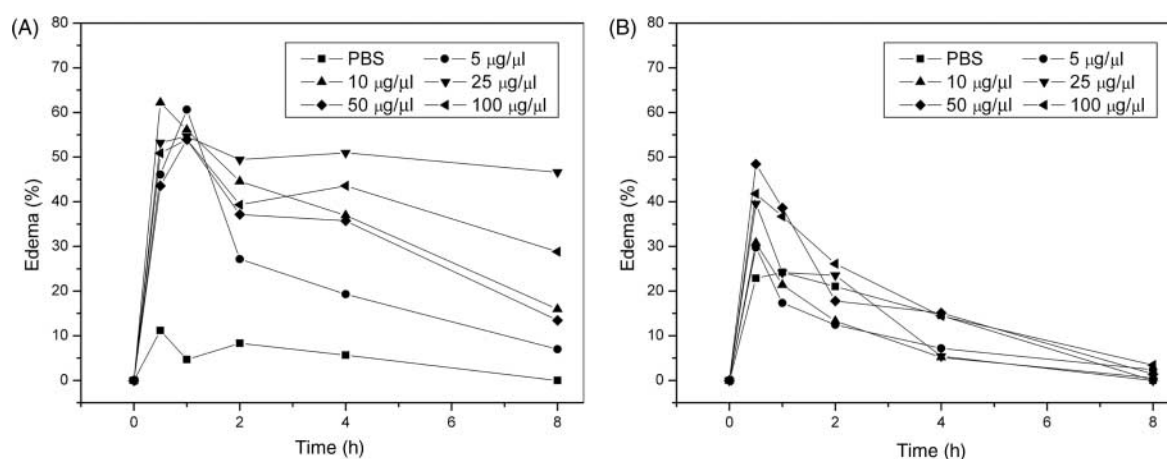


Fig. 4 Time-course of paw edema induced by CdtII-2 (A) and CdcII-3 (B) in 18–22 g male Swiss mice

(from *A. c. contortrix*, PROTAC), and fibrinogen-clotting activity (SVSPs from a number of snake venoms). These observations indicate that the structural determinants for their high substrate selectivity are limited to a few amino acid residues and surface charge alterations in the interfacial face around the catalytic site. Thus, in order to address the molecular basis for substrate recognition of the highly conserved class of TLEs, we have modeled the structure of gyroxin, a fibrinogen-clotting enzyme and compared it to the plasminogen activator from *T. stejnegeri* (TSV-PA) and protein C activator from PROTAC. We propose that TLEs, a subgroup of SVSPs, share very close structural features involved in the catalytic mechanistic and substrate

recognition and structural determinants encountered in gyroxin.

The overall and local quality of the final structural model of gyroxin was assessed by analysing its local and global stereochemical properties using VERIFY3D [19], PROSA [20], and VADAR [21] and by comparison with other crystallographic structures. The analysis of the Ramachandran diagram φ - ψ plots generated by VADAR of the gyroxin structure indicates 95.3% of the residues in most favorable region, 3.0% in additional allowed region, and 1.7% in disallowed region [23]. The calculations indicated an accessible surface area of 10,365.8 Å² and 65% of the residues form hydrogen bonds. Overall model quality assessed by PROSA

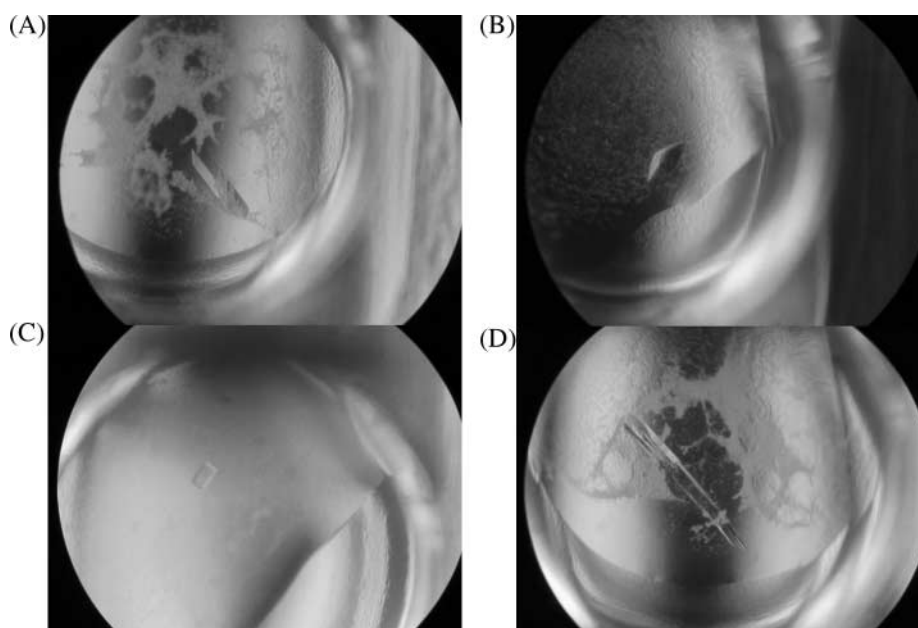


Fig. 5 Microphotograph of CdcII-3 crystals obtained by hanging-drop vapor diffusion

Gyroxin	VI GGDECNINEHRLLAIVY-TNSSQCAGTLINQEWWLTAAHCDGENMDIYLGVHNESVQY
PROTAC	VI GGDECNINEHRFLALVY-ANGSLCGGTLINQEWWLTAARHCDRGNMRYILGMHNLKVLN
Bovine trypsin	IVGGYTCGANTVPYQVSLN-SGYHFCGGSLLNSQWVVAACHYKSGIQVRLGEDNINVVE
TSV-PA	VFGGDECNINEHRSLVLFNSNGFLCGGTLINQDWWVTAACHDSNNFQLLFGVHSHKILN
Gyroxin	DDEEGRVAAEKFFCLSSRNYSKWDKDIMLIRLNIPVRNSTHIAPLSLSPSSPPSVGVSQRV
PROTAC	KDALRRFPKPKYFCLNTRNDTIWDKDIMLIRLNRPVRNSAHIAPLSLSPNPPSVGVSQRI
Bovine trypsin	GNE-QFISASKSIVHPSYNSNTLNNDIMLIKLSAASLNSRVASISLPTSCASAGTQCLI
TSV-PA	EDEQTRDPKPKFFFCPNRKKDDEVKDIMLIKLDSSVSNSEHIAPLSLSPSSPPSVGVSQRI
Gyroxin	MGWGTITSPNETYPDVPHCANINLFDYEVCLAAYPEFGLPATSRITLCAGIQQGGKDTCGS
PROTAC	MGWGTITSPNATLPDVPHCANINILDYAVCQAAYK--GLA--ATTLCAGILEGGKDTCKG
Bovine trypsin	SGWNTKSSGTSYPDVLKCKLAPILSDSSCKSAYPG-QIT--SNMFCAGYLEGGKDSQCG
TSV-PA	MGWGKTIPTKEIYPDVPHCANINILDHAVCRTAYSWRQVA--NTTLCAGILQGGRRDCHF
Gyroxin	DSGGSLICNGQFQGI VSWGDNPCAPHKFALYTKVLDDTEWIQSI IAGNTAVT CPP
PROTAC	DSGGPLICNGQFQGI LSVGGNPCAPRKPGEIYTKVFDYTDWIQSI ISGNTDATCPP
Bovine trypsin	DSGGPVVCSGKLGIVSWGSG-CAQKNKPGVYTKVCNYVSWIKQTIASN-----
TSV-PA	DSGGPLICNGIFQGI VSWGHPGCGPGEIYTKVFDYLDWIKSI IAGNKDATCPP

Fig. 6 Multiple sequence alignment of gyroxin (thrombin-like enzyme from *Crotalus durissus terrificus* venom), PROTAC (protein C activator from *Agkistrodon contortrix contortrix* venom), TSV-PA (plasminogen activator from *Trimeresurus stejnegeri* venom), and bovine trypsin

resulted in a Z-Score of -6.61 , which is in agreement with the Z-Scores of all experimentally determined protein chains in currently deposited with the Protein Data Bank. Local quality analysis assessed by plotting energies as a function of amino acid sequence position using PROSA and VERIFY3D resulted in no positive values for all residues in the energy plot, which indicates no problematic or erroneous parts of the structure. The careful analysis strongly indicates that the molecular model possesses good overall stereochemical quality and is suitable for structural analysis and comparisons.

The modeled structure conserves all structural features present in trypsin-like enzymes consisting of two domains each containing a six-stranded β -barrel and two short α -helices. The catalytic triad (His57, Asp102, and Ser195) is located at the junction of both barrels and is surrounded by the 37-, 60-, 70-, 99-, 148-, 174-, and 218-loops (sequence numbering is based on chymotrypsinogen) [16,24] (**Fig. 7**).

Superpositioning of the atomic coordinates of the PROTAC (protein C activator) on the modeled structure of the gyroxin (fibrinogen-activating enzyme) reveals significant differences in the 174-loop and mainly in the surface charge distribution around the interfacial face. The 174-loop comprising the residues 171–180, previously described as a part of macromolecular recognition mechanism found in SVSPs [16], has a two insertions in the gyroxin structure (PROTAC sequence 174-loop: AYK–GLA–ATTL; gyroxin sequence 174-loop: AYPEFGLPATSRITL) that probably plays an important role in substrate selectivity (**Fig. 7**). Another

critical difference observed between the PROTAC and gyroxin structures is the surface charge distribution pattern; whereas gyroxin has a diffuse negative charge around the active site, PROTAC contains an arginine belt which confers a strong positive charge distribution essential for recognition and binding of the Asp–Glu activation pro-peptide of protein C precursor (**Fig. 8**).

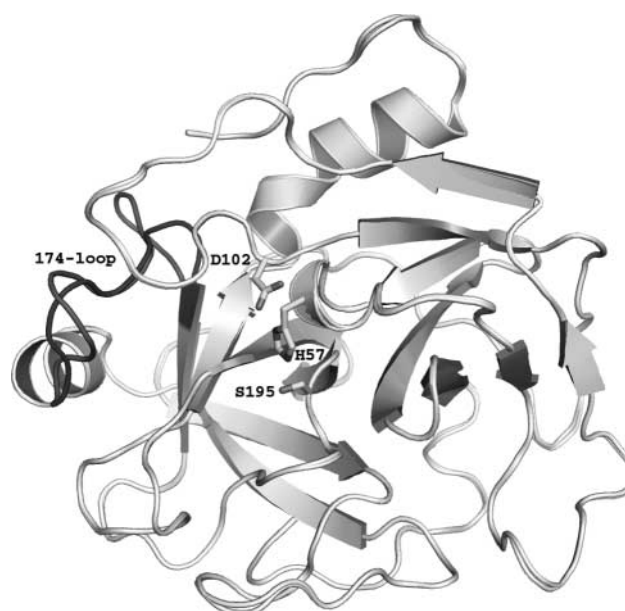


Fig. 7 Superpositioning of the atomic coordinates of the gyroxin modeled structure on the atomic coordinates of the PROTAC crystal structure. The 174-loop of gyroxin and PROTAC are colored in red and blue, respectively. Catalytic triad is represented as sticks with carbon atoms colored in yellow.

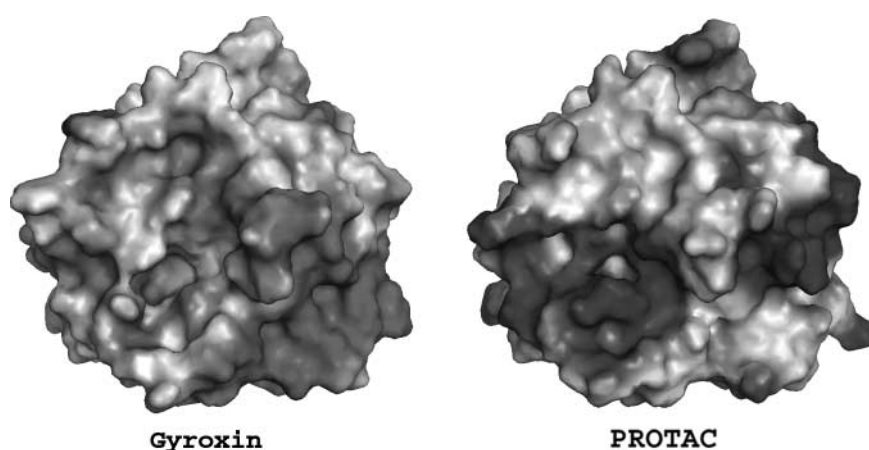


Fig. 8 Surface charge distributions around the active site region of the gyroxin modeled structure and the PROTAC crystal structure

Detailed analysis in a sphere of 10 Å around the catalytic triad reveals four significant differences in the amino acid residues (Arg60Ser; Trp99Glu; Gly193Phe; and Val215Trp) between gyroxin and PROTAC. The presence of Ser and Phe at positions 60 and 193, respectively, indicates high similarity between gyroxin and the mammalian trypsin-like enzymes. Another important amino acid difference between PROTAC and gyroxin is the substitution of Trp99Glu that contributes to the negative charge of the surface at the active site region (**Fig. 6**).

Conclusions

The combination of gel filtration and ion-exchange chromatographies proved to be successful in obtaining milligram quantities of pure samples of TLEs from the venoms of *C. d. terrificus* and *C. d. collilineatus* with estimated molecular weights of 30 kDa similar to that obtained for the TLEs from other species [25–33] (**Fig. 1**).

In summary, both TLEs demonstrated maximum activity at 37°C and a pH optima between 7.0 and 8.0 (data not shown) and were completely inhibited by leupeptin, significantly inhibited by PMSF but were not inhibited by aprotinin, heparin, 1,10-phenanthroline, or EDTA (**Table 1**). Analogous to the TLEs isolated from the venoms of *Bitis gabonica* [34] and *Cerastes vipera* [28] venoms, both enzymes preferentially degraded the Bβ chain of fibrinogen and secondarily the Aα chain, at high enzyme doses (>5 μg) whereas the Cγ chain was unaffected (**Fig. 3**). The purified enzyme from *C. d. collilineatus* venom displayed a twofold higher coagulant activity (0.6 μg/μl) in comparison with the enzyme from *C. d. terrificus* venom (1.5 μg/μl) (**Fig. 2**).

Both enzymes induced an edema of ~50% in a dose-independent manner in the first 30 min with a subsequent decrease in a time-dependent manner which was significantly greater for the enzyme from *C. d. collilineatus* venom (**Fig. 4**). Based on the activity on synthetic chromogenic substrates, both purified enzymes were classified as TLEs without detectable plasmin-like activity, since both displayed significant activity on S-2238 (substrate specific for thrombin) and no residual activity on S-2251 (substrate specific for plasmin and streptokinase).

The inability of heparin to inhibit the esterase activity of both enzymes is interesting and indicates that significant structural differences exist between these enzymes and thrombin, which should be of clinical interest. The crystal structures of many mammalian and bacterial serine proteases both in the native states and with bound inhibitors, substrates, and substrate analogs have been determined. However, the only two crystal structures of SVSPs currently available are the plasminogen activator TSV-PA [35] and PROTAC [16]. Results of our structural study should provide us with an insight into the mechanism by which these enzymes convert fibrinogen into fibrin and their ability to produce abnormal fibrin clots. This ability of these enzymes to form fibrin clots made up of short polymers has potential therapeutic applications for the treatment of patients with occlusive arterial or venous thrombotic diseases without stimulating the endogenous fibrinolysis system [36–38]. Detailed high-resolution structural information of these unique snake venom proteases should also provide useful information for the rational design of drugs that could be applied in the medical and pharmacological fields of homeostasis and thrombosis.

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