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Structure of saxthrombin, a thrombin-like enzyme from *Gloydius saxatilis*

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Snake-venom thrombin-like enzymes (SVTLEs) are serine proteases that are widely distributed in snakes from the Crotalinae subfamily of the Viperidae. In contrast to other snake-venom serine proteases, they have a biochemical activity similar to that of thrombin and play an important role in the process of blood coagulation. However, SVTLEs cannot activate factor VIII, which is essential in blood-clot stabilization. Consequently, blood clots produced by SVTLEs are not stable and are cleared rapidly. This characteristic makes SVTLEs attractive as potential candidates for antithrombotic therapy. Saxthrombin, an SVTLE from *Gloydius saxatilis*, was purified and crystallized to obtain a high-quality crystal, from which data were acquired to 1.43 Å resolution. Preliminary X-ray diffraction analysis showed that the crystal belonged to space group *C*2, with unit-cell parameters $a = 94.2$, $b = 52.2$, $c = 50.1$ Å, $\beta = 96.7^\circ$. The crystal structure was determined by molecular replacement and the final R factor was 18.69%; the $R_{\rm free}$ was 20.01%. This is the first report of a crystal structure of an SVTLE. Saxthrombin belongs to the typical α/β-hydrolase fold of serine proteases. Its structure was compared with those of thrombin and other snake-venom serine proteases. The observed differences in the amino-acid composition of the loops surrounding the active site appear to contribute to different surface-charge distributions and thus alter the shape of the active-site cleft, which may explain the differences in substrate affinity.

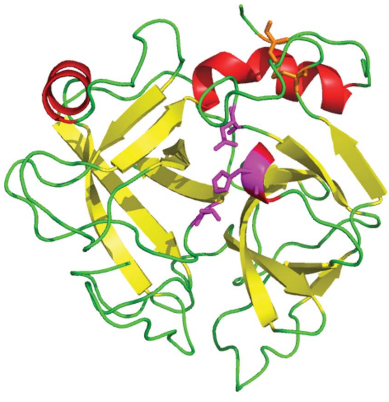
1. Introduction

Snake venoms contain a variety of proteolytic enzymes (Stocker, 1990). Most of these enzymes are serine proteases that can affect the host's coagulation process (Braud *et al.*, 2000; Matsui *et al.*, 2000). Serine proteases with a capacity for blood clotting *in vitro* are widely present in venoms from several genera of snakes. The enzymatic activity of these serine proteases at least partly resembles that of thrombin, a multifunctional protease that plays a key role in blood coagulation. Hence, these enzymes are known as snake-venom thrombin-like enzymes (SVTLEs; Castro *et al.*, 2004; Bell, 1997; Pirkle, 1998; Huang *et al.*, 1999).

Since the first studies of coagulation in 1957, more than 40 SVTLEs have been isolated from various snake venoms and about 30 sequences have been determined (Pirkle, 1998; Pirkle & Stocker, 1991; Blomback *et al.*, 1958). The similarity between SVTLEs and trypsin, their mammalian counterpart, has been clearly indicated by analysis of their primary structures and by their susceptibility to classical serine protease inhibitors (Castro *et al.*, 2004).

Most SVTLEs can cleave fibrinogen to release fibrinopeptide A, but none of them can activate factor VIII (Cho *et al.*, 2001). This is the most important difference between SVTLEs and thrombin. Factor VIII is required for the formation of the fibrin cross-links that stabilize blood clots. Therefore, the blood clots produced by SVTLEs are not cross-linked and can rapidly be cleared (Cho *et al.*, 2001; Furukawa & Ishimara, 1990).

Saxthrombin is a snake-venom serine protease originally isolated from *Gloydius saxatilis*. A sequence analysis of this protease has been provided by Sun *et al.* (2003), while we have described its clotting



activity and successful crystallization (Wei *et al.*, 2007). Saxthrombin exhibits a high degree of sequence identity to other snake-venom proteases: 82% identity to Aav-SP-I, a glycosylated snake-venom serine protease from *Agkistrodon acutus*, 83% to Aav-SP-II, another glycosylated snake-venom serine protease from *A. acutus*, and 70% to TSV-PA, a plasminogen activator from *Trimeresurus stejnejeri*. Although the amino-acid sequences of these enzymes are similar to each other, there are considerable differences in their biochemical activity. There are no reports of coagulation activity for either the Aav-SPs or TSV-PA (Parry *et al.*, 1998; Zhu *et al.*, 2005). However, as an SVTLE, saxthrombin has strong blood-coagulation activity *in vitro* (Wei *et al.*, 2007).

Interestingly, saxthrombin and α-thrombin have a similar capacity for blood coagulation even though the amino-acid sequence identity between them is only 29%, which is much lower than that between saxthrombin and other snake-venom serine proteases. What interests us is the reason why enzymes with higher sequence similarity have different activities while enzymes with poorer similarity have similar activities.

To date, no crystal structure has been reported for an SVTLE. Here, we have purified and crystallized saxthrombin. We examined the structural similarities and differences between saxthrombin and α-thrombin or other snake-venom serine proteases such as the AaV-SPs and TSV-PA. These structure–function analyses will be helpful in revealing the basis of the functional variations at the atomic level.

2. Materials and methods

Crude *G. saxatilis* venom was obtained from the Institute for Snakes and Snake Venom, Dalian Shedao Hospital, Dalian, People's Republic of China. Saxthrombin was purified from this venom using a three-step chromatographic procedure as described previously (Wei *et al.*, 2007). The crystallization and X-ray diffraction analysis of saxthrombin have also been described previously (Wei *et al.*, 2007).

3. Results and discussion

3.1. Overall crystal structure of saxthrombin

The crystal structure was solved by molecular replacement. The structure of AaV-SP-I (PDB entry 1op0; Zhu *et al.*, 2005) was used as a model. Details of crystallographic refinement are given in Table 1. The saxthrombin structure has been refined to an R factor of 0.187 ($R_{\rm free}$ = 0.200) at 1.43 Å resolution. The overall structure (Fig. 1) displays a typical trypsin-like serine protease fold and contains 13 β-strands and three α-helices. The β-strands fold into two β-barrels. One barrel contains $\beta1$–$\beta7$ and the other contains $\beta8$–$\beta13$. The active-site cleft and catalytic residues are located at the junction of the two barrels. One of the three helices is at the C-terminus of the protein and the other two helices are in the middle of each barrel. The structure contains a C-terminal extension sequence consisting of residues 241–249 and the orientation of the backbone is opposite to the C-terminal α-helix, as in TSV-PA and the AaV-SPs. As in these structures, a disulfide bridge between Cys247 and Cys90 results in a defined conformation of the C-terminal extension. However, the salt bridges between Pro245 and Lys101 that are seen in TSV-PA and the AaV-SPs (Zhu *et al.*, 2005; Parry *et al.*, 1998) are absent from the structure of saxthrombin.

Table 1
Summary of crystallographic refinement.

Resolution range (Å)	40–1.43
No. of reflections used	40813
No. of reflections selected for $R_{\rm free}$	3119
R factor (%)	18.69
$R_{\rm free}$ (%)	20.01
No. of atoms in the protein	1752
No. of water molecules	159
Average B factor (Å^2)	23.0

3.2. Loops surrounding the saxthrombin active site

The entrance to the active-site cleft of saxthrombin is restricted by several surface loops. These loops and their distinct properties determine the basis of its substrate specificity. The loops surrounding the active site can be divided into two parts by the active-site cleft. One part contains four loops that determine the left border of the active-site cleft at residues 174, 99, 60 and 37. Similarly, the other part also contains four loops that determine the right border of the active-site cleft at residues 218, 146, 70 and 193.

We did not find any significant differences between the backbones of saxthrombin and other snake-venom serine proteases. An optimal superimposition of saxthrombin with TSV-PA, Aav-SP-I and Aav-SP-II (Fig. 2) resulted in topologically equivalent C$^\alpha$ atoms at residues 194, 204 and 191, with root-mean-square (r.m.s.) deviations of 0.53, 0.31 and 0.31 Å, respectively, if only C$^\alpha$-atom pairs within 1.5 Å were included. Despite the high overall sequence identity between

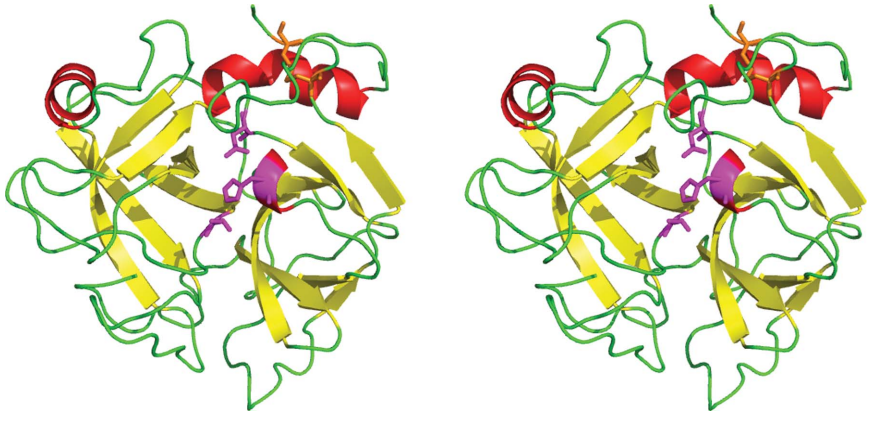


Figure 1
Stereoview of saxthrombin. This view (referred to as the standard orientation) shows the active-site cleft, which runs from left to right across the molecular surface. The catalytic residues His57, Asp102 and Ser196 are depicted as stick models (magenta). The disulfide bridge connecting the C-terminal extension to the 99-loop is depicted as a stick model (orange). β-Sheets are shown in yellow, α-helices in red and loops in green.

saxthrombin and TSV-PA, Aav-SPI and Aav-SPII (70, 83 and 84%, respectively), the amino-acid residues in the loops surrounding the active sites of these proteases are not the same (Fig. 3). Thus, the sequence conservation in the loops surrounding the active site is considerably lower than that across the entire sequences of these proteases. The high variability of the amino-acid sequences in the loops surrounding the active site may result in a differential distribution of the surface charge and an altered shape of the active cleft, which may affect the the substrate affinity and finally result in the observed functional diversity. However, the specific contribution of each amino acid to these differences needs to be further investigated.

3.3. Structural comparison of saxthrombin with thrombin

Previously, we compared the structure of saxthrombin with other available structures of snake-venom serine proteases. While saxthrombin is similar to thrombin in terms of its capacity to hydrolyze and clot fibrin, it cannot activate factor VIII and hence fibrin cross-links are not formed. Here, we compare the amino-acid sequences and tertiary structures of these two enzymes in an attempt to establish a structure–activity relationship.

Saxthrombin has 29% sequence identity to thrombin. There are four insertions in thrombin containing several amino-acid residues which do not exist in saxthrombin: thrombin residues 48–58, 125–129, 150–154 and 220–225 (Fig. 4). The first insertion sequence (48–58) is immediately adjacent to the active site (Fig. 5). The presence of this insertion sequence may result in transmutation of the active-site cleft, making the cleft in thrombin larger than that in saxthrombin. Hence, substrates which have a large side chain will be able to occupy the bottom of the active-site cleft of thrombin but may not be able to do so in the saxthrombin counterpart. Therefore, saxthrombin would have a stricter substrate specificity than thrombin for larger substrates. This may explain why saxthrombin cannot activate factor VIII and bring about fibrin cross-linking.

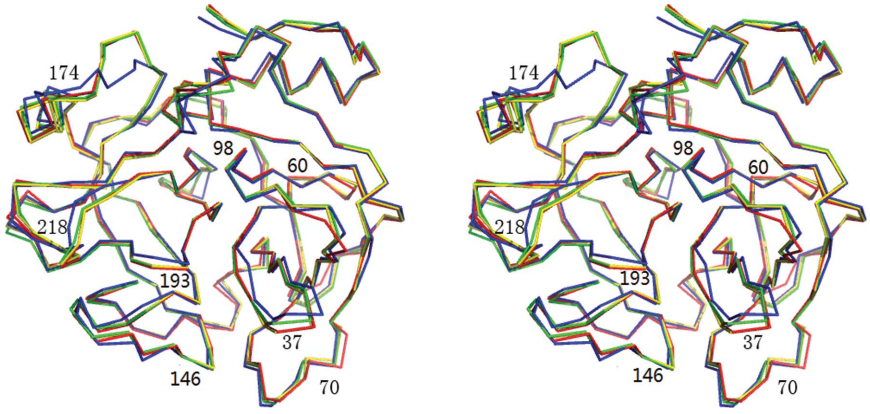


Figure 2
Stereoview superposition of C^{α} plots of the catalytic domains of TSV-PA (blue), AaV-SP-I (yellow), AaV-SP-II (green) and saxthrombin (red). The view and figure labels are as in Fig. 1 (standard orientation). The numbers correspond to the loops surrounding the active-site cleft.

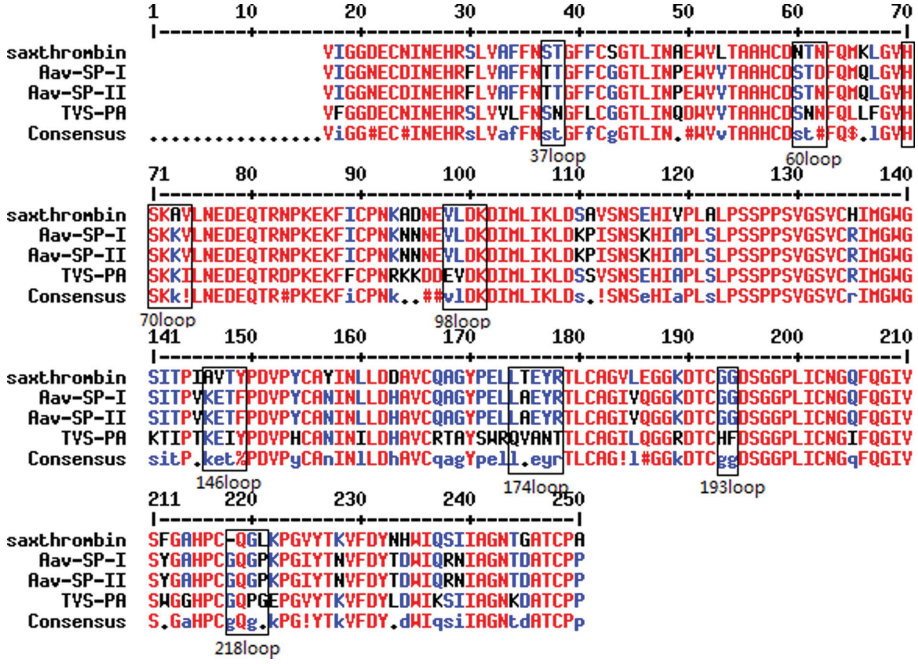


Figure 3
Sequence comparison of snake-venom serine proteases. The amino-acid sequences of saxthrombin, AaV-SP-I, AaV-SP-II and TSV-PA are aligned according to their topological equivalence. The numbering is that of the saxthrombin sequence. Amino-acid residues that are similar in all four proteins are shown in blue and identical residues are shown in red. The sequences of the loops surrounding the active site are indicated in black boxes.

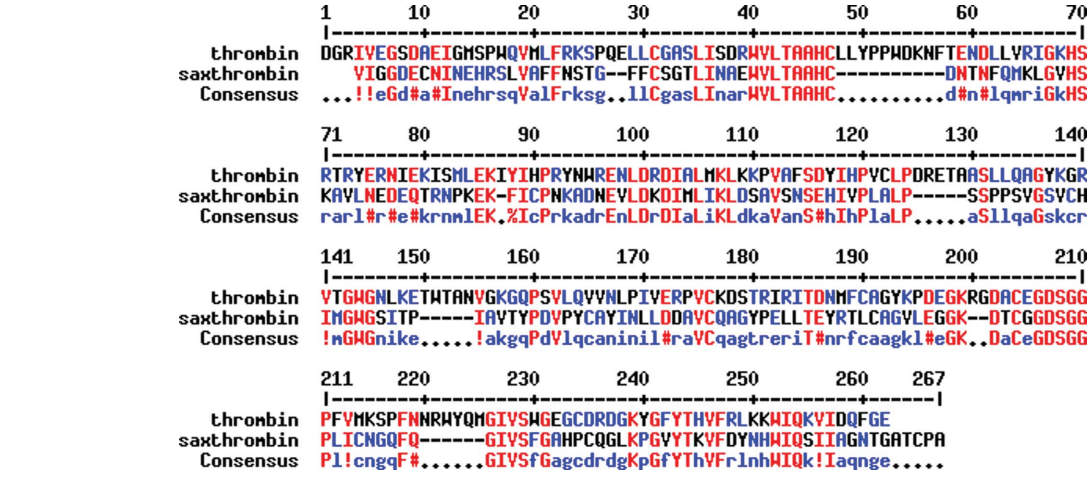


Figure 4
Sequence comparison of thrombin and saxthrombin. The amino-acid sequences of thrombin and saxthrombin were aligned according to their topological equivalence. The numbering is that of the thrombin sequence. Similar amino-acid residues are shown in blue and identical residues are shown in red.

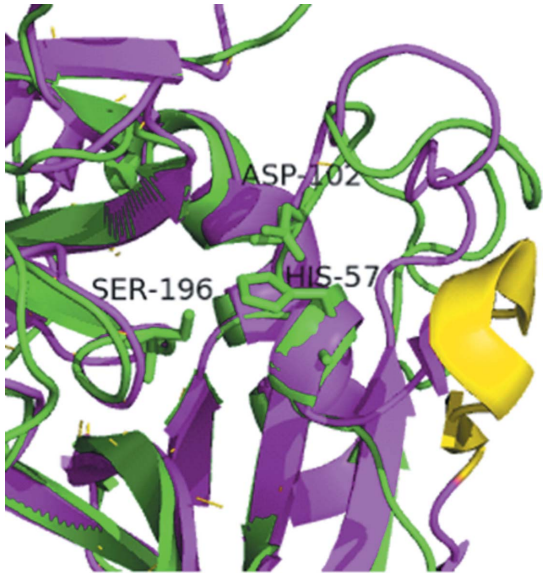


Figure 5
Comparison of the structure around the active sites of saxthrombin (green) and thrombin (pink). Key catalytic residues are labelled. The first insertion sequence in thrombin (residues 48–58) is shown in yellow.

The surface of α-thrombin is rich in positive charge, apart from a small region near the catalytic site. In particular, there is a positively charged region rich in Arg to the left side of the active cleft. This region is considered to bind proteolytic substrates and most inhibitors. Conversely, saxthrombin is rich in negative charge on its surface, in the catalytic centre and on the surface around the active site. There is no Arg-rich region on the saxthrombin surface at a position homologous to that found in α-thrombin. Therefore, thrombin inhibitors that bind this region cannot bind saxthrombin or inhibit its activity. In the same way, substrates cannot bind saxthrombin through electrostatic interactions. It is suggested that the binding of fibrinogen to saxthrombin is mostly dependent on hydrophobic interactions and surface complementarity.

This is the first reported crystal structure of an SVTLE and a comparative structure–function analysis has provided several pointers regarding the functional diversity in this type of serine protease. Further investigations may be able to provide the identities and specific contributions of individual amino acids which could be useful in the application of SVTLEs in antithrombotic therapy.

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