รายงานวิจัยฉบับสมบูรณ์

โครงการ กลไกการออกฤทธิ์ระดับโมเลกุลของโปรตีนจากพิษงูเขียวหางไหม้ (*Cryptelytrops albolabris*)

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15 มิถุนายน 2556

Introduction

Snakebite is the important global public health problem affecting over 5 million victims annually. Furthermore, viper venoms are the rich sources of proteins affecting hemostasis, vascular and cancer biology. Molecular studies not only advance clinical managements, but also yield potentially useful diagnostic and therapeutic compounds. Molecular biology approach was used to investigate the toxic components of the venom by cloning venom genes and expressing proteins in *Pichia pastoris* system. In this study, we cloned and expressed 2 novel snake venom metalloproteinase (SVMPs) from *C. albolabris* venom gland library, albolamin and albocollagenase. Furthermore, we cloned the cDNA of a new snake venom C-type lectin (snaclec), the 120-kDa alboaggregin D, and studied its activities on platelets.

Albolamin

1. Introduction

Green pit viper, *Cryptelytrops albolabris* is the main cause of snakebites in Bangkok (Mahasandana and Jintakune, 1990). Its venom exerts many effects on hemostatic system resulting in hemorrhagic symptoms in patients (Mitrakul and Impun, 1973). In addition to hypofibrinogenemia and thrombocytopenia, green pit viper can cause local tissue damages resulting in pain, swelling and/or tissue necrosis (Rojnuckarin et al, 1998). Notably, antivenom therapy can promptly neutralize systemic effects of viper venom, but tissue necrosis still occurs after antivenom treatment (Chotenimitkhun and Rojnuckarin, 2008). The main mechanism of tissue necrosis is due to the catalytic activity of snake venom metalloproteinases (SVMPs) that degrade extracellular matrix proteins and stimulate tissue inflammation causing further damages around biting wounds (Laing et al., 2003). Furthermore, SVMPs may contribute to systemic bleeding by damaging vascular wall basement membrane and inducing endothelial cell apoptosis, as well as exerting fibrinogenolytic and fibrinolytic activities (Fox and Serrano, 2005, Gutierrez et al. 2005, Swenson and Markland, 2005). Furthermore, SVMPs may activate coagulation factors causing consumptive coagulopathy and inhibit platelet functions (Kamiguti et al, 1996, Fox and Serrano, 2005).

SVMPs are multi-domain proteins that can be classified into 3 major classes depending on its domains components (Fox and Serrano, 2008). A P-I class SVMP comprises a signal peptide, prodomain and metalloproteinase domain that are conserved in all classes. P-II class SVMPs are followed by disintegrin domains, which usually contain either Arg-Gly-Asp

(RGD) or Lys-Gly-Asp (KGD) sequences, the known platelet integrin $\alpha_{IIb}\beta_3$ (glycoprotein IIb/IIIa) binding sites. Instead of disintegrin domain, the P-III class contains disintegrin-like and cysteine-rich domains after metalloproteinase domains. These 2 domains act by binding and targeting the metalloproteinases to the substrates (Takeda et al, 2006).

Studies of snake venom activities may be performed on purified proteins from crude lyophilized venom. Alternatively, molecular biology techniques can be employed. Recombinant expression allows us to obtain unlimited amounts of purified proteins devoid of any contamination to investigate their activities and correlate with the amino acid sequences. In addition, future structure-function relationship experiments can be done.

SVMPs have been expressed in various systems. Although there were reports that active proteins could be obtained from the *Escherichia coli* system (Moura-Da-Silva et al., 1999), it may required a complicated refolding procedure (Ramos et al, 2003). In addition, glycan domains of SVMPs, such as jararrhargin and ACLH, are critical for their hemorrhagic activities (Garcia et al., 2004). By pre-incubation of native jararrhargin and ACLH with an N-deglycosylating enzyme, both SVMPs lost their hemorrhagic activity and underwent autolytic degradation. This finding supports the significance of glycosylation on enzymatic activity of SVMPs. Therefore, eukaryotic expression systems with correct folding and post-translational modifications are required. For examples, expression of Agkistin from *Agkistrodon halys* in baculovirus sytem and the jerdonitin from *Protrobothrops jerdonii* in *Pichia pastoris* successfully produced active proteins (Wang et al, 2003, Zhu et al, 2010) The *Pichia pastoris* system has an additional benefit in yielding large amounts of recombinant proteins (Cereghino and Cregg, 2000).

In this study, we cloned albolamin, a novel SVMP from *C. albolabris*, expressed its metalloproteinase and disintegrin domains in the *Pichia pastoris* system and investigate its activity on collagen and platelet aggregation. This not only gives deeper insight in pathogenesis of green pit viper bites, but also has potentials to provide a novel mean of snakebite therapy and platelet inhibition.

2. Material and method

2.1 Obtaining full-length cDNA of a novel snake venom metalloproteinase from Cryptelytrops albolabris venom gland.

Green pit viper (*C. albolabris*) venom gland library was prepared as previously described (Rojnuckarin, et al. 2006).Three partial 3' sequences of clones of new SVMP were derived from the library. The complete sequences and expression of 2 SVMPs (albolatin and

albocollagenase) were previously published (Singhamatr et al, 2007 and Pinyachart et al, 2011, respectively). The third clone was further investigated in this study.

Rapid amplification of 5' cDNA end (5'RACE) was performed using SMART™ RACE kit (BD Biosciences Clontech, USA) using a gene specific primer 5' AAA TCA AAT CTG AGA GAA GCC AGA GGT TGA T 3' to obtain the full-length cDNA clones. The coding sequence was conceptually translated, analyzed and compared with homologous sequences in GENBANK using BLAST search as well as CLUSTALW multiple sequence alignment.

2.2 *Expression of the metalloproteinase with a disintegrin domain of the SVMP in Pichia pastoris system*

From a complete cDNA clone, the metalloproteinase followed by disintegrin domain was amplified by polymerase chain reaction (PCR). The 5' primer included an *Eco*RI recognition site, 6-histidine tag and the N terminal part of metalloproteinase, 5′ CGG AAT TCC ATC ATC ATC ATC ATC ATA GAT TCA TTG AGC TTG TCA TCA TAA TT 3′. The 3' primer contained the C-terminus of the protein with stop codon and *Xba* I recognition site, 5′ GCT CTA GAT TAG GAA TTT CTG GGA CAG TAC 3′.

The PCR product was electrophoresed on 1.2 % agarose gel. After extraction from the gel, the PCR product was cloned into pGEM®-T Vector (Promega, USA), transformed into JM 109 competent cells (Promega, USA) using a heat-shock method and plated on LB agar plate with 100 μg/ml amplicillin supplemented with 100 mM IPTG and 50 μg/ml of X-gal for blue/white screening.

The construct in pGEM-T was digested using *Eco*R and *Xba* I. The insert was then ligated to the yeast expression vector, pPICZαA (Invitrogen, USA). The vector was, subsequently, transformed into JM 109 incubated in Low Salt LB plate with 25 μg/ml Zeocin at 37 °C, overnight. Zeocin-resistant colonies were picked and inoculated into 3 ml of low salt LB medium with 25 μg/ml Zeocin at 37 °C. The plasmid DNA was isolated by High pure plasmid isolation kit (Roche Diagnostics, Switzerland).

SacI linearized Plasmid DNA was transformed into *Pichia pastoris* X-33 using the *Pichia* EasyComp™ Kit from Invitrogen. The transformed cells were spread on YPDS plate with 100 μg/ml Zeocin and incubated for 3 to 10 days at 30 °C.

A single colony was inoculated in BMGY medium and incubated at 30 °C for 16–18 hours, or until culture reached an OD_{600} of 2 to 6. Subsequently, cell pellets were

harvested and inoculated in 100 ml of BMGY at 30 °C. The methanol concentration was maintained at 0.5 % (v/v) every 24 hours for optimal induction during the entire expression period.

At 24, 48, 72, and 96 hours, an aliquot of the expression culture was transferred to Amicon™ 15 ml tube. The tubes were centrifuged at 25,000 x g for 10 minutes. The supernatant was transferred to a separate tube and concentrated by ultrafiltration using AMICON™ concentrator (Millipore, USA) that had MWCO of 10,000 Da. The assembled concentrator was centrifuged at 25,000 x g for 30 minutes. The remaining sample from the bottom of the concentrated pocket was recovered in aliquots.

2.3 Purification and detection of recombinant SVMP

The recombinant protein with a polyhistidine tag was isolated according to protocol by MagneHis™ Ni- particle affinity purification system (Promega, USA). Protein concentration was determined using Micro BCA™ Protein Assay Reagent Kit (Pierce). The reaction was measured the absorbance at 570 nm on an ELISA plate reader.

The samples were analyzed for expression of the fusion protein by 10 % of resolving and 5 % of stacking acrylamide gel containing 10% sodium dodecyl sulfate (SDS-PAGE). After electrophoresis, the gel was stained in Coomassie brilliant blue solution. The molecular weight of the protein on SDS-PAGE gel was determined on a standard curve plotting the relative migration distances (R_f) against molecular weight standards on a logarithmic scale.

For Western blot analysis proteins were transferred to polyvinylidene fluoride (PVDF) membrane at 40 volts for 40 minutes after SDS-PAGE. Subsequently, the blotted membrane was immediately placed into the blocking solution for 1 hour at room temperature, washed 3 times with 1X PBST and incubated with 1:3,000 dilution of anti-histidine antibody (Roche Diagnostics, Switzerland) in blocking buffer for 1 hour at room temperature. After washing, the membrane was incubated with 1: 1,000 dilution of horse radish peroxidase-conjugated rabbit anti mouse IgG (Dako Cytomation, Denmark) in blocking buffer for 2 hours and then washed. For developing the blot, it was soaked in the visualizing solution (Amersham ECL™ Western Blotting System) for 10 minutes and exposed to Amersham Hyperfilm ECL™ in the dark for an optimal time. The film was developed by developer and fixer from Kodak.

2.4 Collagen degradation

Collagen degradation was performed as previously described (Pinyachart et al, 2011). Type IV collagen (C7521, Sigma, USA) was diluted with 0.25% acetic acid to the final concentration of 5 mg/ml. The performed reactions (20 µL final volumes) were incubated at 37°C and all reagents were pre-warmed for 1 hour before used. The final concentrations of recombinant SVMP and collagen were 141.7 µM and 2.5 mg/L, respectively. At every incubation time (15, 20, 25, 30 minutes; and 1, 2, 24 hours), 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. Collagen degradation was determined on an 8% reducing SDS-PAGE stained with Coomassie-blue R250.

2.5 Activities of recombinant protein on platelets

Platelet aggregation assay was performed using a light transmission aggregometer (Helena Laboratories, USA). Venous blood (9 parts) from a healthy donor who had not received any medication for at least 2 weeks was collected in 3.2% sodium citrate (1 part). The whole blood was centrifuged at 150 xg for 15 minutes to obtain platelet-rich plasma (PRP) and platelet-poor plasma (PPP) was prepared from the remaining whole blood by centrifuging at 800 xg for 10 minutes. PRP was diluted to 250×10^9 platelets/L using PPP. Different amounts of recombinant protein were added to PRP and incubated at 37 °C for 10 minutes.

Platelet aggregation was initiated by adding collagen (2 mg/ml) or ADP (5 µM). Light transmittance was recorded and the maximum aggregation response was obtained. The maximal aggregation in the absence of venom protein was given a value of 100 % aggregation.

3. Results

3.1 Cloning of a novel snake venom metalloproteinase (SVMP) from Cryptelytrops albolabris venom gland

A cDNA of SVMP was cloned from the *C. albolabris* venom gland library. The cDNA sequence and its conceptual translation are shown in **Figure 1**. The sequences had not been reported and, therefore, the SVMP was termed, here, albolamin.

Sequence analysis showed that albolamin protein was a SVMP in the P-II class containing signal peptide, prodomain, metalloproteinase, spacer and disintegrin domains (**Figure 1**). The prodomain had a conserved sequence, which contains a cysteine, to form a

tcacgtatgggttgaaagcaggaagagattgcctgtcttccagccaaatccagcctccaaa
atgatccaagttcttttggtaaccatatgcttagcagtttttccttatcaagggagttct
M I Q V L L V T I C L A V F P Y Q G S S
ataatcctggaatctgggaacgttaatgattatgaagtcgtgtatccacgaaaagtcact
I I L E S G N V N D Y E V V Y P R K V T
ttattacccaaaggagcaattcagccaaagtatgaagacaccatgcaatatgaatttaag
L L P K G A I Q P K Y E D T M Q Y E F K
gtgaatggagagccagtgatccttcacctggaaaaaaataaaggacttttttcagaagat
V N G E P V I L H L E K N K G L F S E D
tacagcgagactcattattcccctgatggcagagaaattacaacataccctcggttgag
Y S E T H Y S P D G R E I T T Y P S V E
gatcactgctattatcatggacgcatccagaatgatgctgacttaactgcaagcatcagt
D H C Y Y H G R I Q N D A D L T A S I S
gcatgcaatggtttgaaaggacatttcaagcttcaaggggagacgtactttattgaaccc
A C N G L K G H F K L Q G E T Y F I E P
ttgaagatttccgacagtgaagcccacgcagtctacaaatatgaaaacatagaaaagcag
L K I S D S E A H A V Y K Y E N I E K Q
gacgaggcctccaaaatgtgtggggtaaccgagactaattgggaatcagatgaacccatc
D E A S K M C G V T E T N W E S D E P I
aaaaagacctcacagttaaatcttactcctgacgaaaaaagattcattgagcttgtcata
K K T S Q L N L T P D E K R F I E L V I
attgcggaccacagaatgtacacgaaatatgaaggtgatgagactgagatatgttcaaga
I A D H R M Y T K Y E G D E T E I C S R
atatatgaaagtgtcaacagtttaaatttgattttcagagctttgtatattactatagct
I Y E S V N S L N L I F R A L Y I T I A
ctgattggcgtagaaatttggtccagcggagatttgatgcctgtgacattatcagcagat
L I G V E I W S S G D L M P V T L S A D
gagactttggagtcatttggagaatggagaagaagacattttctgaagagcaaaagacat
E T L E S F G E W R R R H F L K S K R H
gataatgctcagttactcacgggcatgatcttcaatgaaaaaatcgaaggaagggcttac
D N A Q L L T G M I F N E K I E G R A Y
agaaagagcatgtgtgacccgaagcgttctgtaggaattgttcaagatcatagaagtaga
R K S M C D P K R S V G I V Q D H R S R
tatcattttgttgcaaatagaatggcc***catgagctgggtcataatctgggcattcatcat***
Y H F V A N R M A ***H E L G H N L G I H H***
gacggagattcctgtacttgcggtgctaacacatgcattatgtctgaaacagtaagcaac
D G D S C T C G A N T ***C I M S*** E T V S N
gaaccttccagtcggttcagcgattgtagtcttaatcaatatttgagtgatattattcat
E P S S R F S D C S L N Q Y L S D I I H
attcatcgttgccttttgaatgaaccctcgaagacagatattgtttcacctccagtttgt
I H R C L L N E P S K T D I *V S P P V C*
ggcaattactatgtggaggtgggagaagattgtgactgtggccctcctgcaaattgtcag
G N Y Y V E V G E D C D C G P P A N C Q
aatccatgctgtgatgctacaacctgtaaactgacaccagggtcacaatgtgcagaagga
N P C C D A T T C K L T P G S Q C A E G
ctgtgttgtgcccagtgcaaatttatagaagcaagaaaaatatgtcggaaaggaa**aggggt**
L C C A Q C K F I E A R K I C R K G **R G**
gataatccggatgatcgttgcactggccaatctggtgactgtcccagaaattcctaaaca
D N P D D R C T G Q S G D C P R N S -
acaatggagacagaatggtctgcaacagcaacaggcagtgttggatggactacagcctac
tacttatcaacctctggcttctctcagattgatttggagaccctgttccagaagattcaa
cttccctccaggtccaaagagaaccatcctgcctgcatcttactagtaaatcactcttag
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tcccatccaataaactaattattttcattctgaaaaaaaaaaaaaaaaa

Figure 1 The cDNA sequence and conceptual translation of albolamin. The signal peptide is underlined and followed by the prodomain. The conserved inhibitory motif in prodomain is highlighted in black, The metalloprotease domain is highlighted in grey, while its Zn^{2+} binding motif and methionine turn are not highlighted, but italicized. They are followed by the spacer domain (underlined and italicized) and disintegrin domain, respectively. The RGD sequence is bold and underlined**.**

disulfide bond with the metalloproteinase inhibiting protease activity. The metalloproteinase possessed the Zn^{2+} binding motif, HEXXHXXGXXHD (X represented any amino acids.), and methionine turn, CIMS. Finally, the disintegrin displayed Arg-Gly-Asp (RGD) sequence that was predicted to bind integrins.

Conceptually-translated protein sequences were searched for homology in GENBANK using the BLAST program. Most homologous proteins were metalloproteinase 3 from *Crotalus adamanteus* (GENBANK accession number AEJ31986), albolatin from *Cryptelytrops albolabris* (Swiss Protein number P0C6B6.1), jerdonitin from *Protrobothrops jerdonii* (Swiss protein P83912.1), VMPII from *Crotalus adamanteus* (GENBANK AFS49715.1) and Agkistin from *Gloydius halys* (Swiss Protein Q8AWX7.1) with identity scores of 85%, 82%, 81%, 80% and 78%, respectively. The multiple sequence alignment is shown in **figure 2**. Albolamin was homologous to P-IIb subtype of SVMPs. This group of SVMP contains 2 cysteines in the spacer and disinregrin domains, respectively (**Figure 2**). They were predicted to form a disulfide bone causing disintegrins attached to the metalloproteinases in mature proteins.

3.2 Expression and characterization of albolamin in Pichia pastoris system

The cDNA of albolamin from the metalloproteinase domain down to its stop codon was PCR amplified and ligated into the *Pichia pastoris* expression vector. The prodomain was excluded to obtain an active protein. Six histidines were inserted into the N terminus of the protein making an 859-basepair construct. The calculated molecular weight based on the amino acid sequence was 33.06 kDa.

The culture media after induction with methanol were concentrated and affinity-purified using a nickel column that binds specifically to polyhistidine. Subsequently, the concentration of the protein was measured by the microBCA^{TM} Protein Assay. The final concentration of protein was 84.5 μg/ml. Therefore, the yield of recombinant albolamin produced in *P. pastoris* was 8.5 mg/L of culture medium.

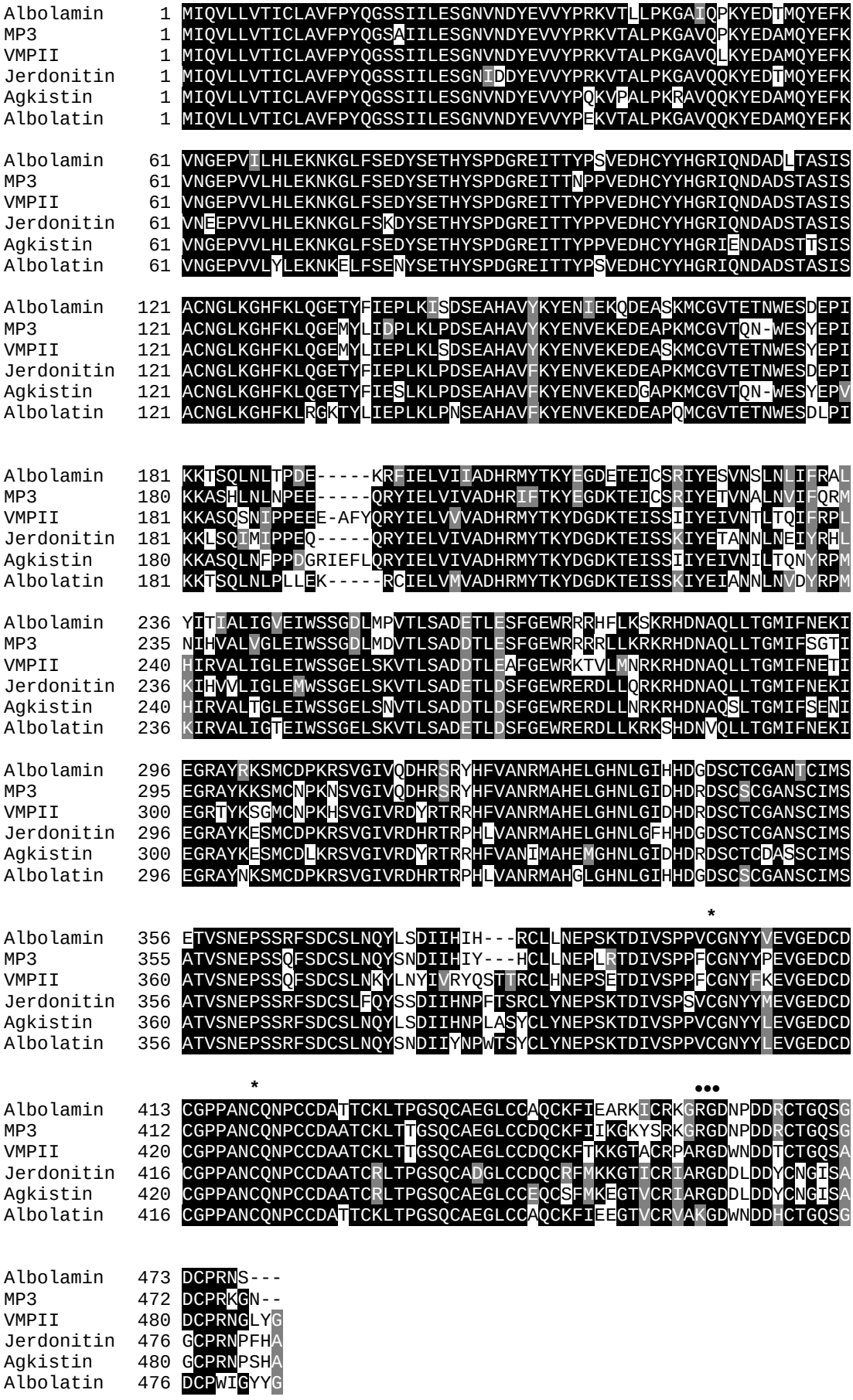
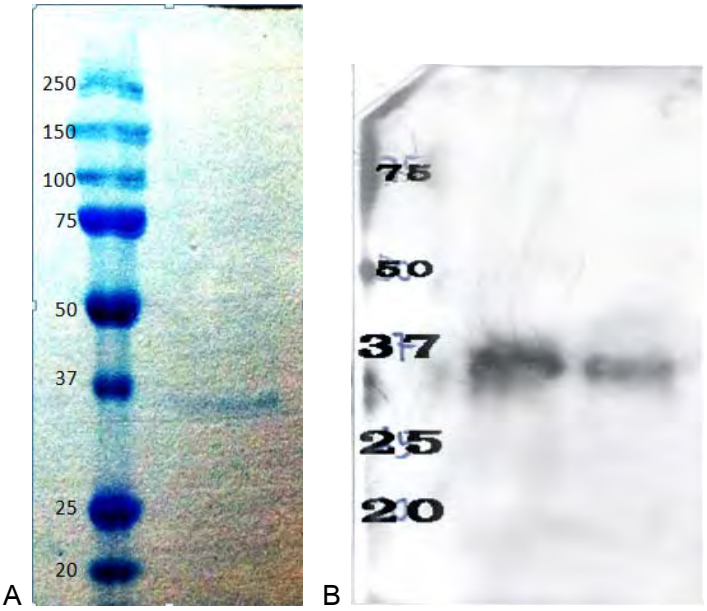

Albolamin 1 MIQVLLVTICLAVFPYQGSSIILESGNVNDYEVVYPRKVTLLPKGAIQPKYEDTMQYEFK
MP3 1 MIQVLLVTICLAVFPYQGSAIILESGNVNDYEVVYPRKVTALPKGAVQPKYEDAMQYEFK
VMPII 1 MIQVLLVTICLAVFPYQGSSIILESGNVNDYEVVYPRKVTALPKGAVQLKYEDAMQYEFK
Jerdonitin 1 MIQVLLVTICLAVFPYQGSSIILESGNIDDYEVVYPRKVTALPKGAVQQKYEDTMQYEFK
Agkistin 1 MIQVLLVTICLAVFPYQGSSIILESGNVNDYEVVYPQKVPALPKRAVQQKYEDAMQYEFK
Albolatin 1 MIQVLLVTICLAVFPYQGSSIILESGNVNDYEVVYPEKVTALPKGAVQQKYEDAMQYEFK
Albolamin 61 VNGEPVILHLEKNKGLFSEDYSETHYSPDGREITTYPSVEDHCYYHGRIQNDADLTASIS
MP3 61 VNGEPVVLHLEKNKGLFSEDYSETHYSPDGREITTNPPVEDHCYYHGRIQNDADSTASIS
VMPII 61 VNGEPVVLHLEKNKGLFSEDYSETHYSPDGREITTYPPVEDHCYYHGRIQNDADSTASIS
Jerdonitin 61 VNEEPVVLHLEKNKGLFSKDYSETHYSPDGREITTYPPVEDHCYYHGRIQNDADSTASIS
Agkistin 61 VNGEPVVLHLEKNKGLFSEDYSETHYSPDGREITTYPPVEDHCYYHGRIENDADSTTSIS
Albolatin 61 VNGEPVVLYLEKNKELFSENYSETHYSPDGREITTYPSVEDHCYYHGRIQNDADSTASIS
Albolamin 121 ACNGLKGHFKLQGETYFIEPLKISDSEAHAVYKYENIEKQDEASKMCGVTETNWESDEPI
MP3 121 ACNGLKGHFKLQGEMYLIDPLKLPDSEAHAVYKYENVEKEDEAPKMCGVTQN-WESYEPI
VMPII 121 ACNGLKGHFKLQGEMYLIEPLKLSDSEAHAVYKYENVEKEDEASKMCGVTETNWESYEPI
Jerdonitin 121 ACNGLKGHFKLQGETYFIEPLKLPDSEAHAVFKYENVEKEDEAPKMCGVTETNWESDEPI
Agkistin 121 ACNGLKGHFKLQGETYFIESLKLPDSEAHAVFKYENVEKEDGAPKMCGVTQN-WESYEPV
Albolatin 121 ACNGLKGHFKLRGKTYLIEPLKLPNSEAHAVFKYENVEKEDEAPQMCGVTETNWESDLPI
Albolamin 181 KKISQLNLTPDE-----KRFIELVIIADHRMYTKYEGDETEICSRIYESVNSLNLIFRAL
MP3 180 KKASHLNLNPEE-----QRYIELVIVADHRIFTKYEGDKTEICSRIYEIVNALNVIFQRM
VMPII 181 KKASQSNIPPEEE-AFYQRYIELVVVADHRMYTKYDGDKTEISSIIYEIVNTLTQIFRPL
Jerdonitin 181 KKLSQIMIPPEQ-----QRYIELVIVADHRMYTKYDGDKTEISSKIYETANNLNEIYRHL
Agkistin 180 KKASQLNFPPDGRIEFLQRYIELVIVADHRMYTKYDGDKTEISSIIYEIVNILTQNYRPM
Albolatin 181 KKTSQLNLPLLEK-----RCIELVMVADHRMYTKYDGDKTEISSKIYEIANNLNVDYRPM
Albolamin 236 YITIALIGVEIWSSGDLMPVTLSADETLESFGEWRRRHFLKSKRHDNAQLLTGMIFNEKI
MP3 235 NIHVALVGLEIWSSGDLMDVTLSADDTLESFGEWRRRRLLKRKRHDNAQLLTGMIFSGTI
VMPII 240 HIRVALIGLEIWSSGELSKVTLSADDTLEAFGEWRKTVLMNRKRHDNAQLLTGMIFNETI
Jerdonitin 236 KIHVVLIGLEMWSSGELSKVTLSADETLDSFGEWRERDLLQRKRHDNAQLLTGMIFNEKI
Agkistin 240 HIRVALTGLEIWSSGELSNVTLSADDTLDSFGEWRERDLLNRKRHDNAQSLTGMIFSENI
Albolatin 236 KIRVALIGTEIWSSGELSKVTLSADETLDSFGEWRERDLLKRKSHDNVQLLTGMIFNEKI
Albolamin 296 EGRAYRKSMCDPKRSVGIVQDHRSRYHFVANRMAHELGHNLGIHHDGDSCTCGANTCIMS
MP3 295 EGRAYKKSMCNPKNSVGIVQDHRSRYHFVANRMAHELGHNLGIDHDRDSCSCGANSCIMS
VMPII 300 EGRTYKSGMCNPKHSVGIVRDYRTRRHFVANRMAHELGHNLGIDHDRDSCTCGANSCIMS
Jerdonitin 296 EGRAYKESMCDPKRSVGIVRDHRTRPHLVANRMAHELGHNLGFHHDGDSCTCGANSCIMS
Agkistin 300 EGRAYKESMCDLKRSVGIVRDYRTRRHFVANIMAHEMGHNLGIDHDRDSCTCDASSCIMS
Albolatin 296 EGRAYNKSMCDPKRSVGIVRDHRTRPHLVANRMAHGLGHNLGIHHDGDSCSCGANSCIMS
Albolamin 356 ETVSNEPSSRFSDCSLNQYLSDIIHIH---RCLLNEPSKTDIVSPPVCGNYYVEVGEDCD
MP3 355 ATVSNEPSSQFSDCSLNQYSNDIIHIY---HCLLNEPLRTDIVSPPFCGNYYPEVGEDCD
VMPII 360 ATVSNEPSSQFSDCSLNKYLNYIVRYQSTTRCLHNEPSETDIVSPPFCGNYFKEVGEDCD
Jerdonitin 356 ATVSNEPSSRFSDCSLFQYSSDIIHNPFTSRCLYNEPSKTDIVSPSVCGNYYMEVGEDCD
Agkistin 360 ATVSNEPSSRFSDCSLNQYLSDIIHNPLASYCLYNEPSKTDIVSPPVCGNYYLEVGEDCD
Albolatin 356 ATVSNEPSSRFSDCSLNQYSNDIIYNPWTSYCLYNEPSKTDIVSPPVCGNYYLEVGEDCD
Albolamin 413 CGPPANCQNPCCDATTCKLTPGSQCAEGLCCAQCKFIEARKICRKGRGDNPDDRCTGQSG
MP3 412 CGPPANCQNPCCDAATCKLTTGSQCAEGLCCDQCKFIIKGKYSRKGRGDNPDDRCTGQSG
VMPII 420 CGPPANCQNPCCDAATCKLTTGSQCAEGLCCDQCKFTKKGTACRPARGDWNDDTCTGQSA
Jerdonitin 416 CGPPANCQNPCCDAATCRLTPGSQCADGLCCDQCRFMKKGTICRIARGDDLDDYCNGISA
Agkistin 420 CGPPANCQNPCCDAATCRLTPGSQCAEGLCCEQCSFMKEGTVCRIARGDDLDDYCNGISA
Albolatin 416 CGPPANCQNPCCDATTCKLTPGSQCAEGLCCAQCKFIEEGTVCRVAKGDWNDDHCTGQSG
Albolamin 473 DCPRNS---
MP3 472 DCPRKGN--
VMPII 480 DCPRNGLYG
Jerdonitin 476 GCPRNPFHA
Agkistin 480 GCPRNPSHA
Albolatin 476 DCPWIGYYG

Figure 2 Multiple protein sequence alignment of albolamin

Albolamin reported in this study is aligned with *Crotalus adamanteus* Metalloproteinase 3 (MP3), VMPII from *Crolaus adamanteus*, Jerdonitin from *Protrobothrops jerdonii*, Agkistin from *Gloydius halys* and Albolatin from *C. albolabris* using CLUSTALW and BoxShade programs.

The purified protein was electrophoresed on SDS-PAGE (10% gel) and subjected to Western blot on PVDF membrane. They revealed the pure recombinant protein at the size of approximately 35 kDa (**Figure 3A and 3B**).



A B

Figure 3 Recombinant albolamin expressed in *Pichia pastoris*

A. SDS-PAGE of the purified albolamin stained with Coomassie Blue.

B. Western blot analysis of albolamin probed with anti-histidine antibody

3.3 *The activities of recombinant albolamin*

After incubation for different periods of time, the recombinant albolamin could digest human type IV collagen in a time-dependent manner as shown in **Figure 4**. Albolamin began collagen degradation within 15 minutes and smaller bands were progressively more apparent during the period of 24 hours.

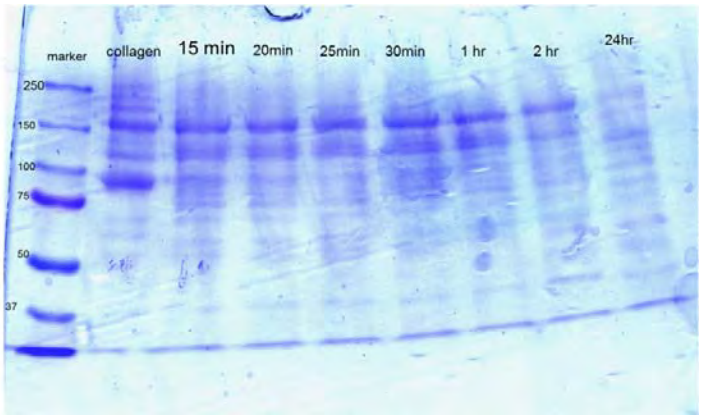


Figure 4 Collagen degradation by recombinant albolamin

The recombinant albolamin was incubated with human type IV collagen for the indicated periods of time at 37 $^{\circ}$C before the reactions were stopped and underwent SDS-PAGE. The digested smaller bands were progressively increased with time.

The purified recombinant albolamin was then investigated for the activities on platelet aggregation. Albolamin could inhibit collagen-induced platelets aggregation in a dose dependent manner. At the concentrations of 0, 4.25, 9.04 and 13.5 μM, there was 65%, 30% 10% and 0% aggregation, respectively (**Figure 5**A). Using a curve fitting calculation, the 50% inhibitory concentration (IC_{50}) of recombinant protein was 5.62 μM (**Figure 5B**). However, there was no effect of albolamin on ADP-induced platelet aggregation (the data was not shown).

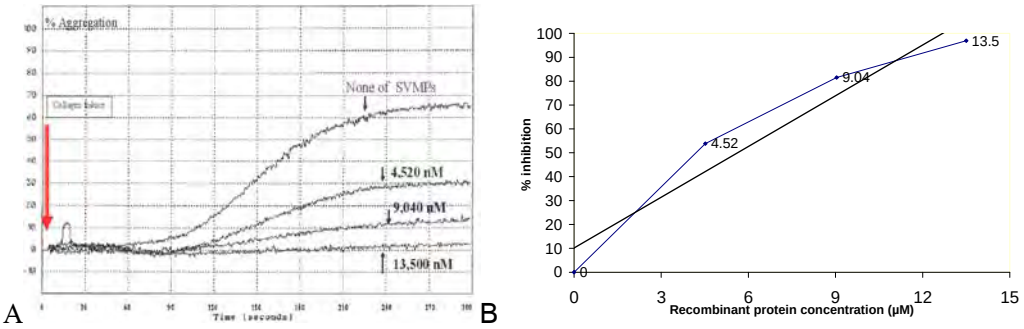


Figure 5 The effects of recombinant albolamin on platelet aggregation

A. The inhibition of collagen-induced platelet aggregation by variable concentrations of recombinant albolamin

B. Albolamin dose-dependently inhibited collagen-induced human platelets aggregation. The IC_{50} as determined from curve fitting was 5.62 μM

4. Discussion

In this study, we first reported the cDNA and protein sequences of albolamin, a class P-II snake venom metalloprotese (SVMP) from *C. albolabris*. SVMPs of P-II class contain a disintegrin domain that lie after a spacer domain. In the majority of P-II SVMPs, disintegrins are release from metalloproteinase domain becoming 2 separate proteins, such as atrolysin E and MVD-D (Chen et al, 2003, Fujii et al., 2003). On the other hand, the P-IIa subclass possesses an additional cysteines that may cause attachment of metalloproteinase and disintegrin domains together in the mature protein (Fox and Serrano, 2005). Upon sequence alignment (**Figure 2**), the characteristic of cysteine residues were found at position 221 and 240 similar to jerdonitin from *P. jerdonii* and agkistin from *G. halys*, but different from the other P-II. Therefore, they were classified as P-IIa SVMPs class. Consistent with this prediction, the recombinant albolamin was expressed as the disintegrin domain attached to the mature protein, similar to jerdonitin and agkistin, with the molecular weight of 35 kDa.

The recombinant protein expression was used in this study instead of protein purification from crude venom as the cDNA clone will be very helpful for future protein engineering in order to obtain useful agents research, diagnostic or therapeutic uses. Using the *Pichia pastris* system, we could obtain a functional protein that was secreted in to the media suggesting correct folding and post-translational modification. The yield of recombinant protein was 8.5 mg/L of culture medium, which was higher than that of albolatin (Singhamatr and Rojnuckarin, 2006), which yielded 3.3 mg/L of media and close to that of rhodostomin (Guo et al*,* 2001), which was produced from *P. pastoris* at 5 – 10 mg/L of media. Therefore, this system can produce sufficient yields of proteins for research.

The metalloproteinase domain of SVMPs has hemorrhagic activity degrading various substrates in extracellular matrix in vascular basement membrane and can cause endothelial cell apoptosis (Kamiguti et al, 1996, Gutierrez et al. 2005). From sequence comparison, albolamin is likely to contain the hemorrhagic activity contributing to local tissue damages in green pit viper bite patients. In this study, we found that albolamin could digest human type IV collgen starting from 15 minutes and progressively up to 24 hours. Therefore, albolamin probably contributes to local tissue damages in patients bitten by green pit vipers. Therapy targeting this protein may be helpful in alleviating this debilitating complication in snakebite patients.

Disintegrins can inhibit platelet aggregation. The 3-dimensional structure shows that the C terminus of disintegrin is in close proximity to the RGD or KGD conserved sequences.

Therefore, the sequences around the R/KGD and the C terminus were reported to determine integrin specificity (Marcinkiewicz et al, 1997). As the histidine insertion was used to aid protein purification, the N-terminal polyhistidine was utilized to avoid an interference with the C-terminus of the disintegrin.

The recombinant albolamin displayed a dose-dependent inhibitory activity on collagen-induced platelets aggregation, but there was no effect on platelet aggregation after adding ADP as an agonist. This might have been mediated by the binding to the $\alpha_2\beta_1$ integrin, an integrin that is a collagen receptor on the platelet surface. If a disintegrin binds to platelet $\alpha_{IIb}\beta_3$ integrin, it should affect all platelet agonists including ADP. The amino acid sequence of albolamin was homologous to jerdonitin from *P. jerdonii*. However, jerdonitin expressed in *Escherichai coli* system using pMD18-T vector dose-dependently inhibited ADP-induced human platelets aggregation (Chen et al, 2003). Similarly, native jerdonitin and recombinant jerdonitin expressed in *P. pastoris* also inhibited ADP-induced platelet aggregation (Zhu et al, 2010). The mechanism of jerdonitin inhibition was dissimilar to that of albolamin, although the sequences were highly homologous including the RGD motif. This may be explained by the different C terminal sequences of the disintegrin domains (**Figure 2**) that may confer their binding specificities (Fujii et al, 2003). Albolabrin, an RGD-containing disintegrin purified from *C. albolabris* venom, also inhibited fibrinogen binding to integrin $\alpha_{IIB}\beta_3$ (Cavette et al, 1991). On the other hand, albolatin from *C. albolabris* displayed the same activity on collagen-induced platelet aggregation similar to albolamin. However, it contained KGD motif (Singhamatr and Rojnuckarin, 2006). The molecular mechanisms of platelet inhibition by these SVMPs remain to be determined.

Uncontrolled platelet activation has been implicated in arterial thrombosis including peripheral, myocardial and cerebral ischemia. Current anti-platelet agents block either ADP receptor or $\alpha_{IIb}\beta_3$ integrin on platelet surface. A drug that targets collagen-induced platelet activation is a novel mean that is potentially useful for the prevention and/or treatments of these thrombotic problems. Several SVMPs can antagonize collagen-mediated signaling in platelets. For example, acurhagin from *A. actus* inhibits collagen- and von Willebrand factor-induced platelet aggregation by inhibiting tyrosine phosphorylation of various signaling proteins (Wang et al., 2005). The effects of albolamin and albolamin on platelet signal transduction remain to be investigated. Furthermore, Zhu et al (2010) reported that recombinant jerdonitin affected growth of several cancer cell lines including Bel7402, human leukemia cell (K562), and human gastric carcinoma cell (BG823). Therefore, SVMPs have potentials to be new cancer therapy.

In conclusion, we cloned a P-IIa class SVMP, albolamin, from *C. albolabris* venom gland. Recombinant expression using *P. pastoris* yielded an active protein with proteolytic and anti-platelet activities. The mechanisms of albolamin on platelet functions should be investigated in order to develop a new mode of platelet inhibition for thrombotic diseases.

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Albocollagenase

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 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 *C. 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 *E. 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, *P. 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 pGEM T easy vector (Promega, Madison, WI) before transformation into *Escherichia coli* (*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 CAA TTG 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 $^{\circ}$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 $^{\circ}$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 hours). 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 hour.

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 hour before used. Ten 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 minutes and 1, 4, 8, 24 hours), 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 (Stroka et al., 2005, Muanpasitporn and Rojnuckarin, 2007). 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 minutes and 5, 12 hours), 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 $x10^9$ 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 (**Figure 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 *Crotalus atrox* (Zhou et al., 1995) and jararhagin from *Bothrops 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%- 91.8%, while those of the mature proteins were from 54.5%-67.7%.

The mature albocollagenase was then aligned with jararhagin from *Bothrops jararaca*, catrocollastatin and atrolysin A (Hite et al., 1994) from *Crotalus atrox*, and kaouthiagin from *Naja kaouthia* (Ito et al., 2001) using CLUSTALW program as shown in **Figure 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 **Figure 2**.

3.2 Albocollagenase was expressed at a low level in Pichia 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 pPICZ∝A 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 6histidine-tagged albocollagenase.

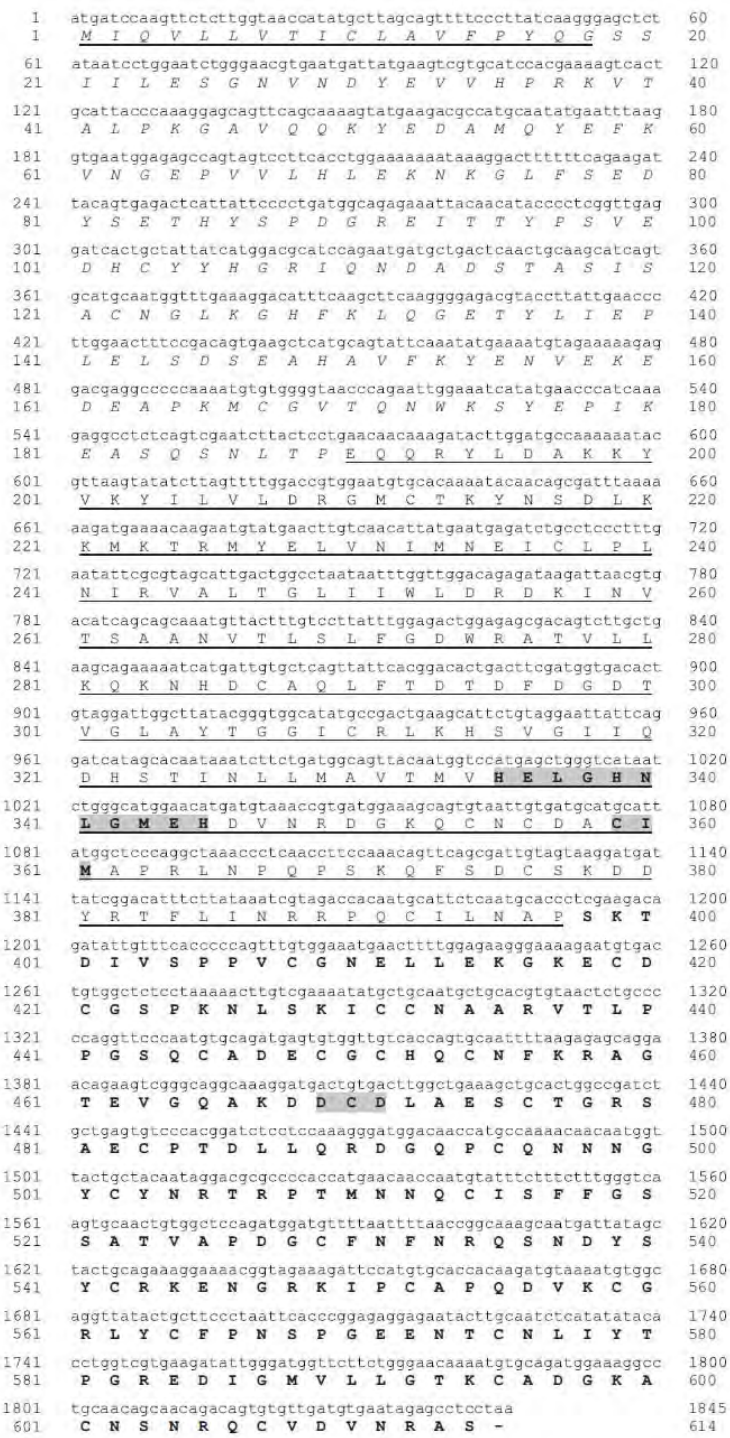

Fig. 1. The coding nucleotide and deduced protein sequences of albocollagenase. They comprise 1845 base pairs and 614 amino acid residues, respectively. The protein sequence comprises the signal peptide, propeptide, metalloproteinase domain, disintegrin-like and cysteine-rich domains, respectively. The 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.

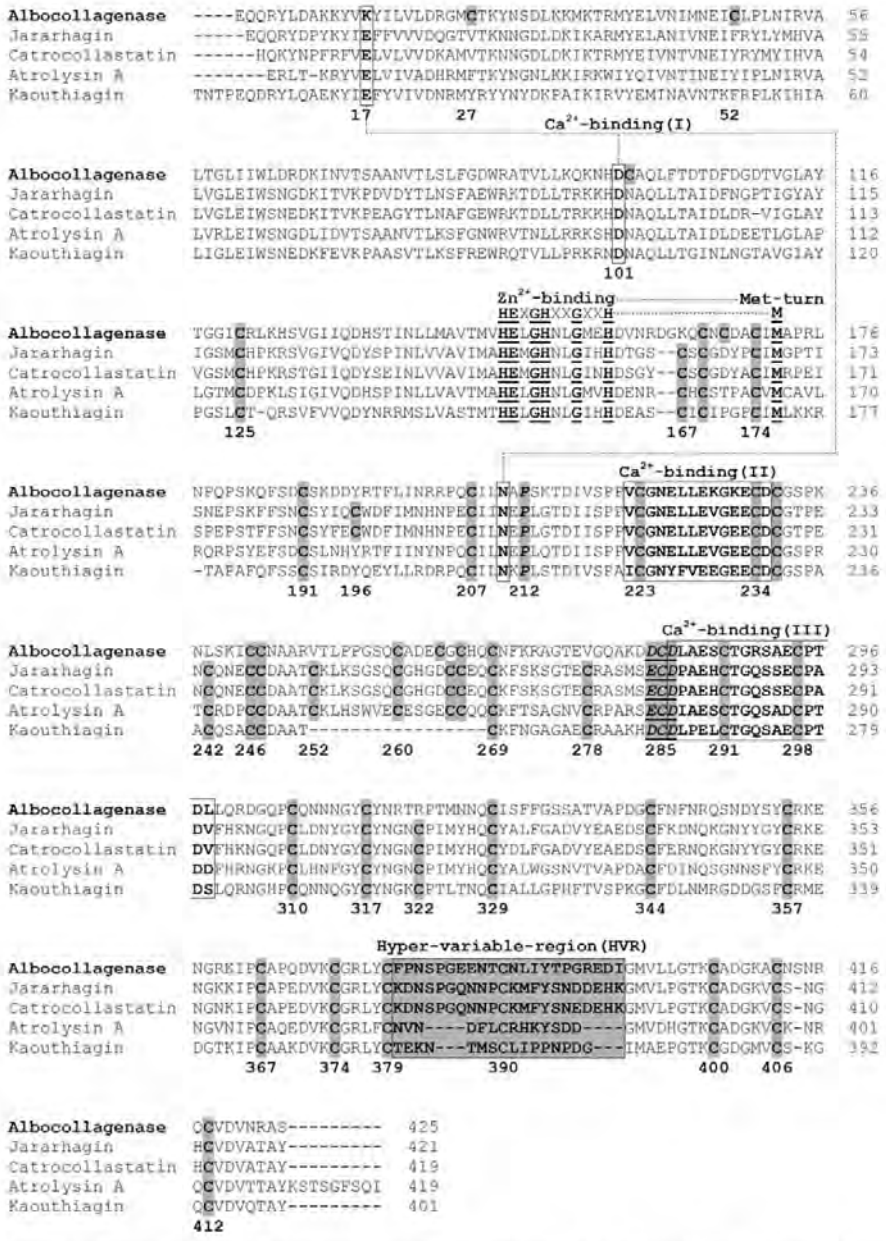

Fig. 2. The multiple sequence alignment of the P-III SVMP putative mature proteins compared with other SVMPs Cysteine residues are numbered according to kaouthiagin. The cysteine residues are highlighted. The Zn^{2+}-binding sequences are underlined. The putative self-post-translational processing position, 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.

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 **Figure 3A and 3B**.

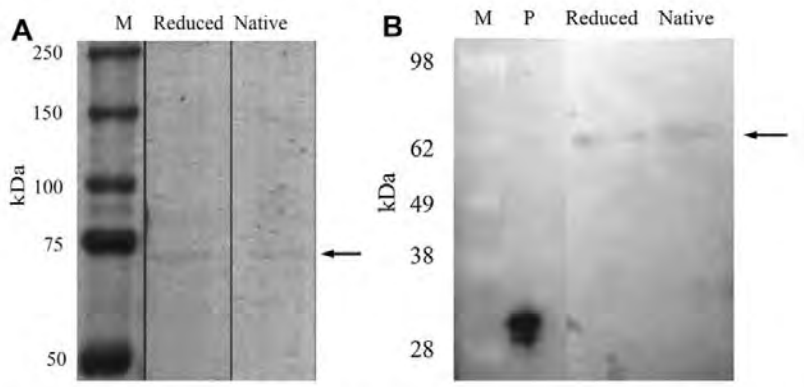

Fig. 3. Recombinant albocollagenase (Arrows) (A) The recombinant protein was purified, electrophoresed on 8% SDS-PAGE in reduced and native conditions and stained with Coomassie-blue R250. The molecular weight markers are shown in lane M. (B) The purified protein was electrophoresed as described in (A) 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.

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 **Figure 4A**. Albocollagenase degraded human type IV collagen in a time-dependent manner. While the degradation of type IV collagen began at 1 minute, more digested bands were progressively more visible during the period of 24 hours.

In **Figure 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 **Figure 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).

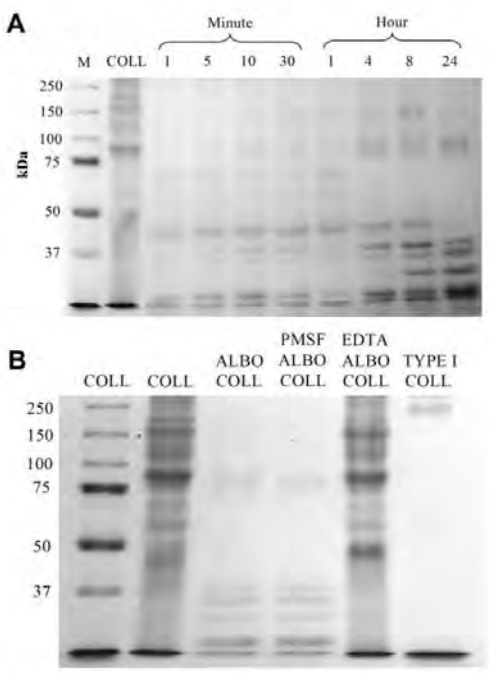

Fig. 4. Time-dependent type IV collagen degradation by albocollagenase (A) Type IV collagen was incubated with albocollagenase at different time points as indicated in each lane. Each reaction was subjected to 8% reducing SDS-PAGE and stained with Coomassie-blue. M and COLL represent the molecular weight 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.

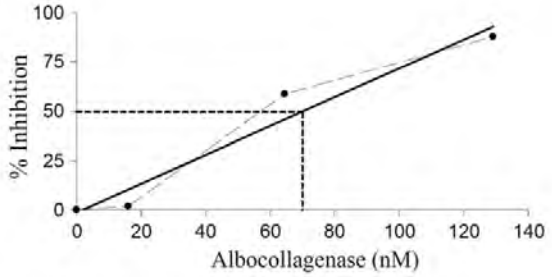

Fig. 5. Albocollagenase inhibited platelet aggregation. Various concentrations of the albocollagenase (0, 0.25, 1, and 2 mg/L) were pre-incubated with platelet-rich plasma (PRP) before adding collagen. The maximal aggregation without albocollagenase was regarded as 100%. The PRP without agonist was set 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.

4. Discussion

Snake venom metalloproteinases (SVMPs) are the main venom components responsible for local tissue injury after 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 **Figure 2** revealed that several cysteine residues were conserved 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 (Igarashi et al., 2007, Fox and Serrano, 2008). Nevertheless, the first cysteinyl residues in disintegrin-like domain, C223, was usually lacking in disintegrin domains (Fox and Serrano, 2008). **Figure 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 **Figure 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 pro-domain 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 **Figure 4**, the degradation of type IV collagen began at 1 minute 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 (**Figure 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 to $\alpha_2\beta_1$ (Kamiguti et al., 2003). Acurhagin interacted with GPVI (Wang et al., 2005). Secondly, others could degrade or interact with adhesive proteins involved in haemostasis, 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.

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Alboaggregin D

1. Introduction

The geography and climate of Thailand support more than 160 species of snakes. Among these, there are 11 types of green pit vipers. White-lipped pit viper or *Cryptelytrops albolabris* is the most prevalent (Viravan et al., 1992; Mahasandana and Jintakune, 1990). Its venom contains a large number of toxins exerting powerful inhibitory or stimulatory effects on platelets.

Platelet-aggregating agents in venoms activate platelets *in vivo* resulting in platelet consumption and thrombocytopenia in biting victims (Mahasandana et al., 1980). These platelet activators in viper venoms are usually in the family of C-type lectin-like proteins (Weis et al., 1992; Drickamer, 1999), which are currently termed snaclecs (Clemetson et al., 2008). A number of snaclecs affecting platelet functions have been isolated and characterized. Some of them inhibit platelet aggregation by binding to platelet glycoprotein (GP) Ib-IX-V and blocking von Willebrand factor (vWF) binding (Fukuda et al., 2000), whereas the others promote platelet aggregation by targeting vWF (Brinkhous et al., 1983), GPIb-IX-V (Andrews et al., 1996), GPIV (Polgár et al., 1997) and possibly other platelet receptors.

Snaclecs are found only in snake venoms and belong to the group VII of C-type lectins. C-type lectins are non-enzymatic proteins that Ca^{2+}-dependently bind to saccharides. They contain one or more copies of a highly conserved carbohydrate recognition domain (CRD) consisting of 115 to 130 amino acids. Although snaclecs are structurally homologous to C-type lectins, they lack carbohydrate binding activity and most of them do not depend on calcium (Drickamer, 1999; Morita, 2004b).

Snake venom snaclecs show diverse pharmacological activities against coagulation factors and platelets. A prominent feature of snaclecs is that they are heterodimers or oligomeric complexes of heterodimers comprising 2 subunits: a subunit a (α chain) of 14–15 kDa and a subunit b (β chain) of 13–14 kDa linked by inter-chain disulfide bonds. Snaclecs appear in a variety of oligomeric forms, including $\alpha\beta$, $(\alpha\beta)_2$ or $(\alpha\beta)_4$ (Atoda et al., 1991; Fukuda et al., 2000; Morita, T., 2005; Wang and Huang, 2001; Wang et al., 2003).

Snaclecs are important proteins and should be thoroughly investigated in order to understand pathogenesis of snake venom-induced platelet

abnormalities and find effective treatments for snakebites. Furthermore, studying the molecular mechanisms of platelet activation by snaclecs will give us deeper insights in platelet signaling pathways. Finally, these proteins display potentials to be diagnostic agents for platelet function tests in patients with bleeding or thrombotic disorders.

In this study, we cloned a novel snaclec gene, alboaggregin D, and purified the protein to characterize its functions, as well as dissected molecular mechanisms involving platelet surface glycoproteins (GPs) and signal transduction.

2. Materials and methods

2.1 Obtaining full-length cDNA of subunits of snaclecs

Green pit viper venom gland library was prepared as previously described (Rojnuckarin, et al. 2006). Two unique partial snaclec cDNA sequences were cloned. 5'RACE was performed using SMART^{TM} RACE kit (BD Biosciences Clontech, USA). A snaclec-specific primer was designed based on snaclec sequences from library (5'CCAGACTTCAGACAGCTGGATCTT-3). PCR products were electrophoresed, purified and ligated to the pGEM T vector (Promega, USA) before transformation into *Escherichai coli*. Clones were sequenced using ABI Prism 310 Genetic Analyzer (Perkin-Elmer, USA). We found that the sequences 5' to the start codon of all *C. albolabris* snaclecs were conserved. Therefore, a 5' common snaclec primer (5'-CCAGACTTCAGACAGCTGGATCTT-3') was designed to obtain additional snaclec clones using 3'RACE.

2.2 Alignment and computational sequence analysis

The nucleotide sequences and their conceptual translation obtained from the clones of interest were compared against nucleotide or protein sequences in online databases using BLAST N program via the World Wide Web. Alignments of sequence were made using the CLUSTALW multiple sequence alignment program.

2.3 Purification of platelet-aggregating proteins

Lyophilized *C. albolabris* venom from Queen Saovabha memorial institute was fractionated by gel filtration chromatography (Sephacryl S-200 (16/60),

Amersham Pharmacia, PRC), using an automate LP system (Biorad, USA). Each fraction was detected for A280 and human platelet-agglutinating activity. Protein markers (12–200 kD) (Sigma, USA) were used for molecular weight calculation. Positive fractions were pooled and concentrated using Vivaspin (Sartorius AG, Germany). The sample was then separated on an ion exchange column (Resource Q, Amersham Pharmacia, PRC) with a linear 0–0.5M NaCl gradient. Purified protein was analyzed on Coomassie-stained SDS-PAGE gel and its concentration was determined using Micro BCA kit (Pierce, USA).

2.4 Protein identification by tandem mass spectrometry

The trypsin-digested peptides were resuspended in 0.1% formic acid then analyzed by liquid chromatography coupled with tandem mass spectrometry (LC/MS/MS). Peptide ions were detected in a survey scan from 400 to 1600 amu followed by one data-dependent MS/MS scan. All MS/MS spectra were searched using Biowork^{TM} 3.3 software (Sequest algorithm) to compare ion spectra between experimental products and theoretical products in the non-redundant database.

All MS/MS spectra were searched against the database using the following criteria: enzyme trypsin, static modification of cysteine (+57.05130 Da), differential modification of methionine (+15.99940). The results of searching were filters by Xcorr versus charge state (+1≥1.5, +2≥2.0, +3≥2.5) and protein probability (minimum 1.00E-3).

2.5 Platelet aggregation assay

Human platelet-rich plasma was isolated by successive centrifugation steps. The platelet-rich supernatant was centrifuged at 1942xg for 10 min to obtain a pellet. Platelets were resuspended in 113 mM NaCl, 4.3 mM K_2HPO_4, 24.4 mM NaH_2PO_4, 5.5 mM glucose, pH6.5 (buffer B), centrifuged at 180xg for 10 min and washed once more with buffer B. Washed platelets were resuspended in 20 mM HEPES, 140 mM NaCl, 4 mM KCl, 5.5 mM glucose, pH 7.4 (buffer C) The platelet count was adjusted to 350 x10^9/L in buffer C.

Before analysis, platelets were incubated at 37°C for 2 min. Different amounts of purified snaclecs were added to washed platelets and incubated with continuous magnetic stirring at 37°C for 10 minutes. Platelet aggregation

was measured by changes in light transmission using the platelet aggregation chromogenic kinetic system-4 (PACKS-4) aggregometer (Helena Laboratories, USA).

For inhibitory studies, washed platelets were incubated with various concentrations of monoclonal anti GPIbα, clone SZ2 (Beckman Coulter company, France.), and/or polyclonal anti GPVI antibody (Abcam plc, UK.) for 2 min before adding alboaggregin D.

2.6 *Platelet activation assay*

Fifty μl of purified snaclecs were added to 450 μl of washed normal platelets (350 $x10^9$/L). Aliquots were taken at fixed times (5 min) and the platelet suspension was lysed by adding 5 μl of HEPES containing 10% SDS, 10 mM N-ethylmaleimide, 20 mM Na_3VO_4 and 20 mM EDTA.

After centrifugation, the supernatant were electrophoresed on an 8% SDS-polyacrylamide gel and then blotted onto polyvinylidene fluoride (PVDF) membrane. The monoclonal antibody to phosphotyrosine, 4G10 (Biodesign, USA), was applied to detect tyrosine phosphorylated protein as primary antibody followed by peroxide-coupled rabbit anti-mouse secondary antibody (Dako Cytomation, Denmark). Bound antibody was detected using chemiluminescence.

3. Results

3.1 Molecular cloning of full-length snaclecs from C. albolabris venom gland library

5' RACE using snaclec-specific primers was performed on the cDNA library of *C. albolabris* venom glands. EcoR I digestion was used to screen for inserts of more than 100 clones derived from 5'RACE. Insert-containing clones were sequenced. The cDNA inserts encoding snaclecs could be divided into 2 major groups, the factor IX binding protein homologs and those similar to platelet-binding proteins.

A common snaclec primer was designed based on the 5'RACE results for the 3'RACE. We found 3 additional major groups of snaclecs. Three clones were homologous to the α subunit of alboaggregin B. Furthermore, 8 clones

were related to the α subunit and 8 clones were homologous to the β subunit of the other platelet-binding snaclec, which will be focused in this study. This novel snaclec was termed, here, alboaggregin D (**Figure 1**).

There were 2 groups of alboaggregin D α (4 clones each) with differences in 3 amino acid residues as illustrated in **figure 1**. The clone showed in the figure was fitter to the mass spectrometry data and, therefore, chosen. In addition, there were 3 additional nucleotide differences between these 2 clones, but there was no change in amino acid residues.

There were also 2 groups of the β subunit (4 clones each) with variations in 5 separate nucleic acid sequences. However, all amino acids were the same.

3.2 Sequence alignment and computational searching analysis

Using BLASTN, the nucleotide sequence of the α subunit of alboaggregin D showed the highest homology to *Protobothrops mucrosquamatus* mucrocetin α chain mRNA and the β subunit showed the highest identity to *Crotalus durissus* convulxin β mRNA.

An alignment between conceptually translated sequences of alboaggregin D α and mucrocetin α chain (Huang et al, 2004) showed 86.7% amino acid sequence identity, while the translated sequences of the β chain and *Crotalus durissus* convulxin β showed 77.0% identity.

Subsequently, the amino acid sequence of alboaggregin D α and β were compared with other snake venom snaclecs using Clustal W multiple sequence alignment. The results revealed that the identity scores of alboaggregin D α and β subunits with those of the other known platelet collagen receptor-binding snaclecs as shown in **Figure 2**. The scores were higher for multimeric snaclecs (mucrocetin, stejnulxin and convulxin) as compared with dimeric ones (EMS16 and aggretin).

3.3 Protein purification and SDS-PAGE analysis

Gel filtration chromatography of 0.5 g of crude *C. albolabris* venom was performed on Sephacyl G-200 column resulting in 7 protein peaks (**Figure 3A**). Peak 1 and 4 showed strong platelet-aggregating activity. The low molecular

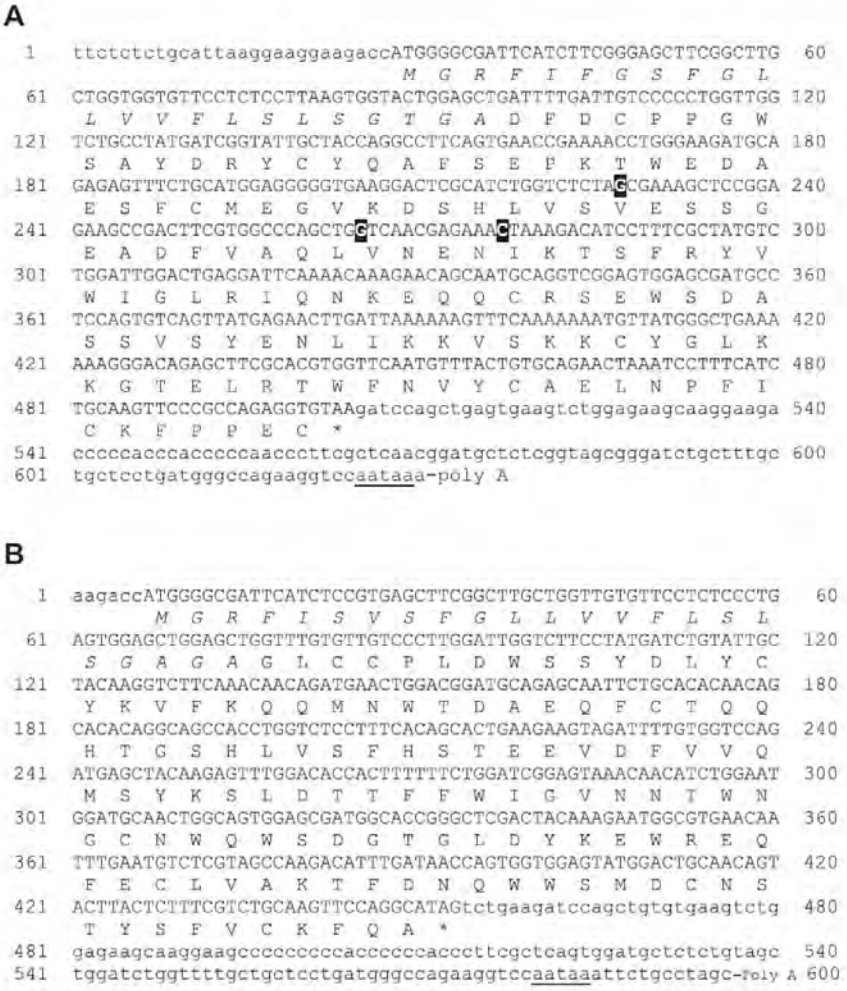

Fig. 1. The cDNA and conceptually translated sequences of α subunit (A) and β subunit (B) of alboaggregin D. The signal peptides are italicized and polyadenylation signals are underlined. The other clone of α subunit contains 3 nucleotide changes (highlighted) all to adenine resulting in amino acid substitution with isoleucine, isoleucine and lysine respectively.

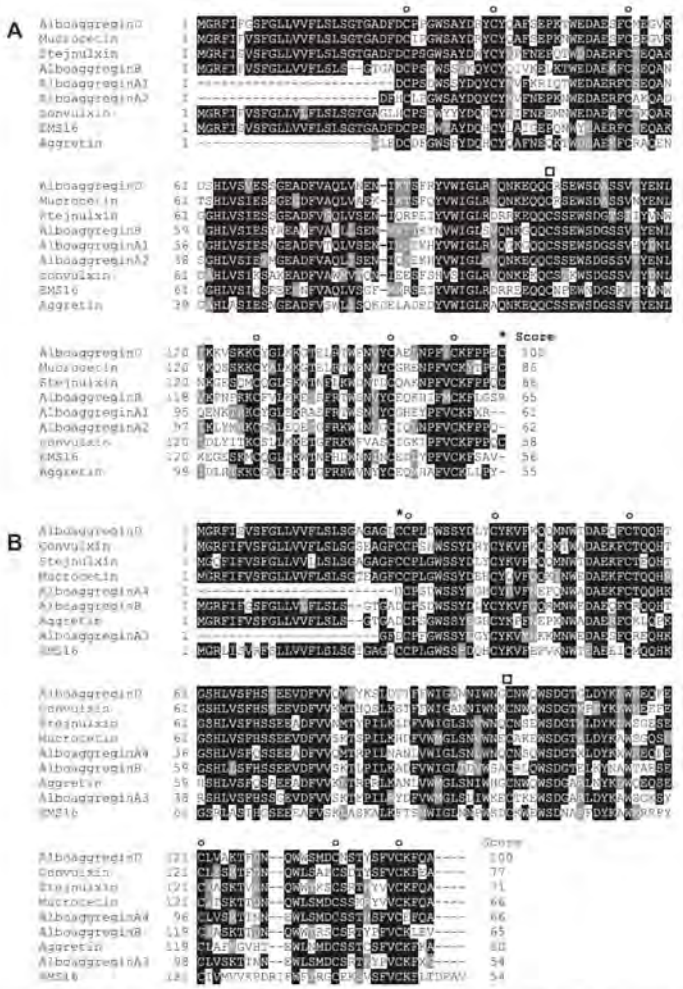

Fig. 2. The comparison of the deduced amino acid sequences of alboaggregin D α subunit (A) and β subunit (B) with those of other platelet collagen receptor-binding snaclecs from snake venoms. The conserved cysteine residues that form intra-chain, dimeric inter-chain and multimeric inter-chain disulfide bonds are marked by circles, squares and asterisks, respectively.

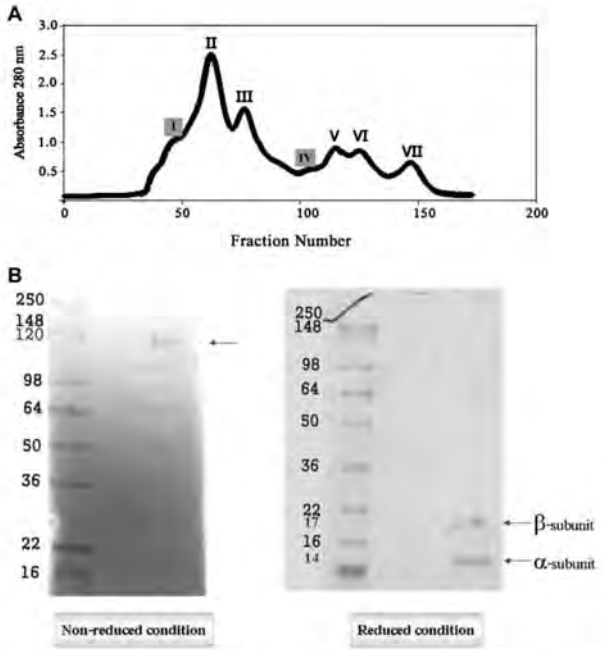

Fig. 3. Purification of alboaggregin D from *C. albolabris* venom. A. Gel filtration chromatogram of the venom on a Sephacyl G-200 column. Protein concentrations were estimated from the absorbance at 280 nm. Highlighted peaks represent the proteins with platelet-aggregating activity. The peak I is alboaggregin D and the peak IV is alboaggregin B. B. The purified protein was analyzed on SDS-PAGE under native and reduced conditions. The molecular weight markers are shown on the left lane of each gel.

weight peak 4 was identified as alboaggregin B and previously reported (Arpijuntarangkoon et al., 2007). The high molecular weight platelet-aggregating protein (Peak 1) was further investigated in this study.

The pooled peak 1 was fractionated on Resourse Q ion-exchange column using a linear gradient of NaCl from 0 to 0.5 M. This protein was eluted at approximately 0.38 M NaCl as determined by platelet aggregating activity. The purified protein was analyzed by SDS-PAGE (**Figure 3B**). It had an apparent molecular mass of approximately 120 kDa under non-reducing condition. Upon reduction, it separated into two bands with apparent molecular weights of 14 kDa and 17 kDa consistent with α and β subunits of a snaclec.

3.4 Protein identification by LC/MS/MS

The nature of purified *C. albolabris* snaclec α and β subunits were determined by LC/MS/MS. The peptide mass fingerprints were shown in **table 1**. Some large fragments might not be detectable on mass spectrometry. In addition, the fragments from α and β subunits sequence were R.YCYQAFSEPK.N and R.EQFECLVA.K. They were matched with deduced amino acid sequences of α and β subunits obtained from the cDNA library as described above.

The molecular mass of α and β subunits acquired from LC/MS/MS were 15728.4 Da and 16963.1 Da. This purified snaclec was, therefore, alboaggregin D.

Table 1
The peptide mass fingerprints of alboaggregin D.

	Expected masses[a]	Actual masses	Sequences
	α Subunit		
1	1529.62	1560.92	DFDCPPGWSAYDR
2	1236.38	Sequenced[b]	YCYQAFSEPK
3	1632.82	1626.85	TWEDAESFCMEGVK
4	2688.91	2808.09	DSHLVSVESSGEADFVAQLVNENIK
	2489.70	–	DSHLVS**I**ESSGEADFVAQL**I**NE**K**[c]
5	1390.68	1391.80	YVWIGLRIQNK
6	1815.94	1814.81	SEWSDASSVSYENLIK
7	1944.12	1940.94	TWFNVYCAELNPFICK
	β Subunit		
1	2404.85	2402.96	GLCCPLDWSSYDLYCYKVFK
2	4606.03	–	QQMNWTDAEQFCTQQHTGSHLVSFHSTEEVDFVVQMSYK
3	3639.98	–	SLDTTFFWIGVNNIWNGCNWQWSDGTGLDYK
4	1067.25	Sequenced[b]	EQFECLVAK
5	2810.10	2808.09	TFDNQWWSMDCNSTYSFVCKFQA

[a] Fragments with too small molecular masses are not included.
[b] The sequences on LC/MS/MS were perfectly matched with the conceptual translations of the cDNAs.
[c] The variant of α subunit is found on cDNA sequencing.

3.5 The effects of alboaggregin D on washed human platelets

The purified alboaggregin D caused aggregation of washed platelets (**Figure 4**). The dose that induced maximal platelet aggregation was 4.2 nM. The concentration that brought about 50% aggregation of washed platelets (EC_{50}) was 0.25 nM.

The direct human platelet aggregation activity of alboaggregin D (8.3 nM final concentration) was inhibited by either monoclonal antibody (mAb) to GPIbα or polyclonal GPVI antibody. The anti-GPIbα antibody dose-dependently inhibited alboaggregin D-induced platelet aggregation with the 50% inhibitory concentration (IC_{50}) of 87.4 nM. In addition, anti-GPVI antibody also inhibited alboaggregin D-induced platelet aggregation with the IC_{50} of 44.8 nM (**Figure 5**).

Either of the antibodies did not completely inhibit aggregation. Therefore, we incubated anti-GPIbα and anti-GPVI antibody together with the highest concentration of each. The combination of antibodies completely inhibited platelet aggregation induced by alboaggregin D.

3.6 Platelet signal transduction induced by alboaggregin D

Alboaggregin D is a potent platelet agonist similar to collagen. Based on the light transmission aggregation curve slopes of washed platelet suspensions when treated with either the alboaggregin D or collagen, the final concentration of 10 μg/ml of collagen and 5 μg/ml of the snaclec gave comparable platelet aggregation rates.

Subsequently, tyrosine phosphorylation of platelet signaling proteins after stimulation by the equivalent doses of alboaggregin D and collagen was determined. After 5-min incubation, platelet lysate was subjected to Western blotting probed by anti-phosphotyrosine antibody. Compared with platelets activated by collagen, alboaggregin D-stimulated platelets yielded stronger tyrosine phosphorylation of signaling proteins, including phospholipase C (PLC)γ2, phosphoinositide 3 kinase (PI3K), Syk, Src, LAT and p26 (**Figure 6**).

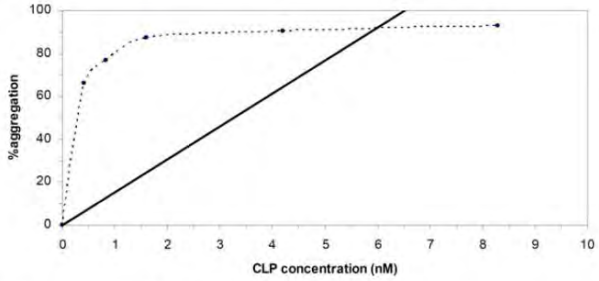

Fig. 4. The effect of alboaggregin D on aggregation of washed platelets. The EC_{50} (the concentration that induced 50% of maximal aggregation) is 0.25 nM. The diagonal line is the linear regression.

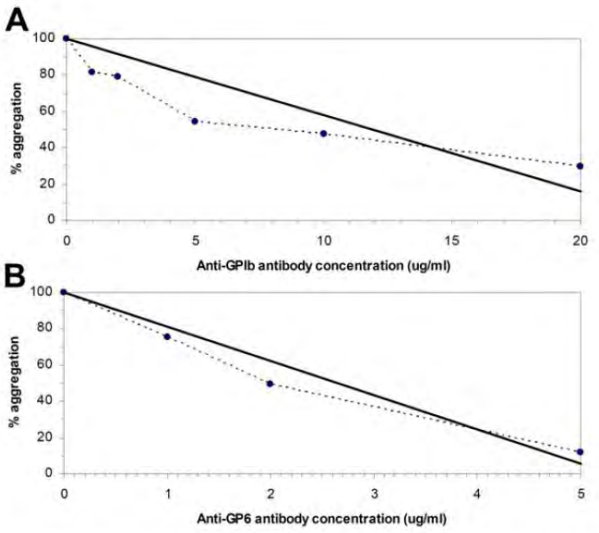

Fig. 5. The effects of platelet glycoprotein (GP) antibodies on alboaggregin D-induced platelet aggregation. A. Inhibitory effect of monoclonal anti-GPIb antibody. B. Inhibitory effect of polyclonal GPVI antibody. The IC_{50} was determined using curve fitting with linear regression (diagonal line).

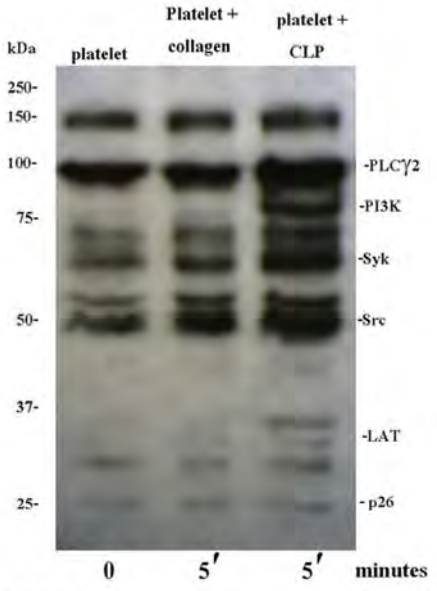

Fig. 6. Tyrosine phosphorylation of human platelet proteins after 5-min activation by 5 μg/ml of alboaggregin D (platelet CLP) compared with resting platelets (platelet) and platelets activated by 10 μg/ml of collagen (platelet + collagen). The band positions for various signaling proteins are labeled on the right. The blot with longer film exposure is shown in supplementary material.

4. Discussion

In this study, we have purified and characterized a novel 120-kDa C-type lectin like-protein (snaclec) from *C. albolabris* crude venom that has an ability to induce platelet aggregation through both GPIbα and GPVI. From the sequences, molecular weights of its subunits and activities (**Table 2**), this snaclec has not been previously reported because they are not consistent with alboaggregin A (Dörmann et al., 2001), alboaggregin B (Peng et al. 1991; Arpijuntarangkoon et al., 2007), alboaggregin C or alboluxin (Du et al., 2002). Therefore, it is called, here, alboaggregin D because it is one of the Gp Ib binding proteins (Clemetson et al., 2008).

The molecular masses suggest that alboaggregin D is an octameric heterodimers composed of $(\alpha\beta)_4$. Comparison of the disulfide patterns with other homologous snake venoms, there are 7 conserved cysteine residues in each subunit (**Figure 2**). The inter-chain disulfide bond of alboaggregin D is predicted to be between Cys 158 of α subunit and Cys 26 of β subunit. In addition, there is an additional cysteine residue in each subunit in high molecular weight snaclecs to form an extra link resulting in a larger multimeric structure (**Figure 2**).

The purified snaclec is analyzed by SDS-PAGE. It exhibits a single band with molecular weight of 120 kDa under a non-reduced condition and 2 bands with apparent molecular weights of 14 and 17 kDa under a reduced condition for α and β subunits, respectively. Moreover, we use LC/MS/MS to confirm the identity amino acid sequence of the purified snaclec. The fragments of α and β subunits show peptide mass fingerprints that are identical to the sequences of those respective subunits of snake venom snaclecs obtained from cDNA library suggesting that they are the same protein. Excluding signal peptides, the theoretical molecular masses of α and β subunits are 15658.61 and 14922.59 Da, while the respective molecular masses obtained from LC/MS/MS are 15728.4 and 16963.1Da. The actual molecular masses are higher than predicted masses, possibly due to post-translational modifications.

As summarized in **table 2**, alboaggregin A (Dörmann et al., 2001) and alboaggregin B (Arpijuntarangkoon et al., 2007) have the total molecular masses of 50 and 25 kDa, respectively. Alboaggregin C and alboaggregin D show similar molecular masses. However alboaggregin D can activate platelet

signal transduction, while alboaggregin C only agglutinates platelets (Du et al., 2002). Therefore, our snaclec is unlikely to be alboaggregin C. Alboluxin and alboaggregin D have similar masses and activities, but the reported N-terminal sequence of the β subunit of alboluxin is NFSPPDWYAYD.

Alboaggregin D is a potent platelet activator. Furthermore, it displayed a lag phase before the start of aggregation similar to collagen. This lag time is probably the period of platelet signal transduction. This high potency that causes agglutination is thought to come from cooperative action of multiple binding sites of its octameric structure similar to convulxin, stejnulxin and alboluxin (Polgár et al, 1997; Murakami et al., 2003; Lee et al., 2003; Du et al., 2002). This large polyvalent structure may cross-link and cluster several molecules of platelet glycoprotein resulting in strong activation.

From inhibitory studies, an anti-GPVI polyclonal antibody more strongly inhibits alboaggregin D-induced platelet aggregation than the anti-GPIbα antibody. This provides the evidence that alboaggregin D activates platelets through both GPIbα and GPVI. The other study (Du et al., 2002) has shown that another *C. albolabris* snaclec, alboluxin, also induced platelet aggregation via both GPVI and GPIbα. The IC_{50} of monoclonal GPIb antibody and polyclonal GPVI that inhibit alboluxin are 296 nM (final concentration of alboluxin 0.25 nM) and 31 μM (final concentration of alboluxin 0.33 nM). Therefore, alboluxin appears to be a stronger agonist because it requires larger quantities of antibodies to inhibit platelet aggregation.

Furthermore our experimental data suggest that alboaggregin D binds to both GPIbα and GPVI because it induces phosphorylation of tyrosine residues in signaling proteins that are usually activated by these 2 platelet GPs. These 2 receptors are likely to function synergistically because only one of the antibodies almost completely inhibits the activity of snaclec. However, only one of the receptor is sufficient for platelet activation by alboaggregin D because both antibodies are required to completely inhibit snaclec-induced platelet aggregation.

Examining the alboaggregin D-induced tyrosine phosphorylation profile in platelets, we found that it was fairly similar to those produced by collagen and alboluxin, except that platelets activated by alboluxin showed particularly strong Fcγ phosphorylation.

We hypothesize that alboaggregin D draws GPIb-IX-V and GPVI into close proximity resulting in synergistic signal transduction between these 2 receptors. Therefore, one snaclec molecule should contain separate GPIb and GPVI binding sites. These sites on the snaclec remain to be identified. Further studies on expression, characterization and mutagenesis of recombinant *C. albolabris* snaclecs are essential to elucidate this structure-function relationship. These experiments will give us deeper insights in molecular mechanisms of platelet activation induced by these 2 receptors.

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Summary

Our findings suggest that *C. albolabris* venom disrupts platelet-collagen interactions resulting in abnormal platelet adhesion and delayed wound healing. These proteins are targets of snakebite therapy and useful in studies on platelet activation.

Outputs

1. Publications

1. Pinyachat A, Rojnuckarin P, Muanpasitporn C, Singhamatr P, Nuchprayoon S. *Albocollagenase, a novel recombinant P-III snake venom metalloproteinase from green pit viper (Cryptelytrops albolabris), digests collagen and inhibits platelet aggregation*. Toxicon **2011**; 57:772-780.

2. Mekchay P, Rojnuckarin P. *Molecular cloning and characterization of alboaggregin D, a novel platelet activating protein, from Green pit viper (Cryptelytrops albolabris) venom.* Toxicon **2012**; 59:59-67.

3. Pongpit J, Limpawittayakul P, Juntiang J, Akkawat B, Rojnuckarin P. *The role of prothrombin time (PT) in evaluating green pit viper (Cryptelytrops sp) bitten patients.* Transactions of the Royal Society of Tropical Medicine and Hygiene **2012**; 106: 415-418.

2. Guidelines for management of snakebites

The recommendations are based on the results of publication #3.

1. Manual of Practical Management of snake-bites and animal toxin poisoning 2012, Queen Saovabha Memorial Institute, Thai Red Cross Society, ISBN 978-616-7287-70-6
2. **Rojnuckarin P**, Suteparuk S, Sibunruang. Diagnosis and management of venomous snakebites in Southeast Asia. Asian Biomed 2012 Dec; 6(6): 795-805.

3. Guest lectures

1. Snakebite-induced coagulopathy. The 2nd TSH International Symposium: Thrombosis and Hemostasis. Centrala Grand Convention Center, Bangkok, May 24th, 2013.

2. Green pit viper bites: from bedside to bench. Department of Pathology, Faculty of Medicine, Songklanakarin University, Songkla, September 26th, 2012.

3. Snakebites in Thailand. Short Course in Tropical Medicine, School of Tropical Medicine, Mahidol University, Bangkok July 2nd 2012.

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5. Snakebites, Hematology for non-hematologists, Nakaburi Resort, Udornthani, August 28th, 2010.

6. Snakebites, Intensive Course in Hematology, Department of Medicine, Chulalongkorn University, Bangkok, July 14th, 2010.

7. Snakebites in Thailand. Short Course in Tropical Medicine, School of Tropical Medicine, Mahidol University, Bangkok July 5th, 2010.

4. Book Chapters

1. Ponlapat Rojnuckarin “Snakebite-Induced Coagulopathy and Bleeding Disorders” In: R. Manjunatha Kini, Kenneth J. Clemetson, Francis S. Markland, Mary Ann McLane, Takashi Morita (Editors). Toxins and Hemostasis: from Bench to Bedside 767 pages New York: Springer 2010; p 699-710.

2. พลภัทร โรจน์นครินทร์ “การวินิจฉัยและรักษาผู้ป่วยที่ถูกงูกัด” ใน พลภัทร โรจน์นครินทร์ บรรณาธิการ หนังสือ **โรคเลือดออกง่ายและหลอดเลือดอุดตันในเขตร้อน** จำนวน 255 หน้า กรุงเทพมหานคร: โรงพิมพ์แห่งจุฬาลงกรณ์มหาวิทยาลัย 2553; หน้า 106-139.

3. พลภัทร โรจน์นครินทร์ “พิษงูที่มีผลต่อระบบห้ามเลือด: วิทยาศาสตร์พื้นฐานและการนำไปใช้ประโยชน์” ใน พลภัทร โรจน์นครินทร์ บรรณาธิการ หนังสือ **โรคเลือดออกง่ายและหลอดเลือดอุดตันในเขตร้อน** จำนวน 255 หน้า กรุงเทพมหานคร: โรงพิมพ์แห่งจุฬาลงกรณ์มหาวิทยาลัย 2553; หน้า 140-156.

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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

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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 CAA TTG 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.

```
   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  ataatcctggaatctgggaacgtgaatgattatgaagtcgtgcatccacgaaaagtcact  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  gtgaatggagagccagtagtccttcacctggaaaaaaataaaggacttttttcagaagat  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  gcatgcaatggtttgaaaggacatttcaagcttcaaggggagacgtaccttattgaaccc  420
 121   A  C  N  G  L  K  G  H  F  K  L  Q  G  E  T  Y  L  I  E  P   140

 421  ttggaactttccgacagtgaagctcatgcagtattcaaatatgaaaatgtagaaaaagag  480
 141   L  E  L  S  D  S  E  A  H  A  V  F  K  Y  E  N  V  E  K  E   160

 481  gacgaggcccccaaaatgtgtggggtaacccagaattggaaatcatatgaaccccatcaaa  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  aatattcgcgtagcattgactggcctaataatttggttggacagagataagattaacgtg  780
 241   N  I  R  V  A  L  T  G  L  I  I  W  L  D  R  D  K  I  N  V   260

 781  acatcagcagcaaatgttactttgtcctatttggagactggagagcgacagtcttgctg  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  gatcatagcacaataaatcttctgatggcagttacaatggtccatgagctgggtcataat 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  atggctcccaggctaaaccctcaaccttccaaacagttcagcgattgtagtaaggatgat 1140
 361   M  A  P  R  L  N  P  Q  P  S  K  Q  F  S  D  C  S  K  D  D   380

1141  tatcggacatttcttataaatcgtagaccacaatgcattctcaatgcacgcctcgaagaca 1200
 381   Y  R  T  F  L  I  N  R  R  P  Q  C  I  L  N  A  P  S  K  T   400

1201  gatattgtttcacccccagtttgtggaaatgaacttttggagaagggaaaagaatgtgac 1260
 401   D  I  V  S  P  P  V  C  G  N  E  L  L  E  K  G  K  E  C  D   420

1261  tgtggctctcctaaaaacttgtcgaaaatatgctgcaatgctgcacgtgtaactctgccc 1320
 421   C  G  S  P  K  N  L  S  K  I  C  C  N  A  A  R  V  T  L  P   440

1321  ccaggttcccaatgtgcagatgagtgtggttgtcaccagtgcaattttaagaagagcagga 1380
 441   P  G  S  Q  C  A  D  E  C  G  C  H  Q  C  N  F  K  R  A  G   460

1381  acagaagtcggcaggcaaaggatgactgtgacttggctgaaagctgcactggccgatct 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  tactgctacaataggacgcgccccaccatgaacaaccaatgtatttcttttctttgggtca 1560
 501   Y  C  Y  N  R  T  R  P  T  M  N  N  Q  C  I  S  F  F  G  S   520

1561  agtgcaactgtggctccagatggatgttttaattttaaccggcaaagcaatgattatagc 1620
 521   S  A  T  V  A  P  D  G  C  F  N  F  N  R  Q  S  N  D  Y  S   540

1621  tactgcagaaaggaaaacggtagaaagattccatgtgcaccacaagatgtaaaatgtggc 1680
 541   Y  C  R  K  E  N  G  R  K  I  P  C  A  P  Q  D  V  K  C  G   560

1681  aggttatactgcttccctaatttcacccggagaggagaatacttgcaatctcatatataca 1740
 561   R  L  Y  C  F  P  N  S  P  G  E  E  N  T  C  N  L  I  Y  T   580

1741  cctggtcgtgaagatattgggatggttcttctggggaacaaaatgtgcagatggaaaggcc 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
```

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, propeptide, 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 pPICZ∝A 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

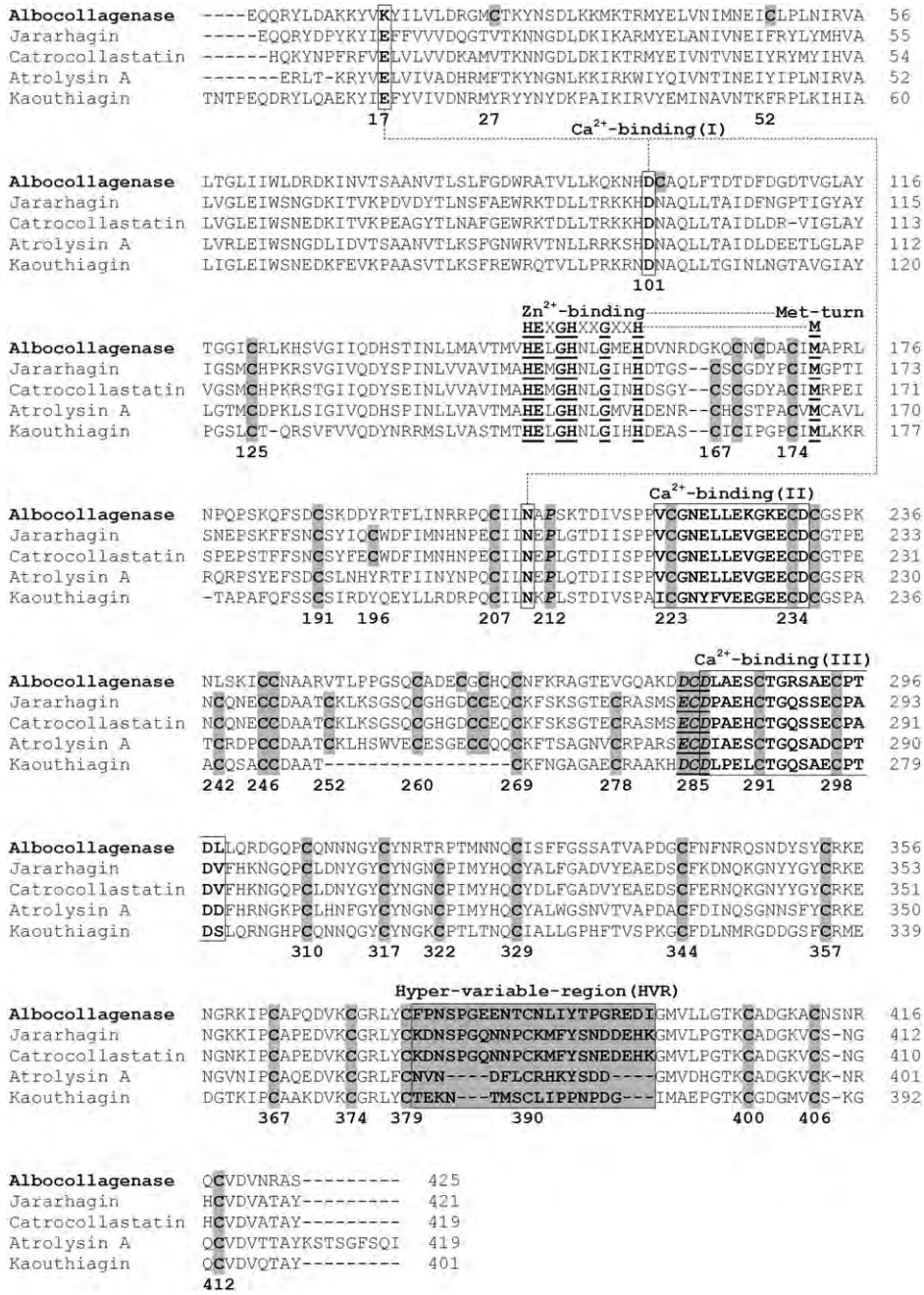

Fig. 2. The multiple sequence alignment of the P-III SVMP putative mature proteins compared with other SVMPs Cysteine residues are numbered according to kaouthiagin. The cysteine residues are highlighted. The Zn^{2+}-binding sequences are underlined. The putative self-post-translational processing position, 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

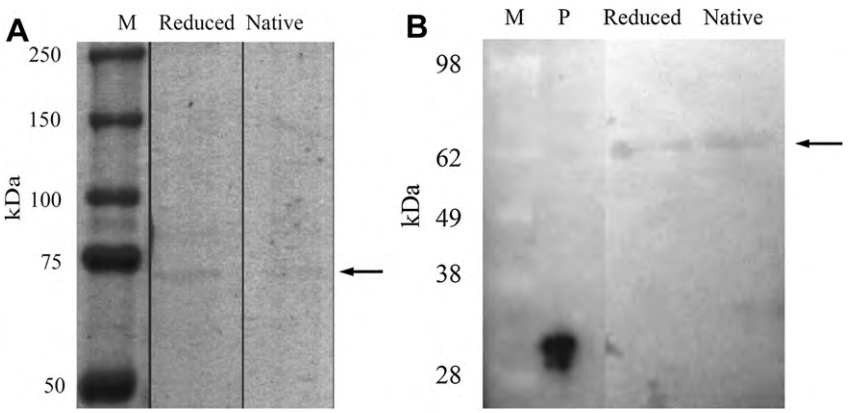

Fig. 3. Recombinant albocollagenase (Arrows) (A) The recombinant protein was purified, electrophoresed on 8% SDS-PAGE in reduced and native conditions and stained with Coomassie-blue R250. The molecular weight markers are shown in lane M. (B) The purified protein was electrophoresed as described in (A) 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.

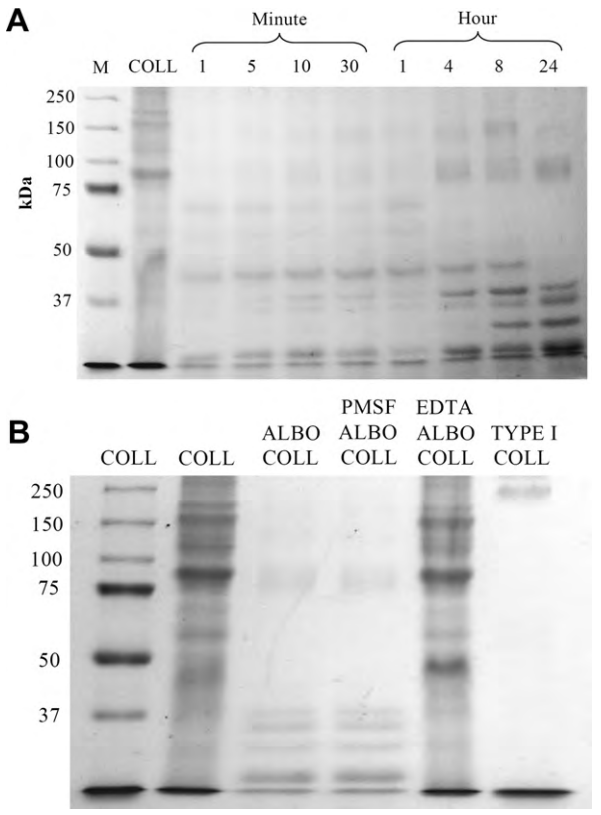

Fig. 4. Time-dependent type IV collagen degradation by albocollagenase (A) Type IV collagen was incubated with albocollagenase at different time points as indicated in each lane. Each reaction was subjected to 8% reducing SDS-PAGE and stained with Coomassie-blue. M and COLL represent the molecular weight 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).

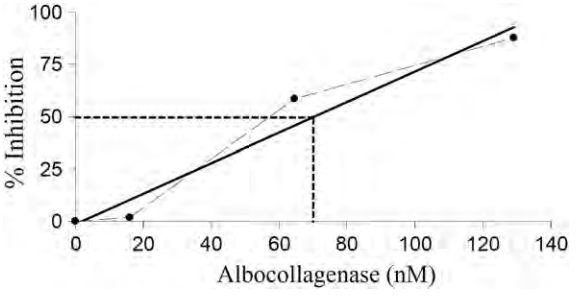

Fig. 5. Albocollagenase inhibited platelet aggregation. Various concentrations of the albocollagenase (0, 0.25, 1, and 2 mg/L) were pre-incubated with platelet-rich plasma (PRP) before adding collagen. The maximal aggregation without albocollagenase was regarded as 100%. The PRP without agonist was set 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.

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.

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Conflict of interest

The authors have no conflict of interest.

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Molecular cloning and characterization of alboaggregin D, a novel platelet activating protein, from Green pit viper (*Cryptelytrops albolabris*) venom

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ABSTRACT

Viper venoms are abundant sources of proteins affecting hemostasis. This study aimed to clone and purify a high-molecular-weight C-type lectin-like protein (snaclec) from Green pit viper (*Cryptelytrops albolabris*) venom, as well as to characterize its effects on human platelets.

Based on the partial sequences from the *C. albolabris* venom gland library, we cloned full-length cDNAs encoding the snaclec subunits using 5′RACE and 3′RACE methods. The cDNA sequence of the α subunit contained 477 base pairs (bp) that were translated into 23 amino acid residue signal peptide and a 135-residue mature protein. The cDNA sequence of the β subunit contained 447 bp that were translated into 23-residue signal peptide and a 125-residue mature protein. Compared with known sequences of dimeric snaclecs, these peptides contained extra cysteines that probably formed a high-order multimer. In parallel, a snaclec was isolated from *C. albolabris* crude venom using gel filtration followed by ion-exchange chromatography. The purified *C. albolabris* snaclec on SDS-PAGE showed the apparent molecular mass of 120 kDa under native condition and 2 bands of 14 and 17 kD under reduced condition suggesting a tetramer of heterodimers $(\alpha\beta)_4$. Liquid chromatography-tandem mass spectrometry analysis of the peptides found perfect matches with the conceptually translated sequences from the cDNA library. This protein was unique from any other snaclecs previously purified from *C. albolabris* and named alboaggregin D. It induced human platelet aggregation in the absence of any cofactor with the EC_{50} of 0.25 nM and caused tyrosine phosphorylation in human platelets. Antibodies against either platelet glycoprotein (GP) Ib or GPVI could inhibit alboaggregin D-induced platelet aggregation. This snaclec may be useful for dissecting the mechanisms of platelet activation.

1. Introduction

The geography and climate of Thailand support more than 160 species of snakes. Among these, there are 11 types of green pit vipers. White-lipped pit viper or *Cryptelytrops albolabris* is the most prevalent (Viravan et al., 1992; Mahasandana and Jintakune, 1990). Its venom contains a large number of toxins exerting powerful inhibitory or stimulatory effects on platelets.

Platelet-aggregating agents in venoms activate platelets *in vivo* resulting in platelet consumption and thrombocytopenia in biting victims (Mahasandana et al., 1980). These platelet activators in viper venoms are usually in the family of C-type lectin-like proteins (Weis et al., 1992; Drickamer, 1999), which are currently termed snaclecs (Clemetson et al., 2008). A number of snaclecs affecting platelet functions have been isolated and characterized. Some of them inhibit platelet aggregation by binding to platelet

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glycoprotein (GP) Ib-IX-V and blocking von Willebrand factor (vWF) binding (Fukuda et al., 2000), whereas the others promote platelet aggregation by targeting vWF (Brinkhous et al., 1983), GPIb-IX-V (Andrews et al., 1996), GPVI (Polgár et al., 1997) and possibly other platelet receptors.

Snaclecs are found only in snake venoms and belong to the group VII of C-type lectins. C-type lectins are non-enzymatic proteins that Ca^{2+}-dependently bind to saccharides. They contain one or more copies of a highly conserved carbohydrate recognition domain (CRD) consisting of 115–130 amino acids. Although snaclecs are structurally homologous to C-type lectins, they lack carbohydrate binding activity and most of them do not depend on calcium (Drickamer, 1999; Morita, 2004).

Snake venom snaclecs show diverse pharmacological activities against coagulation factors and platelets. A prominent feature of snaclecs is that they are heterodimers or oligomeric complexes of heterodimers comprising 2 subunits: a subunit a (α chain) of 14–15 kDa and a subunit b (β chain) of 13–14 kDa linked by inter-chain disulfide bonds. Snaclecs appear in a variety of oligomeric forms, including αβ, $(\alpha\beta)_2$ or $(\alpha\beta)_4$ (Atoda et al., 1991; Fukuda et al., 2000; Morita, 2005; Wang and Huang, 2001; Wang et al., 2003).

Snaclecs are important proteins and should be thoroughly investigated in order to understand pathogenesis of snake venom-induced platelet abnormalities and find effective treatments for snakebites. Furthermore, studying the molecular mechanisms of platelet activation by snaclecs will give us deeper insights in platelet signaling pathways. Finally, these proteins display potentials to be diagnostic agents for platelet function tests in patients with bleeding or thrombotic disorders.

In this study, we cloned a novel snaclec gene, alboaggregin D, and purified the protein to characterize its functions, as well as dissected molecular mechanisms involving platelet surface glycoproteins (GPs) and signal transduction.

2. Materials and methods

2.1. Obtaining full-length cDNA of subunits of snaclecs

Green pit viper venom gland library was prepared as previously described (Rojnuckarin et al., 2006). Two unique partial snaclec cDNA sequences were cloned. 5′RACE was performed using SMART™ RACE kit (BD Biosciences Clontech, USA). A snaclec-specific primer was designed based on snaclec sequences from library (5′CCAGACTTCAGACAGCTG GATCTT-3). PCR products were electrophoresed, purified and ligated to the pGEM T vector (Promega, USA) before transformation into *Escherichia coli*. Clones were sequenced using ABI Prism 310 Genetic Analyzer (Perkin–Elmer, USA). We found that the sequences 5′ to the start codon of all *C. albolabris* snaclecs were conserved. Therefore, a 5′ common snaclec primer (5′-CCAGACTTCAGACAGCTGGATCTT-3′) was designed to obtain additional snaclec clones using 3′RACE.

2.2. Alignment and computational sequence analysis

The nucleotide sequences and their conceptual translation obtained from the clones of interest were compared against nucleotide or protein sequences in online databases using BLAST N program via the World Wide Web. Alignments of sequence were made using the Clustal W multiple sequence alignment program.

2.3. Purification of platelet-aggregating proteins

Lyophilized *C. albolabris* venom from Queen Saovabha memorial institute was fractionated by gel filtration chromatography (Sephacryl S-200 (16/60), Amersham Pharmacia, PRC), using an automate LP system (Biorad, USA). Each fraction was detected for A280 and human platelet-agglutinating activity. Protein markers (12–200 kD) (Sigma, USA) were used for molecular weight calculation. Positive fractions were pooled and concentrated using Vivaspin (Sartorius AG, Germany). The sample was then separated on an ion-exchange column (Resource Q, Amersham Pharmacia, PRC) with a linear 0–0.5 M NaCl gradient. Purified protein was analyzed on Coomassie-stained SDS-PAGE gel and its concentration was determined using Micro BCA kit (Pierce, USA).

2.4. Protein identification by tandem mass spectrometry

The trypsin-digested peptides were resuspended in 0.1% formic acid then analyzed by liquid chromatography coupled with tandem mass spectrometry (LC/MS/MS). Peptide ions were detected in a survey scan from 400 to 1600 amu followed by one data-dependent MS/MS scan. All MS/MS spectra were searched using Biowork™ 3.3 software (Sequest algorithm) to compare ion spectra between experimental products and theoretical products in the non-redundant database.

All MS/MS spectra were searched against the database using the following criteria: enzyme trypsin, static modification of cysteine (+57.05130 Da), differential modification of methionine (+15.99940). The results of searching were filters by Xcorr versus charge state ($+1 \geq 1.5$, $+2 \geq 2.0$, $+3 \geq 2.5$) and protein probability (minimum 1.00E-3).

2.5. Platelet aggregation assay

Human platelet-rich plasma was isolated by successive centrifugation steps. The platelet-rich supernatant was centrifuged at 1942 × *g* for 10 min to obtain a pellet. Platelets were resuspended in 113 mM NaCl, 4.3 mM K_2HPO_4, 24.4 mM NaH_2PO_4, 5.5 mM glucose, pH6.5 (buffer B), centrifuged at 180 × *g* for 10 min and washed once more with buffer B. Washed platelets were resuspended in 20 mM HEPES, 140 mM NaCl, 4 mM KCl, 5.5 mM glucose, pH 7.4 (buffer C) The platelet count was adjusted to 350×10^9/L in buffer C.

Before analysis, platelets were incubated at 37 °C for 2 min. Different amounts of purified snaclecs were added to washed platelets and incubated with continuous magnetic stirring at 37 °C for 10 min. Platelet aggregation was measured by changes in light transmission using the platelet aggregation chromogenic kinetic system-4 (PACKS-4) aggregometer (Helena Laboratories, USA).

For inhibitory studies, washed platelets were incubated with various concentrations of monoclonal anti GPIbα,

clone SZ2 (Beckman Coulter company, France.), and/or polyclonal anti GPVI antibody (Abcam plc, UK.) for 2 min before adding alboaggregin D.

2.6. Platelet activation assay

Fifty microliters of purified snaclecs were added to 450 μl of washed normal platelets (350×10^9/L). Aliquots were taken at fixed times (5 min) and the platelet suspension was lysed by adding 5 μl of HEPES containing 10% SDS, 10 mM *N*-ethylmaleimide, 20 mM Na_3VO_4 and 20 mM EDTA.

After centrifugation, the supernatant were electrophoresed on an 8% SDS-polyacrylamide gel and then blotted onto polyvinylidene fluoride (PVDF) membrane. The monoclonal antibody to phosphotyrosine, 4G10 (Biodesign, USA), was applied to detect tyrosine phosphorylated proteins as primary antibody followed by peroxide-coupled rabbit anti-mouse secondary antibody (Dako Cytomation, Denmark). Bound antibody was detected using chemiluminescence.

3. Results

3.1. Molecular cloning of full-length snaclecs from C. albolabris venom gland library

5′RACE using snaclec-specific primers was performed on the cDNA library of *C. albolabris* venom glands. EcoR I digestion was used to screen for inserts of more than 100 clones derived from 5′RACE. Insert-containing clones were sequenced. The cDNA inserts encoding snaclecs could be divided into two major groups, the factor IX binding protein homologs and those similar to platelet-binding proteins.

A

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 1   ttctctctgcattaaggaaggaagaccATGGGGCGATTCATCTTCGGGAGCTTCGGCTTG  60
                                 M  G  R  F  I  F  G  S  F  G  L
 61  CTGGTGGTGTTCCTCTCCTTAAGTGGTACTGGAGCTGATTTTGATTGTCCCCCTGGTTGG 120
      L  V  V  F  L  S  L  S  G  T  G  A  D  F  D  C  P  P  G  W
121  TCTGCCTATGATCGGTATTGCTACCAGGCCTTCAGTGAACCGAAAACCTGGGAAGATGCA 180
      S  A  Y  D  R  Y  C  Y  Q  A  F  S  E  P  K  T  W  E  D  A
181  GAGAGTTTCTGCATGGAGGGGGTGAAGGACTCGCATCTGGTCTCTACGCGAAAGCTCCGGA 240
      E  S  F  C  M  E  G  V  K  D  S  H  L  V  S  V  E  S  S  G
241  GAAGCCGACTTCGTGGCCCAGCTGGTCAACGAGAAACTAAAGACATCCTTTCGCTATGTC 300
      E  A  D  F  V  A  Q  L  V  N  E  N  I  K  T  S  F  R  Y  V
301  TGGATTGGACTGAGGATTCAAAACAAAGAACAGCAATGCAGGTCGGAGTGGAGCGATGCC 360
      W  I  G  L  R  I  Q  N  K  E  Q  Q  C  R  S  E  W  S  D  A
361  TCCAGTGTCAGTTATGAGAACTTGATTAAAAAAGTTTCAAAAAAATGTTATGGGCTGAAA 420
      S  S  V  S  Y  E  N  L  I  K  K  V  S  K  K  C  Y  G  L  K
421  AAAGGGACAGAGCTTCGCACGTGGTTCAATGTTTACTGTGCAGAACTAAATCCTTTCATC 480
      K  G  T  E  L  R  T  W  F  N  V  Y  C  A  E  L  N  P  F  I
481  TGCAAGTTCCCGCCAGAGGTGTAAgatccagctgagtgaagtctggagaagcaaggaaga 540
      C  K  F  P  P  E  C  *
541  ccccacccaccccccaacccttcgctcaacggatgctctcggtagcgggatctgctttgc 600
601  tgctcctgatgggccagaaggtccaataaa-poly A
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B

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  1  aagaccATGGGGCGATTCATCTCCGTGAGCTTCGGCTTGCTGGTTGTGTTCCTCTCCCTG   60
           M  G  R  F  I  S  V  S  F  G  L  L  V  V  F  L  S  L
 61  AGTGGAGCTGGAGCTGGTTTGTGTTGTCCCTTGGATTGGTCTTCCTATGATCTGTATTGC  120
      S  G  A  G  A  G  L  C  C  P  L  D  W  S  S  Y  D  L  Y  C
121  TACAAGGTCTTCAAACAACAGATGAACTGGACGGATGCAGAGCAATTCTGCACACAACAG  180
      Y  K  V  F  K  Q  Q  M  N  W  T  D  A  E  Q  F  C  T  Q  Q
181  CACACAGGCAGCCACCTGGTCTCCTTTCACAGCACTGAAGAAGTAGATTTTGTGGTCCAG  240
      H  T  G  S  H  L  V  S  F  H  S  T  E  E  V  D  F  V  V  Q
241  ATGAGCTACAAGAGTTTGGACACCACTTTTTTCTGGATCGGAGTAAACAACATCTGGAAT  300
      M  S  Y  K  S  L  D  T  T  F  F  W  I  G  V  N  N  I  W  N
301  GGATGCAACTGGCAGTGGAGCGATGGCACCGGGCTCGACTACAAAGAATGGCGTGAACAA  360
      G  C  N  W  Q  W  S  D  G  T  G  L  D  Y  K  E  W  R  E  Q
361  TTTGAATGTCTCGTAGCCAAGACATTTGATAACCAGTGGTGGAGTATGGACTGCAACAGT  420
      F  E  C  L  V  A  K  T  F  D  N  Q  W  W  S  M  D  C  N  S
421  ACTTACTCTTTCGTCTGCAAGTTCCAGGCATAGtctgaagatccagctgtgtgaagtctg  480
      T  Y  S  F  V  C  K  F  Q  A  *
481  gagaagcaaggaagccccccccccaccccccaccttcgctcagtggatgctctctgtagc  540
541  tggatctggttttgctgctcctgatgggccagaaggtccaataaattctgcctagc-Poly A 600
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Fig. 1. The cDNA and conceptually translated sequences of α subunit (A) and β subunit (B) of alboaggregin D. The signal peptides are italicized and polyadenylation signals are underlined. The other clone of α subunit contains 3 nucleotide changes (highlighted) all to adenine resulting in amino acid substitution with isoleucine, isoleucine and lysine respectively.

A common snaclec primer was designed based on the 5′RACE results for the 3′RACE. We found 3 additional major groups of snaclecs. Three clones were homologous to the α subunit of alboaggregin B. Furthermore, 8 clones were related to the α subunit and 8 clones were homologous to the β subunit of the other platelet-binding snaclec, which will be focused in this study. This novel snaclec was termed, here, alboaggregin D (Fig. 1).

There were two groups of alboaggregin D α (4 clones each) with differences in three amino acid residues as illustrated in Fig. 1. The clone showed in the figure was fitter to the mass spectrometry data and, therefore, chosen. In addition, there were three additional nucleotide differences between these two clones, but there was no change in amino acid residues.

There were also two groups of the β subunit (four clones each) with variations in 5 separate nucleic acid sequences. However, all amino acids were the same.

3.2. Sequence alignment and computational searching analysis

Using BLAST N, the nucleotide sequence of the α subunit of alboaggregin D showed the highest homology to *Protobothrops mucrosquamatus* mucrocetin α chain mRNA and the β subunit showed the highest identity to *Crotalus durissus* convulxin β mRNA.

An alignment between conceptually translated sequences of alboaggregin D α and mucrocetin α chain (Huang et al., 2004) showed 86.7% amino acid sequence identity, while the translated sequences of the β chain and *C. durissus* convulxin β showed 77.0% identity.

Subsequently, the amino acid sequences of alboaggregin D α and β were compared with other snake venom snaclecs using Clustal W multiple sequence alignment. The results revealed that the identity scores of alboaggregin D α and β subunits with those of the other known platelet collagen receptor-binding snaclecs as shown in Fig. 2. The scores were higher for multimeric snaclecs (mucrocetin, stejnulxin and convulxin) as compared with dimeric ones (EMS16 and aggretin).

3.3. Protein purification and SDS–PAGE analysis

Gel filtration chromatography of 0.5 g of crude *C. albolabris* venom was performed on Sephacyl G-200 column resulting in 7 protein peaks (Fig. 3A). Peaks 1 and 4 showed strong platelet-aggregating activity. The low molecular weight peak 4 was identified as alboaggregin B and previously reported (Arpijuntarangkoon et al., 2007). The high molecular weight platelet-aggregating protein (peak 1) was further investigated in this study.

The pooled peak 1 was fractionated on Resourse Q ion-exchange column using a linear gradient of NaCl from 0 to 0.5 M. This protein was eluted at approximately 0.38 M NaCl as determined by platelet aggregating activity. The purified protein was analyzed by SDS-PAGE (Fig. 3B). It had an apparent molecular mass of approximately 120 kDa under non-reducing condition. Upon reduction, it separated into two bands with apparent molecular weights of 14 kDa and 17 kDa consistent with α and β subunits of a snaclec.

3.4. Protein identification by LC/MS/MS

The nature of purified *C. albolabris* snaclec α and β subunits were determined by LC/MS/MS. The peptide mass fingerprints were shown in Table 1. Some large fragments might not be detectable on mass spectrometry. In addition, the fragments from α and β subunits sequence were R.YCYQAFSEPK.N and R.EQFECLVA.K. They were matched with deduced amino acid sequences of α and β subunits obtained from the cDNA library as described above.

The molecular mass of α and β subunits acquired from LC/MS/MS were 15 728.4 Da and 16 963.1 Da. This purified snaclec was, therefore, alboaggregin D.

3.5. The effects of alboaggregin D on washed human platelets

The purified alboaggregin D caused aggregation of washed platelets (Fig. 4). The dose that induced maximal platelet aggregation was 4.2 nM. The concentration that brought about 50% aggregation of washed platelets (EC_{50}) was 0.25 nM.

The direct human platelet aggregation activity of alboaggregin D (8.3 nM final concentration) was inhibited by either monoclonal antibody (mAb) to GPIbα or polyclonal GPVI antibody. The anti-GPIbα antibody dose-dependently inhibited alboaggregin D-induced platelet aggregation with the 50% inhibitory concentration (IC_{50}) of 87.4 nM. In addition, anti-GPVI antibody also inhibited alboaggregin D-induced platelet aggregation with the IC_{50} of 44.8 nM (Fig. 5).

Either of the antibodies did not completely inhibit aggregation. Therefore, we incubated anti-GPIbα and anti-GPVI antibody together with the highest concentration of each. The combination of antibodies completely inhibited platelet aggregation induced by alboaggregin D.

3.6. Platelet signal transduction induced by alboaggregin D

Alboaggregin D is a potent platelet agonist similar to collagen. Based on the light transmission aggregation curve slopes of washed platelet suspensions when treated with either the alboaggregin D or collagen, the final concentration of 10 μg/ml of collagen and 5 μg/ml of the snaclec gave comparable platelet aggregation rates.

Subsequently, tyrosine phosphorylation of platelet signaling proteins after stimulation by the equivalent doses of alboaggregin D and collagen was determined. After 5-min incubation, platelet lysate was subjected to Western blotting probed by anti-phosphotyrosine antibody. Compared with platelets activated by collagen, alboaggregin D-stimulated platelets yielded stronger tyrosine phosphorylation of signaling proteins, including phospholipase C (PLC)γ2, phosphoinositide 3 kinase (PI3K), Syk, Src, LAT and p26 (Fig. 6).

4. Discussion

In this study, we have purified and characterized a novel 120-kDa C-type lectin like-protein (snaclec) from *C. albolabris* crude venom that has an ability to induce platelet

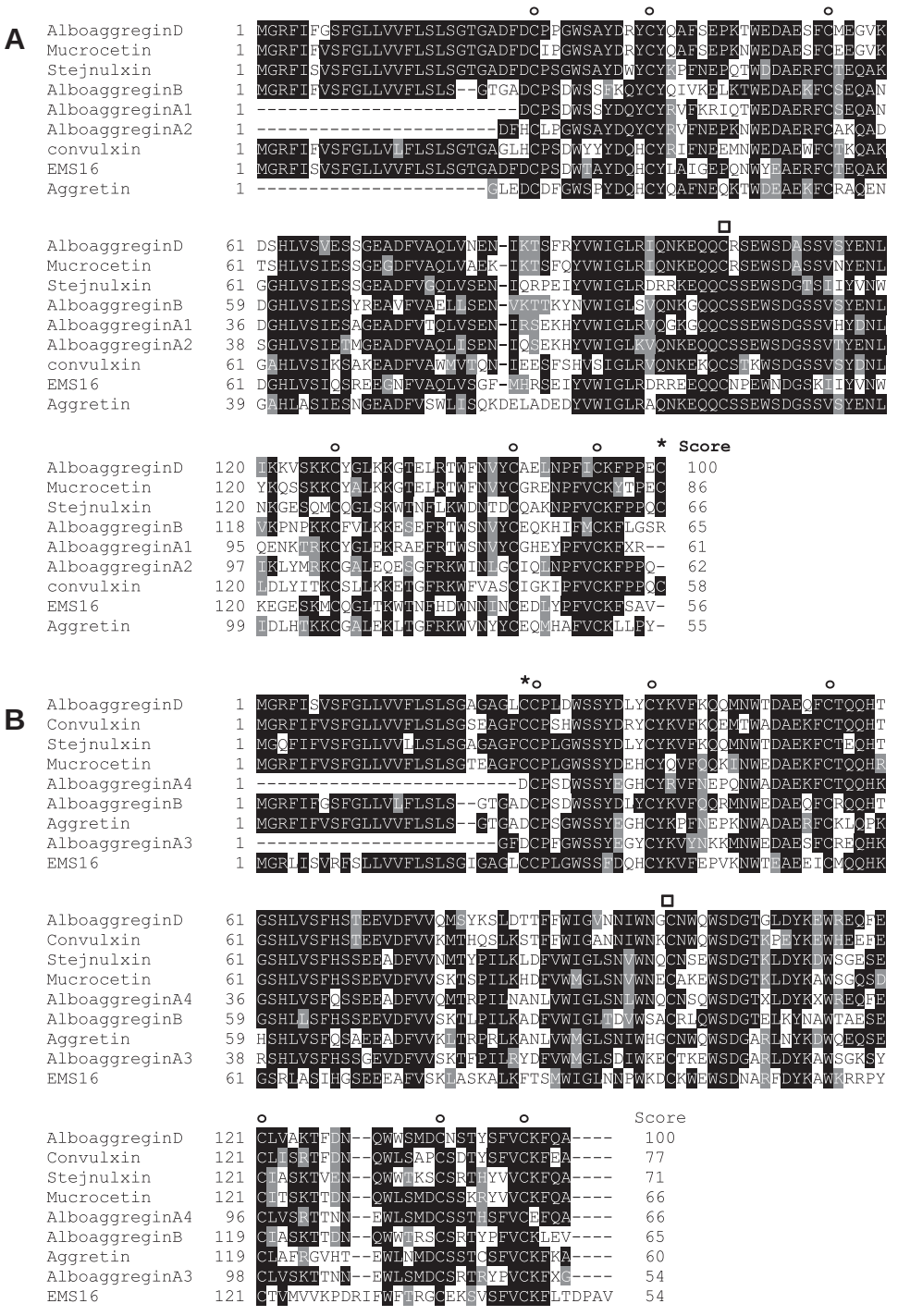

Fig. 2. The comparison of the deduced amino acid sequences of alboaggregin D α subunit (A) and β subunit (B) with those of other platelet collagen receptor-binding snaclecs from snake venoms. The conserved cysteine residues that form intra-chain, dimeric inter-chain and multimeric inter-chain disulfide bonds are marked by circles, squares and asterisks, respectively.

aggregation through both GPIbα and GPVI. From the sequences, molecular weights of its subunits and activities (Table 2), this snaclec has not been previously reported because they are not consistent with alboaggregin A (Dörmann et al., 2001), alboaggregin B (Peng et al., 1991; Arpijuntarangkoon et al., 2007), alboaggregin C or alboluxin (Du et al., 2002). Therefore, it is called, here, alboaggregin D because it is one of the Gp Ib binding proteins (Clemetson et al., 2008).

The molecular masses suggest that alboaggregin D is an octameric heterodimers composed of $(\alpha\beta)_4$. Comparison of the disulfide patterns with other homologous snake

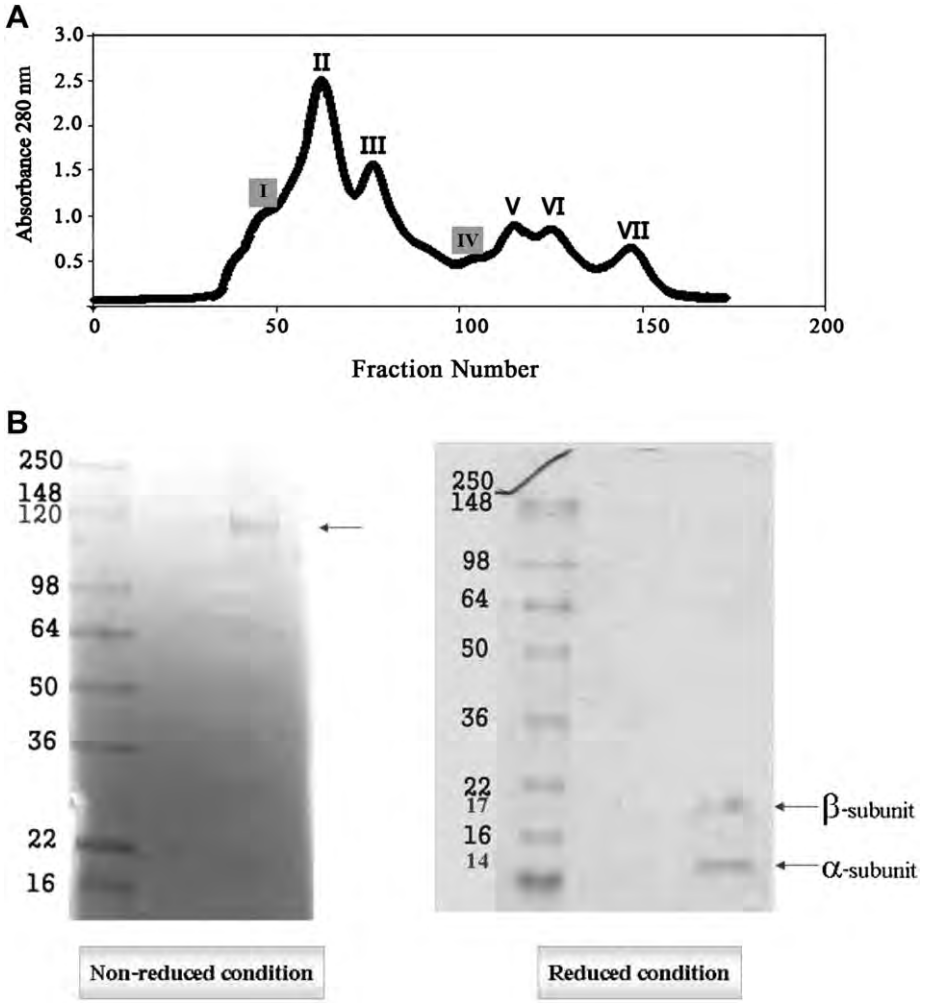

Fig. 3. Purification of alboaggregin D from *C. albolabris* venom. A. Gel filtration chromatogram of the venom on a Sephacyl G-200 column. Protein concentrations were estimated from the absorbance at 280 nm. Highlighted peaks represent the proteins with platelet-aggregating activity. The peak I is alboaggregin D and the peak IV is alboaggregin B. B. The purified protein was analyzed on SDS-PAGE under native and reduced conditions. The molecular weight markers are shown on the left lane of each gel.

Table 1
The peptide mass fingerprints of alboaggregin D.

	Expected masses[a]	Actual masses	Sequences
	α Subunit		
1	1529.62	1560.92	DFDCPPGWSAYDR
2	1236.38	Sequenced[b]	YCYQAFSEPK
3	1632.82	1626.85	TWEDAESFCMEGVK
4	2688.91	2808.09	DSHLVSVESSGEADFVAQLVNENIK
	2489.70	–	DSHLVS**I**ESSGEADFVAQL**I**NE**K**[c]
5	1390.68	1391.80	YVWIGLRIQNK
6	1815.94	1814.81	SEWSDASSVSYENLIK
7	1944.12	1940.94	TWFNVYCAELNPFICK
	β Subunit		
1	2404.85	2402.96	GLCCPLDWSSYDLYCYKVFK
2	4606.03	–	QQMNWTDAEQFCTQQHTGSHLVSFHSTEEVDFVVQMSYK
3	3639.98	–	SLDTTFFWIGVNNIWNGCNWQWSDGTGLDYK
4	1067.25	Sequenced[b]	EQFECLVAK
5	2810.10	2808.09	TFDNQWWSMDCNSTYSFVCKFQA

[a] Fragments with too small molecular masses are not included.
[b] The sequences on LC/MS/MS were perfectly matched with the conceptual translations of the cDNAs.
[c] The variant of α subunit is found on cDNA sequencing.

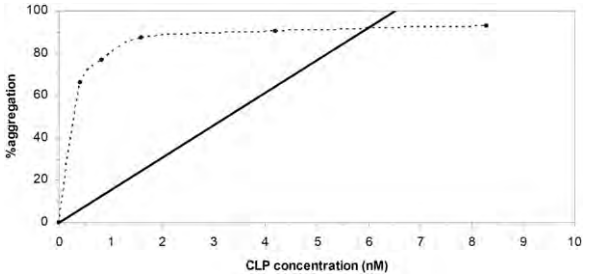

Fig. 4. The effect of alboaggregin D on aggregation of washed platelets. The EC_{50} (the concentration that induced 50% of maximal aggregation) is 0.25 nM. The diagonal line is the linear regression.

venoms, there are seven conserved cysteine residues in each subunit (Fig. 2). In addition, there is an additional cysteine residue in each subunit in high molecular weight snaclecs to form an extra link resulting in a larger multimeric structure (Fig. 2). The inter-chain disulfide bond of alboaggregin D is predicted to be between Cys 158 of α subunit and Cys 26 of β subunit.

The purified snaclec is analyzed by SDS–PAGE. It exhibits a single band with molecular weight of 120 kDa under a non-reduced condition and two bands with apparent molecular weights of 14 and 17 kDa under a reduced condition for α and β subunits, respectively. Moreover, we use LC/MS/MS to confirm the identity amino acid sequence of the purified snaclec. The fragments of α and β subunits show peptide mass fingerprints that are identical to the sequences of those respective subunits of snake venom snaclecs obtained from cDNA library suggesting that they are the same protein. Excluding signal peptides, the theoretical molecular masses of α and β subunits are 15 658.61 and 14 922.59 Da, while the respective molecular masses obtained from LC/MS/MS are 15 728.4 and 16 963.1 Da. The actual molecular masses are

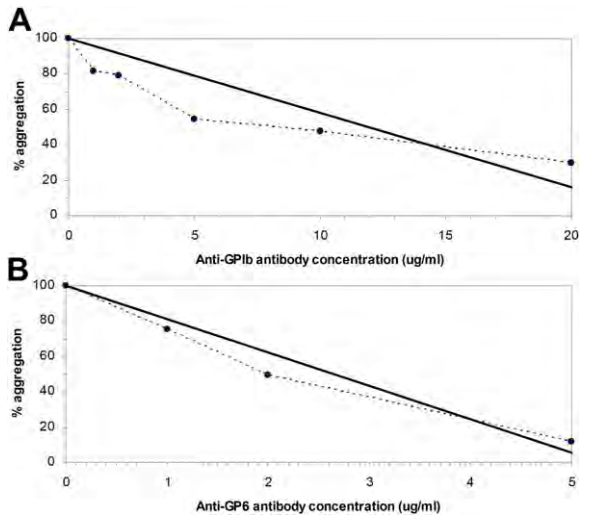

Fig. 5. The effects of platelet glycoprotein (GP) antibodies on alboaggregin D-induced platelet aggregation. A. Inhibitory effect of monoclonal anti-GPIb antibody. B. Inhibitory effect of polyclonal GPVI antibody. The IC_{50} was determined using curve fitting with linear regression (diagonal line).

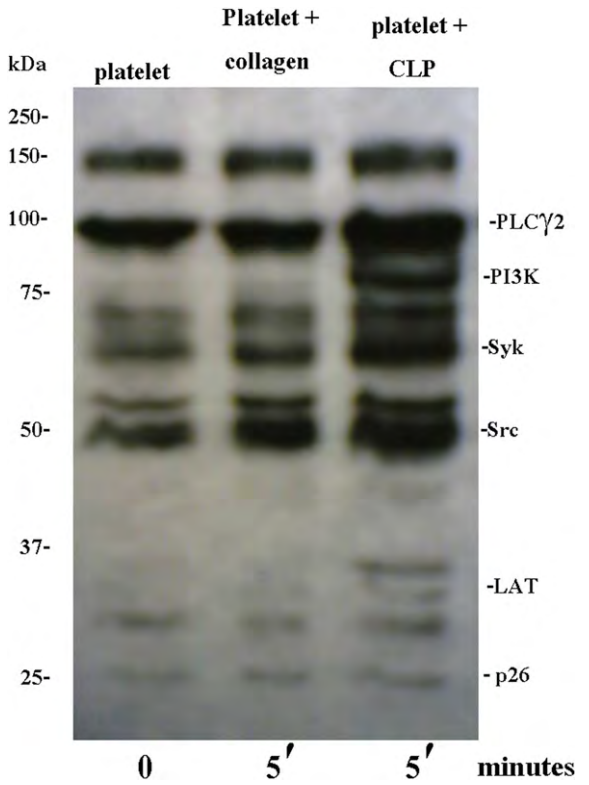

Fig. 6. Tyrosine phosphorylation of human platelet proteins after 5-min activation by 5 μg/ml of alboaggregin D (platelet CLP) compared with resting platelets (platelet) and platelets activated by 10 μg/ml of collagen (platelet + collagen). The band positions for various signaling proteins are labeled on the right. The blot with longer film exposure is shown in supplementary material.

higher than predicted masses, possibly due to post-translational modifications.

As summarized in Table 2, alboaggregin A (Dörmann et al., 2001) and alboaggregin B (Arpijuntarangkoon et al., 2007) have the total molecular masses of 50 and 25 kDa, respectively. Alboaggregin C and alboaggregin D show similar molecular masses. However alboaggregin D can activate platelet signal transduction, while alboaggregin C only agglutinates platelets (Du et al., 2002). Therefore, our snaclec is unlikely to be alboaggregin C. Alboluxin and alboaggregin D have similar masses and activities, but the reported N-terminal sequence of the β subunit of alboluxin is NFSPPDWYAYD.

Alboaggregin D is a potent platelet activator. Furthermore, it displayed a lag phase before the start of aggregation similar to collagen. This lag time is probably the period of platelet signal transduction. This high potency that causes agglutination is thought to come from cooperative action of multiple binding sites of its octameric structure similar to convulxin, stejnulxin and alboluxin (Polgár et al., 1997; Murakami et al., 2003; Lee et al., 2003; Du et al., 2002). This large polyvalent structure may cross-link and cluster several molecules of platelet glycoprotein resulting in strong activation.

From inhibitory studies, an anti-GPVI polyclonal antibody more strongly inhibits alboaggregin D-induced

Table 2
Comparison of the properties of snaclecs from *Cryptelytrops albolabris* venoms.

	MW (kDa)	Structure	Subunits (kDa)	Targets[a]	Platelet activation	Reported sequences
Alboaggregin A	50	$\alpha\alpha'\beta\beta'$	14/14/15/16	GPIb/GPVI	Yes	Partial
Alboaggregin B	25	$\alpha\beta$	15/17	GPIb	No	Complete
Alboaggregin C	120	unknown	17/19	unknown	No	None
Alboaggregin D	120	$(\alpha\beta)_4$	14/17	GPIb/GPVI	Yes	Complete
Alboluxin	120	$(\alpha\beta)_3$	17/24	GPIb/GPVI	Yes	N terminus

[a] GP: Platelet glycoprotein

platelet aggregation than the anti-GPIbα antibody. This provides the evidence that alboaggregin D activates platelets through both GPIbα and GPVI. The other study (Du et al., 2002) has shown that another *C. albolabris* snaclec, alboluxin, also induced platelet aggregation via both GPVI and GPIbα. The IC_{50} of monoclonal GPIb antibody and polyclonal GPVI that inhibit alboluxin are 296 nM (final concentration of alboluxin 0.25 nM) and 31 μM (final concentration of alboluxin 0.33 nM). Therefore, alboluxin appears to be a stronger agonist because it requires larger quantities of antibodies to inhibit platelet aggregation.

Furthermore, our experimental data suggest that alboaggregin D binds to both GPIbα and GPVI because it induces phosphorylation of tyrosine residues in signaling proteins that are usually activated by these two platelet GPs. These two receptors are likely to function synergistically because only one of the antibodies almost completely inhibits the activity of snaclec. However, only one of the receptor is sufficient for platelet activation by alboaggregin D because both antibodies are required to completely inhibit snaclec-induced platelet aggregation.

Examining the alboaggregin D-induced tyrosine phosphorylation profile in platelets, we found that it was fairly similar to those produced by collagen and alboluxin, except that platelets activated by alboluxin showed particularly strong Fcγ phosphorylation.

We hypothesize that alboaggregin D draws GPIb-IX-V and GPVI into close proximity resulting in synergistic signal transduction between these two receptors. Therefore, one snaclec molecule should contain separate GPIb and GPVI binding sites. These sites on the snaclec remain to be identified. Further studies on expression, characterization and mutagenesis of recombinant *C. albolabris* snaclecs are essential to elucidate this structure–function relationship. These experiments will give us deeper insights in molecular mechanisms of platelet activation induced by these two receptors.

Conflict of interest statement

None.

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Appendix. Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.toxicon.2011.10.009.

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The role of prothrombin time (PT) in evaluating green pit viper (*Cryptelytrops* sp) bitten patients

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ABSTRACT

Viper bites cause consumptive coagulopathy resulting in hypofibrinogenaemia. Whole-blood clotting time is a standard test used to assess bleeding risk. Prothrombin time (PT) and activated partial thromboplastin time (APTT) are better standardised assays that are widely available, but their diagnostic accuracy in viper bites remains unknown. Adult patients presumed bitten by green pit vipers (*Cryptelytops* sp.) were enrolled. Conventional venous clotting time (VCT), 20 min whole-blood clotting time (20WBCT), PT with international normalized ratio (INR) and APTT were determined. A fibrinogen level below 1.0 g/litre was used as the gold standard. There were 97 patients. The average age was 46.1 years and 49.5% were men. VCT >30 min, INR >1.2 and fibrinogen level <1.0 g/litre were found in 9.3, 10.3 and 7.2%, respectively. The sensitivities of VCT >30 min, 20WBCT (N = 55), INR and APTT were 57.0%, 85.7%, 85.7% and 57.1%, respectively. The respective specificities were 94.4%, 95.8%, 95.6% and 72.4%. Three hypofibrinogenaemic patients who did not receive antivenom because of VCT <30 min had persistently normal VCT and went home without clinical bleeding. In conclusion, PT with INR can be an alternative test for evaluation of coagulopathy in green pit viper bitten patients with potentially improved inter-laboratory standardisation.

1. Introduction

Worldwide, snakebite is a major, yet neglected, public health problem.[1] One of the main effects of snake venoms is on the human haemostatic system. Most of the viperid, and some elapid, venoms activate common pathways of blood coagulation resulting in consumptive coagulopathy.[2,3] Fibrinogen, which is the common final substrate of the coagulation cascade, is invariably low in this condition. However, fibrinogen assay is not generally available, especially in remote areas where snakebites are prevalent. Therefore, alternative blood tests are required for initial evaluation, follow-ups and monitoring responses after antivenom therapy in snakebite victims.

A simple 20-min whole-blood clotting test (20WBCT) has been shown to correlate with fibrinogen levels in patients bitten by *Bothrops* sp. in South America.[4] Consequently, the WHO recommended this method for evaluation of coagulopathy in snakebite patients.[5] In addition, a standard Lee and White venous clotting time (VCT), in conjunction with platelet counts, was shown to be predictive for systemic bleeding in a multivariate analysis of green pit viper bitten patients.[6] While these tests are relatively rapid and simple, their accuracy has rarely been evaluated. Furthermore, they are usually performed at the bedside by personnel not well trained in laboratory medicine and, therefore, they are subject to error. Training

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of the treatment team is critical for the reliability of these tests.

Prothrombin time (PT) and activated partial thromboplastin time (APTT) are better standardised tests with well-established quality control systems. They are available in many hospitals and can be automated, thus assuring their precision. In addition, the international normalized ratio (INR) values harmonise PT tests performed in different laboratories using different commercial reagents. However, data on their uses in viper bitten patients are lacking. We hypothesised that PT and APTT would be effective for the assessment of viper bitten patients.

2. Material and methods

This was a prospective observational study. The subjects were adults (age ≥15 years) bitten by presumed green pit vipers (*Cryptelytrops albolabris* or *C. macrops*) and presenting to the emergency department or snakebite clinic at King Chulalongkorn Memorial Hospital within 72 h of the bite from October 2008 to December 2010. The diagnosis of green pit viper bite was made on the basis of the dead snake brought with the patient or the victim's clear identification of a green snake with a red tail in conjunction with occurrence of the bite in Bangkok. The red tail is a specific and easily recognizable identifying characteristic of *C. albolabris* and *C. macrops*, the only two green pit viper species found in Bangkok.[7] Clinical manifestations of patients bitten by these species were previously described.[6] The exclusion criterion was the presence of any underlying coagulopathy, e.g. cirrhosis, anticoagulant use, haemophilia or von Willebrand disease.

The patients were managed according to the Thailand guideline for management of venomous snakebites.[8] After history taking and physical examinations, blood was sent for complete blood count (CBC) and three-tube Lee and White VCT. Patients with normal test results were followed as outpatients for a total of 3 days. The indications for antivenom were systemic bleeding, VCT >30 min, platelet count $<50 \times 10^9$/litre or impending compartment syndrome. VCT was performed by treating physicians in the emergency department or by laboratory technicians in the snakebite clinic.

The demographic data, information about the biting snakes, and local and systemic signs and symptoms of snakebite were recorded. After informed consent, an additional amount of blood was collected at the same time as the routine venepuncture of the patients. A subgroup of patients (from October 2008 to December 2009) also had 20-min whole blood clotting test (20WBCT) performed by physicians. The plasma was separated and kept at −70 °C until tested. APTT, PT and fibrinogen levels were determined using Sysmex CA 1500 Coagulation analyser (Dade Behring, Milton Keynes, UK). Fibrinogen levels were determined using a clotting-based Clauss method.[9] These special laboratory tests were performed only once. The treating physicians did not know these special test results except for the 20WBCT. The patients were managed according to the conventional VCT results.

A fibrinogen level of 1.0 g/litre was set as a gold standard as it has been accepted to be sufficient for haemostasis.[10] Additional analyses using hypofibrinogenaemia (fibrinogen below 1.7 g/litre) as a gold standard were calculated. The sensitivity and specificity of candidate cut-off points derived from the receiver operator characteristics (ROC) were also determined. Statistical analysis was carried out using SPSS version 16.0 statistical software (SPSS Inc., Chicago, IL, USA).

3. Results

There were a total of 109 patients. Twelve individuals were excluded from the study because the type of snake was unknown (7), there was underlying cirrhosis (2) or current warfarin intake (2), or the specimen collected was inadequate (1).

Ninety-seven individuals were included in the analysis. The mean age was 46.1 ± 16.6 years (mean ± SD), ranging from 15 to 82 years. Forty-eight of them (49.5%) were men. Twenty patients (20.6%) brought the dead snake, and the investigators (JP or PL) identified 16 snakes as *C. albolabris* and four as *C. macrops*.

Most of the patients (85.7%) came to the hospital within 24 h of the bite incident. The common sites of bites were hands (42.3%), feet (38.1%) and ankles (14.4%). Some patients (22.7%) were asymptomatic and 77.3% had local pain and swelling. Two of the patients (2.1%) had bleeding from the gums. A total of 12 patients (12.4%) received antivenom, 9 because their VCT was >30 min with or without bleeding and 3 because of marked oedema causing concern for possible compartment syndrome. All local and systemic effects recovered after antivenom. No patient in the study died.

Conventional VCTs of >20 min and >30 min were found in 18 (18.6%) and 9 (9.3%), patients respectively. Sixty-four (66.0%) of the VCTs were done by treating physicians. A 20WBCT was carried out by a doctor in 55 patients, in 8 of whom (14.5%) the WBCT was unclotted. Seven individuals (7.2%) had prolonged INR >1.2. Seven patients (7.2%) had a fibrinogen level <1.0 g/litre. The percentages of other laboratory abnormalities are listed in Table 1. As the table shows, the conventional VCT displayed the highest correlation coefficient with fibrinogen levels. Thrombocytopenia was found in three patients. All of them had prolonged VCT and PT and low fibrinogen levels. These three patients received antivenom.

Using fibrinogen levels below 1.0 g/litre as the gold standard, PT and INR showed the highest area under the ROC curve, while APTT was less able to distinguish hypofibrinogenaemic patients from the others (Table 1). Subsequently, sensitivity and specificity at various cut-off points of VCT and INR were calculated (Table 2). The diagnostic accuracy of 20WBCT and INR (>1.2) was superior to that of VCT (> 30 min) and APTT (at any cut-off points). However, PT (INR) was not sensitive when the fibrinogen level of 1.7 g/litre was used as a cut-off point.

Three patients had fibrinogen levels <1.0 g/litre but VCT <30 min. Because the treating doctors did not know the fibrinogen results, none of these three individuals received antivenom. They were followed for a total of 3 days and all daily VCTs remained normal without clinical bleeding.

Table 1
Correlation of the results of tests to assess bleeding risk with fibrinogen levels in 97 patients bitten by green pit vipers (*Cryptelytrops* sp)

Test	Normal range	Abnormality (%)	Correlation coefficient with fibrinogen levels	Area under ROC curves for fibrinogen <1.0 g/litre* (95% CI)
VCT	<15 min	31 (32.0%)	−0.330 (p = 0.001)	0.772 (0.591–0.954, p = 0.007)
APTT	23.08–30.12 s	27 (27.8%)	−0.232 (p = 0.022)	0.638 (0.409–0.867, p = 0.173)
PT	10.44–13.23 s	12 (12.4%)	−0.254 (p = 0.012)	0.866 (0.728–1.003, p < 0.001)
INR	0.92–1.16	15 (15.5%)	−0.252 (p = 0.013)	0.870 (0.731–1.009, p < 0.001)
Fibrinogen	1.7–4.0 g/litre	21 (21.6%)	1.0	Not applicable

* 7.2% of patients had fibrinogen <1.0 g/litre. APTT: activated partial thromboplastin time; INR: international normalized ratio; PT: prothrombin time; ROC: receiver operator characteristics; VCT: Lee and White venous clotting time.

Table 2
Sensitivity and specificity for fibrinogen levels of tests to assess bleeding risk (VCT, 20WBCT, INR and APTT) in patients bitten by green pit vipers (*Cryptelytrops* sp)

Laboratory cut-off point (N = 97)	Fibrinogen <1.0 g/litre		Fibrinogen <1.7 g/litre	
	Sensitivity	Specificity	Sensitivity	Specificity
VCT >15 min	71.4	71.1	66.7	77.6
VCT >20 min	57.1	84.4	47.6	89.5
VCT >30 min	57.1	94.4	33.3	97.4
INR ≥1.2	85.7	95.6	28.6	94.7
INR ≥1.3	83.3	98.9	23.8	98.7
INR ≥1.4	71.4	97.8	23.8	100
Prolonged APTT	57.1	72.4	28.6	72.4
APTT >31 s	57.1	77.8	28.6	76.3
APTT >32 s	42.9	81.1	23.8	80.3
APTT >33 s	42.9	87.8	19.0	86.8
20WBCT (n = 55)	85.7	95.8	50.0	97.6

APTT: activated partial thromboplastin time; INR: international normalized ratio; VCT: Lee and White venous clotting time; 20WBCT: 20-min whole blood clotting test.

Each was contacted by telephone a few weeks after the event, and none reported any late complication.

4. Discussion

In this study, prolongation of PT (INR >1.2) was found to be related to severe hypofibrinogenaemia (fibrinogen <1.0 g/litre), suggesting that it could be used as an indication for antivenom administration in green pit viper bitten patients. Prolongation of PT is caused by low fibrinogen, and by hyperfibrinolysis[11] releasing fibrin degradation products (FDPs) that interfere with fibrin polymerisation. The other clotting factors were not depressed in green pit viper victims.[12] Similar to previously reported data, PT was usually normal in mild hypofibrinogenaemia,[13] resulting in low sensitivity for the cut-off fibrinogen of 1.7 g/litre (Table 2). Therefore, a normal INR cannot exclude mild systemic envenomation or coagulopathy after viper bites. A point-of-care device is widely used for rapid determination of PT, especially in warfarin monitoring.[14] As the test measures generated thrombin, independent of fibrinogen levels, it is probably not useful for evaluation of viper bites, which affect mainly fibrinogen levels. In contrast, APTT showed lower diagnostic accuracy, in terms of sensitivity and specificity, than PT. In addition, inter-laboratory variation prohibits the use of a universal cut-off point for APTT, in contrast to the use of INR for PT. An interesting observational study showed the combination of APTT and PT to be useful for the evaluation and treatment monitoring of coagulopathy caused by elapid bites in Australia.[15]

Our data confirmed the accuracy of 20WBCT in predicting severe hypofibrinogenaemia.[4,5] The test is simple, rapid and inexpensive, and therefore applicable in most healthcare facilities. However, the present study included fewer 20WBCT than PT and VCT.

We used conventional VCT in this study because it is a quantitative test for which we might have been able to find a better cut-off point. We chose to have the test carried out by treating persons to reflect the real-life use of VCT as a bedside test. However, in our study the sensitivity of VCT (>30 min) for hypofibrinogenaemia was low (Table 2). One potential explanation is that VCT depends on both fibrinogen quantity and the FDP levels from fibrinolysis. In addition to hypofibrinogenaemia, hyperfibrinolysis,[11] thrombocytopenia[6] and vascular damage[16] probably contribute to clinical haemorrhage, and the three hypofibrinogenaemic patients with VCT <30 min in our series all recovered uneventfully. A fibrinogen level of 1.0 g/litre was used as a surrogate marker for haemostatic level.[10] The ideal gold standard would be clinical bleeding but this outcome is rare in green pit viper bites as the goal of treatment is to prevent this complication. Both of our bleeding patients had obvious prolongation of all the tested coagulation times. In our experience, VCT has been used in thousands of snakebite patients with no unexpected bleeding problems. Another possible reason for the insensitivity of VCT was that some of the tests were performed by physicians instead of laboratory personnel. The procedure was more complicated than the 20WBCT, and subject to analytical errors.

In conclusion, either PT or 20WBCT can be used for evaluation of green pit viper bitten patients depending on the convenience and availability of the tests. Prolongation of either test suggests severe hypofibrinogenaemia that requires antivenom therapy.

Authors' contributions: PR conceived the study. JP, PL and PR designed the study. JP, PL, JJ and BA analysed the data. JP, PL and PR interpreted the data. JP, PL and PR drafted the manuscript. JJ and BA critically revised the manuscript for intellectual content. All authors read and approved the final manuscript. PR is the guarantor of the paper.

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