FEBS Letters 581 (2007) 2965–2973

Identification and characterization of Harobin, a novel fibrino(geno)lytic serine protease from a sea snake (*Lapemis hardwickii*)

Junyun He[1], Shiyong Chen[1], Jun Gu*

National Key Laboratory of Protein Engineering and Plant Gene Engineering, LSC, Peking University, Beijing, China

Received 30 March 2007; revised 8 May 2007; accepted 18 May 2007

Available online 29 May 2007

Edited by Veli-Pekka Lehto

Abstract **A gene encoding a novel serine protease designated as Harobin is cloned and identified from a sea snake venom gland bacteriophage T7 library. It has 265 amino acids and shares 50–70% similarity to terrestrial snake serine proteases. In addition to the 12 conservative Cys, it has three more Cys residues that may contribute to its higher enzymatic stability. Harobin is expressed in *Pichia pastoris* and purified.**

Recombinant Harobin exhibits an amidolytic activity, and specifically degrades Aα, Bβ-chain of fibrinogen. It functions as a defibrase both in vitro and in vivo, and reduces thrombosis. Harobin prolongs the coagulation time and the bleeding time of mice and reduces the fibrinogen levels of rats as well. Meanwhile, intravenous injection of Harobin leads to the reduction of blood pressure in SHR rats. It results from the ability of Harobin that cleaves angiotensin I and release bradykinin from plasma kininogen in vitro and in vivo. These data suggest that Harobin is a novel defibrase and has a potential to be an agent for the therapy of thrombosis and hypertension.

Keywords: Sea snake; Serine protease; Defibrase; Fibrinogen; Antithrombosis; Hypertension

1. Introduction

Snake venoms, especially from crotalidae and viperidae families, are abundant in proteolytic enzymes. According to the difference of the enzymatic active site, these proteases are divided into two groups: serine protease and metalloprotease. Both of them can interact with at least one sort of coagulation factors or the other protein components in plasma and cleave specific peptide bond in their substrates. These substrates are including coagulation factor II, V, IX, X, plasminogen, protein C, kininogen and fibrinogen, etc. [1]. Among these venom proteases, some hydrolyze N-terminal end of fibrinogen releasing fibrinopeptide A or B or both resulting in the formation of fibrin. Such an activity resembles that of thrombin and thus is named thrombin-like enzyme (TLE). Some degrade the Aα-, Bβ- or both chains of fibrinogen at the C-terminus making it unclottable by thrombin, and therefore, are called fibrinogenase [2]. Some protease can cleave kininogen releasing bradykinin or kallidin, which is a strong vasodilator and has ability to reduce blood pressure [3]. These protease are regarded as kinin-releasing enzyme or kininogenase [4].

Generally, each of these proteases exhibits only one specific enzyme activity. However, up to now, six multifunctional proteases that possess double enzymatic activities have been reported, including cratalase from *Crotalus adamanteus*, KN-BJ from *Bothrops jararaca*, flavovilase from *Trimeresurus flavoridis* (habu), β-fibrinogenase from *Tremeresurus mucrosquamatus*, halyse from *Agkistrodon halys blomhoffii* and Jerdonase from *Tremeresurus jerdonii* [5]. The former three are kinin-releasing and fibrinogen clotting enzyme and the later three are kinin-releasing and fibrinogenolytic enzymes. Their enzymatic characteristics have been elucidated but their physiological functions in experimental animals have not been fully investigated.

Up to now, most of venom serine proteases characterized are from the terrestrial snakes [6,7]. Venom serine proteases from sea snake are seldom reported in the literature. We here report a novel serine protease from sea snake venom. This protease, named Harobin, are expressed in yeast and characterized. It exerts antithrombotic activity by cleaving fibrinogen. Furthermore, Harobin cleaved high molecular weight (HMW) kininogen, angiotensin I and angiotensin II, led to reduced blood pressure in hypertensive rats. The results demonstrate that Harobin may have a potential application in the therapy of thrombosis and hypertension.

2. Methods

2.1. Cloning and preparation of recombinant Harobin

Sea snake *Lapemis hardwickii* was from the coast area of Guangxi province of the People's Republic of China. Venom gland was taken and subjected to the extraction of total RNA. mRNA was prepared using mRNA Purification Kit (Amersham Pharmacia). Following the protocols of OrientExpress Oligo(dT) cDNA synthesizing Kit (Novagen) and T7 Select packaging Kit (Novagen), a T7 phage display library was constructed. Human fibrinogen (Sigma) was used as a bait to screen the library. A positive clone was identified after screening. The gene was cloned and sequenced.

The mature enzyme gene of Harobin (without the signal peptide and the pro-peptide) was cloned into *P. pastoris* expressing vector pPIC9K (Invitrogen) at the sites of XhoI and EcoRI. The reconstructed vector was transformed into GS115 by electroporation after linearized by SalI. Expression of recombinant enzyme was performed according to the instruction by manufacturer (Invitrogen). Yeast expressed Harobin was purified with Benzamidine-Sepharose CL-6B column (Amersham Biosciences, USA) using modified method by Débora [8].

*Corresponding author. Address: Department of Biochemistry and Molecular Biology, College of Life Sciences, Peking University, Beijing 100871, China. Fax: +86 10 62756174.
E-mail address: gj@pku.edu.cn (J. Gu).

[1]These authors contribute equally to this work.

0014-5793/$32.00
doi:10.1016/j.febslet.2007.05.047

2.2. Assay for enzymatic activity

Ten micrograms of purified wild type or mutated recombinant Harobin were mixed with 0.15 mM of the substrate *N*-*p*-tosyl-Gly-Pro-Arg-*p*-nitroanilide (Sigma, USA) in 30 mM Tris–HCl buffer (pH 8.0), and incubated at 37 °C for 10 min. The amount of *p*-nitroaniline released was determined by measuring the changes in absorbance at 405 nm. One unit of amidolytic activity was defined as the amount of enzyme needed to hydrolyze 1 μmol substrate per min. Appropriate amount of the enzyme was incubated with different concentration of substrates ranging from 20 μM to 200 μM, the enzyme reaction was plotted in a Lineweaver–Burk manner to obtain the Michaelis constant K_m.

Arginine esterase activity was measured as described by Yabuki et al. [9], using *N*-*p*-tosyl-L-arginine methylesterase (TAME, Sigma) as a substrate. The absorbance change was monitored at 247 nm for 10 min.

The optimal pH and temperature for the amidolytic activity of Harobin was determined at pH range between 4 and 9, and different temperatures (25, 35, 45, 55, 65, 75, and 85 °C), respectively. For optimal pH assay, Harobin was dissolved in either citrate buffer (30 mM, pH 4–6) or Tris–HCl buffer (30 mM, pH 7–9) and preincubated at room temperature for 2 h. For optimal temperature assay, Harobin was dissolved in Tris–HCl buffer (pH 8.0). The enzyme was preincubated at each temperature for 15 min and the reaction was performed for 10 min to measure its amidolytic activity.

PMSF, EDTA and DTT were used to inhibit the enzyme activity of Harobin. One micrograms of Harobin was incubated with the indicated concentration of inhibitors in 0.4 ml 30 mM Tris buffer, amidolytic activity was measured.

Fibrinogen clotting activity was measured as described previously [10]. The time of coagulation of 0.4 ml human fibrinogen (2 mg/ml, Sigma product) in 50 mM Tris–HCl (pH 8.0) was determined after 2 μg Harobin were added.

The fibrinogenolytic activity was determined by incubating 5 μg fibrinogen with 0.5 μg Harobin in 20 μl Tris–HCl buffer (pH 8.0) at 37 °C. An aliquot was taken at 15 min, 30 min, 3 h, 12 h, 18 h, 24 h and 40 h intervals, respectively, and analyzed on SDS–PAGE.

Fibrinolytic activity was assayed using the fibrin plate technique [11].

2.3. Cleavage of angiotensin I

Fifty microliters of angiotensin I (1 mg/ml) was incubated with 10 μg Harobin in Hepes buffer (50 mM, pH 7.5) at 37 °C for 3 h. The mixture was then analyzed by HPLC (Bio-Rad Bio-Sil ODS-5S C_{18} column). HPLC was run for 35 min in a linear gradient of 0–75% solvent B (95% acetonitrile containing 0.1% trifluoroacetic acid (TFA)) with 5% acetonitrile/0.1% TFA (solvent A) as the starting and equilibration eluent. The flow rate was set at 1 ml/min. Peak fractions were monitored at UV 214 nm and collected. Amino acid sequences were analyzed by ABI Precise 491 Protein/Peptide Sequencer.

2.4. Cleavage of single chain HMW kininogen

Five micrograms of single chain HMW kininogen (from human plasma, The Enzyme Research Laboratory) was incubated at 37 °C with 0.2 μg Harobin in a total volume of 20 μl buffer (50 mM Tris–HCl, pH 8.0) for various time intervals. The proteolytic products were resolved on SDS–PAGE. For the assay of released kinin, the above reacting mixtures were subjected to HPLC. Each peak fraction was collected and sequenced.

2.5. Animals and animal models

Male Sprague–Dawley (SD) rats, spontaneously hypertensive rats (SHR) and male Balb/c mice were obtained from the Vitalriver Experimental Animals Center, Beijing, China. All animals were specific pathogen free (SPF). They were bred at the animal center of Peking University. Procedures involving animals and their care were conducted in accordance with the guidelines for the use of animals in biochemical research.

For tail thrombus model, mice were injected intravenously of drugs. Thirty minutes later, 300 mg/kg of carrageenan (Sigma, Co) was subcutaneously injected to induce thrombus. The length of infarction was measured after 48 h according to the literature [12].

For the measurement of fibrinogen, 1 ml citrated blood was collected from the carotid artery of rats before administration of drug and 6 h later, plasma was prepared by centrifugation and used for the measure of fibrinogen following the protocols of the manufacturer (Shanghai Suntech. Co.).

For arterio-venous shunt model, it was performed according to the method of Umetsu and Sanai [13]. Male SD rat (270–310 g) was anesthetized with sodium pentobarbital and fixed in the supine position. A cervical incision was made in the midline to expose the left carotid artery and right jugular vein. A 20 cm long polyethylene tube with a 6 cm long silk thread fixed in its lumen was filled with physiological saline. One end of the tube was inserted into the right jugular vein and tied. Harobin (400 μg/kg), heparin (50 IU/kg) or physiological saline (2 ml/kg) was injected from the other end of the tube. The proximal side of the left carotid artery was clamped to block the blood flow temporarily, while the free end of the tube was inserted into the artery and tied. The clamp was released and the blood flow through the tube was confirmed. After 15 min, the silk thread was removed from the tube and its wet weight was immediately measured. The dry weight was measured after 24 h at 37 °C.

For vena cava model, the method described by Reyers et al. [14] was used. Briefly, 50, 200 μg/kg Harobin, 0.5 μg/kg defibrase (from *agkistrodon halys ussuriensis emelinov*, Beijing Institute of Biological Products, China) or saline was injected from the tail venous just before the surgery. After anesthetization, the abdomen was opened, the vena cava was isolated and tied just caudally to the left renal vein and the incision closed. Six hours later, animals were re-anesthetized, abdominal incision was reopened. When thrombus appeared, it was removed from the segment, blotted on filter paper. The wet weight of the thrombus was immediately measured. Its dry weight was measured after 24 h at 37 °C.

For the hemorrhagic activity assay, mice were killed 24 h after i.p administration of 5 mg/kg Harobin and checked for subcutaneous hemorrhage.

For the clotting time (CT) measurements, a drop of whole blood was added to a glass slide and stirred up every 30 s with a dried needle until fibrin appeared. The clotting time was recorded in minutes. Bleeding time (BT) of mice was measured by a modification of the method described by Kung et al. [15].

2.6. In vivo hypotension assay

Blood pressure was assayed by the method as described previously [16]. Male SHR rats (250–300 g) were grouped randomly and anesthetized with sodium pentobarbital. The right carotid artery were cannulated with polyethylene tube and attached to a pressure transducer (Model YPJ01). The blood pressure was recorded on a RM6240 system (Chengdu Keyi Factory). Harobin (2 mg/kg), captopril (1 mg/kg) or saline was injected from the femoral vein. After the measurement of blood pressure, rats were killed and blood was collected. The level of angiotensin II in the blood was measured by radioimmunoassay kit (North Biotechnology Research Institute, Beijing).

2.7. Statistical analysis

All data was expressed as the mean ± S.D. Student's *t*-test was used to assess the statistical differences. $P < .05$ was considered to be statistically significant.

3. Results

3.1. Cloning of Harobin

One phage clone from a sea snake venom gland bacteriophage T7 library was identified after screening using fibrinogen as bait. The gene was then cloned and designated as Harobin. It had an open reading frame of 795 nucleotides coding for 265 amino acid residues (GenBank accession AY835844, Fig. 1A). It was homologous to terrestrial snake serine enzymes, sharing 78% similarity with kallikrein-phi4 from *Philodryas olfersii* [17], 63% similarity with mucrofibrase from *Trimeresurus mucrosquamatus* [18], VSP-3 (Venom serine protease 3) from *Trimeresurus gramineus* [19] and Pallabin from *Gloydius halys*

[20], 62% with CPI-enzyme-2 from *Gloydius ussuriensis* [21], 60% with calobin from *Gloydius ussuriensis* [22], 58% with Tsv-Pa from *Trimeresurus stejnegeri* [23] and PA-BJ from *Bothrops jararaca* [24], 48% with Ancrod from *Calloselasma rhodostoma* [25], respectively, indicating that Harobin was a new member of the serine protease family (Fig. 1B). A putative signal peptide of 18 residues was predicted according to the method by Nielsen [26]. The pre-zymogen also had a proposed pro-peptide of 15 amino acid residues (19–33aa). The predicted molecular mass of mature enzyme (34–265aa) was 25414 Da. Based on the sequence similarity, we deduced that the catalytic triad residues were common to serine proteases. They were His74, Asp119 and Ser212. Three putative *N*-glycosylation sites in Asn-Xaa-Ser/Thr, a Ser and a Thr residue as putative *O*-glycosylation sites were identified in the protein through the analysis using CBS Prediction Server, which was a online tool and convenient for the analysis of glycosylation site in protein [27]. Interestingly, this enzyme contained 15 Cys residues, among which 12 residues were identical to other venom serine proteases (Fig. 1B). Most of snake venom serine proteases, including TLE [18,20,28], fibrino(geno)lytic serine protease [29,30], plasminogen activators [31,32] and so on, had six disulfide bonds which were critical for the maintenance of protein structure.

3.2. Harobin was a thermostable serine protease

To characterize its biological function, recombinant Harobin was successfully expressed in *P. pastoris* (Fig. 2A) and easily purified by column chromatograph (Fig. 2B). The molecular weight of Harobin on SDS–PAGE was as deduced as about 25 kD. Since Harobin shared high homology with known serine proteases from terrestrial snake venom, we first determined if it had enzymatic activity. Harobin was incubated with *N*-*p*-tosyl-Gly-Pro-Arg-*p*-nitroanilide or TAME, respectively. The results showed that Harobin had an amidolytic activity and no arginine esterase activity. The amidolytic activity was about 8.3 ± 0.3 U/mg protein with a Michaelis constant K_m of 0.39 mmol/L, which was higher than naturally purified defibrase from terrestrial snake venom (Table 1). The optimum conditions for enzyme activity were pH 8.0 and 65 °C (Fig. 2C). The effect of the known serine protease inhibitors on the amidolytic activity of Harobin was evaluated. As shown in Fig. 2D, PMSF inhibited the enzyme activity nearly 100%, while EDTA did not affect at all, indicating that Harobin

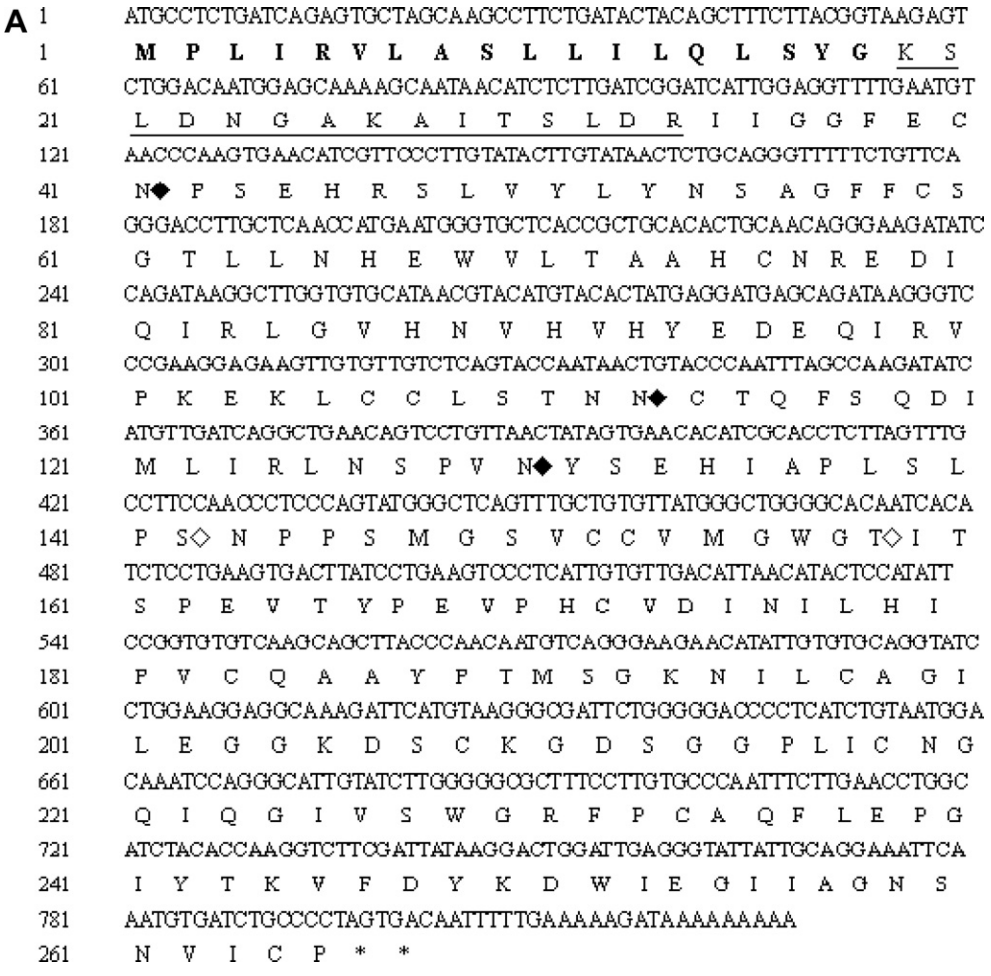

Fig. 1. Sequence analysis. (A) Gene sequence and deduced amino acid sequence of Harobin. Signal sequence was denoted in boldface. Pro-peptide was underlined. The translation stop codon was indicated by asterisk. Solid and open diamonds indicate putative *N*-glycosylation and *O*-glycosylation sites, respectively. The sequence data had been submitted to the GenBank with the accession number AY835844. (B) Sequence alignment. Three conserved critical catalytic sites were highlighted by asterisks. Twelve identical cysteine residues and three different cysteine residues of the serine proteases were indicated as capital C and C′ under the aligned sequence, respectively.

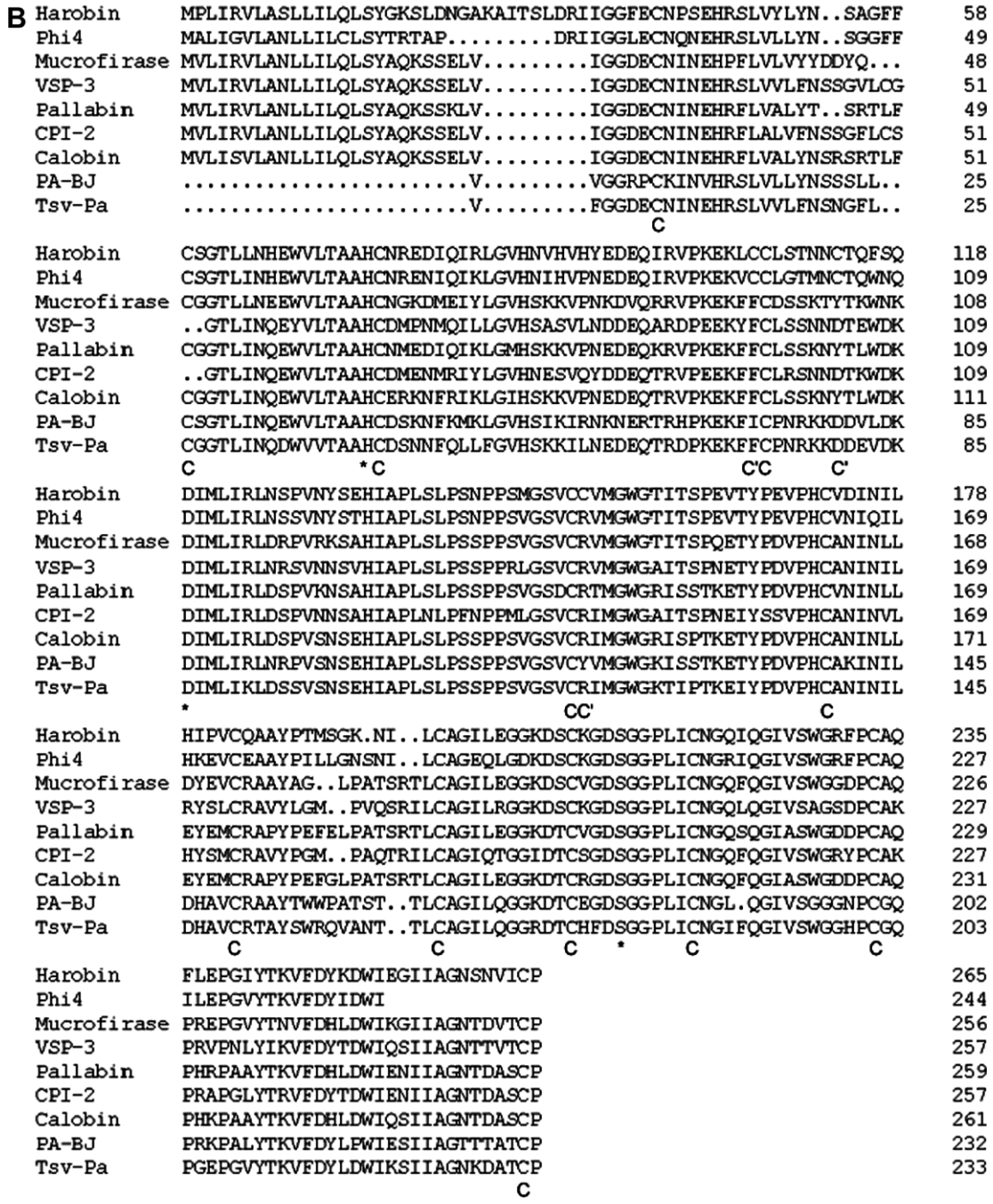

Fig. 1 (*continued*)

was a serine protease but not a metal protease. DTT could deoxidize disulfate-bond so that it inhibited disulfate-bond containing serine protease. Defibrase and Calobin were inhibited 80% by 10 mM DTT [33,34], while Harobin was not affected at the same concentration, indicating that Harobin was more stable than other six-disulfate-bond serine protease. To confirm the notion, we made mutation in Harobin. Two of the additional three Cys in Harobin (106 Cys-Val, 152 Cys-Ser) were mutated and expressed in *P. pastoris*. The activity of wild type and mutant was compared. Harobin mutant was more sensitive to DTT and less stable in 65 °C (Fig. 2D). It demonstrated that 106 Cys and 152 Cys participated in the formation of disulfate-bonds and were required for the stability of the enzyme. Furthermore, the amidolytic activity of Harobin in 30 mM Tris–HCl buffer maintained for six months at 4 °C without distinct decrease (Data not shown), indicating that Harobin was a higher stable protease.

3.3. Harobin functioned as a defibrase in vitro and in vivo

Since Harobin was cloned as a fibrinogen binding protein and identified to be a serine protease, it was expected to be a defibrase. Its fibrino(gen)lytic activity was first tested in vitro. It showed that Harobin digested Bβ-chain of fibrinogen first and then the Aα-chain. The intensity on the γ-chain of

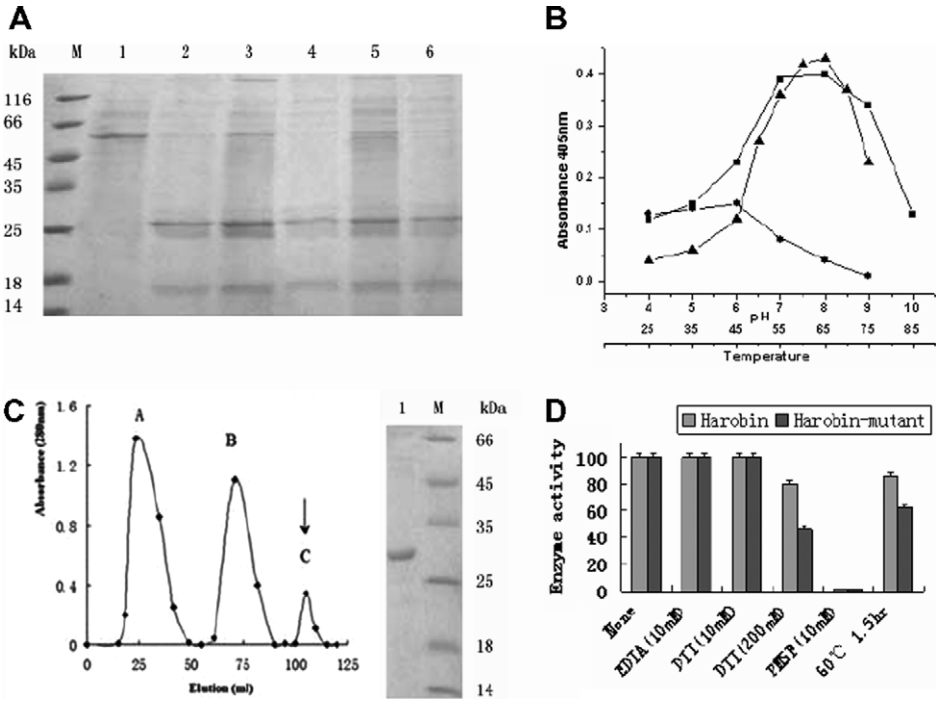

Fig. 2. Expression and activity analysis of Harobin. (A) Expression of Harobin in *P. pastoris* GS115. Supernatants from the cell culture of clones were resolved on SDS–PAGE. Lane M: protein markers; Lane 1: parental GS115; Lane 2–6: different clones. (B) Affinity chromatography on Benzamidine-Sepharose CL-6B column (left panel). The elution C was resolved on SDS–PAGE (right panel). (C) Effects of pH and temperature on the amidolytic activity of Harobin and Defibrase. Symbols: ▲ pH optimum of Harobin; ■ temperature optimum of Harobin; ● temperature optimum of Defibrase. (D) Effect of inhibitors on the amidolytic activity of Harobin and Harobin mutant (106 Cys-Val, 152 Cys-Ser).

Table 1
Comparison of enzymatic activity between Harobin and defibrase

	Enzyme activity (U/mg)	
	Harobin	Defibrase
Amidolytic activity	8.3	1.5
Arginine esterase activity	<0.01	<0.01
TLE activity	0	1250

fibrinogen was increased with the decrease of the intensity of fibrinogen Aα-, Bβ-chain, indicating that the Aα-chain or Bβ-chain of fibrinogen was cleaved to smaller pieces that were close to the γ-chain size. Finally, the y-chain of fibrinogen was digested at 18 h and fibrinogen was cleaved to 42 kD fragment. There was no band corresponding to fibrinogen on SDS–PAGE at 40 h, suggesting that it was completely degraded by Harobin (Fig. 3a). As to the specificity of the enzyme, we tested its cleavage ability on BSA and found that it did not cleave BSA. Harobin had no fibrinogen clotting ability and the coagulation of fibrinogen by thrombin was significantly retarded when fibrinogen was incubated with Harobin before addition of thrombin (data not shown). Fibrinolytic activity

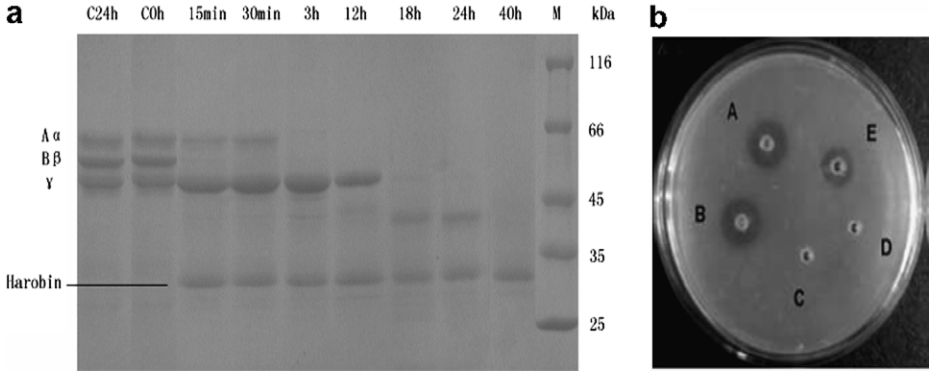

Fig. 3. Analysis of fibrino(geno)lytic activity of Harobin. (a) Cleavage pattern of fibrinogen by Harobin. Five micrograms of fibrinogen from human plasma was incubated with 0.5 μg Harobin in 20 μl Tris buffer (pH 8.0) at 37 °C for 15 min, 30 min, 3 h, 12 h, 18 h, 24 h and 40 h, respectively. Lane C0 and Lane C24 represent fibrinogen alone at 37 °C for 0 h or 24 h. Lane M: molecular weight markers. (b) Detection of the enzymatic activity of Harobin on fibrin plate. Twenty microliters of purified Harobin (0.1 mg/ml) was dropped onto the fibrin plate and incubated at 37 °C for 6 h in the presence of 5 μl, 2 U/ml human plasminogen (A) or in the absence of plasminogen (B). As controls, 20 μl Tris–HCl buffer (C), 5 μl, 2 U/ml plasminogen solution (D) and the same quantity of plasminogen plus 2 μl, 1 U/ml urokinase (E) were used.

Table 2
Effect of Harobin on mice tail thrombosis ($n = 10$)

Groups	Dose (μg/kg)	Length of thrombus (mm)
Saline	–	16 ± 8
Defibrase	0.5	3 ± 2*
Harobin	50	4 ± 2*
Harobin	200	0**

*$P < 0.05$.
**$P < 0.001$ vs. saline treated groups.

Table 3
Effect of Harobin on the wet weight of thrombus in vena cava model ($n = 10$)

Groups	Dose (μg/kg)	Wet weight of thrombus (mg)	Inhibition rate (%)
Saline	–	31 ± 7.3	–
Defibrase	0.5	20.5 ± 3.6*	34
Harobin	50	18.2 ± 2.1*	41
Harobin	200	11.4 ± 5.0**	63

*$P < 0.05$.
**$P < 0.001$ vs. saline treated groups.

Table 4
Effect of Harobin on the wet weight of thrombus in arterio-venous shunt model ($n = 10$)

	Dose	Wet weight of thrombosis (mg)
Saline	–	41.5 ± 8
Heparine	50 IU/kg	22.1 ± 4.9**
Harobin	400 μg/kg	43.3 ± 5.4

**$P < 0.001$ vs. saline treated groups.

was assayed using fibrin plate method. It showed that addition of Harobin led to the formation of a clear hollow (Fig. 3b). The size of the clear hollow kept unchanged even in the presence of plasminogen, suggesting that Harobin was not a plasminogen activator.

We next addressed whether Harobin could function as a defibrase and had antithrombotic effect in vivo. Three animal models were established and used for the tests. Injection of Harobin significantly decreased the length of tail thrombus at the concentration of 50 μg/kg, similar to the effect by commercial defibrase in mouse tail thrombosis model (Table 2). In the vena cava model of rats, Harobin caused the wet weight of thrombus decrease by 41% at concentration of 50 μg/kg, and by 63% at the concentration of 200 μg/kg (Table 3). The proportion of wet weight to dry weight of thrombus was 27% approximately. In the arterio-venous shunt model of rats, Harobin had no effect on the wet weight of thrombus at concentration of 400 μg/kg, while heparin, the positive control, could inhibit 50% of the wet weight of thrombus at the concentration of 50 IU/kg (Table 4). The effect of Harobin on the hemorrhagic system was also evaluated. The clotting time and bleeding time of mice were prolonged significantly by Harobin (Table 5), the level of fibrinogen of rats was also decreased significantly (Table 6). However, Harobin did not show hemorrhagic activity in mice at a concentration of 5 mg/kg (data not shown), indicating it was safe as a therapeutic agent.

3.4. Angiotensin I and kininogen were the substrates of Harobin

It had been suggested that defibrase from snake might had the ability to reduce blood hypertension [16]. The effects of Harobin on blood pressure were therefore investigated. Injection of Harobin into SHR rats caused a significant blood pressure drop at a concentration of 2 mg/kg (Fig. 4A). Reduction of blood pressure started 30 min after injection of Harobin and lasted at least for 4 h. The change of systolic blood pressure (SBP) was demonstrated in Table 7.

To explore the mechanism of its anti-hypertensive effect, we addressed whether angiotensin I and kininogen were the substrates of Harobin. When kininogen was incubated with Harobin, the disappearance of kininogen coupled with the formation of the major fragment of 58 kDa was similar to that of kallikrein (Fig. 4B). The products after reaction were further evaluated by reverse-phase HPLC. One of the released peptides was identified as bradykinin (data not shown). Incubation of angiotensin I with Harobin resulted in the formation of four major peptide fragments. The peptides were sequenced, respectively. They were His-Leu, Asp-Arg-Val-Tyr, Ile-His-Pro-Phe, and Asp-Arg-Val-Tyr-Ile-His-Pro-Phe (corresponding to the four major peaks in Fig. 4C). The result suggested

Table 5
Effect of Harobin on coagulation time (CT) and bleeding time (BT) on mice ($n = 10$)

Groups	Dose (μg/kg)	CT (min)	Prolonged CT (%)	BT (min)	Prolonged BT (%)
Saline	–	2.41 ± 0.47	–	3.5 ± 1.13	–
Defibrase	0.5	3.81 ± 0.61**	52.4	4.43 ± 0.83*	26.57
Harobin	50	3.50 ± 0.39*	39.44	4.61 ± 0.54*	31.71
Harobin	200	4.22 ± 0.75**	68.13	5.37 ± 1.32**	53.43

*$P < 0.05$.
**$P < 0.001$ vs. saline treated groups.

Table 6
Effect of Harobin on the plasma level of fibrinogen of rats after 6hr of treatment ($n = 10$)

Groups	Dose (μg/kg)	Fibrinogen (g/L)	Reducing rate (%)
Saline	–	2.17 ± 0.12	—
Defibrase	0.5	1.62 ± 0.11**	25
Harobin	50	1.72 ± 0.11**	21
Harobin	200	1.3 ± 0.32**	40

*$P < 0.05$.
**$P < 0.001$ vs. saline-treated groups.

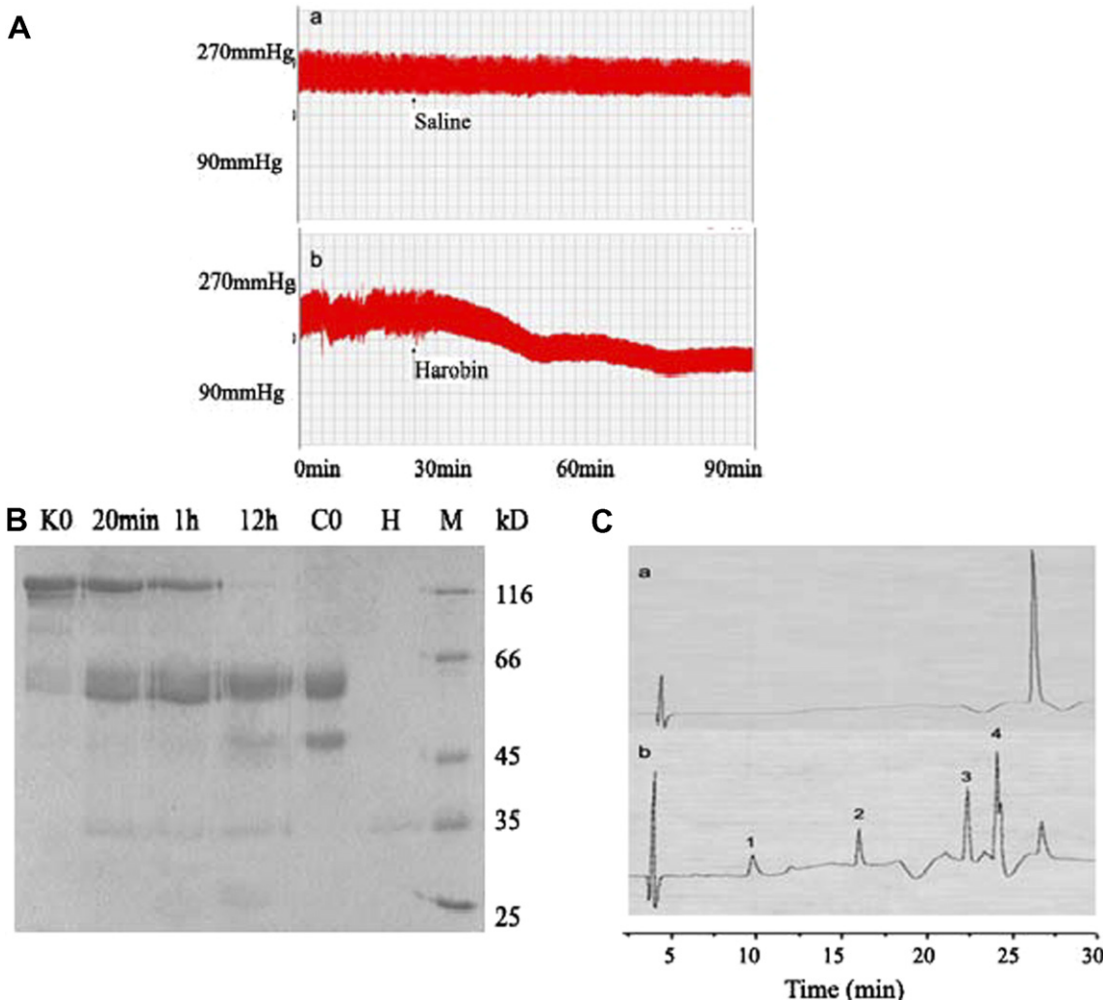

Fig. 4. Effect of Harobin on hypertension and its mechanism. (A) A typical example of blood pressure change in SHR rats after treatment of Harobin (2 mg/kg). (B) SDS–PAGE analysis of kininogen cleaved by Harobin. 5 μg single chain HMW kininogen (from human plasma) were incubated at 37 °C with 0.2 μg Harobin in a total volume of 20 μl Tris–HCl buffer (50 mM, pH 8.0) for various time intervals. Lane K0: kininogen alone; Lane 20 min 1 h 12 h represent the time of kininogen cleaved by Harobin. Lane C0 represents kininogen cleaved by kallikrein; Lane H: Harobin alone. Lane M: molecular weight markers. (C) Analysis of angiotensin I cleaved by Harobin. Angiotensin I (1 mg/ml) was incubated with 10 μg Harobin in 50 μl Hepes buffer (50 mM, pH 7.5) at 37 °C for 3 h. The reaction mixtures were then analyzed by HPLC. Peak fractions were collected and sequenced. (a) represented angiotensin I alone, (b) represented angiotensin I after digestion by Harobin. The labeled peaks indicated four major proteolytic fragments from angiotensin I.

Table 7
Effect of Harobin on the SBP of SHR rats ($n = 8$)

Groups	Dose (mg/kg)	Before i.v. (mmHg)	4 h after i.v. (mmHg)
Saline	–	242 ± 52	227 ± 40
Captopril	1	260 ± 10	151 ± 15**
Harobin	2	241 ± 45	161 ± 35*

*$P < 0.05$.
**$P < 0.001$ vs. before treatment.

Table 8
Effect of Harobin on the level of plasma angiotensin II of SHR rats ($n = 8$)

Groups	Dose (mg/kg)	Angiotensin II (pg/ml)
Saline	–	368 ± 44
Captopril	1	172 ± 39**
Harobin	2	246 ± 79*

*$P < 0.05$.
**$P < 0.001$ vs. saline-treated groups.

that angiotensin I was first cleaved to angiotensin II and subsequently cleaved to two fragments by Harobin. We further tested if such reaction occurred in vivo. The level of angiotensin II in the plasma was significantly reduced after the injection of Harobin (Table 8). It suggested that angiotensin II was the target of Harobin in vivo.

4. Discussion

In this study, a novel sea snake serine protease that targets fibrinogen, angiotensin and kininogen is described. To our knowledge, this is the first report of isolation, cloning and characterization of a fibrino(geno)lytic and kinin-releasing serine protease from sea snake venom.

Snake serine proteases usually contain very conservative 12 Cys besides their highly conservative catalytic sites. However, the three additional Cys residues in Harobin are very unique and may contribute greatly to the enzyme stability. 106 Cys and 152 Cys were selected to mutate because these two are more close to the conservative Cys and may disturb the normal disulfate-bonds. The mutation leads to the instability of the enzyme (Fig. 2D). Further studies on its crystal structure will highlight its relationship between the structure and function. Nevertheless, the finding of Harobin enriches the knowledge of the family of serine protease.

Harobin functions as a defibrase in vitro and in vivo. It digests fibrinogen efficiently in vitro (Fig. 3a). Bβ-chain of the fibrinogen is degraded first followed by the degradation of Aa-chain. This digestion pattern is consistent to that by Tm-VIG [16]. It is interesting to show that γ-chain is degraded as well after 18 h incubation with Harobin. Digestion of γ-chain of fibrino(gen) by defibrase has been seldom reported. It might be one of the differences between the serine protease from terrestrial and sea snake. The antithrombotic activity of Harobin has been evaluated in three animal models. Mice tail thrombus induced by carageenan is a peri-venous thrombus model and rat vena cava thrombus is a deep venous thrombus model. Harobin shows the same effects on antithrombus as natural purified defibrase does in both animal models (Fig. 4), but the amount of Harobin is about 100-folds to that of Defibrase. This is because Harobin has no TLE activity though it possesses higher amidolytic activity than Defibrase (Table 1). The mechanism of antithrombus by Harobin can be explained by that it degrades fibrinogen or fibrin, and leads to the decrease of blood viscosity, thus prevents the formation of thrombus or dissolves the existed thrombus. The antithrombus effect is not due to the activation of plasminogen (Fig. 3b). Harobin does not affect the thrombus in the arterio-venous thrombus model (Table 4). The structure of thrombus formed in the model is similar to that of white thrombus in vivo model. This model is widely used for the evaluation of anti-platelet drugs [13]. It has been suggested that the inability of the drug in this model can be explained no effect on the aggregation of the platelet. Therefore, the failure of Harobin in this model suggests that it has no effect on the aggregation of the platelet.

It is interesting to show that Harobin significantly reduced the hypertension of SHR rats (Fig. 4A). Since some peptides from snake venom are able to inhibit the activity of angiotensin converting enzyme (ACE) [35]. We first exam whether Harobin is an ACE inhibitor. It turns out that Harobin does not affect ACE (data not shown). We then test the effects of Harobin on angiotensin I and kininogen, both of which are important factors regulating blood pressure. Angiotensin I is first cleaved to angiotensin II and the latter is further cleaved to two tetrapeptides by Harobin in vitro and in vivo (Fig. 4B, C). Angiotensin II is hypertensive peptide while the tetrapeptide is not. Cleavage of angiotensin II to small peptides could be one way that Harobin reduces blood pressure. On the other hand, Harobin has inherent kallikrein-like activity, it can release bradykinin from plasma kininogen in vitro. This kallikrein-like activity is especially intriguing since a-fibrinogenases like ancrod does not show such a specificity against kininogen [16]. Bradykinin is a strong vasodilator, this could be another way that Harobin regulate the blood pressure. Together with the factor that Harobin is a defibrase to degrade the fibrinogen, we conclude that anti-hypertension effect of Harobin at least results from the following three independent ways. (1) It functions as a defibrase that decreases the concentration of blood fibrinogen leading to the alteration of blood rheology. (2) It degrades angiotensin I and angiotensin II to lower the concentration of angiotensin II; (3) It works as kallikrein to cleave kininogen and release bradykinin that promotes vasodilatation.

Ancrod and batroxobin are all TLE purified from venom for clinical usage. Harobin has no TLE activity, but its antithrombotic activity is equivalent to naturally purified defibrase. The unique structure features and the abilities to digest fibrinogen, angiotensin and kininogen with higher stability suggest that Harobin is a novel serine protease. It holds a potential to be an antithrombosis and hypertension agent.

Acknowledgements: This work was supported by the grants from Ministry of Science and Technology, China (2006AA09Z407), National Basic Research Program of China (2006CB503802) and Award of China Postdoctoral Science Foundation (2004035253) to Shiyong Chen.

References

[1] Matsui, T., Fujimura, Y. and Titani, K. (2000) Snake venom proteases affecting hemostasis and thrombosis. Biochim. Biophys. Acta 1477, 146–156.
[2] Mutton, R.A. and Warrell, D.A. (1993) Action of snake venom components on the haemostatic system. Blood Rev. 7, 176–189.
[3] Dimitropoulou, C., Chatterjee, A., McCloud, L., Yetik-Anacak, G. and Catravas, J.D. (2006) Angiotensin, bradykinin and the endothelium. Handbook Exp. Pharmacol. 176 Pt 1, 255–294.
[4] Rocha, C.S.M., Beraldo, W.T. and Rosenfeld, G. (1949) Bradykinin, a hypotensive and smooth muscle stimulating factor released from plasma globulin by snake venoms and trypsin. Am. J. Physiol. 156, 261–273.
[5] Jia, Y.H., Jin, Y., Lu, Q.M., Li, D.S., Wang, W.Y. and Xiong, Y.L. (2003) Jerdonase, a novel serine protease with kinin-releasing and fibrinogenolytic activity from *Trimeresurus jerdonii* venom. Acta Biochim. Biophys. Sinica 35 (8), 689–694.
[6] Stocker, K.F. (1990) in: Medical Use Of Snake Venom Proteins (Stocker, K.F., Ed.), pp. 97–160, CRC Press, Boston, MA.
[7] Markland, F.S. (1998) Snake venoms and the hemostatic system. Toxicon 36 (12), 1749–1800.
[8] Vieira, D.F., Watanabe, L., Sant'Ana, C.D., Marcussi, S., Sampaio, S.V., Soares, A.M. and Ami, R.K. (2004) Purification and characterization of Jararassin-I, a thrombin-like enzyme from *Bothrops jararaca* snake venom. Acta Biochim. Biophys. Sinica 36, 798–802.
[9] Yabuki, Y., Oguchi, Y. and Takahashi, H. (1991) Purification of kininogenase-2 from the venom of Agkistrodon caliginosus (Kankoku-mamushi). Toxicon 29, 73–84.
[10] Hofmann, H., Dumarey, C. and Bon, C. (1983) Blood coagulation induced by *Bothrops atrox* venom: identification and properties of a factor X activator. Biochimie 65, 201–210.
[11] Astrup, T. and Mullertz, S. (1952) The fibrin plate method for estimating fibrinolytic activity. Arch. Biochem. Biophys. 40, 346–351.
[12] Bekemeier, H., Hirschelmann, R. and Giessler, A. (1985) Carrageenin-induced thrombosis in rats and mice: a model for testing antithrombotic substances? Agent Actions 16 (5), 446–451.
[13] Umetsu, T. and Sanai, K. (1978) Effect of L-methyl-2-mercapto-5(3-pyridyl)-imidazole (KC-6141), an anti-aggregating compound, on experimental thrombosis in rats. Thromb. Haemostasis 39 (1), 74–83.
[14] Reyers, I., Mussoni, L., Donati, M.B. and de Gaetano, G. (1980) Failure of aspirin at different doses to modify experimental thrombosis in rats. Thromb. Res. 18, 669–674.
[15] Kung, S.H., Hagstrom, J.N., Cass, D., Tai, S.J., Lin, H.F., Stafford, D.W. and High, K.A. (1998) Human factor IX corrects the bleeding diathesis of mice with hemophilia B. Blood 91, 784–790.

[16] Hung, C.C. and Chiou, S.H. (2001) Fibrinogenolytic proteases isolated from the snake venom of Taiwan Habu: serine proteases with kallikrein-like and angiotensin-degrading activities. Biochem. Biophys. Res. Commun. 281, 1012–1018.
[17] Fry, Bryan G., Vidal, Nicolas, Norman, Janette A., Vonk, Freek J., Scheib, Holger, Ramjan, S.F. Ryan, Kuruppu, Sanjaya, Fung, Kim, Hedges, S. Blair, Richardson, Michael K., Hodgson, Wayne. C., Ignjatovic, Vera, Summerhayes, Robyn and Kochva, Elazar (2006) Early evolution of the venom system in lizards and snakes. Nature 439, 584–588.
[18] Hung, C.C., Huang, K.F. and Chiou, S.H. (1994) Characterization of one novel venom protease with beta-fibrinogenase activity from the Taiwan habu (*Trimeresurus mucrosquamatus*): purification and cDNA sequence analysis. Biochem. Biophys. Res. Commun. 205, 1707–1715.
[19] Deshimaru, M., Ogawa, T., Nakashima, K., Nobuhisa, I., Chijiwa, T., Shimohigashi, Y., Fukumaki, Y., Niwa, M., Yamashina, I., Hattori, S. and Ohno, M. (1996) Accelerated evolution of crotalinae snake venom gland serineproteases. FEBS Lett. 397, 83–88.
[20] Fan, C.Y., Qian, Y.C., Yang, S.L. and Gong, Y. (1999) Cloning, sequence analysis and expression in *E. coli* of the cDNA of the thrombin-like enzyme (pallabin) from the venom of Agkistrodon halys pallas. Mol. Biol. Int. 47, 217–225.
[21] Hahn, B.S., Baek, K., Kim, W.S., Lee, C.S., Chang, I.L. and Kim, Y.S. (1998) Molecular cloning of capillary permeability-increasing enzyme-2 from *Agkistrodon caliginosus* (Korean viper). Toxicon 36, 1887–1893.
[22] Hahn, B.S., Yang, K.Y., Park, E.M., Chang, I.M. and Kim, Y.S. (1996) Purification and molecular cloning of calobin, a thrombin-like enzyme from *Agkistrodon caliginosus* (Korean viper). J. Biochem. 119, 835–843.
[23] Parry, M.A., Jacob, U., Huber, R., Wisner, A., Bon, C. and Bode, W. (1998) The crystal structure of the novel snake venom plasminogen activator TSV-PA: a prototype structure for snake venom serine proteinases. Structure 6, 1195–1206.
[24] Serrano, S.M., Mentele, R., Sampio, C.A. and Fink, E. (1995) Purification, characterization, and amino acid sequence of a serine proteinase, PA-BJ, with platelet-aggregating activity from the venom of *Bothrops jararaca*. Biochemistry 34, 7186–7193.
[25] Burkhart, W., Smith, J.F., Su, J.L., Parikh, I. and LeVine, H. (1992) Amino acid sequence determination of ancrod, the thrombin-like alpha-fibrinogenase from the venom of *Akistrodon rhodostoma*. FEBS Lett. 297, 297–301.
[26] Nielsen, H., Engelbrecht, J., Brunak, S. and von Heijne, G. (1997) Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. Protein Eng. 10, 1–6.
[27] Julenius, K., Møgaard, A., Gupta, R. and Brunak, S. (2005) Prediction, conservation analysis and structural characterization of mammalian mucin-type *O*-glycosylation sites. Glycobiology 15, 153–164.
[28] Itoh, N., Tanaka, N., Mihashi, S. and Yamashina, I. (1987) Molecular cloning and sequence analysis of cDNA for batroxobin, a thrombin-like snake venom enzyme. J. Biol. Chem. 262, 3132–3135.
[29] Cheng, X., Qian, Y., Liu, Q., Li, B.X., Zhang, M. and Liu, J. (1999) Purification, characterization, and cDNA cloning of a new fibrinogenlytic venom protein, Agkisacutacin from *Agkistrodon acutus* venom. Biochem. Biophys. Res. Commun. 265, 530–535.
[30] Guo, Y.W., Chang, T.Y., Lin, K.T., Liu, H.W., Shih, K.C. and Cheng, S.H. (2001) Cloning and functional expression of the Mucrosobin protein, a β-fibrinogenase of *Trimeresurus mucrosquamatus* (Taiwan Habu). Protein Exp. Purif. 23, 483–490.
[31] Zhang, Y., Wisner, A., Xiong, Y. and Bon, C. (1995) A novel plasminogen activator from snake venom: purification, characterization and molecular cloning. J. Biol. Chem. 270, 10246–10255.
[32] Park, D., Kim, H., Chung, K., Kim, D.S. and Yun, Y. (1998) Expression and characterization of a novel plasminogen activator from *Agkistrodon halys* venom. Toxicon 36, 1807–1819.
[33] Cho, S.Y., Hahn, B.S., Yang, K.Y. and Kim, Y.S. (2001) Purification and characterization of calobin II, a second type of thrombin-like enzyme from *Agkistrodon caliginosus* (Korean viper). Toxicon 39 (4), 499–506.
[34] Jiao, H.M., Yang, L.X., Lu, B., Wu, Y.Q. and Zhou, Y.C. (2005) Shedaoenase, a novel fibrinogenase from the venom of *Agkistrodon shedaoenthesis* Zhao. Acta Biochim. Biophys. Sinica 37 (12), 835–842.
[35] L'vov, V.M. and Sadykov, E.S. (2000) Activity of angiotensin-converting enzyme inhibitors in venoms of central Asian snakes. Chem. Natural Compd. 36 (3), 307–314.