Albocollagenase, a novel recombinant P-III snake venom metalloproteinase from green pit viper (*Cryptelytrops albolabris*), digests collagen and inhibits platelet aggregation

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ABSTRACT

Molecular cloning and functional characterization of P-III snake venom metalloproteinases (SVMPs) will give us deeper insights in the pathogenesis of viper bites. This may lead to novel therapy for venom-induced local tissue damages, the complication refractory to current antivenom. The aim of this study was to elucidate the *in vitro* activities of a new SVMP from the green pit viper (GPV) using recombinant DNA technology. We report, here, a new cDNA clone from GPV (*Cryptelytrops albolabris*) venom glands encoding 614 amino acid residues P-III SVMP, termed albocollagenase. The conceptually translated protein comprised a signal peptide and prodomain, followed by a metalloproteinase domain containing a zinc-binding motifs, HEXGHXXGXXH-CIM and 9 cysteine residues. The disintegrin-like and cysteine-rich domains possessed 24 cysteines and a DCD (Asp-Cys-Asp) motif. The albocollagenase deduced amino acid sequence alignments showed approximately 70% identity with other P-III SVMPs. Notably, the prodomain was highly conserved, while the metalloproteinase, disintegrin-like and cysteine-rich domains contained several differences. Albocollagenase without the signal peptide and prodomain was expressed in *Pichia pastoris* with an N-terminal six-histidine tag. After affinity purification from the supernatant of methanol-induced media, SDS-PAGE and Western blot analysis in both reducing and non-reducing conditions showed a protein band of approximately 62 kDa. The recombinant albocollagenase could digest human type IV collagen from human placenta basement membrane within 1 min. After 10-min incubation, it also inhibited collagen-induced platelet aggregation with 50% inhibitory concentration (IC_{50}) of 70 nM. This is the first report of the active recombinant SVMP enzymes expressed in *P. pastoris*. The results suggest the significant roles of P-III SVMP in local and systemic pathology of envenomated patients. Inhibitors of this SVMP will be investigated in further studies to find a better treatment for viper bites.

1. Introduction

Green pit viper (GPV) bites are very common in Southeast Asia including Thailand (Mahasandana and Jintakune, 1990). The clinical manifestations include local symptoms that are edema, ecchymosis, blister and skin necrosis, as well as systemic hypofibrinogenemia and thrombocytopenia (Mahasandana et al., 1980). In an analysis of 271 Thai GPV envenomated patients, 6.6% of the patients had disabling necrosis of fingers that might necessitate surgery (Rojnuckarin et al., 1998). A retrospective study from our

group had shown that intravenous antivenom, which was effective for systemic coagulopathy, could not prevent dermonecrosis in these patients (Chotenimitkhun and Rojnuckarin, 2008).

Snake venom metalloproteinases (SVMPs) are considered to be one of the major causes of extracellular matrix (ECM) degradation and induce both local damages and systemic bleeding in viper bite patients (Bjarnason and Fox, 1994). SVMPs are classified into three groups according to their domain structures (Hite et al., 1992). The P-I class is composed of a metalloproteinase domain, while the P-II class consists of a metalloproteinase and a disintegrin domain. The P-III class comprises metalloproteinase, disintegrin-like and cysteine-rich domains. The previous P-IV class containing additional disulfide-linked C-type lectin-like domains compared with P-III SVMPs (Jia et al., 1996), has been reclassified as part of group III (Fox and Serrano, 2008).

In addition to ECM degradation, SVMPs also affect proteins of hemostatic system. For example, the purified P-I BlaH1 from *Bothrops lanceolatus* (Stroka et al., 2005), the P-III hemorrhagin from *Trimeresurus pupureomaculatus* (Khow et al., 2002) and P-III VaH1 and VaH2 from *Vipera ammodites* (Leonardi et al., 2002) degraded fibrinogen, collagen, and elastin *in vitro* inducing hemorrhage *in vivo*. Furthermore, the purified the P-III SVMP, jerdohagin, also cleaved fibrinogen and prothrombin (Chen et al., 2004).

There has been a report of purified and characterized alborhagin, a 60 kDa SVMP from *Cryptelytrops albolabris*. It was a platelet agonist and, subsequently, induced ectodomain shedding of the platelet collagen receptor glycoprotein VI (Andrews et al., 2001; Wijeyewickrema et al., 2007). However, its full sequence is not yet available.

Many structure-function studies of the SVMPs have been reported using venom purification correlating with cDNA cloning to obtain the sequences. While previous data showed that P-III SVMP was more strongly hemorrhagic than P-I and P-II SVMPs (Moura-da-Silva et al., 2008), very few reports on recombinant expression of P-III SVMPs with active protease domains were published. This may be partly due to the difficulties in protein production, purification and/or autolysis of P-III SVMP (Kamiguti et al., 2000; Moura-da-Silva et al., 2003; Oliveira et al., 2009). Recombinant P-III catrocollastatin from *Crotalus atrox* expressed using baculovirus expression system in insect cells inhibited collagen-induced platelet aggregation, but the enzymatic activity was not reported (Zhou et al., 1995). The other studies expressed the disintegrin-like domain of P-III jararhagin (Moura-da-Silva et al., 1999) and P-III DC-HF3 from *Bothrops jararaca* (Silva et al., 2004) in *Escherichia coli* and found that they reacted with platelets.

The goal of this study was to identify a new P-III SVMP gene to investigate the functions related with cDNA sequences and to elucidate its *in vitro* activities that corresponded to systemic effects and local tissue damages in snakebite victims. The SVMP cDNA was cloned and expressed in the methylotrophic yeast, *Pichia pastoris*. The system was chosen because it enables correct eukaryotic post-translational modifications that may be essential for enzymatic activities.

2. Materials and methods

2.1. Obtaining the full-length cDNA sequence of the P-III SVMP gene

Total RNA was isolated from venom glands of *C. albolabris* using TRIzol LS reagent (Gibco BRL, Grand Island, NY, USA). The mRNA was then purified using Poly AT Tract system (Promega, Madison, WI) using magnetic beads coated with poly T. According to the manufacturer's instructions of the 5′ rapid amplification of cDNA ends (5′ RACE, BD Biosciences Clontech, Palo Alto, CA), the first-strand cDNA was synthesized by a modified oligo (dT) primer and reverse transcriptase coupled with a 3′ poly C tail. We designed the gene specific primer, 5′AGA GGT TGA TTA GGA GGC TCT ATT CAC ATC AAC ACA 3′, based on the sequences from the cDNA library constructed as previously described (Rojnuckarin et al., 2006). The cDNA was then amplified using the Advantage2 PCR kit (BD Biosciences Clontech, Palo Alto, CA), priming by the gene specific primer and the SMART II A oligonucleotide (a 5′ primer linked with poly G).

The RACE products were, subsequently, ligated to the pGEMT easy vector (Promega, Madison, WI) before transformation into *E. coli* JM109 using a blue–white selection system and sequenced. The nucleotide sequences and its conceptual translation obtained from the clones of interest were compared with other sequences using BLAST (Basic Local Alignment Search Tool) and CLUSTALW multiple sequence alignment program.

2.2. Expression of P-III SVMP from C. albolabris in P. pastoris

The inserted P-III SVMP cDNA in pGEMT easy vector was cut with *Eco* RI to generate the DNA fragment and cloned into pGEMT vector (Promega, Madison, WI), which did not contain *Eco* RI and *Xba* I restriction sites. The forward primer, 5′ CGG AAT TCC ATC ATC ATC ATC ATC ATG AAC AAC AAA GAT ACT TGG ATG CCA AAA AAT ACG TTA AGT ATA TCT TAG TT 3′, and reverse primer, 5′ GCT CTA GAT TAG GAG GCT CTA TTC ACA TCA ACA CAC TGT CTG TTG 3′, were designed to generate the DNA fragment with the N-terminal *Eco* RI and 6His tag sites as well as the C terminal stop codon and an *Xba* I site using the Advantage2 PCR kit.

The construct was then cloned into the yeast vector, pPicZ∝A (Invitrogen, Carlsbad, CA), using the Zeocin-resistant selection system in *E. coli*, JM109. The plasmid from pPicZ∝A was sequenced and cut with *Sac* I restriction enzyme to linearized before transforming into *P. pastoris*, KM71H strain (Invitrogen, Carlsbad, CA). The PCR analysis of *Pichia* integrants using 5′ GAC TGG TTC AAT TG ACA AGC 3′ and 5′ GCA AAT GGC ATT CTG ACA TCC 3′ primers was performed in *Pichia* Zeocin-resistant colonies on a YPDS/Zeocin agar plate to verify the inserts.

Pichia colonies with positive PCR results were inoculated in YPD broth containing 800 μg/ml Zeocin with shaking 250 rpm at 30 °C overnight. The growing colonies were selected for expression. A small-scale expression was performed in 50 ml conical tubes. The *Pichia* colonies from YPDS/Zeocin plate were inoculated in 10 ml BMGY with shaking 250 rpm at 30 °C overnight. Then, the cells were

incubated in 10 ml BMMY for 6 days. Methanol was added daily at the 0.5% final concentration. The supernatant from each day was concentrated using membrane filtration with 10 kDa molecular weight cut off (MWCO) purchased from Vivascience, Sartorius AG, Goettingen, Germany and subjected to Western blot probed with 1:3000 murine anti-His antibody (Amersham Pharmacia, Hong Kong, PRC).

For large scale expression, a selected *Pichia* colony was inoculated in YPD/Zeocin broth using a 50-ml baffled flask in a shaking incubator (250 rpm) at 30 °C overnight. Expression was performed in a 2-L baffled flask. Harvested cells were resuspended in BMGY medium with starting $OD_{600 \, nm}$ of 0.1 and grown in shaking incubator (250 rpm) at 30 °C until the culture reached an $OD_{600 \, nm}$ of 8 (approximately 15 h). Subsequently, the harvested cells were resuspended in BMMY medium with starting $OD_{600 \, nm}$ of 20 and grown in shaking incubator (250 rpm) at 30 °C for 3 days. In addition, methanol induction of protein expression was used and the concentration was maintained at 5% (v/v) every 24 h.

The supernatant was concentrated using membrane filtration with 10 kDa MWCO. The recombinant P-III SVMP was purified according to the protocol from the BD TALON Metal affinity resins user manual (BD Biosciences, Mountain view, CA). The recombinant protein was re-purified with MagneHis protein purification system (Promega, Madison, WI), electrophoresed on SDS-PAGE in either native or reduced (5% v/v of β-mercaptoethanol) conditions and subjected to Western blotting. Protein concentration was determined using the Micro BCA protein assay reagent (Pierce, Rockford, IL).

2.3. Collagen degradation

Type IV collagen was selected to be the substrate according to the protocol from previous studies (Khow et al., 2002; Oliveira et al., 2010). Collagen powder (C7521, Sigma, USA) was diluted to 5 mg/ml with 0.25% acetic acid. The performed reaction and all substances were incubated in a water bath at 37 °C for 1 h before used. 10 μL of the recombinant P-III SVMP (100 μg/ml) and 10 μL of soluble collagen were then mixed together for each reaction. The final concentrations were 0.8 μM and 2.5 mg/L, respectively. At every incubation time (1, 5, 10, 30 min and 1, 4, 8, 24 h), an aliquot of each reaction was stopped using a SDS-PAGE sample buffer containing β-mercaptoethanol and 10 mM EDTA and immediately frozen at −80 °C until tested.

To determine collagen degradation, the reactions were run on an 8% reducing SDS-PAGE and then stained with Coomassie-blue R250. We used the collagenase type I (Gibco BRL, USA) as a positive control for collagen digestion.

2.4. Fibrinogen degradation

The method was previously described (Muanpasitporn and Rojnuckarin, 2007; Stroka et al., 2005). Briefly, human fibrinogen (Sigma, USA) and the recombinant P-III SVMP were mixed together at 0.8 μM and 2.5 mg/L final concentrations, respectively. At every incubation time (5, 15, 30, 60, 120 min and 5, 12 h), an aliquot of each reaction was stopped and subjected to SDS-PAGE as described above. Pepsin (Sigma, USA) was used as a positive control for fibrinogen degradation.

2.5. Platelet aggregation

Platelet aggregation assay was performed using the Helena aggregometer (Beaumont, TX). The citrated platelet-rich plasma (PRP) from two healthy donors was adjusted to 250×10^9 platelets/L. The reaction was pre-incubated in an aggregometer holder at 37 °C for 10 min with different concentrations of the recombinant P-III SVMP before adding platelet agonist (collagen or ADP). Bovine serum albumin was used as a negative control for platelet aggregation inhibition. Platelet aggregation was initiated by collagen (Helena Laboratory, TX) at the final concentration of 40 mg/L or ADP (Sigma, USA) at the concentration of 1 μM. Light transmittance reflecting percentage aggregation was measured. The maximal aggregation in the absence of the recombinant P-III SVMP was given as 100% aggregation.

3. Results

3.1. Albocollagenase showed approximately 70% identity with other P-III SVMPs

Using a partial sequence from *C. albolabris* venom gland cDNA library, we designed a P-III SVMP specific primer and performed the 5′ RACE to obtain the full-length cDNA for sequence analysis and expression. Six similar clones of P-III SVMP were recovered. This SVMP from *C. albolabris* was first identified in this study and termed albocollagenase.

The conceptually translated sequence was analyzed. Albocollagenase was classified as a class III SVMP since it comprised metalloproteinase domain containing the conserved Zn^{2+}-binding sequences (Fig. 1) and together with disintegrin-like and cysteine-rich domains. There were 9 and 24 cysteine residues in the metalloprotease, disintegrin-like and cysteine-rich domains, respectively. The deduced protein sequence was most closely related to the P-II SVMP agkistin from *Agkistrodon contratruticus* (Wang et al., 2003) with 73% identity using the BLAST program in NCBI database.

The full-length albocollagenase protein was compared with other P-III SVMPs including ACLD (Selistre de Araujo et al., 1997) and VMP-III (Jia and Perez, 2010) from *Agkistrodon contortrix laticinctus*, Met-isofrom 1 from *Sistrurus catenatus edwardsi* (Pahari et al., 2007), Berythractivase from *Bothrops erythromelas* (Silva et al., 2003), catrocollastatin from *C. atrox* (Zhou et al., 1995) and jararhagin from *B. jararaca* (Paine et al., 1992). The percentage of identity with albocollagenase was 74%, 74%, 74%, 70%, 65% and 63%, respectively. Notably, the identity in the prodomain ranged from 89.4% to 91.8%, while those of the mature proteins were from 54.5% to 67.7%.

The mature albocollagenase was then aligned with jararhagin from *B. jararaca*, catrocollastatin and atrolysin A (Hite et al., 1994) from *C. atrox*, and kaouthiagin from *Naja kaouthia* (Ito et al., 2001) using CLUSTALW program as shown in Fig. 2. The conserved Zn^{2+}-binding sequences

were found to be identical to other active SVMPs. However, several cysteines were different from the other P-III SVMPs. Albocollagenase disintegrin-like domain contained DCD (aspartate cysteine aspartate) that was homologous to ECD (glutamate cysteine aspartate) motif, a putative collagen binding site. Additionally, it contained 3 putative Ca^{2+}-binding sites as shown in Fig. 2.

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   1   atgatccaagttctcttggtaaccatatgcttagcagttttcccttatcaagggagctct    60
   1    M  I  Q  V  L  L  V  T  I  C  L  A  V  F  P  Y  Q  G  S  S    20
  61   ataatcctggaatcgggaacgtgaatgattatgaagtcgtgcatccacgaaagtcact   120
  21    I  I  L  E  S  G  N  V  N  D  Y  E  V  V  H  P  R  K  V  T    40
 121   gcattacccaaaggagcagttcagcaaaagtatgaagacgccatgcaatatgaatttaag  180
  41    A  L  P  K  G  A  V  Q  Q  K  Y  E  D  A  M  Q  Y  E  F  K    60
 181   gtgaatggagagccagtagtcctttcacctggaaaaaaataaaggactttttttcagaagat  240
  61    V  N  G  E  P  V  V  L  H  L  E  K  N  K  G  L  F  S  E  D    80
 241   tacagtgagactcattattcccctgatggcagagaaattacaacatacccctcggttgag  300
  81    Y  S  E  T  H  Y  S  P  D  G  R  E  I  T  T  Y  P  S  V  E   100
 301   gatcactgctattatcatggacgcatccagaatgatgctgactcaactgcaagcatcagt  360
 101    D  H  C  Y  Y  H  G  R  I  Q  N  D  A  D  S  T  A  S  I  S   120
 361   gcatgcaatggtttgaaaggacatttcaagcttcaagggggacgtaccttattgaaccc   420
 121    A  C  N  G  L  K  G  H  F  K  L  Q  G  E  T  Y  L  I  E  P   140
 421   ttggaacttttccgacagtgaagctcatgcagtattcaaatatgaaaatgtagaaaaagag  480
 141    L  E  L  S  D  S  E  A  H  A  V  F  K  Y  E  N  V  E  K  E   160
 481   gacgaggccccaaaatgtgtggggtaacccagaattggaaatcatatgaacccatcaaa   540
 161    D  E  A  P  K  M  C  G  V  T  Q  N  W  K  S  Y  E  P  I  K   180
 541   gaggcctctcagtcgaatcttactcctgaacaacaaagatacttggatgccaaaaaatac  600
 181    E  A  S  Q  S  N  L  T  P  E  Q  Q  R  Y  L  D  A  K  K  Y   200
 601   gttaagtatatcttagttttggaccgtggaatgtgcacaaaatacaacagcgatttaaaa  660
 201    V  K  Y  I  L  V  L  D  R  G  M  C  T  K  Y  N  S  D  L  K   220
 661   aagatgaaaacaagaatgtatgaacttgtcaacattatgaatgagatctgcctccctttg  720
 221    K  M  K  T  R  M  Y  E  L  V  N  I  M  N  E  I  C  L  P  L   240
 721   aatattcgcgtagcattgactggcctaataatttggttggacagagtaagattaacgtg  780
 241    N  I  R  V  A  L  T  G  L  I  I  W  L  D  R  D  K  I  N  V   260
 781   acatcagcagcaaatgttacttttgtcctttatttggagactggagagcgacagtcttgctg  840
 261    T  S  A  A  N  V  T  L  S  L  F  G  D  W  R  A  T  V  L  L   280
 841   aagcagaaaaatcatgattgtgctcagttattcacggacactgacttcgatggtgacact  900
 281    K  Q  K  N  H  D  C  A  Q  L  F  T  D  T  D  F  D  G  D  T   300
 901   gtaggattggcttatacgggtggcatatgccgactgaagcattctgtaggaattattcag  960
 301    V  G  L  A  Y  T  G  G  I  C  R  L  K  H  S  V  G  I  I  Q   320
 961   gatcatgacacaataaatcttctgatggcagttacaatggtccatgagctggtcataat 1020
 321    D  H  S  T  I  N  L  L  M  A  V  T  M  V  *H*  *E*  *L*  *G*  *H*  *N*  340
1021   ctgggcatggaacatgatgtaaaccgtgatggaaagcagtgtaattgtgatgcatgcatt 1080
 341    *L*  *G*  *M*  *E*  *H*  D  V  N  R  D  G  K  Q  C  N  C  D  A  *C*  *I*  360
1081   atggctcccaggctaaaccctcaaccttccaaacagttcagcgattgtagtaaagacgat 1140
 361    *M*  A  P  R  L  N  P  Q  P  S  K  Q  F  S  D  C  S  K  D  D  380
1141   tatcggacatttcttataaatcgtagcaccaatgcattctcaatgcaccctcgaagaca 1200
 381    Y  R  T  F  L  I  N  R  R  P  Q  C  I  L  N  A  P  *S*  *K*  *T*  400
1201   gatattgtttcaccccccagtttgtgggaaatgaactttggagaagggaaaagaatgtgac 1260
 401    D  I  V  S  P  V  C  G  N  E  L  L  E  K  G  K  E  C  D  420
1261   tgtggctctcctaaaaacttgtccgaaaatatgctgcaatgctgcacgtgtaactctgccc 1320
 421    C  G  S  P  K  N  L  S  K  I  C  C  N  A  A  R  V  T  L  P  440
1321   ccaggttcccaatgtgcagatgagtgtggttgtcaccagtgcaattttaagagagcagga 1380
 441    P  G  S  Q  C  A  D  E  C  G  C  H  Q  C  N  F  K  R  A  G  460
1381   acagaagtcgggcaggcaaaggatgactgtgacttggctgaaagctgcactggccgatct 1440
 461    T  E  V  G  Q  A  K  D  *D*  *C*  *D*  L  A  E  S  C  T  G  R  S  480
1441   gctgagtgtcccacggatctcctccaaagggatggacaaccatgccaaaacaacaatggt 1500
 481    A  E  C  P  T  D  L  L  Q  R  D  G  Q  P  C  Q  N  N  N  G  500
1501   tactgctacaataggacggcgccccaccatgaacaaccaatgtatttctttctttgggtca 1560
 501    Y  C  Y  N  R  T  R  P  T  M  N  N  Q  C  I  S  F  F  G  S  520
1561   agtgcaactgtggctccagatggatgtttttaattttaaccggcaaagcaatgattatagc 1620
 521    S  A  T  V  A  P  D  G  C  F  N  F  N  R  Q  S  N  D  Y  S  540
1621   tactgcagaaaggaaaacgtagaaaagattccatgtgcaccacaagatgtaaatgtggc 1680
 541    Y  C  R  K  E  N  G  R  K  I  P  C  A  P  Q  D  V  K  C  G  560
1681   aggttatactgctctcctaattcacccggagaggagaaatacttgcaatctcatatataca 1740
 561    R  L  Y  C  F  P  N  S  P  G  E  E  N  T  C  N  L  I  Y  T  580
1741   cctgtcgtgaagatattgggatggttcttctgggaacaaaatgtgcagatggaaaggcc 1800
 581    P  G  R  E  D  I  G  M  V  L  L  G  T  K  C  A  D  G  K  A  600
1801   tgcaacagcaacagacagtgtgttgatgtgaatagagcctcctaa 1845
 601    C  N  S  N  R  Q  C  V  D  V  N  R  A  S  -  614
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Fig. 1. The coding nucleotide and deduced protein sequences of albocollagenase. They comprise 1845 base pairs and 614 amino acid residues, respectively. The protein sequence comprises the signal peptide, pro-peptide, metalloproteinase domain, disintegrin-like and cysteine-rich domains, respectively. The putative signal peptide is italicized and underlined. The putative pro-peptide is *italicized*. The metalloproteinase domain is underlined. The Zn^{2+}-binding sequences followed by methionine turn (Met-turn) (HELGHNLGMEH-CIM) are underlined and highlighted. The disintegrin-like and cysteine-rich domains are bold. The putative collagen binding sequences, DCD, are bold and highlighted. The recombinant protein composed of metalloproteinase, disintegrin-like and cysteine-rich domains was expressed in this study.

3.2. Albocollagenase was expressed at a low level in P. pastoris

We constructed the mature albocollagenase containing metalloproteinase, disintegrin-like and cysteine-rich domains, as well as an N-terminal 6 histidine tag, in the pPICZαA vector. P. pastoris KM71H was used as the host cells. The Zeocin-resistant colonies of Pichia were randomly selected for small-scale expression and Western blot.

The chosen Pichia colony was optimized for suitable expression conditions. We cultured the Pichia for 6 days showing protein expression during day 2–6 and the third day of induction was selected. The recombinant protein was purified using 2 tandem techniques, a Ni^{2+}-resin column and a Ni^{2+}-magnetic bead to bind recombinant 6 histidine-tagged albocollagenase.

The protein purification yielded 100 μg of recombinant albocollagenase from 400 ml of culture media. It was stored at −80 °C until used. On SDS-PAGE and Western blot, the protein bands of albocollagenase in both native and reduced conditions were approximately 62 kDa as shown in Fig. 3A and B.

3.3. Albocollagenase digested type IV collagen but not fibrinogen

The recombinant albocollagenase was incubated with type IV collagen for different periods of time as shown in Fig. 4A. Albocollagenase degraded human type IV collagen in a time-dependent manner. While the degradation of type IV collagen began at 1 min, more digested bands were progressively more visible during the period of 24 h.

In Fig. 4B, albocollagenase was able to digest type IV collagen like to the collagenase type I, a positive control. EDTA (a metal iron chelator), but not PMSF (a serine protease inhibitor), could inhibit collagen degradation by albocollagenase suggesting that it was a metalloproteinase, not a serine protease.

A human fibrinogen degradation assay was also performed for albocollagenase in the presence and absence of calcium ion. We found that albocollagenase could not digest human fibrinogen in either condition (data not shown).

3.4. Albocollagenase inhibited collagen-induced platelet aggregation

In order to test the effects of the recombinant protein and platelets, we performed platelet aggregation analysis using 10-min pre-incubation of various concentrations of albocollagenase and platelets before adding collagen or ADP as the inducers. We found that albocollagenase could inhibit collagen-induced platelet aggregation in a concentration-dependent manner as shown in Fig. 5. The 50% of inhibitory concentration (IC_{50}) value was 70 nM. However, there was no effect on ADP-induced platelet aggregation (data not shown).

4. Discussion

Snake venom metalloproteinases (SVMPs) are the main venom components responsible for local tissue injury after

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Albocollagenase   ----EQQRYLDAKKYVKYILVLDRGMCTKYNSDLKKMKTRMYELVNIMNEICLPLNIRVA   56
Jararhagin        -----EQQRYDPYKYIEFFVVVDQGTVTKNNGDLDKIKARMYELANIVNEIFRYLYMHVA   55
Catrocollastatin  ------HQKYNPFRFVELVLVVDKAMVTKNNGDLDKIKTRMYEIVNTVNEIYRYMYIHVA   54
Atrolysin A       -------ERLT-KRYVELVIVADHRMFTKYNGNLKKIRKWIYQIVNTINEIYIPLNIRVA   52
Kaouthiagin       TNTPEQDRYLQAEKYIEFYVIVDNRMYRYYNYDKPAIKIRVYEMINAVNTKFRPLKIHIA   60
                          17        27            Ca2+-binding(I)        52
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Albocollagenase   LTGLIIWLDRDKINVTSAANVTLSLFGDWRATVLLKQKNHDCAQLFTDTDFDGDTVGLAY   116
Jararhagin        LVGLEIWSNGDKITVKPDVDYTLNSFAEWRKTDLLTRKKHDNAQLLTAIDFNGPTIGYAY   115
Catrocollastatin  LVGLEIWSNEDKITVKPEAGYTLNAFGEWRKTDLLTRKKHDNAQLLTAIDLDR-VIGLAY   113
Atrolysin A       LVRLEIWSNGDLIDVTSAANVTLKSFGNWRVTNLLRRKSHDNAQLLTAIDLDEETLGLAP   112
Kaouthiagin       LIGLEIWSNEDKFEVKPAASVTLKSFREWRQTVLLPRKRNDNAQLLTGINLNGTAVGIAY   120
                                                                101
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                                        Zn2+-binding                Met-turn
                                        HEXGHXXGXXH                    M
Albocollagenase   TGGICRLKHSVGIIQDHSTINLLMAVTMVHELGHNLGMEHDVNRDGKQCNCDACIMAPRL   176
Jararhagin        IGSMCHPKRSVGIVQDYSPINLVVAVIMAHEMGHNLGIHHDTGS--CSCGDYPCIMGPTI   173
Catrocollastatin  VGSMCHPKRSTGIIQDHSPEINLVVAVIMAHEMGHNLGINHDSGY--CSCGDYACIMRPEI   171
Atrolysin A       LGTMCDPKLSIGIVQDHSPINLLVAVTMAHELGHNLGMVHDENR--CHCSTPACVMCAVL   170
Kaouthiagin       PGSLCT-QRSVFVVQDYNRRMSLVASTMTHELGHNLGIHHDEAS--CICIPGPCIMLKKR   177
                       125                                    167     174
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                                          Ca2+-binding(II)
Albocollagenase   NPQPSKQFSDCSKDDYRTFLINRRPQCIILNAPSKTDIVSPPVCGNELLEKGKECDCGSPK   236
Jararhagin        SNEPSKFFSNCSYIQCWDFIMNHNPECIINEPLGTDIISPPVCGNELLEVGEECDCGTPE   233
Catrocollastatin  SPEPSTFFSNCSYFECWDFIMNHNPECIINEPLGTDIISPPVCGNELLEVGEECDCGTPE   231
Atrolysin A       RQRPSYEFSDCSLNHYRTFIINYNPQCIINEPLQTDIISPPVCGNELLEVGEECDCGSPR   230
Kaouthiagin       -TAPAFQFSSCSIRDYQEYLLRDRPQCIILNKPLSTDIVSPAICGNYFVEEGEECDCGSPA   236
                       191     196           207   212      223        234
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                                                Ca2+-binding(III)
Albocollagenase   NLSKICCNAARVTLPPGSQCADECGCHQCNFKRAGTEVGQAKDDCDLAESCTGRSAECPT   296
Jararhagin        NCQNECCDAATCKLKSGSQCGHGDCCEQCKFSKSGTECRASMSECDPAEHCTGQSSECPA   293
Catrocollastatin  NCQNECCDAATCKLKSGSQCGHGDCCEQCKFSKSGTECRASMSECDPAEHCTGQSSECPA   291
Atrolysin A       TCRDPCCDAATCKLHSWVECESGECCQQCKFTSAGNVCRPARSECDIAESCTGQSADCPT   290
Kaouthiagin       ACQSACCDAAT----------------CKFNGAGAECRAAKHDCDLPELCTGQSAECPT   279
                     242 246    252      260      269     278    285    291    298
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Albocollagenase   DLLQRDGQPCQNNNGYCYNRTRPTMNNQCISFFGSSATVAPDGCFNFNRQSNDYSYCRKE   356
Jararhagin        DVFHKNGQPCLDNYGYCYNGNCPIMYHQCYALFGADVYEAEDSCFKDNQKGNYYGYCRKE   353
Catrocollastatin  DVFHKNGQPCLDNYGYCYNGNCPIMYHQCYDLFGADVYEAEDSCFERNQKGNYYGYCRKE   351
Atrolysin A       DDFHRNGKPCLHNFGYCYNGNCPIMYHQCYALWGSNVTVAPDACFDINQSGNNSFYCRKE   350
Kaouthiagin       DSLQRNGHPCQNNQGYCYNGKCPTLTNQCIALLGPHFTVSPKGCFDLNMRGDDGSFCRME   339
                      310   317  322   329       344        357
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                                    Hyper-variable-region(HVR)
Albocollagenase   NGRKIPCAPQDVKCGRLYCFPNSPGEENTCNLIYTPGREDIGMVLLGTKCADGKACNSNR   416
Jararhagin        NGKKIPCAPEDVKCGRLYCKDNSPGQNNPCKMFYSNDDEHKGMVLPGTKCADGKVCS-NG   412
Catrocollastatin  NGNKIPCAPEDVKCGRLYCKDNSPGQNNPCKMFYSNEDEHKGMVLPGTKCADGKVCS-NG   410
Atrolysin A       NGVNIPCAQEDVKCGRLFCNVN----DFLCRHKYSDD----GMVDHGTKCADGKVCK-NR   401
Kaouthiagin       DGTKIPCAAKDVKCGRLYCTEKN---TMSCLIPPNPDG---IMAEPGTKCGDGMVCS-KG   392
                      367    374  379    390          400    406
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Albocollagenase   QCVDVNRAS---------   425
Jararhagin        HCVDVATAY---------   421
Catrocollastatin  HCVDVATAY---------   419
Atrolysin A       QCVDVTTAYKSTSGFSQI   419
Kaouthiagin       QCVDVQTAY---------   401
                    412
```

Fig. 2. The multiple sequence alignment of the P-III SVMP putative mature proteins compared with other SVMPs Cysteine residues are numbered according to kaouthiagin. The cysteine residues are highlighted. The Zn^{2+}-binding sequences are underlined. The putative self-post-translational processing position, P212, are italicized and highlighted. The putative collagen binding sequences, DCD, are underlined, italicized and highlighted. The 3 putative Ca^{2+}-binding sites are boxed. The putative hyper-variable-regions (HVR) are boxed and highlighted.

viper bites. As type P-III SVMPs display more potent activities than those of P-I and P-II classes (Bjarnason and Fox, 1994; Hite et al., 1994), we focused our study on a novel P-III class SVMP, termed albocollagenase, from *C. albolabris*.

The recombinant expression in *P. pastoris* was used instead of venom purification because the functions of the proteins could be correlated with the sequences. Our previous studies illustrated the potentials of snake venom protein expression in *P. pastoris*, strain X-33 (Muanpasitporn and Rojnuckarin, 2007; Singhamatr and Rojnuckarin, 2007). As there were more successful data of metalloproteinase expression using *P. pastoris*, KM71H (Brouta et al., 2002; Schwettmann and Tschesche, 2001), this strain was selected for the expression in this study.

The sequence analysis of albocollagenase in Fig. 2 revealed that several cysteine residues were conserved
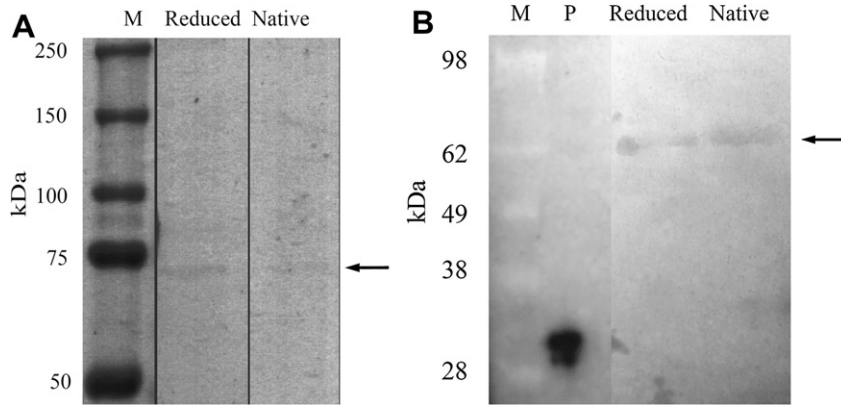
Fig. 3. Recombinant albocollagenase (Arrows) (A) The recombinant protein was purified, electrophoresed on 8% SDS-PAGE in reduced and native conditions and stained with Coomassie-blue R250. The molecular weight markers are shown in lane M. (B) The purified protein was electrophoresed as described in (A) and subjected to Western blot probed with anti-histidine tag antibody. In each lane, 2 μg of protein were loaded. The purified 35 kDa histidine-tagged *Wolbachia* surface protein expressed in *E. coli* was used as a positive control for Western blot was showed in lane P.
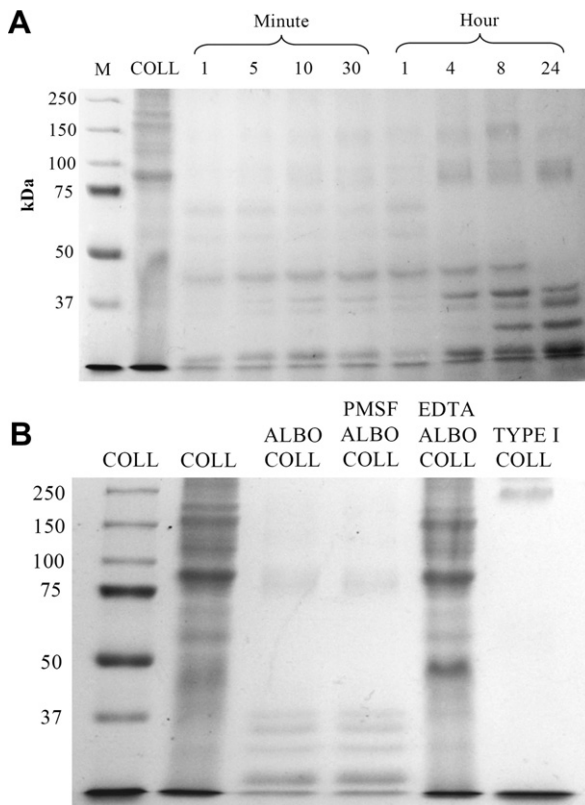
Fig. 4. Time-dependent type IV collagen degradation by albocollagenase (A) Type IV collagen was incubated with albocollagenase at different time points as indicated in each lane. Each reaction was subjected to 8% reducing SDS-PAGE and stained with Coomassie-blue. M and COLL represent the molecular weight markers and undigested collagen, respectively. (B) Type IV collagen alone and collagen plus albocollagenase incubation for 24 h are shown in lane COLL and COLL/ALBO, respectively. In the following lanes, the albocollagenase (ALBO) was pre-incubated with PMSF and EDTA, respectively, at 10 mM final concentration for 1 h before adding collagen (COLL) and incubating for 24 h. Type I collagenase (TYPE I) and collagen (COLL) are shown in the last lane as a positive control.

among P-III SVMPs. However, there were 9 cysteines in albocollagenase metalloproteinase domain compared with 5–7 residues in the other SVMPs. Although the numbers of cysteine residues vary among SVMP metalloproteinase domains, they usually contain only 3 conserved disulfide bonds (Igarashi et al., 2007; Takeda et al., 2006). The 3 'extra' cysteines in albocollagenase were all in the N-terminal portion (C27, C52, and C102) of the protease domain before reaching the highly conserved C125. These N-terminal cysteine residues may not participate in disulfide pairing and, thus, not affect the folding of SVMPs (Fox and Serrano, 2008). For example, a crystal structure of the P-Ia SVMP, adamalysin II, showed an extra unpaired cysteine residue locating N-terminal to the C125 (Gomis-Ruth et al., 1993).

From the crystal structure analysis, adamalysin II contained 2 disulfide bonds (C125-C207 and C167-C174), while the P-IIIb catrocollastatin (Igarashi et al., 2007) contained 3 disulfide pairs (C125-C207, C169-C174, and C167-C191). Interestingly, albocollagenase contained C125, C207, C169, C171, C174 and C191. The disulfide bond pattern of albocollagenase remains to be elucidated.

The disintegrin-like and cysteine-rich domains of albocollagenase contained as many as 24 cysteine residues. Data on disulfide bond pairing within this domain were conflicting between N-terminal sequencing and mass spectrometry analysis (Fox and Serrano, 2008; Igarashi et al., 2007). Nevertheless, the first cysteinyl residues in disintegrin-like domain, C223, was usually lacking in disintegrin domains (Fox and Serrano, 2008). Fig. 2 showed that albocollagenase also contained this conserved C223 as all the other P-III SVMPs.

By comparison with catrocollastatin, albocollagenase contained 3 putative Ca^{2+}-binding sites as shown in Fig. 2. Calcium ions are known to stabilize the tertiary structure of matrix metalloproteinases (MMPs) with collagenase activity (Bode et al., 1994). Alborhagin, another P-III SVMP from *C. albolabris*, could digest human fibrinogen when adding Ca^{2+} to the reaction (Andrews et al., 2001). On contrary, albocollagenase could not digest fibrinogen with or without adding Ca^{2+}.

Albocollagenase was expressed at a low level, 0.25 mg/L of culture media, in our *Pichia* system. Snake codon usage may not be optimal for protein expression (Schmidt-Dannert et al., 1998). Another possible explanation is that the prodomain was not included in expression construct. Pro-domains of enzymes are known to be critical for protein folding (Nagradova, 2004). Alternatively, the recombinant protein may undergo auto-proteolysis (Assakura et al., 2003; Fujimura et al., 2000). SDS-PAGE showed that the recombinant protein was larger (62 kDa) than the molecular weight calculated from the amino acid content (49 kDa). This discrepancy may be due to post-translational modifications (Oliveira et al., 2010).

Collagen is a structural scaffold for connective tissue and blood vessel walls comprising as many as 27 different types. Type IV collagen is the essential component of basal lamina and ocular lens. Previous reports showed that most of P-III SVMPs can degrade human type IV collagen. As shown in Fig. 4, the degradation of type IV collagen began at 1 min implying that the albocollagenase could rapidly degrade extracellular matrix (ECM) of envenomated patients. ECM degradation may result in vascular endothelial damages by inducing endothelial cell anoikis, a specialized form of apoptosis (Tanjoni et al., 2005). Therefore, albocollagenase probably played important roles in rapid local tissue damages in snakebite patients. Inhibition of these enzymatic activities may be helpful in clinical therapy. For example, a small molecule metalloproteinase inhibitor, doxycycline, was found to inhibit the enzyme *in vitro*. Unfortunately, it could not prevent venom hemorrhagic activities *in vivo* (Rucavado et al., 2008).

In addition to the protease domain, the strong proteolytic activity of the P-III SVMP may be resulted from a specific interaction with basement membrane components. Several lines of evidence suggest that the cysteine-rich domain targeted the protease to interact with collagen fiber (Tanjoni et al., 2010) or von Willebrand factor (vWF) (Serrano et al., 2007) contributing to the hemorrhagic activity. In addition, recent crystal structure of catrocollastatin revealed the hyper-variable-region (HVR) located at the C terminal part of the cysteine-rich domain (Fig. 2). This represented

a potential exosite for substrate recognition by binding to ECM proteins (Igarashi et al., 2007). Therefore, cysteine-rich domain may function as substrate targeting to enhance metalloproteinase domain activities. Furthermore, HVR may also play a role in triggering pro-inflammatory effects by promoting leukocyte rolling (Menezes et al., 2008).

Platelet aggregation contributes to hemostasis using complex mechanisms. Binding of subendothelial collagen with platelet receptor glycoprotein (GP) VI (non-integrin) stimulate the signaling pathways and up-regulate platelet integrins (inside-out signaling), such as $\alpha_{IIb}\beta_3$ and $\alpha_2\beta_1$. In addition, stimulated platelets secrete the granule contents, particularly ADP, which promotes platelet activations. Like GPVI, the $\alpha_2\beta_1$ intergrin also binds collagen fibers activating platelet adhesion and spreading, as well as thrombus formation. The integrin $\alpha_{IIb}\beta_3$ plays an exclusive role in linking platelets to one another through the adhesive action of fibrinogen. Engagements of this receptor further activate platelet spreading and enhance platelet aggregation (Adam et al., 2008).

Disintegrin-like domain of SVMPs was the main part interacting with platelets. However, the purified P-I SVMP, lebetase from *Vipera lebetina*, which did not contain disintegrin or disintegrin-like domain, could also inhibit ADP-induced platelet aggregation (Siigur et al., 1998). Therefore, metalloproteinase domains may also react with platelets.

P-III SVMPs could inhibit platelet aggregation through several proposed mechanisms. First, some could degrade or interact with different platelet receptors. For example, jararhagin degraded the β subunit of integrin $\alpha_2\beta_1$ (Kamiguti et al., 1996). Atrolysin A bound and blocked $\alpha_2\beta_1$ (Kamiguti et al., 2003). Acurhagin interacted with GPVI (Wang et al., 2005). Secondly, others could degrade or interact with adhesive proteins involved in hemostasis, e.g. AAV1 (Wang, 2007) and halysase degraded fibrinogen; kaouthiagin (Hamako et al., 1998) and jararhagin (Serrano et al., 2007) destroyed vWF; jararhagin, atrolysin A, and catrocollastatin interacted with vWF domain (Serrano et al., 2007); jararhagin, acurhagin, and catrocollastatin bound collagen fibers. Our results showed that albocollagenase inhibited only collagen (not ADP)-induced platelet aggregation suggesting that the venom protein specifically prevented collagen and collagen receptor (GPVI and/or $\alpha_2\beta_1$ integrin) interactions. Whether this is mediated by enzymatic degradation or non-enzymatic binding mechanisms remain to be determined.

In summary, we cloned, expressed and characterized a novel P-III SVMP, albocollagenase, from *C. albolabris* venom. Like other P-III SVMPs, it displayed a multidomain structure composed of a metalloproteinase, disintegrin-like and cysteine-rich domains. Recombinant albocollagenase exhibited proteolytic activities on collagen and inhibited collagen-induced platelet aggregation. Therefore, it possibly contributed to tissue necrosis and hemorrhage in snakebite patients. Future investigations to identify potent and specific inhibitors to this molecule are warranted.
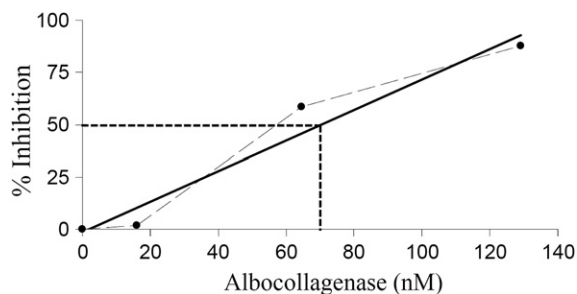

Fig. 5. Albocollagenase inhibited platelet aggregation. Various concentrations of the albocollagenase (0, 0.25, 1, and 2 mg/L) were pre-incubated with platelet-rich plasma (PRP) before adding collagen. The maximal aggregation without albocollagenase was regarded as 100%. The PRP without agonist was set as 0%. The percentage of aggregation was adjusted from maximal aggregation. The percentage of inhibition was calculated from 100 minus the percentage of adjusted aggregation. The 50% of inhibitory concentration (IC_{50}) value of the albocollagenase was 70 nM by linear regression curve fitting algorithm.

Acknowledgments

Conflict of interest

The authors have no conflict of interest.

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