Appl Biochem Biotechnol (2010) 162:1391–1402
DOI 10.1007/s12010-010-8911-6

Cloning and Identification of a Novel P-II Class Snake Venom Metalloproteinase from *Gloydius halys*

Shou-Tao Zhang · Ping Lu · Yun-Fei Qin · San-Jun Chen · Ai-Guang Guo

Received: 10 October 2009 / Accepted: 7 January 2010 /
Published online: 20 February 2010
© Springer Science+Business Media, LLC 2010

Abstract Ahpfibrase was a new snake venom metalloproteinase (SVMP) which was cloned from *Gloydius halys*. The cDNA sequence with 1,891 base pairs encodes an open reading frame of 477 amino acids which includes a 17 amino acid signal peptide, plus a 171 amino acid segment of zymogen-like propeptide, a metalloproteinase domain of 200 amino acids, a spacer of 16 amino acids, and a disintegrin-like peptide of 73 amino acids. The metalloproteinase domain contained a conserved signature zinc-binding motif HEXXHXXGXXH in the catalytic region and a methionine-turn CIM. To determine the activity of ahpfibrase, the coding region including both the metalloproteinase domain and disintegrin region was amplified by PCR, inserted into the pET25b(+) vector, and expressed in *Escherichia coli*. The recombinant protein was recovered from inclusion bodies with 8 M urea and refolding was performed by fed-batch dilution method, and purified recombinant ahpfibrase showed the fibrinolytic activity and platelet aggregation–inhibition ability.

Keywords Expression · cDNA · Cloning · *Gloydius halys* · Refolding · Snake venom metalloproteinase

Introduction

Snake venoms are rich sources of proteases that strongly affect the vascular system by promoting blood coagulation, hemorrhage, and fibrinolysis. The metalloproteinases are major functional snake venom proteins that are synthesized in the venom gland as large multidomain proteins, including a pro-enzyme domain and a highly conserved protease domain. The protease domain is characterized by a conserved zinc-binding extended consensus sequence

S.-T. Zhang (✉) · P. Lu · Y.-F. Qin · S.-J. Chen
Department of Bioengineering, Zhengzhou University, Zhengzhou, Henan 450001, China
e-mail: zhangst@zzu.edu.cn

S.-T. Zhang · A.-G. Guo (✉)
Key Laboratory of Agricultural Molecular Biology in Shaanxi Province, Northwest A&F University, Yangling, Shaanxi 712100, People's Republic of China
e-mail: gaguang@nwsuaf.edu.cn

HEXXHXXGXXHD [1, 2]. A second conserved motif PKMCGVT, which exists in the mammalian matrix metalloprotease pro-enzyme domain, is found in snake venom metalloproteinases (SVMPs) in precursor form. These proteases interfere with the blood coagulation and hemostatic plug formation and degrade the basement membrane or extracellular matrix components of snakebite victims [3–6]. According to their domain structures, SVMPs are members of the reprolysin family of zinc-containing metalloproteinases [6]. Based on the structure of the mature protein, they are classified into four basic structural classes (P-I to P-IV). Group I of SVMPs contains a catalytic domain that is sequentially and C-terminally extended with an RGD (Arg-Gly-Asp) sequence containing a disintegrin domain (group II), a cysteine-rich domain (group III), and a C-type lectin domain (group IV) [6–8]. Most of the metalloproteinases are both fibrinogenolytic and fibrinolytic and can directly split off fragments of α-, β-, and γ-chains of fibrinogen, while degrading fibrin clots made by purified fibrinogen or isolated from blood plasma [9].

In metalloproteinases, almost all disintegrins have the cell binding consensus RGD sequence (Arg-Gly-Asp). The RGD sequence is located in a flexible hairpin loop of 13 amino acid residues that plays a crucial role in inhibition of platelet aggregation through interaction with platelet integrin αIIbβ3 (GP IIb/IIIa) [10, 11]. Sequence analysis of both cDNA of hemorrhagins and disintegrins has shown that they are derived from the same metalloproteinase/disintegrin gene family. Disintegrins are synthesized as large precursor molecules containing the Zinc-dependent proteinase domain found in hemorrhagic toxins. The disintegrin domain is present in large metalloproteinases (50–70 kD), but is absent in low molecular mass hemorrhagins (20–25 kD) [12, 13].

In the present paper, we report on a novel snake venom metalloproteinase/disintegrin, ahpfibrase, which was found in the venom of *Gloydius halys*. The protein is comprised of metalloproteinase, spacer, and RGD-containing disintegrin domains. Recombinant ahpfibrase retains its biological activity and is able to both degrade fibrinogen and inhibit human platelet aggregation.

Materials and Methods

Materials

The snake (*G. halys*) was collected from the province of Guangdong in China. *Escherichia coli* XL1-Blue was used for the construction of recombinant plasmids and BL21(DE3) cells were used for the expression of ahpfibrase. Expression vectors pET25b(+) were purchased from Novagen (Madison, WI, USA) and Superscript III™ Reverse Transcriptase was purchased from Invitrogen. The pGEM-T Easy Kit, T4 DNA ligase, and DNA restriction enzymes were from Promega. Bovine fibrinogen, bovine thrombin, phenylmethanesulfonyl fluoride (PMSF), dithiothreitol (DTT), adenosine 5′-diphosphate (ADP), and azocasein were obtained from Sigma Chemical (St. Louis, MO, USA). Oligonucleotide fragments were synthesized by Bioasia (Shanghai, China). Isopropyl-thiogalactopyranoside (IPTG), HEPES, protein molecular weight standards, and *Pfu* DNA polymerases were from Sangon (Shanghai, China); 5′-Full RACE Core Set was purchased from Takara (Dalin, China).

Design of Hybrid Degenerate Primers

Cloning of the metalloproteinases gene from *G. halys* was performed using a degenerate PCR strategy. This strategy employed the CODEHOP primer design algorithm [14], which was

accessed at http://bioinformatics.weizmann.ac.il/blocks/codehop.html. Primers designed with this program contain a short 3′ core sequence 11–12 bp in length that corresponds to the degenerate region of the primer (lowercase letters) and is followed by a consensus 5′ sequence (uppercase letters) that is non-degenerate and acts as a clamp to stabilize priming.

All non-hemorrhagic SVMPs sequences were obtained from NCBI (http://www.ncbi.nlm.nih.gov/) according to the description in the original work. Seven snake non-hemorrhagic SVMPs [aclf (gi: 603215) [15], adm2 (640082) [2], bneu (gi: 6760464) [16], h2p (gi: 462303) [17], fibrolase (gi: 386076) [18], lebe (gi: 2118144) [19], and fibro (gi: 7428251) [20]] were selected from GenBank and entered into the BLOCKMAKER program [21], which is found at the CODEHOP website and is the first step in primer design. BLOCKMAKER is a multiple alignment program that identifies ungapped segments representing regions of proteins that are the most conserved. The output from BLOCKMAKER was pasted into the CODEHOP algorithm to identify candidate degenerate primers. Two hybrid degenerate primers AhpF1(5′-CCGACCACCGGATGTA Cahnaartayaa-3′) and AhpF2 (5′-CACGACACCCTGAACTCCttyggnrantgg-3′) were selected as the forward primers; each hybrid primer consisted of a short 3′ degenerate core region based on four highly conserved amino acids and a longer 5′ consensus clamp region. The primers were designed by taking into account information on *snake* codon usage.

Total RNA Extraction and cDNA Cloning

When the snake was decapitated, the venom glands were removed immediately, frozen in liquid nitrogen, triturated, and quickly suspended in Trizol reagent (Invitrogen). The extraction of total RNA and synthesis of the cDNAs were performed by using Superscript III™ Reverse Transcriptase and "Oligo-dT-18" DNA primers, according to manufacturer's protocol (Invitrogen). The Oligo-dT-18 as the reverse primer, AhpF1 and AhpF2 as the forward primers, were used to amplify a fragment of the putative metalloproteinases gene. The PCR procedure was conducted under the following conditions: 3 min at 94°C, ten cycles (50 s at 94°C, 50 s at 40°C, 2 min at 72°C), 25 cycles (50 s at 94°C, 50 s at 60°C, 2 min at 72°C) and 5 min at 72°C. The product of PCR (we named this gene fragment ahpfibrase) was cloned to pGEM-T vector and sequenced.

The 5′ RACE was carried out with the 5′-Full RACE Core Set according to the manufacturer's instructions, using a 5′-end phosphorylated primer B3 [5′-(p)CTGCTTGTCAGC-3′] and two special primers: GSP1(5′-ATGCCCAGATTATGACCTAT-3′) and GSP2(5′-TGCCCACATAAGCCAATCCT-3′). The amplified PCR product was purified and cloned into pGEM-T vector and sequenced. By aligning and assembling the products of the 5′ RACE and the conserved fragment, the full-length ahpfibrase gene from *G. halys* was deduced, amplified, and subsequently cloned into pGEM-T vector (designated as pGEM-ahpfibrase).

DNA Sequence Analysis

The DNA sequence data were analyzed primarily by using the websites of http://www.ncbi.nlm.nih.gov and http://cn.expasy.org. Clustalx1.83 and VNTI Suite 8.0 were used for multiple alignment analysis of the full-length ahpfibrase amino acid sequences.

Expression of Ahpfibrase

The coding region for both of the mature protease and disintegrin region were amplified by PCR from pGEM-ahpfibrase. The PCR primers used were (5′-GCGC*CATATG*GCACAT CAAAGATACATTGAG-3′) forward and (5′-GCGC*CTCGAG*ATTAGGCATGGAAGG

GATTTCTG-3′) reverse, the PCR product and the pET25b(+) vector were restricted with *Nde* I and *Xho* I, purified, and ligated with T4 DNA ligase.

The expression plasmid pET25b-ahpfibrase was transformed into BL21(DE3) strains and grown for induction of protein expression, as described in earlier research [22]. To examine if the recombinant protein was expressed in a soluble form, induced cells were disrupted by sonication, centrifuged at 13,000 g, and analyzed by SDS-PAGE.

Purification and Refolding of the Recombinant Proteins

Cells containing the recombinant proteins were harvested by centrifugation at 12,000 g for 10 min at 4°C and were suspended in buffer A (50 mM Tris-HCl, pH 7.0, 1 mM EDTA, 1% Triton X-100, 10 mg/ml lyzozyme) and disrupted by sonication. The insoluble inclusion bodies were recovered by centrifugation and washed four times with buffer B (50 mM Tris-HCl, pH 7.0, 1 mM EDTA, 0.5 mM $ZnCl_2$, 2 M urea, 0.5% Triton X-100). The inclusion bodies were solubilized in 50 mM Tris-HCl (pH 7.0) buffer with 8 M urea and 15 mM DTT. Gel permeation chromatography was then carried out on a Superdex G-75 prep grade gel column equilibrated with buffer C (50 mM Tris-HCl, 0.5 mM $ZnCl_2$, pH 7.0). Fractions were collected at a flow rate of 1.0 ml/min, and protein concentration was monitored at 280 nm.

For the refolding process, the refolding conditions were optimized by adjusting several factors including the refolding method, protein concentration, denaturant, ionic strength, pH, and temperature. The refolding of ahpfibrase was carried out by a batch-fed dilution refolding method at a final concentration of 20 μg/ml. The refolding buffer consisted of 50 mM Tris-HCl, 0.5 M L-arginine, 1 M NaCl, 0.5 mM $ZnCl_2$, 1 mM oxidized glutathione (GSSG), 2 mM reduced glutathione (GSH), and 2 M urea, pH 7.0. After the protein was added, the pH was adjusted to 7.5 and the protein was left to refold at 4°C for about 24 h.

The refolding solution was centrifuged at 12,000 g for 15 min at 4°C to remove any precipitated protein and the supernatant was purified by anion-exchange chromatography using HiTrap 1 ml Q-Sepharose 6 FF. The protein was loaded onto the column equilibrated in buffer D (15 mM Tris-HCl, 0.5 mM $ZnCl_2$, pH 7.5) by the buffer selector valve for pump B1 of an AKTA Purifier (GE Healthcare). After washing with the same buffer, active ahpfibrase was eluted with a linear gradient of sodium chloride from 0 to 600 mM over ten column volumes at a flow rate of 1.0 ml/min. Purified ahpfibrase protein was adjusted to a final concentration of 1 mg/ml.

Azocaseinolytic Activity

Proteolytic activity of ahpfibrase was tested on azocasein according to Escalante et al. [23]. Briefly, various concentrations of ahpfibrase, dissolved in 20 μL of 25 mM Tris, 150 mM NaCl, 5 mM CaCl2, pH 7.5, were incubated with a 5 mg/mL solution of azocasein in the same buffer. After an incubation of 90 min at 37°C, the reaction was stopped by the addition of 200 μL 5% trichloroacetic acid. Samples were then centrifuged at 5,000 g, supernatant (150 μL) was diluted with 150 μL 0.5 M NaOH, and the absorbance was measured at 450 nm. One proteolytic activity unit was defined as the amount of enzyme that would hydrolyze azocasein to induce a change in absorbance of 0.2.

Effects of pH, Temperature, Divalent Cations, and Inhibitors on Ahpfibrase Activity

The effects of pH, temperature, divalent cations, and inhibitors on ahpfibrase activity were tested using the azocaseinolytic assay, as above. The optimum pH of ahpfibrase (20 μg/ml) was determined over a pH range of 6.0–11.0, using HEPES (pH 6.0–7.0) and Tris-HCl (7.5–11.0)

buffers (at 50 mM and containing 100 mM NaCl). Optimum temperature for ahpfibrase (21 μg/ml) was determined by measuring activity at optimum pH and at 25°C, 37°C, or 55°C for 90 min. The effect of 5 mM $MgCl_2$, $CaCl_2$, $ZnCl_2$, Na_2EDTA, or DTT or 2 mM PMSF on ahpfibrase activity was tested by assaying the enzyme (21 μg/ml) in the presence of these compounds.

Fibrinolytic Activity and Fibrinogen Degradation Assays

Fibrinolytic activity analysis was conducted using a fibrin plate method [24] with a slight modification. Fibrin plates were prepared by the addition of 5.0 ml thrombin solution (1 U/ml) to a preheated solution of 2% agarose in 50 mM Tris-HCl, pH 7.5, to which was added 5 ml 0.4% bovine fibrinogen in 50 mM Tris-HCl, pH 7.5, and 0.18 M NaCl. A fibrin clot was formed on a level surface. Sample solution (10 μl; fibrolase and ahpfibrase, about 10 μg,) was placed into holes on a fibrin plate and incubated at 37°C for 18 h, then the areas of clearance were analyzed.

Fibrinogenolytic activity [25] was assayed by incubating 0.1 ml of a 0.2% bovine fibrinogen solution with 0.05 ml recombinant ahpfibrase solution (containing 1–100 ng) in 50 mM Tris-HCl buffer (pH 8.0) at 37°C. Samples were subsequently analyzed by SDS-PAGE.

Platelet Aggregation–inhibition Assay

Platelet aggregation assay was performed by the method of Wang et al. [26]. Human blood was obtained from healthy donors, and 8% sodium citrate solution was added (1:9, *v*/*v*). The mixture was centrifuged at 500 g for 10 min and platelet-rich plasma was transferred into a clean tube. The concentration of platelets used in each assay was adjusted to 200,000 cells per microliter in a final volume of 0.5 ml and the platelet aggregation assay was performed in an aggregometer (Chrono-Log) at 37°C with stirring 1,000 g.

Results

DNA Sequence Analysis

The full-length cDNA sequences of ahpfibrase was 1,891 bp with 5′ and 3′ untranslated regions, polyA tail and a open reading frame, which contained a 17 amino acid signal peptide, a 171 amino acid segment of zymogen-like propeptide, a mature protein of 200 amino acids, a spacer of 16 amino acids, and a disintegrin-like peptide of 73 amino acids. The ahpfibrase proprotein had a sequence of PKMCGV that was stringently conserved among snake venom proteinases and had a cysteine switch motif that was involved in metalloproteinase activation. The metalloproteinase domain contained a conserved signature zinc-binding motif HEXXHXXGXXH in the catalytic region and the CIM turn and shared high similarity with other class P-II snake venom metalloproteinases [6, 13, 20, 27]. Based on the six cysteine residues in the ahpfibrase metalloproteinase domain; the presence of these highly conserved cysteine residues allowed the prediction of the same pattern of disulfide bonds for ahpfibrase (C115-C184, C155-C179, C157-C162, numbering according to ahpfibrase) as determined for fibrolase [28]. These results indicate that ahpfibrase belongs to the three-disulfide bond proteinase class. In addition, the cDNA sequence of ahpfibrase had a high degree of similarity in the 5′ and 3′ untranslated regions with other snake venom metalloproteinases.

There was a disintegrin domain of 73 amino acid residues with a highly conserved RGD sequence at the C-terminal of ahpfibrase. This region functions as an inhibitor of

RGD-dependent integrin adhesion. There was also a space sequence between the metalloproteinase domain and the disintegrin domain of 16 amino acid residues with a conserved T391, T396, S399, which are specific residues of the P-II snake venom metalloproteinases. The full-length cDNA sequences of the ahpfibrase and deduced amino acid sequence (GenBank accession number DQ457057) are described in Fig. 1.

```
1    tgtcttccagccaaatccagcctccaaaatgatccaagttctcttggtgattatatgcttagcagttccttatcaagggagctctataat
                              M  I  Q  V  L  L  V  I  I  C  L  A  V  P  Y  Q  G  S  S  I  I

91   cctggaatctgggaacgtgaatgattatgaagtagtgtatccacgaaaagtcactgcattgcccaaaggagcagttcagccaaagtatga
      L  E  S  G  N  V  N  D  Y  E  V  V  Y  P  R  K  V  T  A  L  P  K  G  A  V  Q  P  K  Y  E

181  agacgccatgcaatatgaattgaaagtgaatggagagccagtggtccttcacctggaaaaaaataaaggacttttttcaaaagattacat
      D  A  M  Q  Y  E  L  K  V  N  G  E  P  V  V  L  H  L  E  K  N  K  G  L  F  S  K  D  Y  I

271  tgagactcattattcccctgatggcagaaaaattacaacaaaccctccggttgaggatcactgctattatcatggacgcatccagaatga
      E  T  H  Y  S  P  D  G  R  K  I  T  T  N  P  P  V  E  D  H  C  Y  Y  H  G  R  I  Q  N  D

361  tgctgactcaactgcaagcatcagtgcatgcaatggtttgaaaggacatttcaagcttcaaggggagacgtaccttattgaacccttgaa
      A  D  S  T  A  S  I  S  A  C  N  G  L  K  G  H  F  K  L  Q  G  E  T  Y  L  I  E  P  L  K

451  gctttccaacagtgaagcccatgcagtctacaaatatgaagatgtagaaaaagaggatgaggcccccaaaatgtgtggggtaacccagaa
      L  S  N  S  E  A  H  A  V  Y  K  Y  E  D  V  E  K  E  D  E  A  P  K  M  C  G  V  T  Q  N

541  ttgggaatcatatgagcccatcaaaaaggcctctcagtcaaatcttactcctgcacatcaaagatacattgagcttgtcatagttgcgga
      W  E  S  Y  E  P  I  K  K  A  S  Q  S  N  L  T  P  A  H  Q  R  Y  I  E  L  V  I  V  A  D

631  ccatggaatgttcacgaaatacgacagcaatttagatactataagaacgtgggtacatgaacttgtcaacagtataaatgagttttacag
      H  G  M  F  T  K  Y  D  S  N  L  D  T  I  R  T  W  V  H  E  L  V  N  S  I  N  E  F  Y  R

721  atctttgaatattgatgtctcactgactgagctagaaatttggtccaacgaagatttgattaacgtgcagccagcagcgcctcatacttt
      S  L  N  I  D  V  S  L  T  E  L  E  I  W  S  N  E  D  L  I  N  V  Q  P  A  A  P  H  T  L

811  ggactcatttggaaaatggagagagagagatttgctgcatcgcatacatcatgataatgctatgttactcacggccattgacttcgatga
      D  S  F  G  K  W  R  E  R  D  L  L  H  R  I  H  H  D  N  A  M  L  L  T  A  I  D  F  D  E

901  accaactataggattggcttatgtgggcaccatgtgcaacccgaagggttctacaggagttgtccaggatcatagcacaataaaccttcg
      P  T  I  G  L  A  Y  V  G  T  M  C  N  P  K  G  S  T  G  V  V  Q  D  H  S  T  I  N  L  R

991  ggttgcagttacaatggcccatgagataggtcataatctgggcattcatcatgacacaggttcctgttcttgcggtggttactcatgcat
      V  A  V  T  M  A  H  E  I  G  H  N  L  G  I  H  H  D  T  G  S  C  S  C  G  G  Y  S  C  I

1081 tatgtctcccgtgataagccatgaaccttccaaatatttcagcgattgtagttatacccaatgttgggactttattatgaatcagaaacc
      M  S  P  V  I  S  H  E  P  S  K  Y  F  S  D  C  S  Y  T  Q  C  W  D  F  I  M  N  Q  K  P

1171 acaatgcattctcaataaacccttgagaacagatactgtttcaactccagtttctggaaatgaacttttggaggccggagaagaatgtga
      Q  C  I  L  N  K  P  L  R  T  D  T  V  S  T  P  V  S  G  N  E  L  L  E  A  G  E  E  C  D

1261 ctgtggctctcctggaaatccatgctgtgatgctgcaacctgtaaactgagacaaggagcacagtgtgcagaaggactgtgttgtgacca
      C  G  S  P  G  N  P  C  C  D  A  A  T  C  K  L  R  Q  G  A  Q  C  A  E  G  L  C  C  D  Q

1351 gtgcagatttatgaaaaaaggaacagtatgccggatagcaaggggcgatgacatggatgattactgcaatggcatatctgctggctgtcc
      C  R  F  M  K  K  G  T  V  C  R  I  A  R  G  D  D  M  D  D  Y  C  N  G  I  S  A  G  C  P

1441 cagaaatcccttccatgcctaaccaacaatggagctggaatggtctgcagcaacaggcagtgtgttgatgcgactacagcctactaatca
      R  N  P  F  H  A  *

1531 acctctggcttctctcagatttgattttggagatcttccttccagaaggtttcacttccctctagtccaaagagacccatctgcctgcat
1621 ccttctagtaaatcacccttagctttcatatggaatctaaattatgcaatatttcttctccacatttaatctgtttacctttgctgtaa
1711 tcaaaccttttccccaccacaaagctccatgggcaaatacaacaccaagggcttatttgctgtcaagaaaaaacaatggccattttacca
1801 tttgccaattgcaaagtacatttaatgcaacaagttctgccttttgagctggtgtattcgaagtcaatgcttcctctcccaaaattttgt
1891 gctggctttccaagatgtagctgcttccatcaataaactaactaatcctcaaaaaaaaaaa
```

Fig. 1 Full-length cDNA sequence and deduced amino acid sequence of ahpfibrase precursor. The full-length sequence is composed of 1,891 nucleotides. Following a 28-nucleotide 5′-end untranslatable region (UTR), an open reading frame is found between nucleotides 29 and 1,459. Nucleotides 1,460 to 1,462 represent the termination codon. The 3′-UTR possesses an AATAAA site and ends with a poly(A) tail. The open reading frame encodes 477 amino acids. The entire opening reading frame is divided into proprotein, metalloproteinase, disintegrin domain with a characteristic RGD region, and a space sequence were *underlined*

Expression, Purification, and Refolding of Ahpfibrase

The cDNA-coding region for the mature ahpfibrase was produced by PCR and inserted into a bacterial expression vector to produce the recombinant protein in *E. coli*. Transformation and induction were performed as described in "Material and Methods" section. The recombinant protein had a molecular weight 32 kDa and was observed 1 h after adding IPTG to cell cultures, with maximal expression at 4 h (data not shown), but almost all the ahpfibrase was recovered as inclusion bodies in the pellet (Fig. 2; lane 2, 3).

The expressed target proteins were solubilized in 8 M urea (pH 7.0), followed by purification with gel permeation chromatography as described in "Materials and Methods" section. After elution with denaturing buffer, the protein was the main component in the inclusion bodies, with a yield of about 300 mg of recombinant protein per liter of culture.

In the refolding process, various factors such as protein concentration, denaturant, ionic strength, pH, and temperature, among others, play an important role in determining protein refolding yields. Thus, the refolding process of the protein was optimized through adjusting these conditions to prevent auto-degradation and aggregation of the protein (data not shown). Finally, good results were obtained when refolding of ahpfibrase was carried out by a batch-fed dilution refolding method at pH 7.5, 4°C, and refolding buffer consisted of 2 M urea, 0.5 mM $ZnCl_2$, 1 mM GSSG and 2 mM GSH, and sample concentration of 1 mg/ml, dilution factor of 50. The refolded protein was purified and eluted as a main sharp peak in anion-exchange chromatography with 300–400 mM sodium acetate (Fig. 2; lane 6), then stabilized with 20% glycerol and stored at −20°C. The recovery of recombinant ahpfibrase was about 25% after refolding and purification (Table 1).

Azocaseinolytic Activity

Ahpfibrase was incubated with azocasein for 90 min at 37°C, then the reaction was stopped by the addition of 5% trichloroacetic acid and the absorbance of the supernatant at 450 nm was recorded, as described in "Materials and Methods" section. Controls containing

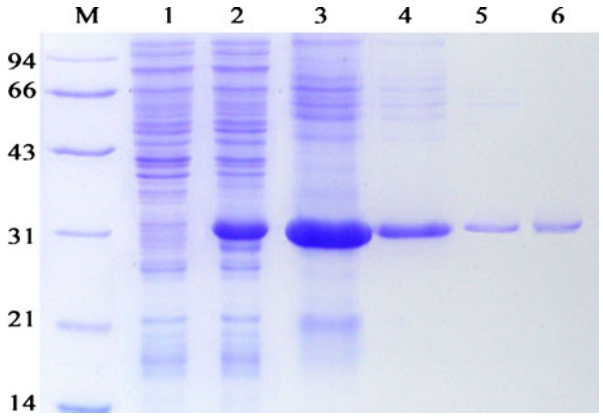

Fig. 2 SDS-PAGE analysis expression and purification of ahpfibrase in *E. coli*. *M* marker, *1* total cell proteins of uninduced, *2* total cell proteins of induced, *3* washing of inclusion bodies, *4* purificated inclusion bodies from Superdex G-75, *5* supernatant of refolding ahpfibrase, *6* purificated refolding ahpfibrase from HiTrap 1 ml Q-Sepharose 6 FF

Table 1 Purification of recombinant ahpfibrase from 1 L of *E. coli* BL21(DE3) cell culture.

	Total protein (mg)	Protein yield (%)
Inclusion bodies	300	100
rAhpfibrase from Superdex G-75	210	70
rAhpfibrase from refolding	52.5	25
rAhpfibrase from Q-Sepharose 6 FF	47.2	90

Protein concentration was estimated by Bradford assay

azocasein without enzyme were run in parallel and their absorbance was subtracted from the sample values. As shown in Fig. 3, ahpfibrase showed dose–response curves of proteolytic activity when tested using azocasein, with a specific activity of 150 units per milligram protein (Fig. 3).

Effects of pH, Temperature, Divalent Cations, and Inhibitors on Ahpfibrase Activity

The optimum pH was pH 7.5, and the optimum temperature was 37°C for the caseinolytic activity of ahpfibrase. This activity was affected by divalent metal ions (Fig. 4). Zn^{2+} caused an increase in activity of about 40%, but Ca^{2+} and Mg^{2+} had no effect. PMSF, DTT, and Na_2EDTA completely abolished the caseinolytic activity of ahpfibrase (Fig. 4). The enzyme was thermolabile, as activity was reduced to about 43% of its original value after incubation at 55°C for 90 min (data not shown).

Fibrinolytic Activity and Fibrinogen Degradation Assays

As shown in Fig. 5, the fibrinolytic activity of ahpfibrase had a similarity to that of fibrolase, a non-hemorrhagic fibrin(ogen)olytic metalloprotease isolated from the venom of the southern copperhead snake (*Agkistrodon contortrix contortrix*) [20].

Fibrinogenolytic activities of the purified proteins were tested by analyzing digestion of fibrinogen. The enzyme preparation was active in the fibrinogen solution, as shown in Fig. 6. The ahpfibrase enzyme initially cleaved the α-chain, degraded the β-chain of fibrinogen much more slowly, and barely cleaved the γ-chain at all.

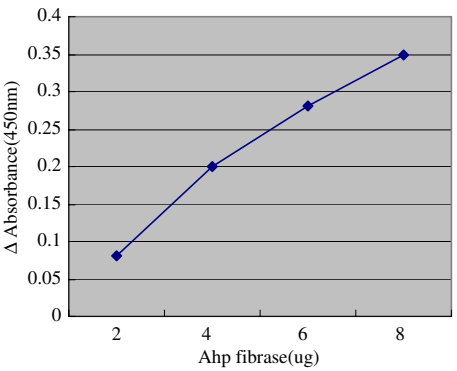

Fig. 3 Hydrolytic activity of ahpfibrase on azocasein

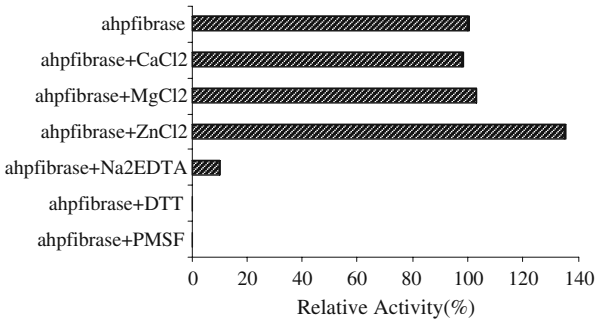

Fig. 4 Enzymatic properties of ahpfibrase. Effect of divalent metals and inhibitors (5 mM, final concentration, except for PMSF, 2 mM) on caseinolytic activity of ahpfibrase

Inhibition of Platelet Aggregation

Inhibition of platelet aggregation by the recombinant metalloprotease ahpfibrase having a disintegrin domain was examined in an ADP-induced platelet aggregation system. Ahpfibrase efficiently inhibited the platelet aggregation in a dose-dependent manner and a prolonged incubation time obviously increased the inhibitory effect (Fig. 7). These results demonstrate clearly that the recombinant ahpfibrase was properly refolded and retained both metalloprotease activity and disintegrin function.

Discussion

SVMPs can cause hemorrhage by degrading the extracellular matrix surrounding capillaries and by inhibiting platelet aggregation. They have been essentially classified into four groups according to their domain structure, except that a new classification of low molecular weight SVMPs has been reported recently by phylogenetic analysis [13, 27].

Fibrin(ogen)olytic enzymes have been described from different snake venoms including members of Crotalidae, Viperidae, and Elapidae families, which can directly split off fragments of α-, β-, and γ-chains of fibrinogen. There are primarily two classes of fibrin (ogen)olytic enzymes: (1) α(β)-fibrinogenases are zinc-metalloproteinases, which also catalyze fibrin hydrolysis; (2) β-fibrinogenases are thermostable serine proteinases which

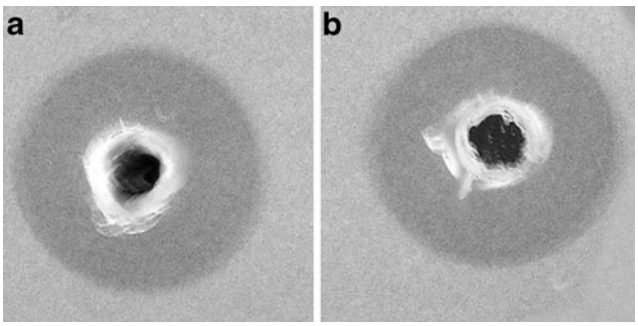

Fig. 5 Fibrinolytic activity of ahpfibrase was determined by the method of fibrin plate. **a** fibrolase. **b** ahpfibrase

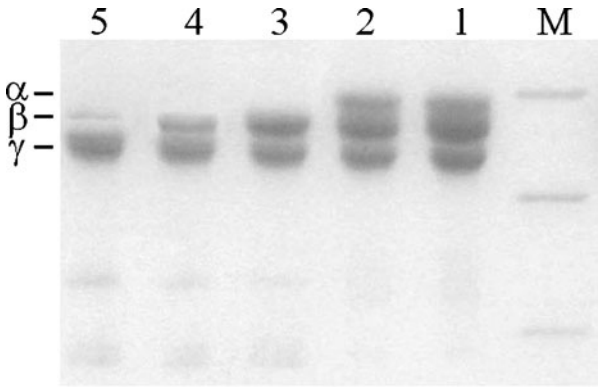

Fig. 6 SDS-PAGE analysis the degradation of fibrinogen by recombinant ahpfibrase. *M* marker, *1* fibrinogen, *2–5* sample incubated with fibrinogen for 1, 2, 4, and 8 h

split the β-chain of fibrinogen. Recently, a novel plasminogen activator was found in snake venom; and (3) it acts as an indirect fibrinolysin [6, 7, 13].

Disintegrins are low molecular weight, cysteine-rich, and Arg-Gly-Asp (RGD)-containing peptides that inhibit platelet aggregation by antagonizing fibrinogen binding to platelet glycoprotein IIb/IIIa [29, 30]. Disintegrins also bind to integrins on other cells, such as vascular endothelial cells and some tumor cells, in an RGD-dependent manner [31].

In the present study, ahpfibrase, a metalloproteinase cDNA was cloned and sequenced from *G. halys* venom from a snake specimen from southern Guangdong Province of China. The sequencing data revealed that ahpfibrase is a new member of P-II class SVMPs and its deduced amino acid sequence has a high similarity with other non-hemorrhagic metalloproteinases such as fibrolase (73%), bneu (73%), and lebe (75%), etc.

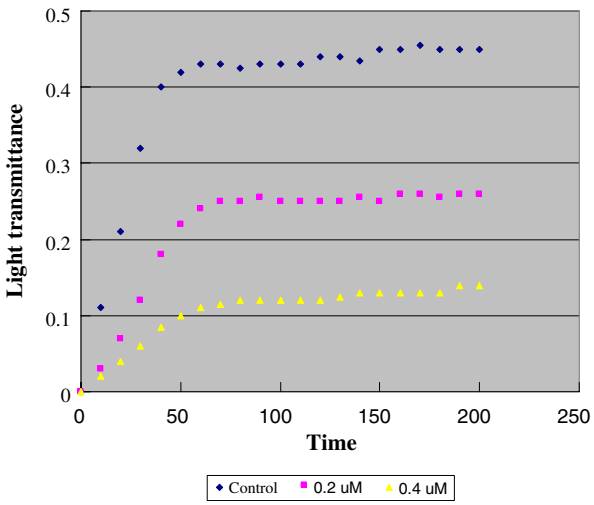

Fig. 7 Inhibition of ADP-induced platelet aggregation by ahpfibrase. The platelet aggregation assay was performed in an aggregometer (Chrono-Log) at 37°C with stirring (900 rpm). The final ADP concentration was 55 nM and the concentration of ahpfibrase was 0.2 and 0.4 μM, respectively

When successfully expressed and purified in *E. coli* cells, the recombinant enzyme was more effective on cleaving the α-chain of fibrinogen than on the β-chain, with no observable cleavage of the γ-chain, and it inhibited the aggregation of human washed platelets induced by ADP. These results are typical of SVMPs and this cleavage of fibrinogen leads to an unclottable product [8].

Acknowledgments This study was supported by the fund of "Created Major New Drugs" of "11th Five-Year Plan" Major National Science and Technology (No. 2009ZX09103-651) and Zhengzhou Scientific Research and Tackle the Key Research Project (No. 083SGYG26122.8).

References

1. Stocker, W., Grams, F., Baumann, U., Reinemer, P., Gomis-Ruth, F. X., McKay, D. B., et al. (1995). The metzincins-topological and sequential relations between the astacins, adamalysins, serralysins, and matrixins (collagenases) define a superfamily of zinc-peptides. *Protein Science, 4*, 823–840.
2. Bode, W., Gomis-Ruth, F. X., & Stockler, W. (1993). Astacins, serralysins, snake venom and matrix metalloproteinases exhibit identical zinc-binding environments (HEXXHXXGXXH and Met-turn) and topologies and should be grouped into a common family, the "metzincins". *FEBS Letters, 331*, 134–140.
3. Taei, M., Yoshihiro, F., & Koiti, T. (2000). Snake venom proteases affecting hemostasis and thrombosis. *Biochimica et Biophysica Acta, 1477*, 146–156.
4. Gremski, L. H., Chaim, O. M., Paludo, K. S., Sade, Y. B., Otuki, M. F., Richardson, M., et al. (2007). Cytotoxic, thrombolytic and edematogenic activities of leucurolysin-a, a metalloproteinase from Bothrops leucurus snake venom. *Toxicon, 50*, 120–134.
5. Pan, H., Zhou, Y. C., Yang, G. Z., & Wu, X. F. (1999). Cloning and expression of cDNA for thrombin-like enzyme from Agkistrodon halys Pallasvenom. *Acta Biochimica et Biophysica Sinica, 31*, 79–82.
6. Bjarnason, J. B., & Fox, J. W. (1995). Snake venom metalloproteinases: reprolysins. *Methods in Enzymology, 248*, 345–368.
7. Iwanaga, S., & Takeya, H. (1992). Structure and function of snake venom metalloproteinase family. *Journal of Protein Chemistry, 11*, 368–369.
8. Hite, L. A., Shannon, J. D., Bjarnason, J. B., & Fox, J. W. (1992). Sequence of a cDNA clone encoding the zinc metalloproteinase hemorrhagic toxin from Crotalus atrox: evidence for signal, zymogen, and disintegrin-like structures. *Biochemistry, 31*, 6203–6211.
9. Markland, F. S. (1998). Snake venom fibrinogenolytic and fibrinolytic enzymes: an updated inventory Registry of Exogenous Hemostatic Factors of the Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis. *Thrombosis and Haemostasis, 79*, 668–674.
10. Shinako, M., Toshiro, O., Kazuhiko, K., & Fox, J. W. (2000). cDNA cloning and characerization of vascular apoptosis-inducing protein. *Biochemical and Biophysical Research Communications, 278*, 197–204.
11. Chung, C. G., Au, L. C., & Huang, T. F. (1999). Molecular cloning and sequence analysis of aggretin, a collagen-like platelet aggregation inducer. *Biochemical and Biophysical Research Communications, 263*, 723–727.
12. Tsai, I. H., Wang, Y. M., & Lee, Y. H. (1994). Characterization of a cDNA encoding the precursor of platelet aggregation inhibition and metalloproteinase from Trimeresurus mucrosquamatus venom. *Biochimica et Biophysica Acta, 1200*, 337–340.
13. Gomis-Ruth, F. X., Kress, L. F., & Bode, W. (1993). First structure of a snake metalloproteinase: a prototype for matrix metalloproteinase/collagenases. *EMBO Journal, 13*, 4151–4157.
14. Rose, T. M., Schultz, E. R., Henikoff, J. G., Pietrokovski, S., McCallum, C. M., & Henikoff, S. (1998). Consensus-degenerate hybrid oligonucleotide primers for amplification of distantly related sequences. *Nucleic Acids Research, 26*, 1628–1635.
15. Selistre-de-Araujo, H. S., & Ownby, C. L. (1995). Molecular cloning and sequence analysis of cDNAs for metalloproteinases from broad-banded copperhead Agkistrodon contortrix laticinctus. *Archives of Biochemistry and Biophysics, 320*, 141–148.

16. Rodrigues, V. M., Soares, A. M., Guerra-Sá, R., Rodrigues, V., Fontes, M. R., & Giglio, J. R. (2000). Structural and functional characterization of neuwiedase, a nonhemorrhagic fibrin(ogen)olytic metalloprotease from Bothrops neuwiedi snake venom. *Archives of Biochemistry and Biophysics, 381*, 213–224.
17. Takeya, H., Arakawa, M., Miyata, T., Iwanaga, S., & Omori-Satoh, T. (1989). Primary structure of H2-proteinase, a non-hemorrhagic metalloproteinase, isolated from the venom of the habu snake, Trimeresurus flavoviridis. *Journal of Biochemistry, 106*, 151–157.
18. Randolph, A., Chamberlain, S. H., Cathy Chu, H. L., Retzios, A. D., Markland, F. S., & Masiarz, F. R. (1992). Amino acid sequence of fibrolase, a direct-acting fibrinolytic enzyme from agkistrodon contortrix contortrix venom. *Protein Science, 1*, 590–600.
19. Siigur, E., Aaspõllu, A., Tu, A. T., & Siigur, J. (1996). cDNA cloning and deduced amino acid sequence of fibrinolytic enzyme (lebetase) from Vipera lebetina snake venom. *Biochemical and Biophysical Research Communications, 224*, 229–236.
20. Ramos, O. H. P., & Selistre-de-Araujo, H. S. (2004). Comparative analysis of the catalytic domain of hemorrhagic and non-hemorrhagic snake venom metallopeptidases using bioinformatic tools. *Toxicon, 44*, 529–538.
21. Henikoff, S., Henikoff, J. G., Alford, W. J., & Pietrokovski, S. (1995). Automated construction and graphical presentation of protein blocks from unaligned sequences. *Gene, 163*, GC17–GC26.
22. Zhang, S. T., Shi, J., Zhao, J., Qi, Y. F., & Guo, A. G. (2006). Expression of soluble and functional snake venom fibrinolytic enzyme fibrolase via the co-expression of DsbC in Escherichia coli. *Protein Peptide Letters, 13*, 559–563.
23. Escalante, T., Shannon, J., Moura-da-Silva, A. M., Gutiérrez, J. M., & Fox, J. W. (2006). Novel insights into capillary vessel basement membrane damage by snake venom hemorrhagic metalloproteinases: a biochemical and immunohistochemical study. *Archives of Biochemistry and Biophysics, 455*, 144–153.
24. Peichoto, M. E., Teibler, P., Mackessy, S. P., & Leiva, L. (2007). Purification and characterization of patagonfibrase, a metalloproteinase showing α-fibrinogenolytic and hemorrhagic activities, from Philodryas patagoniensis snake venom. *Biochimica et Biophysica Acta, 1770*, 810–819.
25. Hung, C. C., Huang, K. F., & Chiou, S. H. (1994). Characterization of one novel venom protease with β-fibrinogenase activity from the Taiwan Habu (Trimeresurus mucrosquamatus): purification and cDNA sequence analysis. *Biochemical and Biophysical Research Communications, 205*, 1707–1715.
26. Wang, S. H., Shen, X. C., Yang, G. Z., & Wu, X. F. (2003). cDNA cloning and characterization of Agkistin, a new metalloproteinase from Agkistrodon halys. *Biochemical and Biophysical Research Communications, 301*, 298–303.
27. Gomis-Ruth, F. X., Kress, L. F., Kellermann, J., Mayr, I., Lee, X., Huber, R., et al. (1994). Refined 20A X-ray crystal structure of the snake venom znic-endopeptidase adamalysin II Primary and tertiary structure determination, refinement, molecular structure and comparison with astacin, collagenaese, and thermolysim. *Journal of Molecular Biology, 239*, 513–544.
28. Jones, G., Ronk, M., Mori, F., & Zhang, Z. Q. (2001). Disulfide structure of alfimeprase: a recombinant analog of fibrolase. *Protein Science, 10*, 1264–1267.
29. Fox, J. W., & Bjarnason, B. (1995). Atrolysins: metalloproteinases from Crotalus atrox venom. *Methods in Enzymology, 248*, 368–387.
30. Gould, R. L., Polokoff, M. A., Friedman, P. A., Huang, T. F., Holt, J. C., Cook, J. J., et al. (1990). Disintegrins: a family of integrin inhibitory proteinsfrom viper venoms. *Proceedings of the Society for Experimental Biology and Medicine, 195*, 168–171.
31. Smith, J. B., Theakston, R. D., Coelho, A. L., Barja-Fidalgo, C., Calvete, J. J., & Marcinkiewicz, C. (2002). Characterization of a monomeric disintergrin, ocllatusin, present in the venom of the Nigerian carpet viper, Echisocellatus. *FEBS Letters, 512*, 111–115.