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

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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].

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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*