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Shedaoenase, a Novel Fibrinogenase from the Venom of *Agkistrodon shedaoenthesis* Zhao

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Abstract Shedaoenase, a serine protease, was isolated from the venom of *Agkistrodon shedaoenthesis* Zhao with an apparent molecular mass of 36 kDa. It was purified by affinity chromatography on arginine Sepharose 4B column and anion exchange on Mono Q fast protein liquid chromatography. Shedaoenase preferentially cleaved the Aα-chain of human fibrinogen and slowly digested the Bβ-chain. It also showed arginyl esterase activity using N_{α}-benzoyl-*L*-arginine ethyl ester as a substrate, and some synthetic chromogentic substrates, such as Chromozym PL, S-2266, and S-2160, could also be hydrolyzed. The enzyme activity of shedaoenase could be completely inhibited by phenylmethylsulphonylfluoride and could be little inhibited by the chelating reagent EDTA. The N-terminal sequence of shedaoenase was determined, and its full-length cDNA encoding a protein of 238 amino acid residues was cloned by reverse transcription-polymerase chain reaction from the total mRNA extracted from the snake venom gland. The deduced primary sequence of shedaoenase shares significant homology with other snake venom serine proteases.

Key words *Agkistrodon shedaoenthesis* Zhao; fibrinogenlytic activity; serine protease

Snake venoms, particularly those belonging to Crotalidae and Viperidae, often induce disorder of the blood coagulation system [1]. In this course, the venom proteases play a crucial role. The endogenous substances in plasma involved in the blood coagulation process, namely coagulation factors, are mostly proteins in the form of inactive precursors. Many snake venom proteases, which are generally classified structurally into serine proteases and metalloproteinases [2], can interact with at least one sort of coagulation factor or other protein components in plasma [3], and cleave specific peptide bond(s) in these protein substrates. These substrates include coagulation factor IX (Christmas factor) [4], factor X (Stuart-Prower factor) [5], factor II (prothrombin) [6,7], plasminogen [8,9], protein C [10,11], factor I (fibrinogen) [9,12–25], kininogen [26–31] and factor V (proaccelerin) [32,33]. Of these, the first five proteins are zymogens that can be converted into active enzymes, the last two can be converted into assistant factors, and fibrinogen can be converted into fibrin clots or fibrin degradation products.

Fibrinogen is a 340 kDa protein which contains two disulfide-linked symmetric half-molecules. Each half contains three chains designated as α, β, and γ with molecular masses of 63 kDa, 56 kDa, and 47 kDa, respectively [34]. Some snake venom proteinases, called thrombin-like enzymes, can hydrolyze the N-terminal end of Aα- or/and Bβ-chain, and release either fibrinopeptide A [12,14–19], fibrinopeptide B [18,20–22] or both [13,23], inducing fibrin clots in a similar way to thrombin. Some other venom proteinases degrade the Aα-, Bβ- or both chains of fibrinogen at the C-terminus without forming fibrin clots, and they are called fibrin(ogen)olytic enzyme or protease with fibrin(ogen)olytic activity [9,24–27,29–31].

Here, we report the purification and characterization of a fibrinogenase. It is a serine protease, and was named shedaoenase from *Agkistrodon shedaoensis* Zhao.

Materials and Methods

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Materials

The crude venoms of *A. shedaoensis* Zhao and the living snakes were provided by the Institute of Snakes and Snake Venom, Dalian Shedao Hospital (Dalian, China). Arginine Sepharose 4B gel was the product of Pharmacia Biotech. Synthetic chromogenic substrates were purchased from Sigma. *Escherichia coli* DH16B BL21(DE3) and expression vector pET-40b(+) were preserved by our own laboratory. pMD18-T vector, DNA restriction enzymes and T4 DNA ligase were obtained from TaKaRa. Trizol, *Taq* DNA polymerase, the total RNA isolation kit and Moloney murine leukemia virus first-strand cDNA synthesis kit were purchased from Sangon. Other reagents were of analytic grade.

Purification of shedaoenase

Crude venom of *A. shedaoenthesis* Zhao was dissolved in 50 mM Tris-HCl buffer (pH 8.0) and centrifuged at 6000 *g* for 10 min. The supernatant was applied onto an arginine Sepharose 4B column pre-equilibrated with the same buffer. The column was washed with this buffer of 4 bed volume at a flow rate of 15 ml/h. A large amount of the aforementioned buffer containing 0.2 M NaCl was used, and the latter fraction of the eluted product was dialyzed, lyophilized and further purified by Mono Q on fast protein liquid chromatography (FPLC).

Electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of Laemmli [35]. Ten percent polyacrylamide gel was run under reducing conditions and stained with Coomassie brilliant blue R-250.

Determination of the amino acid sequence of N-terminal

Amino acid sequencing was performed on an auto-PE 491 protein sequencer.

N_α-benzoyl-*L*-arginine ethyl ester (BAEE) activity and inhibitor assay

The BAEE activity of shedaoenase was determined according to the method of Glazer [36]. Shedaoenase solution of the same concentration was incubated with different inhibitors in the reaction solution of Tris-HCl (50 mM, pH 7.8) at room temperature for 30 min, then the residual BAEE activity was examined. The inhibition ratio of different inhibitors was calculated respectively. Phenylmethylsulfonyl fluoride (PMSF) was dissolved in isopropylalcohol; EDTA and dithiothreitol (DTT) were dissolved in Tris-HCl buffer (50 mM, pH 7.8; containing 0.1 M NaCl).

Amidolytic activity on synthetic chromogenic substrate

The amidolytic activity of the enzyme was measured with a U3010 spectrophotometer (Pharmacia Fine Chemicals, Uppsala, Sweden) in 1 cm path-length plastic cuvette at 25 ºC. Chromogenic substrate assays were performed in 0.1 ml Tris-HCl buffer (50 mM, pH 7.8). The chromogenic substrates S-2266 (*D*-Val-Leu-Arg-*p*NA), Chromozym PL (N-*p*-tosyl-Gly-Pro-Lys-*p*NA), S-2160 (N-benzoyl-Phe-Val-Arg-*p*NA), Chromozym PK (N-benzoyl-Pro-Phe-Arg-*p*NA), Chromozym TH (N-Cbz-Gly-Pro-Arg- *p*NA) and S-2222 (N-benzoyl-Ile-Glu-Gly-Arg-*p*NA) were each dissolved at a final concentration of 100 μM. The reactions were initiated by the addition of shedaoenase (final concentration 3.6 μg/ml), and the formation of *p*-nitroanilide (*p*NA) was monitored continuously at 405 nm. The amount of substrates hydrolyzed was calculated from the absorbance at 405 nm using a molar extinction coefficient of 10,500 $M^{-1}\cdot cm^{-1}$ for free *p*NA.

Fibrin(ogen)olytic and fibrinogen clotting activity

The fibrinolytic activity was assayed on the fibrin plates according to the method of Guan *et al.* [37].

The fibrinogenolytic activity was determined by the method of Utaisincharoen *et al.* [31] with modifications. We mixed 0.1 ml of human fibrinogen solution (0.1 M NaCl, 0.01 M $CaCl_2$, 0.2% human fibrinogen, 50 mM Tris-HCl, pH 7.4) with 0.1 ml shedaoenase solution (50 μg/ml in 50 mM Tris-HCl, pH 7.4) and incubated the mixture at 37 ºC. At various time points, 20 μl of the incubation mixture was withdrawn and added to 10 μl of denaturing solution (6 M urea, 4% SDS, 4% β-mercaptoethanol). The samples were reduced and denatured overnight at room temperature and were analyzed by SDS-PAGE (10% acrylamide).

The fibrinogen clotting activity was determined by the method of Jia *et al.* [30] with modifications. We incubated 5.0×10^{-3} mg of shedaoenase with 0.2 ml of 0.6% human fibrinogen solution (0.1 M NaCl, 0.01 M $CaCl_2$, 50 mM Tris-HCl, pH 7.4) at 37 ºC for 4 h. The formation of fibrin clots was monitored.

Western blot

Western blot was performed as Sambrook *et al.* described previously [38]. The antibody of the thrombin-like enzyme from *Agkistrodon actus* was prepared and preserved by our laboratory.

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Total RNA extraction and cDNA amplification

The snake was decapitated. The venom glands were removed immediately, frozen in liquid nitrogen, triturated, and quickly suspended in Trizol reagent (Sangon). The extraction of total RNA and the cDNA synthesis were performed according to the manufacturer's protocol (Sangon).

cDNA cloning and sequencing

Two primers were designed based on the N-terminal sequence of shedaoenase and the conserved 3′ non-coding region. Primer-upstream was 5′-GCAGTACTATGGT-(ACTG)A(G)T(TCA)GG(ACTG)GG(ACTG)GA(TC)GA-3′, primer-downstream was 5′-TTTCTCCTCTT(GA)A-(GC)(CT)(AT)TTTCAAA-3′.

Polymerase chain reaction (PCR) was carried out to amplify cDNA with total cDNA as the template, and it was performed as follows: first five cycles for denaturation at 94 ºC for 1 min, anneal at 48 ºC for 1 min, elongation at 72 ºC 1.5 min; this process was repeated for 30 cycles under the same conditions, except that the annealing temperature was 52 ºC. The PCR products were cloned into pMD18-T vector and transform into *E. coli* DH16B.

The nucleotide sequence was analyzed by the dideoxy chain-termination method using RV-M and M13-47 universal primers.

The DNA sequence and deduced amino acid sequence were compared with sequences in the GenBank database using BLASTN and BLASTP.

Results and Discussion

Purification of shedaoenase

Shedaoenase was purified by a two-step method of affinity chromatography on arginine Sepharose 4B and anion exchange on Mono Q FPLC [**Fig. 1(A)**]. Analysis using SDS-PAGE gave a single band. The apparent molecular mass was estimated to be 36 kDa [**Fig. 1(B)**].

Immune cross-reaction with the antibody of the thrombin-like enzyme from *A. actus*

Western blot analysis showed that shedaoenase has positive immune cross-reaction with the antibody of the thrombin-like enzyme from *A. actus*, which is a typical serine protease [**Fig. 1(C)**].

N-terminal sequence of shedaoenase

The first 10 N-terminal amino acid residues of shedaoenase were sequenced to be VVGGDECNIN, which showed high

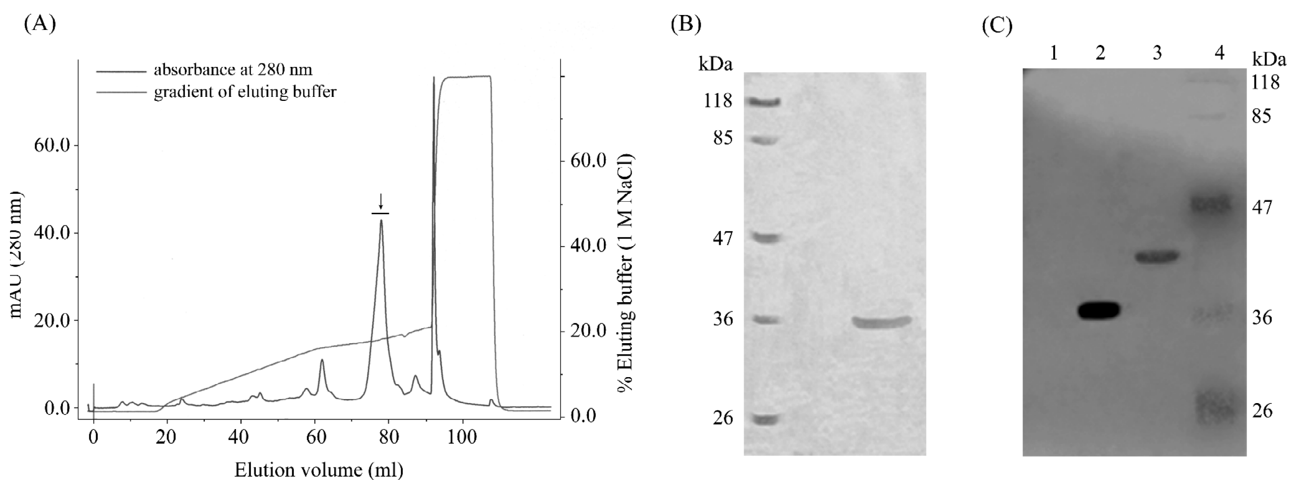

Fig. 1 Purification of shedaoenase

(A) Mono Q FPLC of shedaoenase. Active fractions after affinity chromatography on arginine Sepharose 4B was dissolved in 20 mM Tris-HCl (pH 7.1) and applied to Mono Q FPLC. The column was washed with the same buffer and eluted with a linear gradient from 0 to 1 M NaCl at a flow rate of 2 ml/min. The arrow represents fractions containing shedaoenase. (B) SDS-PAGE of shedaoenase. Following Mono Q FPLC chromatography, the fraction of the front half of the peak containing shedaoenase was performed on 12% polyacrylamide gel. The left lane is the molecular weight marker. The right lane is shedaoenase. (C) Weston blot analysis of shedaoenase. 1, negative control (a disintegrin-like protein from *Agkistrodon shedaoenthesis* Zhao); 2, shedaoenase; 3, positive control (a thrombin-like enzyme from *Agkistrodon actus*); 4, molecular weight marker.

identity to other venom serine proteases.

Fibrin(ogen)olytic and fibrinogen clotting activity

Shedaoenase showed fibrinolytic activity when applied to fibrin plates (data not shown). The transparence under control conditions in a Petri dish was visualized.

No obvious fibrin clot was observed when shedaoenase was incubated with human fibrinogen solution.

Shedaoenase preferentially cleaved the α-chain of human fibrinogen and slowly digested the β-chain, but had no effect on digesting the γ-chain (**Fig. 2**).

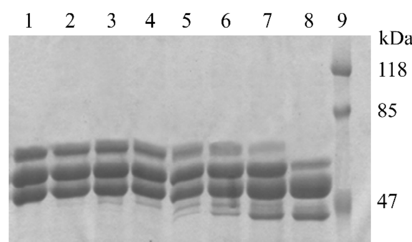

Fig. 2 SDS-PAGE analysis of the human fibrinogen digested by shedaoenase
1–8, 0.1 ml human fibrinogen solution was incubated with 0.1 ml shedaoenase solution at 37 °C for 0, 2, 4, 8, 16, 32, 64 and 128 min, respectively; 9, molecular weight marker.

BAEE activity and inhibitor assay

Shedaoenase showed obvious arginyl esterase activity on BAEE. This activity could be entirely inhibited by PMSF, a serine protease inhibitor, but almost not inhibit by the chelating reagent EDTA, which indicated that shedaoenase is a serine protease. The reducing agent DTT could inhibit the enzyme activity considerably, which suggested that the disulfide bond is crucial for maintaining enzyme activity (**Table 1**).

Table 1 Effects of inhibitors on N_α-benzoyl-*L*-arginine ethyl ester activity of shedaoenase

Inhibitor	Concentration (mM)	Residual activity (%)
Control	0	100±0
PMSF	2	0±0
DTT	10	22±2
EDTA	10	97±4

DTT, dithiothreitol; PMSF, phenylmethylsulphonyl fluoride.

Amidolytic activity on chromogenic substrate

Synthetic chromogenic substrates were used for the protease assay of shedaoenase (**Fig. 3**). Shedaoenase preferentially acts on Chromozym PL (N-*p*-tosyl-Gly-Pro-Lys-*p*NA), the substrate of plasmin. Shedaoenase also shows a substrate preference of S-2266 (*D*-Val-Leu-Arg-*p*NA, substrate of kallikrein) > S-2160 (N-benzoyl-Phe-Val-Arg-*p*NA, substrate of thrombin, trypsin and reptilase) > Chromozym TH (N-Cbz-Gly-Pro-Arg-*p*NA, substrate of thrombin) > Chromozym PK (N-benzoyl-Pro-Phe-Arg-*p*NA, substrate of plasma kallikrein). Shedaoenase shows little activity on S-2222 (N-benzoyl-Ile-Glu-Gly-Arg-*p*NA, substrate of factor Xa). These results suggested that peptides with Lys at the P1 site are probably preferential substrates for shedaoenase than those with Arg at the P1 site.

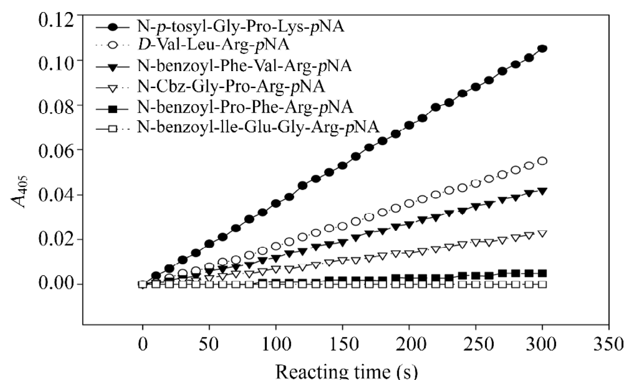

Fig. 3 Comparison of the hydrolyzing activities of shedaoenase toward synthetic chromogenic substrates
The amidolytic activity of the enzyme was measured with a spectrophotometer (U3010) in 1 cm path-length plastic cuvette at 25 °C. Chromogenic substrate assays were performed in 0.1 ml reaction solution with 50 mM Tris-HCl, pH 7.8.

Furthermore, shedaoenase can hydrolyze fibrinogen without forming fibrin clots and also can degrade fibrin. This resembles the physiological effect of plasmin. This result is in accordance with the assay of amidolytic activity on synthetic chromogenic substrates, which showed that shedaoenase hydrolyzes the substrate of plasmin more potently than the substrate of thrombin.

Cloning and sequence determination of shedaoenase

The full-length of the PCR product of shedaoenase cDNA was approximately 750 bp, which was cloned into pMD18-T vector and sequenced (**Fig. 4**). The mature protease covered an open reading frame of 714 nucle-

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      GTGATAGGGGGTGATGAATGTAACATAAATGAACATCGTTTCCTTGTAGCCTTATACACC
  1   V  I  G  G  D  E  C  N  I  N  E  H  R  F  L  V  A  L  Y  T
      TCTAGATTTAGGACTTTGTTCTGCGGTGGGACTTTGATCAACCAGGAATGGGTGCTCACT
 21   S  R  F  R  T  L  F  C  G  G  T  L  I  N  Q  E  W  V  L  T
            ▼
      GCTGCACACTGTGACAGGAAAAATTTCCGGATAAAGCTTGGTATTCATAGCAAAAAGGTA
 41   A  A  H  C  D  R  K  N  F  R  I  K  L  G  I  H  S  K  K  V
      CCAAATGAGGATGAGCAGACAAGAGTCCCAAAGGAGAAGTTCTTTTGTCTCAGTAGCAAA
 61   P  N  E  D  E  Q  T  R  V  P  K  E  K  F  F  C  L  S  S  K
      ◆                    ▼                                   ◆
      AACTATACCCTTTGGGACAAGGACATCATGTTGATCAGGCTGGACAGCCCTGTTAAGAAC
 81   N  Y  T  L  W  D  K  D  I  M  L  I  R  L  D  S  P  V  K  N
      AGTACACACATTGCGCCTTTCAGCTTGCCCTCCAGCCCTCCCAGTGTGGGCTCAGTTTGC
101   S  T  H  I  A  P  F  S  L  P  S  S  P  P  S  V  G  S  V  C
      CGTATTATGGGATGGGGCAGAATCTCACCTACTGAAGAGACTTATCCCGATGTCCCTCAT
111   R  I  M  G  W  G  R  I  S  P  T  E  E  T  Y  P  D  V  P  H
      TGTGTTAACATTAACCTACTCGAATATGAGATGTGTCGAGTACCTTACCCAGAATTTGGG
141   C  V  N  I  N  L  L  E  Y  E  M  C  R  V  P  Y  P  E  F  G
                                                        ▽
      TTGCCAGCGACAAGCAGAACATTGTGTGCAGGTATCCTGGAAGGAGGCAAAGATACATGT
161   L  P  A  T  S  R  T  L  C  A  G  I  L  E  G  G  K  D  T  C
               ▼
      CGGGGTGACTCTGGGGGACCCCTCATCTGTAATGGACAATTCCAGGGCATTGCATCTTGG
181   R  G  D  S  G  G  P  L  I  C  N  G  Q  F  Q  G  I  A  S  W
      GGAGACGATCCTTGTGCCCAACCGCATAAGCCTGCCGCGTACACCAAGGTCTTCGATCAT
201   G  D  D  P  C  A  Q  P  H  K  P  A  A  Y  T  K  V  F  D  H
      CTTGACTGGATCAAGAGCATTATTGCAGGAAATACAGATGCGTCCTGCCCCCCGTGAAAA
221   L  D  W  I  K  S  I  I  A  G  N  T  D  A  S  C  P  P  -
      CTTTTGAAAAAGTCAAGAGGAGAA
```

Fig. 4 The cDNA and deduced amino acid sequence of shedaoenase

Tentative catalytic triad residues, the primary substrate-binding site and the putative glycosylation site are indicated by ▼, ▽ and ◆, respectively.

otides encoding 238 amino acids. This sequence has been submitted to GenBank and its accession number is AAR11859.

The primary structure of shedaoenase has the typical character of the snake venom serine proteases. Based on the sequence similarity to other venom proteases, the reactive sites of the catalytic triad in shedaoenase are presumed to be His^{43}, Asp^{88} and Ser^{194}, corresponding to those of His^{40}, Asp^{84} and Ser^{176} in trypsin. The sequences around these three amino acid residues were found to be highly homologous to other serine proteases. Furthermore, most of the snake venom serine proteases belong to the trypsin-like serine protease family. The bottom of the S1 pocket of trypsin-like serine proteinases is highly conserved with an Asp, which forms a canonical ion pair interaction with the positively charged side chain of the P1 residue of the substrate molecule. This Asp is at the position six residues prior to the active serine.

Shedaoenase contains six disulfide bridges, as do most of the snake venom serine proteases. These disulfide bridges are assigned from the sequence similarity with trypsin, as follows: Cys^{7}-Cys^{141}, Cys^{28}-Cys^{44}, Cys^{76}-Cys^{236}, Cys^{119}-Cys^{190}, Cys^{151}-Cys^{168}, and Cys^{180}-Cys^{206}.

The molecular mass of shedaoenase, based on the deduced amino acid sequence, is approximately 26.4 kDa, whereas the apparent molecular mass according to the result of SDS-PAGE was estimated to be 36 kDa. This discrepancy may be due to the alteration of electrophoresis behavior caused by the oligosaccharide chains linked to shedaoenase. This presumption was corroborated by the sequence analysis. Shedaoenase has two potential N-glycosylation sites at Asn^{80} and Asn^{100}. The Asn^{80}-Tyr^{81}-Thr^{82} and the Asn^{100}-Ser^{101}-Thr^{102} residues might be the N-X-T(S) motif, known to be a potential N-glycosylation site linked to asparagine.

The sequence of shedaoenase was compared with those of other snake venoms, human plasmin and the B-chain of human α-thrombin. The sequences were aligned to maximize mutual homologies (**Fig. 5**). The order of the snake venom serine proteases is arranged according to the substrate specificity. Some of them show multiple enzyme activities. The proteases from Trocarin to TSV-PA exhibit no action on fibrinogen; those from ancrodB to KN-BJ can clot fibrinogen (venombin A, B or AB can release fibrinopeptide A, B or both, respectively); and those from ACC-C to shedaoenase show fibrin(ogen)olytic activity.

Most of the snake venom serine proteases are very similar to each other in amino acid sequence, which implies that they might have evolved from the same precursor protein and possibly adapted to each target protein of the snake's prey. In spite of their high homology in primary structure and tertiary structure, the snake venom serine proteases show various substrate specificities. This may be due to some refined difference of the structure at the substrate-binding site. Some surface loops surrounding the extended substrate-binding site may contribute to this diversity [39–42].

It was reported that special residues or motifs may play important roles in substrate recognition. For example, $D^{101}D^{102}E^{103}$, particularly the D^{102}, in TSV-PA (the residue serial number in this paragraph has been adjusted to correspond with **Fig. 5**, for reading convenience) is proven to be responsible for plasminogen activation. The point mutation D102V led to 125-fold decrease of plasminogen-activating activity [9,39,42], and $F^{100}PNG^{103}$ is only present in factor V activators (RVV-V and VLFVA) [32,33]. The sequences aligned in **Fig. 5** show no distinct difference at the single residue site or motif among the fibrinogen clotting enzymes, fibrin(ogen)olytic enzymes and other enzymes. This suggests that the mechanism of fibrinogen recognition is complicated, and is perhaps a process in which many residues are involved.

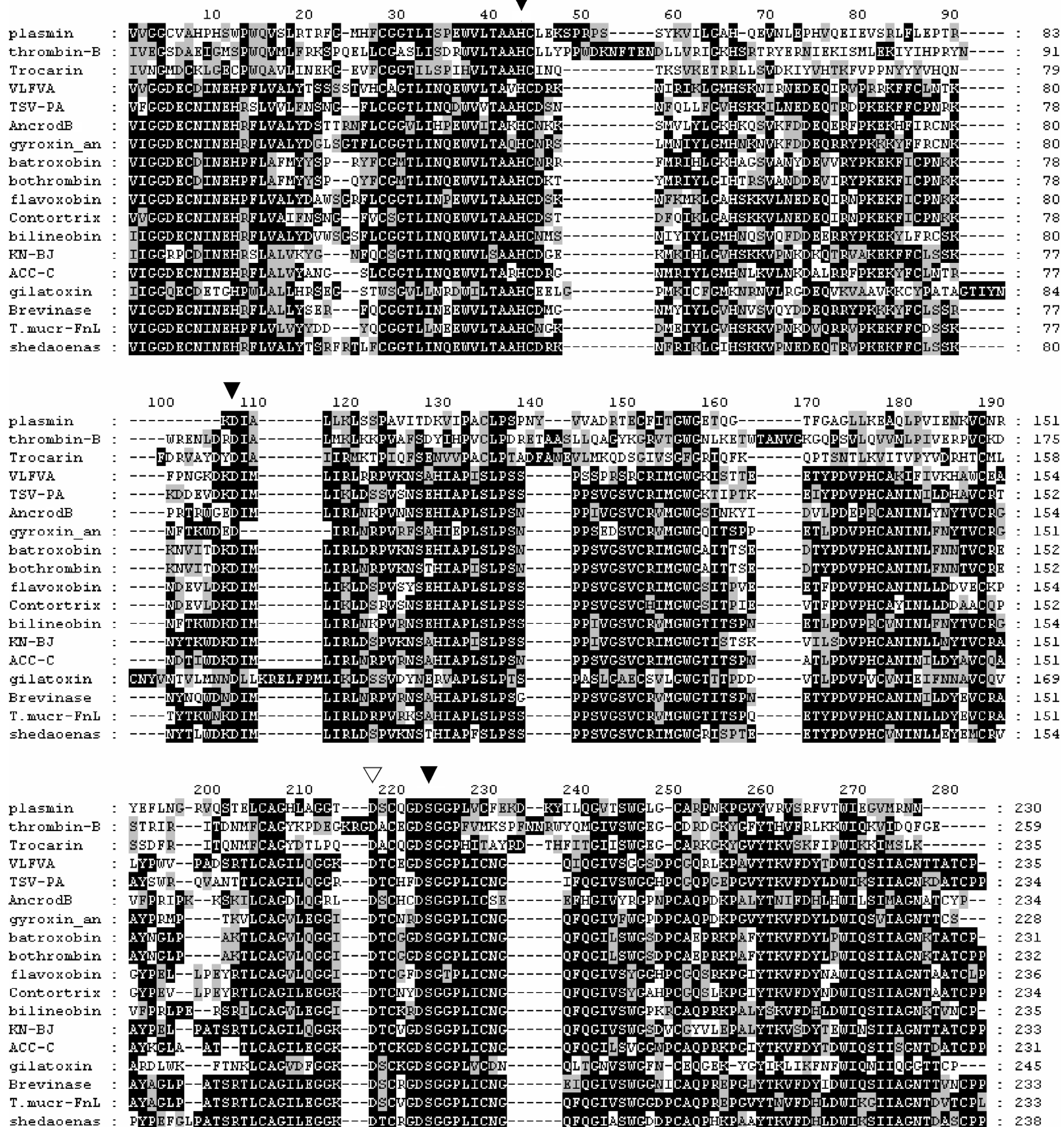

Fig. 5 Comparison of the amino acid sequences of snake venoms and mammalian serine proteases

The sequence of shedaoenase was compared with those of other snake venom, human plasmin and the B-chain of human α-thrombin. The proteases sequences were aligned to maximize mutual homologies. The orders of the snake venom serine proteases are arranged according to the substrate specificity; some of them show multiple enzyme activity. The proteases from Trocarin toTSV-PA exhibit no action on fibrinogen, from ancrodB to KN-BJ can clot fibrinogen (venombin A, B or AB can release fibrinopeptide A, B or both, respectively), from ACC-C to shedaoenase show fibrin(ogen)olytic activity. The tentative catalytic triad residues and the major substrate-binding site are indicated by ▼ and ▽ respectively. Pla (plasmin), human plasmin (REFSEQ NM_000301.1); thr (thrombin), human alpha-thrombin (REFSEQ NM_000506.2); Tro (trocarin), prothrombin activator from *Tropidechis carinatus* [6] (SwissProt P81428); VLF (vl-fva), factor V activator from *Vipera lebetina* [32] (SwissProt Q9PT41); TSF (TSV-PA), plasminogen activator from *Trimeresurus stejnege* [8] (SwissProt Q91516); Anc (ancrodB), venombin A from *Agkistrodon rhodostoma* (Malayan pit viper) (*Calloselasma rhodostoma*) [12] (SwissProt P47797); gyr (gyroxin analog), venombin A from *Lachesis m. muta* [17] (SwissProt P33589); bat (batroxobin), venombin A from *Bothrops atrox* batroxobin [19] (SwissProt P04971); bot (bothrombin), venombin A from *B. jararaca* [14] SwissProt P81661; fla (flavoxobin), venombin A from *Tri. flavoviridis* [16] (SwissProt P05620); Con (Contortrixobin), venombin B from *A. contortrix* contortrix [18] (TrEMBL P82981); bil (bilineobin), venombin AB from *A. bilineatus* [13] (PIR S65621); Knb (KN-BJ), kinin-releasing and venombin A from *B. jararaca* [28] (SwissProt O13069); Acc (ACC-C), protein C activator from *A. contortrix* contortrix, can also hydrolyze many other substrates and show fibrinogenolytic activity [10] (SwissProt P09872); Gil (gilatoxin), kallikrein-like from *Heloderma horridum* horridum, shows fibrinogenolytic activity (Aa preferentially) [31] (SwissProt P43685); Bre (brevinase), a two-chain fibrinolytic enzyme from Korean snake, *A. blomhoffii* brevicaudus [25] (SwissProt Q9PT51); Tmu (T. mucr-FnL), b-fibrinogenase from Taiwan Habu (*Tri. mucrosquamatus*) [26] (SwissProt Q91509); She (shedaoenase), fibrinogenase separated in this work.

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