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ORIGINAL RESEARCH PAPER

Cloning, expression, purification and bioactivity evaluation of a thrombin-like enzyme from *Deinagkistrodon acutus* venom gland library

An Li · Chuan Zhang · Jie Wang · Junjie Wang · Hailong Jiang · Jianzhong Li · Xingyuan Ma · Wen Zhang · Yiming Lu

Abstract

Objectives To identify a new member of serine proteases from *Deinagkistrodon acutus* via phage display technique and appraise its biocatalytic activities.

Results A novel thrombin-like enzyme gene was cloned by screening the phage display library of *D. acutus* venom gland. The gene has a 783 bp ORF encoding 260 amino acids. A recombinant enzyme expression vector was constructed and the fused protein was expressed in *Escherichia coli*. The protein was purified showing a single band of approx. 49.4 kDa after SDS-PAGE. The recombinant enzyme was capable of congealing normal human plasma in vitro with the minimum coagulant dose of 6 µg in 57 s. It exhibited fibrinogenolytic activity by hydrolyzing the Aα-chain of human fibrinogen. It was most active at pH 7.5–8.0 and 35–40 °C with the highest clotting activity of 120 NIH units/mg. It was completely inhibited by PMSF but not by EDTA. Multiple sequence alignments demonstrate that this protein shares high identity with other thrombin-like enzymes from snake venoms.

Conclusions A novel thrombin-like protein from *D. acutus* venom was identified, expressed and biologically characterized in vitro. Its fibrinogenolytic properties make the enzyme applicable for biochemical research and drug development on thrombolytic therapy.

An Li, Chuan Zhang and Jie Wang contributed equally to this work.

A. Li · J. Wang · H. Jiang · J. Li · W. Zhang (✉) · Y. Lu (✉)
School of Pharmacy, Second Military Medical University, Shanghai 200433, China
e-mail: wenzhang1968@163.com

Y. Lu
e-mail: bluesluyi@sina.com

C. Zhang · X. Ma (✉)
State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, Shanghai 200237, China
e-mail: maxy@ecust.edu.cn

J. Wang
Changhai Hospital, Second Military Medical University, Shanghai 200433, China

Introduction

Thrombin-like enzymes (TLEs) are a family of serine proteases that have a similar biochemical activity with thrombin in the process of blood coagulation. They have been the focus of many studies for their clinical and biochemical applications (Koh et al. 2006). TLEs can hydrolyze fibrinogen to release fibrinopeptide A (TLE-A class) or fibrinopeptide B (TLE-B class) or both (TLE-AB class), but are incapable of activating factor XIII, producing soft clots (non-crosslinked fibrins), which are different from the thrombin-induced crosslinked hard clots and are easily degraded by secondary fibrinolysis (Oyama and Takahashi 2002). Snake venom TLEs (SVTLEs) are mainly distributed in Viperidae snakes, and are especially abundant in *Deinagkistrodon acutus* (*Agkistrodon acutus*). Paradoxically, thrombin-like coagulant action has been observed in vitro, but purified TLEs have anticoagulant action in vivo, inducing a benign state of defibrinogenation for which the abnormal soft fibrin clot is thought to be crucial (Davey and Luscher 1965). Based on this characteristic, several SVTLEs, including *Ancrod* (from *Calloselasma rhodostoma* venom, Samsa et al. 2002) and *Batroxobin* (from *Bothrops moojeni* venom, Rajesh et al. 2007), are commercially produced for clinical use as defibrinogenating (anti-coagulant) agents to prevent thrombi and improve blood circulation. Thus the study of new TLE proteins has great prospect for new pharmacology applications.

Serine proteases from *D. acutus* venom have been studied for 30 years during which numerous enzymes were isolated and identified. Only a few, however, have been well-characterized or classified (Komori et al. 1988) due to the limited source of snake venom and low abundance of certain enzymes. In this report, we have cloned a novel thrombin-like enzyme gene designated TLE2, expressed and purified the TLE2 protein derived from the venom gland of *D. acutus*. One of the major challenges is to obtain the enzyme in a soluble and bioactive form. Most of known SVTLEs are cysteine-rich with pairs of intramolecular disulfide bonds, for which expression of these proteins in *Escherichia coli* usually leads to the formation of inclusion bodies resulting in difficulty in refolding (Pan et al. 1999). This research work has used the pET-32a (+) expression vector carrying a fusion protein part of thioredoxin (Trx) tag to increase soluble expression of recombinant TLE2 in the cytoplasm and a $6 \times$ His tag for purification. In addition, its protein sequence and biochemical activities were evaluated and compared with other snake venom serine proteases.

Materials and methods

Biological materials, reagents and plasmids

The snakes used in this study were wild-caught *Deinagkistrodon acutus* from Guangxi province in China. The T7Select 10-3b vector, *E. coli* BLT5403 strain, and T7Select Biopanning Kit were from Novagen. *E. coli* strains Top10 and BL21(DE3) (Novagen) were used for plasmids propagation and protein expression, respectively. The pMD18-T and pET-32a (+) vector were from Takara. Restriction endonucleases and T4 DNA ligase were from Promega. Human fibrinogen and thrombin were from Sigma. Recombinant SVTLE acutobin (expressed in *Pichia pastoris*) was from Cusabio. Other buffers and chemicals were of reagent grade.

Construction of T7 phage display library from *D. acutus* venom gland

Poly(A)+ mRNA was isolated from the fresh venom glands of *D. acutus*, and reverse transcribed into ds-cDNA. The ds-cDNA fragments longer than 400 bp were fractionated by a mini column and assembled into the T7Select 10-3b vector. After being packaged in vitro, the T7Select 10-3b vector was transformed into BLT5403 strain to construct the T7 phage display library. The titer of the library was determined by plaque assay.

Library screening

According to the manual of the T7Select Biopanning Kit, a population of phage were selected out in three rounds of biopanning with high affinity for the target molecule using ELISA plates coated with human

fibrinogen. We amplified the DNA of screened phage by PCR with primer1 (forward): 5′-GGAGCTGTCGT ATTCCAGT-3′ and primer2 (reverse): 5′-TTGGGG AGTTCTGGGCAAAT-3′. The PCR products recovered from agarose gel electrophoresis were cloned into pMD18-T vector, and then transformed into *E. coli* strain Top10. The white transformants were screened by PCR and the positive clones were subjected to sequencing on an Applied Biosystems 3130 Genetic Analyzer.

Recombinant plasmid construction

The plasmid pMD-18T-TLE2 was extracted and used as the template for PCR. The oligonucleotide primers designed to amplify the coding region of TLE2 were primer3 (forward): 5′-GGAATTCATGGTGCTGAT-CAGAGTGCTAGCAAACCTTCT-3′ and primer4 (reverse): 5′-CCCAAGCTTTCACGGGGGGCAAG CCGTAGTTG-3′. After digestion with restriction endonucleases, *Eco*RI and *Hin*dIII, the TLE2 gene fragment was inserted into pET-32a (+) and the ligation product was transformed into *E. coli* Top10 for proliferation. The positive transformants were sequenced to confirm the correct construction of the high-level expression recombinant plasmid, pET-32a-TLE2.

Expression and purification of recombinant TLE2

The plasmid pET-32a-TLE2 was transformed into *E. coli* BL21(DE3) pLysE cells. Individual colonies were grown in 100 ml $2\times$ YT medium (tryptone 16 g/l, yeast extract 10 g/l, NaCl 5 g/l, and ampicillin 100 µg/ml) for 6 h at 37 °C for small scale culture. The culture was then inoculated into 900 ml fresh medium described above at 37 °C until the OD_{600} reached 0.6–0.8, and protein expression was induced by 0.5 mM IPTG at 18 °C. After induction overnight (12–14 h) the cell pellet was collected by centrifugation at $8000\times g$ for 10 min at 4 °C. The drained pellet can be stored at −80 °C, or subjected to subsequent cell disruption with ultrasonication in the lysis buffer (20 mM Tris/HCl pH 7.4, 150 mM NaCl and 0.5 mM EDTA) followed by centrifugation at $18,000\times g$ for 15 min at 4 °C. The supernatant was passed through a Ni–NTA His binding resin and washed with lysis buffer. The recombinant TLE2 with His6-tag was eluted with lysis buffer containing 300 mM imidazole.

Afterwards the protein was purified via HPLC performed on a Protein-Pak 125 column (300×7.8 mm) (Waters) and eluted with 0.1 M sodium phosphate (pH 7.4) at 0.2 ml/min for 120 min. Absorption was measured at 254 nm.

SDS-PAGE and western blotting

Proteins were analyzed by SDS-PAGE using 10% gels and the Tris/glycine system. The separated proteins were stained with Coomassie Brilliant Blue R-250 or electrophoretically transferred to nitrocellulose filters for western blotting. After blocking with 5% (v/v) non-fat milk in TBS, membranes were washed three times with TBS for 10 min and then incubated with the mouse monoclonal antibody against His-tag (Novagen) at a dilution of 1:2000 for 1 h, and the goat anti-mouse fluorescence IgG (LI-COR Biosciences) at a dilution of 1:5000 as the second antibody, washed following the same procedure above. Fluorescent blots were imaged on the Odyssey Infrared Imaging System.

Blood clotting activity

Blood samples were collected from six healthy donors in presence of 3.8% (w/v) sodium citrate and centrifuged at $2500\times g$, 4 °C for 15 min to obtain platelet-poor plasma. Coagulant activity was measured by the method of Biggs (1976). A series of different doses of the purified TLE2 or recombinant SVTLE acutobin (Wang et al. 2001) were added to 0.2 ml citrated human plasma and the clotting time was recorded at 37 °C. Human thrombin (2000 NIH units/mg) was used as the standard. One unit of clotting activity was defined as the amount of protease necessary to induce the same response as the standard thrombin did. Specific activity is expressed as NIH units/mg protein. The minimum coagulant dose (MCD) for plasma was also determined, which represents the least amount of enzyme that induces clotting within 60 s (Perez et al. 2008). The samples were tested three times independently at each dose.

Fibrinogenolytic activity

The fibrinogenolytic activity was determined by incubating the purified protein (50 µg/ml) with human plasminogen-free fibrinogen (5 mg/ml) in 50 mM

Tris/HCl buffer, 0.1 M NaCl, pH 7.8 at 37 °C (Uday et al. 2017). From 5 to 120 min, 20 µl reaction solution was pipetted into another tube containing an equal volume of 2% SDS and 2% β-mercaptoethanol. The fibrinogen degradation products at each time point were then checked on SDS-PAGE. The control was incubated without enzyme for 120 min. We used recombinant SVTLE acutobin as the parallel comparison.

Factor XIII activation activity

The factor XIII activation activity was determined as follows: 0.1 ml of recombinant TLE2 solution (1 mg/ml) and 0.1 ml 25 mM CaCl$_2$ were added to 0.1 ml citrated human plasma. 30 min after the clotting of the reaction mixture, 1 ml 5 M urea was added and the time required for the dissolution of the clot was recorded. Human thrombin served as a control.

Effects of pH, temperature and inhibitors

In order to test the pH-dependence of TLE2's bioactivity, the purified protein was dialyzed and dissolved in 50 mM different buffers with pH from 4 to 10, and the same clotting assay as above was repeated at 37 °C. The optimal enzymatic temperature of TLE2 was determined from 10 to 60 °C in 50 mM HEPES buffer (pH 7.5), and the clotting activity was examined using plasma pre-incubated at each temperature. To investigate potential inhibitory factors affecting TLE's activity, the purified enzyme was incubated for 30 min at 37 °C with (or without) various concentrations of PMSF or EDTA, and subsequently taken to detect the coagulation activity. The experiments were conducted in triplicate.

Results
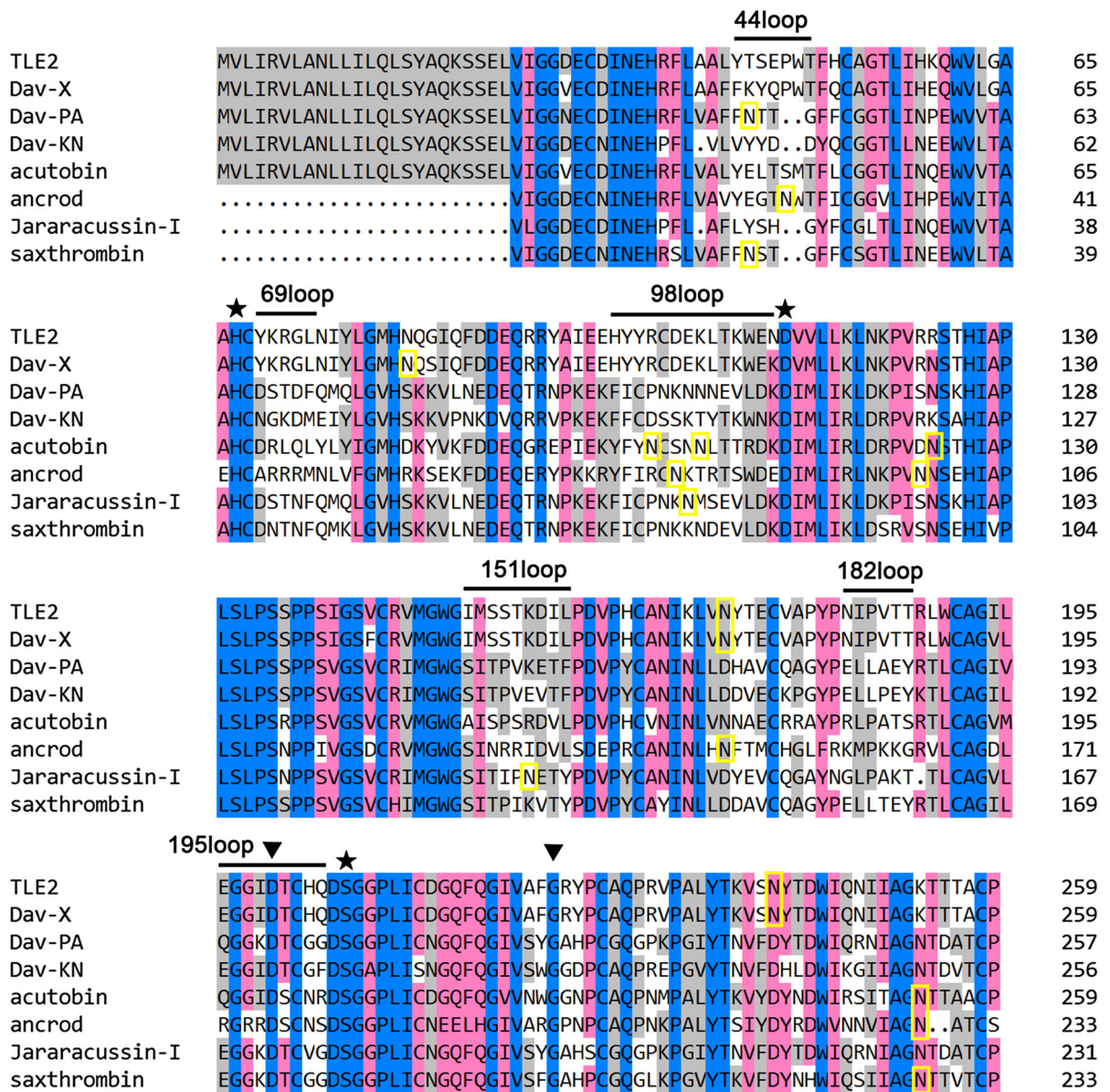
Library construction and biopanning

The initial phage titer was 1.2×10^6 pfu/ml, which suggested that the *D. acutus* venom gland T7 phage display library had been constructed successfully. A population of positive phage were screened out after three rounds of biopanning with high affinity for fibrinogen. We used PCR to amplify the DNA of selected phage. Finally, we cloned a new enzyme gene, designated TLE2, that contains an ORF of 783 bp corresponding to 260 amino acids. The cDNA sequence data of TLE2 has been deposited in the Genbank Data Library under the accession number AY861138.

Molecular characterization

By running a BLAST search in the NCBI databases (http://blast.ncbi.nlm.nih.gov/), we found that the homologous protein sequences of TLE2 covered the majority of snake venom serine proteases (SVSPs), especially the SVTLE family. Interestingly, TLE2 shares the highest sequence identity, 94.6%, with Dav-X from *D. acutus,* but possesses relatively lower similarities of 50–70% with other SVSPs. We compared the protein sequence of TLE2 with those of four enzymes previously discovered in *D. acutus* venom (Wang et al. 2001) and three well-characterized SVTLEs from other Viperidae species using the multiple sequence alignment tool Clustal Omega (Fig. 1). The 24-mer *N*-terminal prepropeptide sequences show complete conformity within the *D. acutus* group of TLE2, Dav-X, Dav-PA, Dav-KN and acutobin. The highly conserved structural features of SVTLEs, including catalytic triad residues (His67, Asp112 and Ser206), the primary (S1) and secondary (S2) substrate specificity sites (Asp200, Gly223), are found in the eight aligned sequences. Other important elements of serine proteases such as disulfide bridges are also preserved in TLE2 deduced as Cys31-Cys165, Cys52-Cys68, Cys102-Cys258, Cys144-Cys212, Cys176-Cys191 and Cys202-Cys227. There exist two potential N-linked glycosylation sites in TLE2 sequence at Asn172 and Asn241, which are not in full accord with the other proteases' except for Dav-X.

To explore the evolutionary relationship of TLE2 and TLEs from other species, a neighbor-joining phylogenic tree was built with the program MEGA6 on the basis of amino acid sequences of 20 representative SVTLEs from Viperidae snakes (Fig. 2). The tree is divided into two basal clusters: the typical coagulating TLEs with the major function of α-fibrinogenase, and the ones which tend to show other activities such as kinin-releasing or coagulation factor-activating. As expected, TLE2 falls into the former cluster adjacent to Dav-X, acutobin (*D. acutus*) and ancrod (*Calloselasma rhodostoma*). Noteworthy, Dav-PA and Dav-KN from *D. acutus* do not cluster

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                                              44loop
TLE2          MVLIRVLANLLILQLSYAQKSSELVIGGDECDINEHRFLAALYTSEPWTFHCAGTLIHKQWVLGA   65
Dav-X         MVLIRVLANLLILQLSYAQKSSELVIGGVECDINEHRFLAAFFKYQPWTFQCAGTLIHEQWVLGA   65
Dav-PA        MVLIRVLANLLILQLSYAQKSSELVIGGNECDINEHRFLVAFFNTT..GFFCGGTLINPEWVVTA   63
Dav-KN        MVLIRVLANLLILQLSYAQKSSELVIGGDECNINEHPFL.VLVYYD..DYQCGGTLLNEEWVLTA   62
acutobin      MVLIRVLANLLILQLSYAQKSSELVIGGVECDINEHRFLVALYELTSMTFLCGGTLINQEWVVTA   65
ancrod        .....................VIGGDECNINEHRFLVAVYEGTNWTFICGGVLIHPEWVITA   41
Jararacussin-I .....................VLGGDECDINEHPFL.AFLYSH..GYFCGLTLINQEWVVTA   38
saxthrombin   .....................VIGGDECNINEHRSLVAFFNST..GFFCSGTLINEEWVLTA   39

              ★  69loop                             98loop           ★
TLE2          AHCYKRGLNIYLGMHNQGIQFDDEQRRYAIEEHYYRCDEKLTKWENDVVLLKLNKPVRRSTHIAP  130
Dav-X         AHCYKRGLNIYLGMHNQSIQFDDEQRRYAIEEHYYRCDEKLTKWEKDVMLLKLNKPVRNSTHIAP  130
Dav-PA        AHCDSTDFQMQLGVHSKKVLNEDEQTRNPKEKFICPNKNNNEVLDKDIMLIKLDKPISNSKHIAP  128
Dav-KN        AHCNGKDMEIYLGVHSKKVPNKDVQRRVPKEKFFCDSSKTYTKWNKDIMLIRLDRPVRKSAHIAP  127
acutobin      AHCDRLQLYLYIGMHDKYVKFDDEQGREPIEKYFYNISNNLTTRDKDIMLIRLDRPVDNSTHIAP  130
ancrod        EHCARRRMNLVFGMHRKSEKFDDEQERYPKKRYFIRCNKTRTSWDEDIMLIRLNKPVNNSEHIAP  106
Jararacussin-I AHCDSTNFQMQLGVHSKKVLNEDEQTRNPKEKFICPNKNMSEVLDKDIMLIKLDKPISNSKHIAP  103
saxthrombin   AHCDNTNFQMKLGVHSKKVLNEDEQTRNPKEKFICPNKKNDEVLDKDIMLIKLDSRVSNSEHIVP  104

                              151loop                      182loop
TLE2          LSLPSSPPSIGSVCRVMGWGIMSSTKDILPDVPHCANIKLVNYTECVAPYPNIPVTTRLWCAGIL  195
Dav-X         LSLPSSPPSIGSFCRVMGWGIMSSTKDILPDVPHCANIKLVNYTECVAPYPNIPVTTRLWCAGVL  195
Dav-PA        LSLPSSPPSVGSVCRIMGWGSITPVKETFPDVPYCANINLLDHAVCQAGYPELLAEYRTLCAGIV  193
Dav-KN        LSLPSSPPSVGSVCRIMGWGSITPVEVTFPDVPYCANINLLDDVECKPGYPELLPEYKTLCAGIL  192
acutobin      LSLPSRPPSVGSVCRVMGWGAISPSRDVLPDVPHCVNINLVNNAECRRAYPRLPATSRTLCAGVM  195
ancrod        LSLPSNPPIVGSDCRVMGWGSINRRIDVLSDEPRCANINLHNFTMCHGLFRKMPKKGRVLCAGDL  171
Jararacussin-I LSLPSNPPSVGSVCRIMGWGSITIFNETYPDVPYCANINLVDYEVCQGAYNGLPAKT.TLCAGVL  167
saxthrombin   LSLPSSPPSVGSVCHIMGWGSITPIKVTYPDVPYCAYINLLDDAVCQAGYPELLTEYRTLCAGIL  169

              195loop           ★                    ▼
TLE2          EGGIDTCHQDSGGPLICDGQFQGIVAFGRYPCAQPRVPALYTKVSNYTDWIQNIIAGKTTTACP  259
Dav-X         EGGIDTCHQDSGGPLICDGQFQGIVAFGRYPCAQPRVPALYTKVSNYTDWIQNIIAGKTTTACP  259
Dav-PA        QGGKDTCGGDSGGPLICNGQFQGIVSYGAHPCGQGPKPGIYTNVFDYTDWIQRNIAGNTDATCP  257
Dav-KN        EGGIDTCGFDSGAPLISNGQFQGIVSWGGDPCAQPREPGVYTNVFDHLDWIKGIIAGNTDVTCP  256
acutobin      QGGIDSCNRDSGGPLICDGQFQGVVNWGGNPCAQPNMPALYTKVYDYNDWIRSITAGNITAACP  259
ancrod        RGRRDSCNSDSGGPLICNEELHGIVARGPNPCAQPNKPALYTSIYDYRDWVNNVIAGN..ATCS  233
Jararacussin-I EGGKDTCVGDSGGPLICNGQFQGIVSYGAHSCGQGPKPGIYTNVFDYTDWIQRNIAGNTDATCP  231
saxthrombin   EGGKDTCGGDSGGPLICNGQFQGIVSFGAHPCGQGLKPGVYTKVFDYNHWIQSIIAGNITVTCP  233
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Fig. 1 Amino acid sequences alignment of TLE2 with other serine proteases. Sequence identities of the proteases compared to TLE2 are as follows: Dav-X (94.6%, *D. acutus*), Dav-PA (56.9%, *D. acutus*), Dav-KN (58.1%, *D. acutus*), acutobin (65.4%, *D. acutus*), ancrod (53.4%, *C. rhodostoma*), Jararacussin-I (51.7%, *B. jararacussu*), and saxthrombin (52.5%, *Gloydius saxatilis*). Numbers on the right represent the residue numbers of the last amino acid in each line. Identical residues are shaded in blue, conserved residues with identity above 75% and between 50 and 75% are shaded in pink and gray, respectively. Residues of the catalytic triad are indicated by pentagrams. The S1 and S2 substrate specificity sites are indicated by triangles. Potential and experimentally determined glycosylation sites are in yellow boxes. The sequences of the loops surrounding the active site are shown by upper lines

together with TLE2, but are laid at remote branches of the latter cluster.

To investigate the structural properties of TLE2, we predicted its tertiary structure on the webserver I-TASSER (http://zhanglab.ccmb.med.umich.edu/I-TASSER/, Yang et al. 2015) and the best model was compared with the crystal structure of Dav-PA from *D. acutus* (PDB code: 1op0, Zhu et al. 2005). The
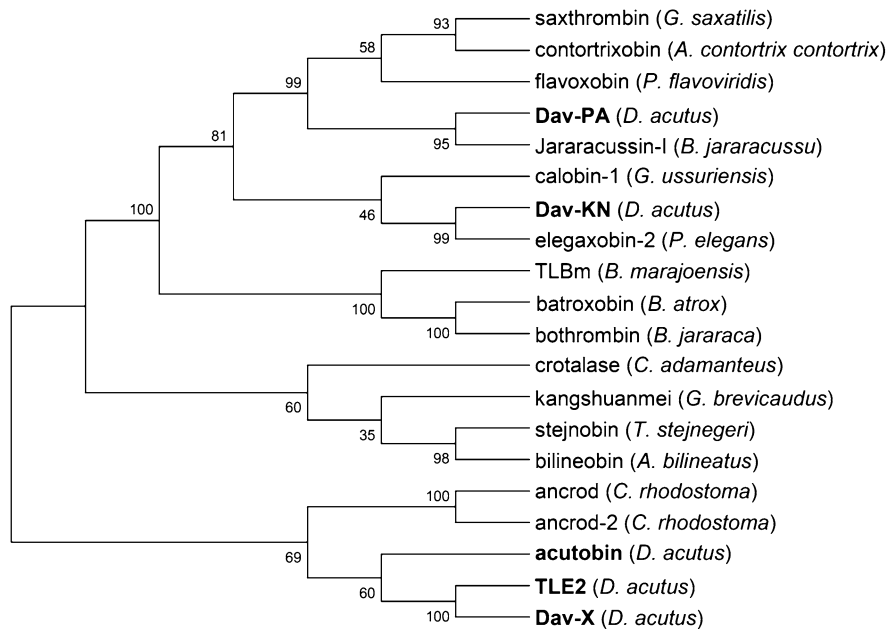
Fig. 2 Phylogenetic tree of TLEs in Viperidae snake venom. The tree was constructed using the neighbor-joining method on the basis of complete amino acid sequences of 20 representative SVTLEs from Viperidae family. The genus abbreviations are as follows: *Gloydius* (*G.*), *Agkistrodon* (*A.*), *Protobothrops* (*P.*), *Deinagkistrodon* (*D.*), *Bothrops* (*B.*), *Calloselasma* (*C.*) and *Trimeresurus* (*T.*). A total of 1000 bootstrap replicates were used to test the reliability of each branch, and the values (%) on the branch indicate the proportions of the bootstrap replicates. The *D. acutus* SVTLEs are in bold type

overall structures of the two proteins present a typical fold of trypsin-like serine proteases and comprise two β-barrels and three α-helices. Also, the spatial organization of the catalytic triad residues His67, Asp112, and Ser206 at the active site cleft between the two β-barrels is preserved. Superposition of the Cα atoms of the TLE2 model with Dav-PA reveals similar backbones (Fig. 3a) with low root-mean-square deviations (RMSD) of 0.42 Å. The main differences are located at the loops surrounding the active site. The varied amino acids substitutions in six loops (Fig. 1) may result in a differential distribution of the surface charge at the catalytic interface and different degrees of entrance limit to the active site pocket for substrates. Despite the same residues at the S1 and S2 sites in TLE2 and Dav-PA, the S3 specificity site as well as the key amino acids involving substrate binding around the active site pocket are unique to each other at the corresponding positions of alignment (Fig. 3b). These distinctions are likely to give rise to distinguishing substrate recognition patterns and different range of substrate selectivity between TLE2 and Dav-PA, which might also account for their separated relationship on the phylogenic tree (Fig. 2).

Plasmid construction, expression and purification of recombinant TLE2

The pET-32a-TLE2 plasmid was sequenced to be in accordance with the previous results. After being expressed in *E. coli* and purified using His-tag affinity chromatography and HPLC (Supplementary Fig. 1), the recombinant protein displayed a single band after SDS-PAGE with an apparent MW of 49.4 kDa containing the 20.4 kDa Trx tag. This was confirmed through western blotting with the primary antibody against His-tag fused on TLE2 and the fluorescence secondary antibody (Fig. 4).

Plasma coagulant activity

The plasma clotting time was measured versus the dose of proteinase. As illustrated in Fig. 5, 6 μg recombinant TLE2 could make the normal human plasma clot in 57 s, while the MCD of recombinant acutobin was below 0.5 μg. (The clotting time is converted into NIH units with reference to human thrombin standard calibration curve). The specific clotting activities of recombinant TLE2 and acutobin
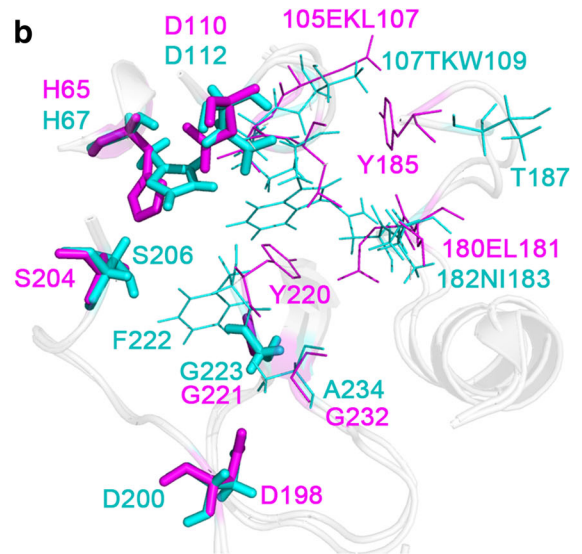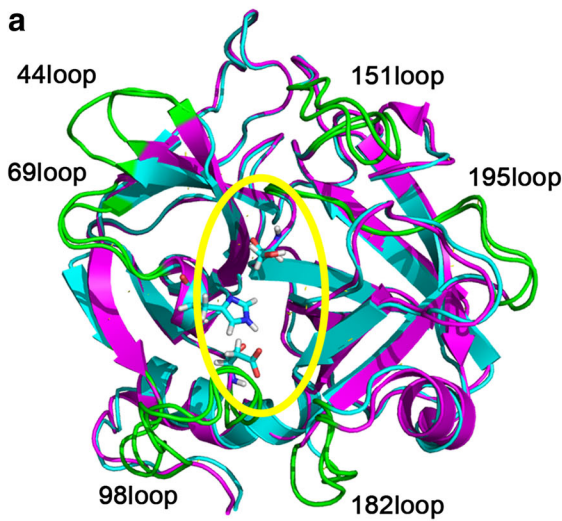
Fig. 3 Comparison of the predicted structure model of TLE2 with the crystal structure of Dav-PA (PDB code: 1OP0). **a** Overall structures superimposition of TLE2 (cyan) and Dav-PA (magenta), the loops surrounding the active site are in green and the catalytic residues (His67, Asp112 and Ser206) are shown as stick models in yellow ellipse indicating the active site cleft. **b** Structure around the active site pocket, the catalytic residues, S1 (Asp200) and S2 (Gly223) specificity sites are depicted as cyan (TLE2) or magenta (Dav-PA) stick models, while the S3 (234) site and the key residues for substrate-binding (107, 108, 109, 182, 183, 187, 222) are shown with cyan (TLE2) or magenta (Dav-PA) line models
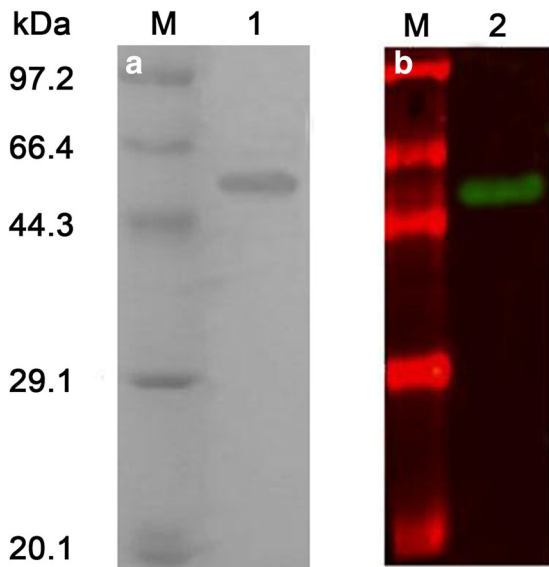
Fig. 4 10% SDS-PAGE assay (**a**) and Western blot analysis (**b**) for recombinant TLE2 purified by His-tag affinity chromatography and HPLC. Lane M, protein molecular weight maker; lane 1, purified TLE2; lane 2, immunoblot of TLE2 probed with anti-His antibody
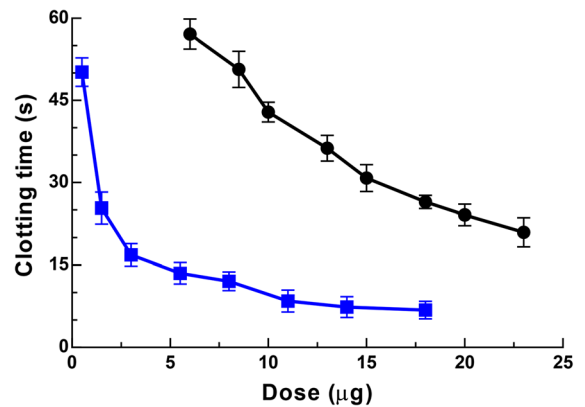
Fig. 5 Effect of various doses of recombinant SVTLEs on clotting time of normal human plasma. The minimum coagulant dose (MCD) corresponds to the least amount of enzyme that induces clotting within 60 s. Recombinant acutobin was assayed as a control. Human thrombin was tested as the standard, and clotting times were transformed into NIH units from dilution curves of human thrombin. The tests were repeated three times at each concentration. (Black circle) recombinant TLE2, (blue square) recombinant acutobin

were 120 NIH units/mg and 1400 NIH units/mg, respectively.

Fibrinogenolytic activity

Figure 6 shows the results of SDS-PAGE analysis of human fibrinogen incubated with recombinant TLE2 (Fig. 6a) or acutobin (Fig. 6b). As shown in the control lane, reduced fibrinogen separated into three bands: Aα, Bβ and γ-chain. When mixed with TLE2, the Aα-chain started to degrade after 5 min of incubation and gradually disappeared within 60 min. When incubated with recombinant acutobin, the Aα-chain was completely degraded just after 5 min of incubation. Identically in the two group, the Bβ-chain and γ-chain of fibrinogen were scarcely cleaved during 120 min incubation.

Optimal enzymatic conditions and inhibitory studies

The recombinant TLE2 was most active at pH 7.5–8 (defined as 100% activity), and the relative coagulant activity at pH 10.0 were nearly 27% but decreased to 6% at pH 4.0 (Fig. 7a). The optimal temperature of TLE2 was 35–40 °C, and the enzyme retained 35 and 12% of its activity at 10 and 60 °C, respectively (Fig. 7b). For effects of protease inhibitors, changes of relative activities are shown in Fig. 7c, d. Exposure to 2 mM PMSF completely inhibited the clotting activity of TLE2 while, in contrast, its activity was not affected by EDTA, which revealed that TLE2 belonged to serine proteases and did not contain metal ions.

Determination of factor XIII activation activity

The plasma clots formed by the action of recombinant TLE2 dissolved 5 min after adding of 5 M urea. As control, the clots formed by human thrombin were not able to dissolve in 5 M urea after 24 h. This suggested that the enzyme had no factor XIII activation activity.

Discussion

A new gene from *D. acutus* venom gland library was screened and cloned. It was designated as TLE2 (Genbank Number AY861138). Homology search and protein sequence alignment reveals that TLE2 is analogous to known SVTLEs and shares the highest identity of 94.6% with Dav-X from *D. acutus*. The featured catalytic triad, cysteines, S1 and S2 substrate specificity sites of typical SVTLEs are highly conserved in TLE2 sequence. The recombinant protein was expressed in a soluble form in *E. coli* and purified to homogeneity. It had a net MW of approx. 29 kDa. The recombinant protease exhibited plasma-clotting activity of 120 NIH units/mg with a minimum
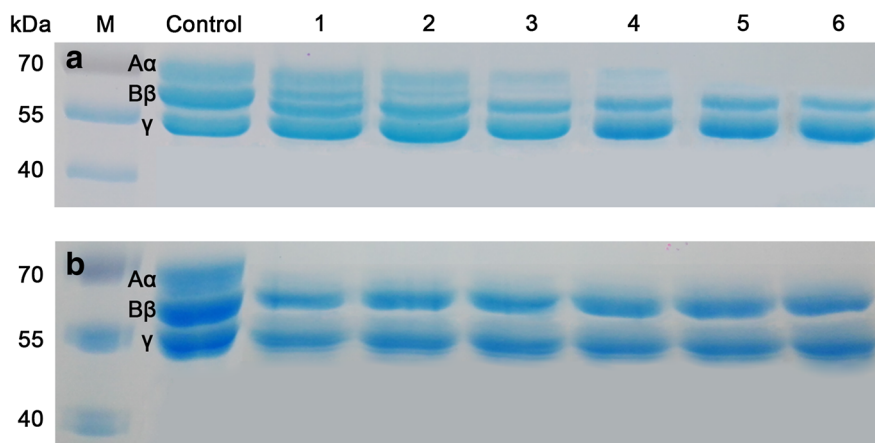
Fig. 6 Fibrinogenolytic activity analysis of recombinant TLE2 (**a**) and acutobin (**b**) by SDS-PAGE. Plasminogen-free human fibrinogen (5 mg/ml) was incubated with 50 µg/ml purified enzymes in 50 mM Tris/HCl buffer, pH 7.8 containing 0.1 M NaCl at 37 °C. At various time points from 5 to 120 min, aliquots of the reaction mixture were collected to determine fibrinogen degradation on 10% SDS-PAGE. The control was incubated without enzyme for 120 min. Lane M, molecular weight maker; lane 1–6, incubation for 5, 10, 20, 40, 60 and 120 min, respectively. Bands of Aα, Bβ and γ chain of fibrinogen were labeled in the figure
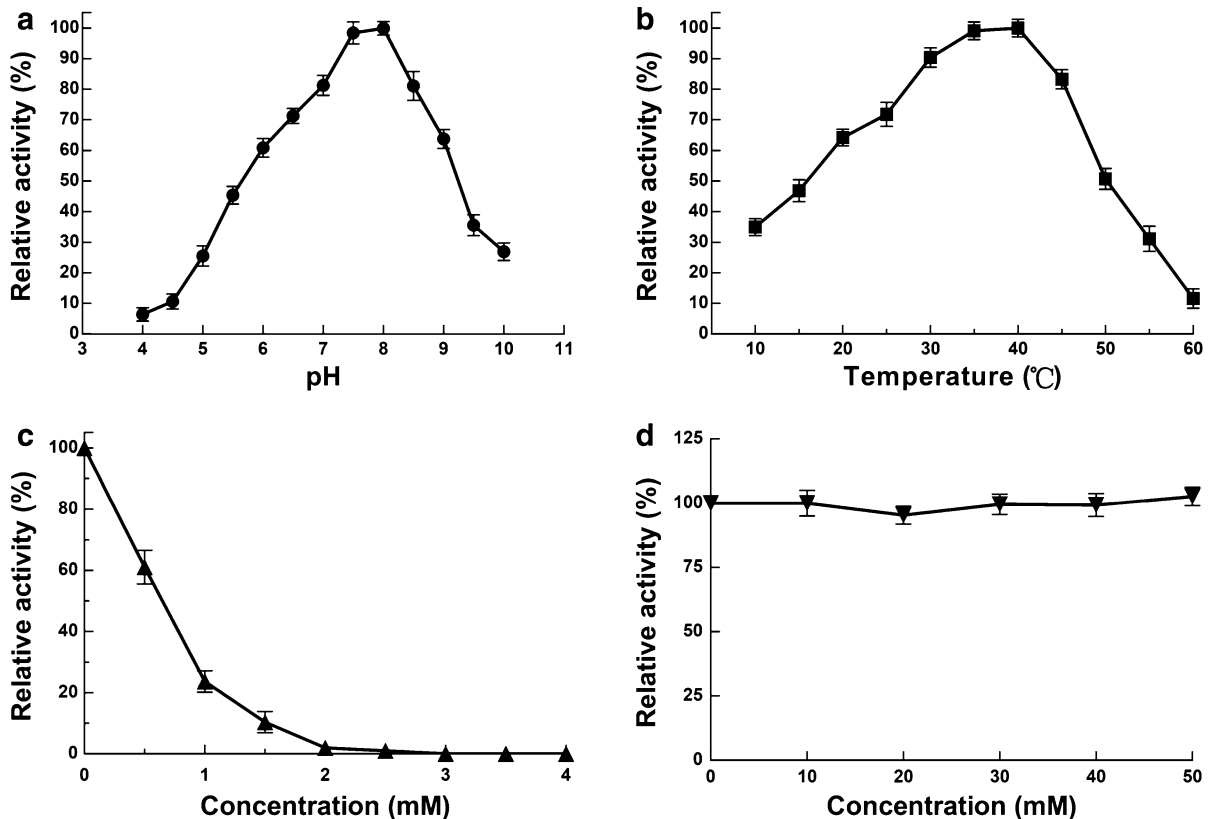
Fig. 7 Biochemical characterization of recombinant TLE2. **a** Optimal pH, the effect of pH on TLE2 activity was determined at 37 °C in each of the following solutions (50 mM): sodium acetate/acetic acid buffer (pH 4.0, 4.5, 5.0), MES buffer (pH 5.5, 6.0, 6.5), Tris/HCl buffer (pH 7.0, 7.5, 8.0, 8.5) and glycine/NaOH buffer (pH 9.0, 9.5, 10.0). **b** Optimal temperature, the optimal temperature was estimated at different temperatures (10–60 °C) using 50 mM HEPES buffer (pH 7.5). For the effects of inhibitors, purified TLE2 was incubated in buffer containing 0–4 mM PMSF (**c**) or 0–50 mM EDTA (**d**) at 37 °C for 30 min, and then the residual clotting activities were tested. The highest activity was treated as 100%

coagulant dose of 6 μg. It hydrolyzed the Aα-chain of fibrinogen without the Bβ-chain or γ-chain being cleaved. The recombinant TLE2 was optimally active at 35–40 °C and at pH 7.5–8. Its coagulant activity was completely inhibited by PMSF, whereas EDTA had no effect. Moreover, recombinant TLE2 displayed no activating activity towards factor XIII. Therefore, this enzyme is identified as a novel SVTLE and belongs to the SVTLE-A class.

Comparison of the predicted three-dimensional model of TLE2 and the crystal structure of Dav-PA demonstrates the common thrombin-like domains in SVSPs and the identical backbones. Although their overall structures are similar, there are several blocks of distinctive residue substitutions located at the loops around the active site pocket. While SVSPs share a common active site and enzymatic mechanism,

variation of primary sequences out of the active center results in differences of substrate specificities and, further, the difference of biological activities (Zhang et al. 1998). Likewise, the phylogenetic analysis revealed that TLE2 and Dav-PA seemed to arise from an ancient ancestor sequence, and early genetic events of key residue mutations led to evolutionary divergence under two separated clusters, which caused the diversity of functional subtypes within one single species.

Acutobin, the main TLE in *D. acutus* venom (Wang et al. 2001), shares 65% sequence identity with TLE2 and they are close on the phylogenic tree. Consistently, both enzymes displayed obvious plasma-clotting and α-fibrinogenase activities. However, the specific coagulation potency of recombinant TLE2 (120 NIH units/mg) was almost 10 times lower than

that of recombinant acutobin (1400 NIH units/mg), and the Aα-chain of fibrinogen degraded much slower in the presence of recombinant TLE2 than of recombinant acutobin. The significant deviation of enzymatic activity is assumed to stem from the presence or absence of glycosylation, which plays an important role in the stabilization and catalytic function of some SVTLEs (Castro et al. 2004), but could not be produced in prokaryotic expression system. On the other hand, it should be noted that since multiple subspecies of *D. acutus* may exist in different geographical localities, the loss of bioactivities or releasing amount of certain venom enzyme isoforms can be regarded as some kind of evolutionary behavior of snakes to adapt to different ecological environments or periods of growth.

In summary, we have identified a novel thrombin-like protein TLE2 from *D. acutus* venom. The defibrinogenating effects of TLE2 make it potential candidate for drug discovery on antithrombotic and anticoagulant treatment. Meanwhile, this system of phage display with protein expression and purification provides good references for technological development concerning large-scale screening and industrial production of commodity enzymes and therapeutic proteins.

References

Biggs R (1976) Human blood coagulation: haemostasis and thrombosis. Blackwell, Philadelphia

Castro HC, Zingali RB, Albuquerque MG, Pujol-Luz M, Rodrigues CR (2004) Snake venom thrombin-like enzymes: from reptilase to now. Cell Mol Life Sci 61:843–856

Davey MG, Luscher EF (1965) Actions of some coagulant snake venoms on blood platelets. Nature 207:730–732

Koh DC, Armugam A, Jeyaseelan K (2006) Snake venom components and their applications in biomedicine. Cell Mol Life Sci 63:3030–3041

Komori Y, Nikai T, Sakairi Y, Sugihara H (1988) Characterization of clotting factors from *Agkistrodon acutus* venom. Int J Biochem 20:387–392

Oyama E, Takahashi H (2002) Amino acid sequence of a thrombin like enzyme, elegaxobin, from the venom of *Trimeresurus elegans* (Sakishima-habu). Toxicon 40:959–970

Pan H, Du X, Yang G, Zhou Y, Wu X (1999) cDNA cloning and expression of acutin. Biochem Biophys Res Commun 255:412–415

Perez AV, Rucavado A, Sanz L, Calvete JJ, Gutierrez JM (2008) Isolation and characterization of a serine proteinase with thrombin-like activity from the venom of the snake *Bothrops asper*. Braz J Med Biol Res 41:12–17

Rajesh R, Shivaprasad HV, Gowda CD, Nataraju A, Dhananjaya BL, Vishwanath BS (2007) Comparative study on plant latex proteases and their involvement in hemostasis: a special emphasis on clot inducing and dissolving properties. Planta Med 73:1061–1067

Samsa GP, Matchar DB, Williams GR, Levy DE (2002) Cost-effectiveness of ancrod treatment of acute ischaemic stroke: results from the Stroke Treatment with Ancrod Trial (STAT). J Eval Clin Pract 8:61–70

Uday P, Maheshwari M, Sharanappa P, Nafeesa Z, Kameshwar VH, Priya BS, Swamy SN (2017) Exploring hemostatic and thrombolytic potential of heynein—a cysteine protease from *Ervatamia heyneana* latex. J Ethnopharmacol 199:316–322

Wang YM, Wang SR, Tsai IH (2001) Serine protease isoforms of *Deinagkistrodon acutus* venom: cloning, sequencing and phylogenetic analysis. Biochem J 354:161–168

Yang J, Yan R, Roy A, Xu D, Poisson J, Zhang Y (2015) The I-TASSER Suite: protein structure and function prediction. Nat Methods 12:7–8

Zhang Y, Gao R, Lee WH, Zhu SW, Xiong YL, Wang WY (1998) Characterization of a fibrinogen-clotting enzyme from *Trimeresurus stejnegeri* venom, and comparative study with other venom proteases. Toxicon 36:131–142

Zhu Z, Liang Z, Zhang T, Zhu Z, Xu W, Teng M, Niu L (2005) Crystal structures and amidolytic activities of two glycosylated snake venom serine proteinases. J Biol Chem 280:10524–10529