Purification and Characterization of a Novel Metalloproteinase, Acurhagin, from Agkistrodon acutus Venom

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Keywords

Snake venom metalloproteinase, Agkistrodon acutus, fibrinogenase, autoproteolysis, platelet aggregation

Summary

Acurhagin, a high-molecular mass hemorrhagic metalloproteinase, was purified from the crude venom of Agkistrodon acutus using anion-exchange and hydrophobic interaction chromatography. Acurhagin is a monomer with a molecular mass of 51.4 kDa under non-reducing conditions on SDS-PAGE and 48,133 Da by mass spectrometry. Partial amino acid sequence of its metalloproteinase domain is homologous to other high-molecular mass metalloproteinases from snake venoms. It preferentially cleaved Αα chain of fibrinogen, followed by Ββ chain, while γ chains was minimally affected. Monitored by RP-HPLC, it extensively degraded fibrinogen into various peptide fragments. In aqueous solution, acurhagin autoproteolysed to a 30 kDa fragment at 37°C. The N-terminal sequence of the 30 kDa fragment of acurhagin showed a high homology to those proteins consisting of disintegrin-like and cysteine-rich domains. Caseinolytic assay showed that the proteinase activity of acurhagin was slightly enhanced by Ca²⁺ and Mg²⁺, but completely inhibited by Zn²⁺. When treated with metal chelators, acurhagin was completely inactivated. Furthermore, acurhagin exerts an inhibitory effect on ADP-induced platelet aggregation of platelet-rich plasma in an incubation-time dependent manner. It also impairs collagen- and ristocetin-induced platelet aggregation by cleaving collagen and vWF, respectively.

Introduction

The major four families of venomous snakes on the earth are Hydrophidae, Elapidae, Viperidae and Crotalidae. Generally, neuron toxins are found in the Hydrophidae and Elapidae venoms while hemorrhagic and myonecrotic toxins are found in the venoms of the other two families of snakes. However, proteases are present in most of the venoms except for the Hydrophidae venoms (1). Proteases so far isolated are generally classified by structure into serine proteases and metalloproteinases. Some of the serine proteases have both fibrinogenolytic and fibrinolytic activities, but a number of them have only fibrinogenolytic activity and also called “thrombin-like” proteinases (2). Snake venom metalloproteinases (SVMPs) belong to the metzincin family among several zinc-containing metalloproteinases (3, 4). They are characterized by the presence of a conservative zinc-binding sequence, HEXX-HXXGXXH, as an essential motif for the proteolytic activity. Chelation of the Zn²⁺ with EDTA or 1,10-o-phenanthroline deprives their enzyme activities.

SVMPs are classified according to their domain structure into four basic groups, P-I (protein class I) to P-IV (1). All four groups share a metalloproteinase domain (about 200 residues) containing the Zn²⁺-binding motif. The P-I class (low molecular mass metalloproteinase) only has a metalloproteinase-domain structure, such as HR2a (5), HT-2 (6), arthrotin (Ht) B, C, E (7, 8), LHF-II and trimereleisin II (1). The P-II class (disintegrin precursor) has an additional domain carboxy to the proteinase domain, a disintegrin or disintegrin-like domain, such as arthrotin E and precursor proteins of trigamin and rhodostomin (1). The P-III class (high-molecular mass metalloproteinase) has both a disintegrin-like domain and a high-cysteine domain carboxy to the proteinase domain, such as trimereleisin I (HR1B) (9), arthrotin A (Ht-a) (10), breviliesin H6 (11), catrocollastatin (12), moccarnhagin (13, 14), kaouthiagin (15, 16) and jararhagin (17). All of these proteinases are potent hemorrhagic toxins, exhibiting stronger hemorrhagic activity than the lower molecular mass toxins, strongly suggesting the importance of the disintegrin-like and high-cysteine domain for the hemorrhagic activity of these toxins. Another class, P-IV (high-molecular mass metalloproteinase with C-type lectin domain), has a similar domain structure to the P-III class, but with an additional lectin-binding domain, such as russelysin (RVV-X), carinactase-I and mucrotoxin A (1, 3).

In addition to degradation of plasma proteins such as serine proteinase inhibitors (serpins), fibrinogen and cross-linked fibrin, metalloproteinases are able to degrade many constituents of the extracellular matrix such as basement membrane, collagen, proteoglycans, fibronectin, nidogen and laminin. Thus, they are implicated in connective tissue remodeling and blood coagulation processes associated with tissue invasion, hemorrhage, necrosis, apoptosis, embryonic development, cell growth and wound repair (18-20). Furthermore, these proteins have potential clinical use in dissolving thrombi than thrombin-like proteinases, which only decrease fibrinogen but do not affect thrombi (21).

In this paper, we purified a high-molecular mass metalloproteinase, termed acurhagin, from Taiwanese A. acutus (22). We found that it is a monomer with an apparent molecular mass of about 51.4 kDa and cleaves preferentially the Αα- and Ββ- chains of fibrinogen with minimal

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Abbreviations: A. acutus, Agkistrodon acutus; B. jararaca, Bothrops jararaca; C. atrox, Crotalus atrox; G. h. brevicaudus, Gloydius halys brevicaudus; T. flavoviridis, Trimeresurus flavoviridis; T. gramineus, Trimeresurus gramineus; MHD, minimum hemorrhagic dose; GP, glycoprotein; PRP, platelet-rich plasma; WPS, washed platelet suspension; vWF, von Willebrand factor; SVMP(s), Snake venom metalloproteinase(s)
attack to the γ-chain. This 51.4-kDa enzyme exhibits autoproteolytic degradation, generating one fragment with a molecular mass of 30 kDa. Partial N-terminal sequence of 30 kDa-fragment showed a high homology with the spacer region connecting metalloproteinase and disintegrin-like domains. In addition, the effects of acurhagin on platelet aggregation induced by ADP, collagen and ristocetin were investigated.

Materials and Methods

Reagents. The crude venom of Formosan A. acutus was provided by Dr. M. Y. Liau at National Institute of Disease Control, Taipei, Taiwan. DEAE-Sephadex A-50, FPLC/Mono-Q HR 5/5, FPLC/RESOURCE PHE and FPLC/Superdex 200 columns were purchased from Pharmacia (Sweden). Acrylamide, BSA, SDS, dithiothreitol (DTT), tris (hydroxymethyl) aminomethane (Tris), collagen (type I, bovine achilles tendon), PGE1, EDTA, azocasein, 1,10-phenanthroline, ristocetin, apyrase and elastase were obtained commercially from Sigma Chemical Co. (St. Louis, MO, USA). Molecular-mass standards for electrophoresis were purchased from Bio-Rad. Acutobin (a thrombin-like proteinase) and agglucetin (a platelet GPIb agonist) were purified from the venom of A. acutus as previously described (23, 24). Human vWF and fibrinogen were obtained from Calbiochem-Novabiochem Corporation (La Jolla, CA). Bovine thrombin was obtained from Parke Davis (Detroit, MI). Fluorescein isothiocyanate (FITC) was purchased from Molecular Probes (Eugene, OR). Monoclonal antibody (mAb) 7E1 against platelet GP IIb/IIIa complex and mAb 6F1 against GPIIb/IIa were kindly supplied by Dr. Coller (The Mount Sinai Medical Center, New York, NY, USA). MAb AP1 against GP Iba was generously provided by Dr. Montgomery (The Blood Center of Southeastern Wisconsin, Milwaukee, WI).

Isolation and purification of acurhagin. Crude venom of A. acutus was applied to a DEAE-Sephadex A-50 column (1.6 × 20 cm) equilibrated with 0.05N ammonium acetate (pH 8.5). The column was eluted at a flow rate of 20 ml/hr with buffer gradient. The eluates were monitored continuously by LKB Uvicord (278 nm) at 4°C. Gradient elution was carried out in three stages: (1) 0.05N ammonium acetate (pH 8.5), 240 ml; (2) 0.05N ammonium acetate (pH 8.5) vs. 0.25N ammonium acetate (pH 6.5), 160 ml; (3) 0.25N ammonium acetate (pH 6.5) vs. 0.8N ammonium acetate (pH 5.5), 240 ml, and 2 ml/tube were collected. The fraction exhibiting fibrinogenolytic and caseinolytic activity was collected, dialyzed and lyophilized for further purification. Secondly, the active fraction was applied to a FPLC / Mono-Q HR 5/5 column (0.5 × 30 mm) pre-equilibrated with 0.02N Tris-HCl buffer, pH 7.8. Elution was carried out with B buffer (1 N NaCl in 0.02 N Tris-HCl buffer, pH 7.8) gradient at room temperature. The elution was monitored at 280 nm, and fractions were collected. Finally, the active fractions were subjected to a FPLC/RESOURCE PHE column (6.4 × 30 mm) equilibrated with 50 mM potassium phosphate, pH 7.0, containing 1.7M ammonium sulfate. The flow rate was adjusted to 0.5 ml/min with a linear gradient of 1.7 – 0 M ammonium sulfate in 50 mM potassium phosphate, pH 7.0. The active fractions found in the major peak were collected and termed as acurhagin.

SDS-PAGE. SDS-PAGE was performed according to the method of Mini Protein II dual slab gel (Bio-Rad) and the gel was subjected to electrophoresis and stained with Coomassie Blue.

Protein assay. Protein concentration was measured by the BCA protein assay (Pierce Chemical Co.) using bovine serum albumin as a standard.

Determination of hemorrhagic activity. Hemorrhagic activity was assayed by the previously reported method (25). 50 μl of test samples dissolved in 0.9% saline (26) was injected subcutaneously into the depilated skin of the back trunk of SCI mice (22-27 g). Twenty hr later, the animals were sacrificed under ether anesthesia and the skins were removed. The hemorrhagic spots were measured from the inside of skin. The amount of venom that produced a hemorrhagic spot was determined. Human vWF and fibrinogen (type I, bovine achilles tendon), PGE1, EDTA, azocasein, 1,10-phenanthroline, ristocetin, apyrase and elastase were obtained commercially from Sigma Chemical Co. (St. Louis, MO, USA). Molecular-mass standards for electrophoresis were purchased from Bio-Rad. Acutobin (a thrombin-like proteinase) and agglucetin (a platelet GPIb agonist) were purified from the venom of A. acutus as previously described (23, 24). Human vWF and fibrinogen were obtained from Calbiochem-Novabiochem Corporation (La Jolla, CA). Bovine thrombin was obtained from Parke Davis (Detroit, MI). Fluorescein isothiocyanate (FITC) was purchased from Molecular Probes (Eugene, OR). Monoclonal antibody (mAb) 7E1 against platelet GP IIb/IIIa complex and mAb 6F1 against GPIIb/IIa were kindly supplied by Dr. Coller (The Mount Sinai Medical Center, New York, NY, USA). MAb AP1 against GP Iba was generously provided by Dr. Montgomery (The Blood Center of Southeastern Wisconsin, Milwaukee, WI).

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Fibrinogenolytic activity assay. Twenty μg of human fibrinogen (1 mg/ml) in 0.9 % saline was incubated at 37°C for 5, 15, 30 or 60 min in the presence of 20 μg/ml (0.5 μM) acurhagin. The reaction was terminated by adding sample buffer and boiled for 10 min at 90°C. Samples were subjected to 12% SDS-PAGE under reducing conditions and stained with Coomassie Blue.

Fig. 1 Purification profile of acurhagin from A. acutus venom. (A) The crude venom of A. acutus was subjected to a DEAE-Sephadex A-50 column equilibrated with 0.05 N ammonium acetate, pH 8.5. Elution was performed at 20 ml/hr with a buffer gradient as indicated. Fraction X (tube number, * 282-294) possessing caseinolytic and fibrinogenolytic activity was collected and lyophilized. (B) Anion-exchange chromatography on a FPLC/Mono-Q HR 5/5 column eluted with B buffer (1 N NaCl in 0.02 N Tris-HCl buffer, pH 7.8) gradient as indicated (a dashed line) at a flow rate of 1 ml/min. The active fractions (*) were pooled for further purification. (C) Hydrophobic interaction chromatography on a FPLC/RESOURCE PHE column eluted with a gradient from 1.7 to 0 M ammonium sulfate in 50 mM potassium phosphate, pH 7.0 at a flow rate of 0.5 ml/min. The major fractions were pooled and concentrated. mAU, milli absorbence unit.
Autodigestion assay. Five μg acurhagin in 0.02N Tris-HCl buffer (pH 7.8) was incubated at 37 °C in the absence or presence of 5 mM EDTA for 1–12 h and then the reaction was quenched by adding sample buffer under non-reducing and reducing conditions. The autoproteolytic pattern shown on 15% SDS-PAGE was visualized by Coomassie staining.

Caseinolytic activity assay. Caseinolytic activity was measured colorimetrically by a modified procedure described by Chowdhury et al. (27). The reaction mixture, consisting of 85 μl acroazocasein (4.25 mg/ml) in 50mM Tris-HCl buffer (pH 8.0), 10 μl acurhagin (100 μg/ml) in Tris buffer and 5 μl Tris buffer or solutions (5 mM) of various metal ions or chelators, was incubated for 90 min at 37 °C. The reaction was quenched by the addition of 200 μl of 5% trichloroacetic acid at room temperature. After centrifugation at 1,000 × g for 5 min, 150 μl of the supernatant was mixed with the equal volume of 0.5 M NaOH and absorbance was measured at 450 nm.

Preparation of fibrinopeptides from human fibrinogen. According to the method described previously (28), twenty μg of human fibrinogen was incubated with 2 U/ml thrombin, 1 μg/ml of acutobin or 10 μg/ml of acurhagin for 4 hr at 37 °C. Then the mixture was heated for 5 min at 95 °C to terminate the reaction and to precipitate any fibrinogen or fibrin not incorporated into the clot. The mixture was centrifuged and the supernatant obtained was lyophilized and analyzed by reverse-phase HPLC. Separation of fibrinopeptides were performed with a Waters C18 column (3.9 × 150 mm) using a linear gradient from 5 to 50% acetonitrile in 0.1% trifluoroacetic acid, at a flow rate of 0.8 ml/min.

Mass spectrometry analysis. Mass measurements were performed with a Finnigan MAT ion-trap LCQ mass spectrometer. Ten μg of acurhagin dissolved in 50% acetonitrile solution containing 1% acetic acid was injected into analyzer for positive mode analysis. The spectra were analyzed with a software (LCQ BioWorks) from the manufacturer.

Reductive alkylation of acurhagin and acurhagin-30K. The reaction was performed according to the protocol described by Wu et al. (29) with a minor modification. Acurhagin (200 μg) was reduced for 1 hr with 20 mM dithiothreitol in 6M guanidinium chloride/0.5 M Tris/HCl (pH 8.0)/1mM EDTA at 37 °C and subsequently S-carboxymethylated by vinylpyridine (20 mM) for 1 hr. The alkylated protein was cleaved for 15 hr by CNBr in 70% (v/v) formic acid at 37 °C. The resultant peptides were purified by reverse-phase HPLC on a Waters C8 column (3.9 × 150 mm) with a gradient from 5-45% acetonitrile in 0.1% (v/v) trifluoroacetic acid and then the sequences of peptide fragments were determined. After reductive alkylation, fifteen μg of acurhagin-30K, the autoproteolytic fragment with a molecular mass of 30 kDa from acurhagin, was separated by 15% SDS-PAGE and electroblotted to a polyvinylidene fluoride (PVDF) membrane. After staining with Coomassie Blue, the 30K-fragment was subjected to N-terminal sequence analysis.

Amino acid sequence analysis and sequence identity search. Amino acid sequencing was performed on a PROCISE 494 protein sequencer (Perkin Elmer/ABL, Foster City, CA, USA) equipped with an on-line detection of phenylthiohydantoin derivatives. Sequence identity was searched in the protein sequence database of Swiss-Prot. This alignment of N-terminal amino acid sequence and sequence identity were calculated by the GCG program (Wisconsin Package Version 10.1 Genetics Computer Group, 2000).

Preparation of human platelets and aggregation assay. Human blood collected in acid citrate dextrose (9:1, v/v) or in 3.8% sodium citrate (9:1, v/v) was centrifuged at 100 × g at room temperature for 10 min to obtain platelet-rich plasma (PRP). Washed platelets were suspended in Tyrode’s solution and the platelet count was adjusted to 3 × 10^10 platelets/ml. In the elastase-treated platelet preparations, the concentration of elastase in the incubation mixture was 1.25 U/10^9 platelets/ml. After reductive alkylation, fifteen μg of acurhagin-30K, the autoproteolytic fragment with a molecular mass of 30 kDa from acurhagin, was separated by 15% SDS-PAGE and electroblotted to a polyvinylidene fluoride (PVDF) membrane. After staining with Coomassie Blue, the 30K-fragment was subjected to N-terminal sequence analysis.

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Flow Cytometry. Human washed platelets (3 × 10^10/m) were reconstituted with phosphate buffer saline or acurhagin (10 μg/ml) at 37 °C for 30 min and then the primary monoclonal antibody against GPIb/IIIa (7E3; 20 μg/ml), GPIa/IIa (6F1; 20 μg/ml), GPIb (AP1; 20 μg/ml) or mouse nonimmune IgG as negative control; 1:50 dilution) was added at room temperature for a further 30 min-incubation, then followed by probing with secondary FITC- goat antimouse IgG (CALTAG Lab. Burlingame, CA) at 4 °C for 30 min. Platelets were then analyzed with FACS Calibur (Becton Dickinson, Mountain View, CA) using excitation and emission wavelengths of 488 and 525 nm, respectively. Fluorescence signals from 10,000 gated cells were collected to calculate mean fluorescence intensity of single platelet.

Statistics. All data were presented as mean ± SEM (n). Paired Student’s t-test was used to assess the significant statistical differences.

Results

Isolation and purification of acurhagin. The crude venom of A. acutus was separated into ten fractions by DEAE-Sephadex A-50 column (Fig. 1A). The last fraction exhibiting caseinolytic and fibrinolytic activity was collected for further purification. The desalted, lyophilized fraction was further refractonated by anion-exchange chromatography on FPLC/Mono-Q column (Fig. 1B). The active fractions were collected and concentrated. The final step of purification was performed by hydrophobic interaction chromatography on FPLC/RESOURCE PHE column (Fig. 1C). The active component corresponding to an elution volume of 12 ml exhibited a minimum hemorrhagic dose (MHD) of 1 μg in causing hemorrhage as administered subcutaneously. Based on the consideration of species origin (Acu-) and the hemorrhagic activity (-rhagin) of the metalloproteinase, we named it as acurhagin. As shown in Fig. 2A, the apparent molecular mass of acurhagin was 51.4 and 56 kDa, respectively, as determined by SDS-PAGE under nonreducing and reducing conditions. Its molecular mass of native acurhagin was estimated to be 53.4 kDa by gel filtration on FPLC/Superdex 200 column (data not shown) and 48,133 Da by electrospray ionization mass spectrometry (Fig. 2B), respectively. Thus, it exists as a single-chain protein.
Comparison of amino acid sequence of acurhagin with members of snake venom metalloproteinases. The failure of a direct N-terminal sequencing suggests that this proteinase was probably N-terminally blocked. Therefore, S-pyridylalkylated acurhagin was subsequently digested by CNBr. The cleaved fragments were further purified by reverse-phase HPLC for sequence analysis. One particular sequence was aligned in Table 1. An identity of 50~86% of acurhagin with members of snake venom metalloproteinases was observed. Of particular interest is that this partial sequence is highly homologous to brevilysin H6 (11), catrocollastatin (30) and jararhagin (31), which all belong to P-III class (possessing a three-domain structure, namely metalloproteinase, disintegrin-like and cysteine-rich domain) of metalloproteinases.

Fibrinogenolytic and fibrinopeptide-degrading activity of acurhagin. Fibrinogen is a 340-kDa dimeric glycoprotein consisting of a pair of three polypeptide chains Aα, Bβ and γ which are interconnected by disulfide bonds and can be cleaved into an insoluble fibrin clot as a consequence of thrombin-catalyzed removal of fibrinopeptides A (FPA, Aα-(20-35)) and B (FPB, Bβ-(31-44)) from the Aα and Bβ chains, respectively (18). Accordingly, proteolytic enzymes of snake venoms have been defined as α-, β- or γ-fibrinogenases based strictly on their direct cleaving activity in vitro on fibrinogen molecules (32). Therefore, we assayed the fibrinogenolytic activity of acurhagin to characterize its proteolytic activity towards this matrix protein. Human fibrinogen was incubated with acurhagin at 37° C for various time intervals. The reaction products were then analysed by SDS-PAGE patterns under reduced conditions. In order to distinguish the different cleaving activity between serine proteinase and metalloproteinase, the SDS-PAGE patterns of acutobin, a 40 kDa thrombin-like Aα fibrinogenase from A. acutus (23), were also examined. SDS-PAGE analysis showed acurhagin degraded predominantly the Aα- and Bβ-chains of fibrinogen whereas the γ-chain was apparently unaffected (Fig. 3B). Thus, it belongs to the members of α-, β-fibrinogenase family. As shown in Fig. 3A and 3B, a band with a molecular mass of ~ 43 kDa appeared after 15 min-incubation and its intensity gradually increased with time lapse in case of acutobin-treated fibrinogen whereas several new bands around 43~35 kDa appeared after 5 min-incubation in case of acurhagin-treated fibrinogen.

Table 1  Comparison of the partial amino acid sequences of the metalloproteinase domain and intact molecular mass of acurhagin with other members of snake venom metalloproteinases

<table>
<thead>
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<th>Venom protein</th>
<th>Amino acid sequence</th>
<th>Mw (kDa)</th>
<th>% Identity</th>
<th>Protein source</th>
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<td>A. acutus</td>
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<td>52</td>
<td>79</td>
<td>A. carinata (31)</td>
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</table>

The 14 residues of acurhagin were obtained by RP-HPLC isolation following the CNBr digestion of purified acurhagin. The apparent molecular mass of venom protein was estimated by SDS-PAGE under non-reducing conditions. Highly homologous residues are shaded. Bold-typed residues are conserved sequence.
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gen, indicating different cleaving sites on fibrinogen molecule for acutobin and acurhagin. Further confirmed by degrading patterns on reverse-phase HPLC, incubation of fibrinogen with acurhagin resulted in producing many fibrinopeptide fragments, indicating an extensive hydrolysis of the molecule (Fig. 4C). Its profile is apparently different from hydrolysis products caused by thrombin, i.e., two major peptides corresponding to fibrinopeptides A and B (Fig. 4A), and by acutobin, releasing only fibrinopeptide A (Fig. 4B).

Autoproteolytic digestion of acurhagin. It is well-known that hemorrhagic metalloproteinases with a molecular mass range of 50–60 kDa may undergo autoproteolysis, releasing a fragment with a molecular mass of ~30 kDa in the absence of Ca²⁺, such as catrocollastatin, HR1A, HT-1, brevilysin H6 and jararhagin (11, 33-35). The identified sequences of these fragments were derived from the disintegrin-like domain and cysteine-rich domain of their parent proteins. As shown in Fig. 5, we also found that acurhagin autodegraded and released a 30 kDa-fragment in the aqueous solution at 37° C upon 1 hr incubation, identified by SDS/PAGE patterns. However, EDTA apparently suppressed the formation of the 30 kDa after an incubation time of 12 hr. In addition, we observed that two additional higher molecular mass proteins of 191.3 and 129 kDa and one additional smaller molecular mass protein of 6.9 kDa were observed under gel filtration chromatography on a FPLC/Superdex 200 column in the presence of EDTA (data not shown), implying reassembling and polymerization of the native and/or proteolytic fragments may occur in the presence of EDTA under native conditions.

Comparison of N-terminal sequence of 30 kDa-fragment of acurhagin with snake venom metalloproteinases containing disintegrin-like and cysteine-rich domains. The N-terminal sequence of the 30 kDa-fragment of acurhagin (acurhagin-30K) was similar to the spacer region between the metalloproteinase and disintegrin-like/cysteine-rich domain of snake venom metalloproteinase. As shown in Table 2, an identity of 69–88% of acurhagin-30K with those of snake venom me-

Fig. 4 Elution profiles of fibrinopeptides using RP-HPLC. Elution profiles of fibrinopeptides released from human fibrinogen by (A) human thrombin (2 U/ml), (B) acutobin (1 μg/ml) and (C) acurhagin (10 μg/ml). FPA and FPB refer to fibrinopeptide A and B, respectively. Chromatography was performed by using buffer A and B according to a buffer gradient described in Methods.

Fig. 5 SDS-PAGE analysis of the autoproteolysis of acurhagin. Acurhagin (5 μg) was incubated at 37° C in aqueous solution in the absence or presence of 5 mM EDTA for indicated time intervals (1–12 h). The reaction was quenched by adding sample buffer and boiled for 10 min at 90° C. Aliquots were subjected to 15% SDS-PAGE under non-reducing (NR) and reducing (R) conditions. Note that high molecular mass bands shown by an arrow could be observed in the presence of EDTA under non-reducing conditions, indicating complex formation of acurhagin.
talloproteinases mentioned above was observed. Surprisingly, this partial sequence has the highest homology to catrocollastatin and jararhagin, which belong to class P-III metalloproteinase and also undergo autoproteolytic digestion. It indicates that acurhagin consists of disintegrin-like, cysteine-rich as well as metalloproteinase domains within its molecule, based on their homologous partial sequence and molecular mass. Further comparison of sequence alignment, those proteinases cleave preferentially at the N-terminal of Leu or Ile residue (Table 2). Moreover, it has been demonstrated that P-III venom hemorrhagic metalloproteinases, such as HR1B as well as Ht-a (atrolysin A), are susceptible to autoproteolysis in generating fragments comprised of various domain structure. Of note, the N-termini of those proteins begin at Leu residue, which usually located at the junction of the metalloproteinase and spacer domains (36). Additionally, a conserved Val residue was displaced by Leu residue in acurhagin-30K molecule.

**Caseinolytic activity of acurhagin.** The effect of various reagents on the caseinolytic activity of acurhagin was investigated in 50 mM Tris-HCl buffer at pH 8.0. As shown in Fig. 5, the % relative enzymatic activity of acurhagin was slightly enhanced by the addition of 5 mM Ca\(^{2+}\) (127.5 ± 0.8%; n = 3) or 5 mM Mg\(^{2+}\) (118.7 ± 1.4%; n = 3), but was completely inhibited by the addition of 5 mM Zn\(^{2+}\) (residual activity 2.3 ± 0.1%; n = 3), and by 5 mM chelating agents such as EDTA (residual activity 15.0 ± 0.2%; n = 3) and 1,10-o-phenanthroline (4.4 ± 0.2%; n = 3), indicating acurhagin is a divalent metal ion-dependent metalloproteinase. Compared with intact acurhagin, 30 kDa-fragment of acurhagin showed little caseinolytic activity (4.5 ± 0.3% of control; n = 3).

**Effect of acurhagin on platelet aggregation.** Upon its pretreatment with platelet-rich plasma (PRP), acurhagin exhibited an inhibitory effect on ADP-induced platelet aggregation in a time-dependent manner (Fig. 7A), which may result from the plasma fibrinogen degradation by

![Fig. 6](image_url)
Fig. 7  Effects of acurhagin on ADP-, fibrinogen- and collagen-induced platelet aggregation. (A) Acurhagin was preincubated with human platelet-rich plasma (PRP) at 37° C for 15, 30 or 60 min, and then ADP was added to trigger platelet aggregation. The control (CTL) tracing was shown at right panel. (B) Acurhagin was preincubated with fibrinogen at 37° C for 10 min, and then the acurhagin-treated fibrinogen was added to trigger aggregation of elastase-treated platelets. Intact fibrinogen (50 μg/ml) was re-added 3 min later to elicit aggregation response. The control tracing was shown at left panel. (C) Acurhagin was preincubated with PRP for 10 or 60 min, and then collagen was added to trigger platelet aggregation. The control (CTL) tracing (60 min in the absence of acurhagin) was shown at middle panel. The right two panels showed the tracing patterns of platelet aggregation caused by acurhagin-treated collagen at 37° C for 5 or 10 min, respectively.

Fig. 8  Effects of acurhagin on ristocetin- and agglucetin-induced platelet agglutination. (A) Human vWF was preincubated with washed platelet suspension (WPS) in the absence (trace CTL; for 6 min) or presence of acurhagin (for 6 or 12 min) at 37° C, and then ristocetin was added to trigger platelet aggregation. In the case of 12 min pretreatment, intact vWF was re-added at the time shown by an arrow (↑). (B) vWF was preincubated with washed platelets for 12 min, and then acurhagin was added immediately prior to the addition of ristocetin. (C) vWF was co-incubated with WPS and EDTA (5 mM)-treated acurhagin at 37° C for 12 min, and then ristocetin was added to induce platelet aggregation. (D) Human WPS was preincubated with Tyrode’s buffer (CTL) or acurhagin at 37° C for 12 min, and then agglucetin (10 μg/ml), a platelet GP Ib agonist, was added to trigger platelet agglutination.
acurhagin. Thus we also examined its effect on fibrinogen-induced platelet aggregation in elastase-treated platelets, which expose the functional GPIIb/IIIa complex on platelet membrane upon elastase treatment (37). After being incubated for 10 min with acurhagin, fibrinogen apparently lost its activity to aggregate platelets (Fig. 7B). However, the re-addition of intact fibrinogen restored full platelet aggregation. This result indicates that the degradation of fibrinogen by acurhagin is responsible for its inhibitory activity, and it apparently has little effect on the binding activity of GPIIb/IIIa toward fibrinogen. In contrast to catrocollastatin (30), acurhagin exhibited no inhibitory effect on collagen-induced platelet aggregation when it was incubated with PRP for 60 min (Fig. 7C). However, as we pretreated collagen with acurhagin for 5 or 10 min, collagen-induced platelet aggregation was profoundly impaired, indicating that acurhagin may possess a collagen-cleaving and/or binding activity. Crovidisin, a collagen-binding metalloprotease, inhibits collagen-induced platelet aggregation without the need for collagen-pretreatment (38). On the other hand, SDS-PAGE analysis of the degradation of collagen type I by acurhagin revealed that three degradation fragments (119, 105 and 101 kDa) were observed (data not shown). Therefore, the collagen-cleaving activity of acurhagin is predominantly responsible for its effect. In order to check if it affects vWF-GP Ib interaction, pre-incubation of vWF with acurhagin (10 µg/ml) for 12 min deprived completely of the ristocetin-induced aggregation of washed platelets (Fig. 8A). However, re-addition of vWF restored the ristocetin-induced platelet aggregation (Fig. 8A, right panel). The inhibitory effect of acurhagin was not observed when it was added immediately prior to the addition of ristocetin (Fig. 8B). EDTA-pretreated acurhagin failed to inhibit ristocetin-induced platelet aggregation after an incubation time of 12 min (Fig. 8C). Additionally, acurhagin showed little effect on agglucetin (a platelet membrane GP Ib agonist)-induced platelet agglutination (Fig. 8D), indicating that acurhagin did not affect GP Ib function even after a 12 min-incubation, in agreement with the results obtained from Fig. 8A. Like jararhagin (39), SDS-PAGE analysis showed that acurhagin rapidly decreased the band intensity of 225 kDa-vWF monomer and concomitantly increased a band intensity of vWF-degrading fragment with a molecular mass of 58 kDa after 5 min-incubation under reducing conditions (data not shown). On the other hand, flow cytometric analysis showed that acurhagin-
treated platelets (30 min-incubation) were found with little alterations of GPⅡb/Ⅲa, GP Ia/Ⅱa or GP Ibα on platelet membrane, as monitored by the binding capacity of the respective monoclonal antibody 7E3, 6F1 or AP1 (Fig. 9). Thus, we conclude that acurhagin is a matrix-cleaving rather a membrane glycoprotein-cleaving proteinase, and its vWF, collagen, or fibrinogen-cleaving activity is responsible for its inhibitory effect on ristocetin, collagen or ADP-induced platelet aggregation, respectively.

Discussion

In this study, we purified a novel metalloproteinase, acurhagin, from venom of A. acutus. On the basis of several lines of evidence listed below, we suggest that acurhagin is a member of high-molecular mass metalloproteinase family. First, the molecular mass of acurhagin on SDS/PAGE (51.4 kDa) is similar to those of P-III class proteinases containing three-domain structure. Second, the partial sequence of metalloproteinase domain of acurhagin revealed a high homology to those of P-III proteinases such as catrocollastatin and jararhagin. Third, acurhagin underwent autoproteolytic degradation, generating a 30-kDa fragment, and its N-terminal sequences are homologous to those sequences of naturally occurring catrocollastatin- or jararhagin-derived fragments, which all contain disintegrin-like and cysteine-rich domains. Autoproteolysis was inhibited in the presence of EDTA, suggesting that it involves with the metalloproteinase enzymatic activity of acurhagin and might be a general mechanism for the processing of venom metalloproteinase of high-molecular mass in venomous gland.

The exact mechanism of complex formation of acurhagin in the presence of EDTA under non-reducing conditions is not clear. Based on the suggestions by Zhang et al. (40) that both Ca2+ and Zn2+ are tightly bound to AaII molecule, an acidic hemorrhagic metalloproteinase from A. acutus venom, we proposed that the complex formation (i.e. high-molecular mass of 191.3, 129 kDa) may be due to formation of a ternary complex among EDTA, metal ions and approximately 3 to 4 molecules of acurhagin or its proteolytic fragments, resulting from intrinsic metal ions tightly bound to acurhagin. Under reduced system, the high-molecular mass complex formation was negligible, implying that the steric conformation maintained by disulfide bonds is essential for the complex formation. Our data regarding the effect of Ca2+, Mg2+ and chelating agents on the caseinolytic activity of acurhagin were consistent with the view that most snake venom hemorrhagins are divalent ion-dependent metalloproteinases. It was unexpected to observe a complete inhibition of caseinolytic activity of acurhagin by adding Zn2+. In addition to having an intrinsic essential Zn2+-binding site for its proteolytic activity, we postulate that acurhagin might have other Zn2+-binding sites with different binding affinity, which may be occupied by high concentration of Zn2+, resulting in a conformational change and loss of proteolytic function. However, the exact mechanism remains to be elucidated.

Acurhagin antagonized ADP-induced platelet aggregation under a prolonged incubation of PRP with acurhagin (Fig. 7A). This effect might be the consequence of depletion of fibrinogen since fibrinogen is indispensable to cross-link the platelet membrane GPⅡb/Ⅲa in causing platelet aggregation. It is well-documented that fibrinogen binding to activated GPIIb/Ⅲa is mediated through two specific binding motifs. One binding site contains an Arg-Gly-Asp sequence including Ao 95-97 and Ao 572-574, and the other is the dodecapeptide 400-411 (HHLGGAKQAGDV) at the C-terminal end of the γ-chain (39). The nonspecific α, β-fibrinogenolytic activity of acurhagin may affect the binding capacity of fibrinogen toward GPⅡb/Ⅲa. Regarding its effect on vWF-GPⅡb interaction, although we did not thoroughly investigate the proteolysis of vWF in plasma, pre-treatment of platelet-rich plasma with acurhagin also inhibited platelet agglutination caused by ristocetin (data not shown), consistent with our observations with washed platelet preparations (Fig. 8A). It is possible that acurhagin cleaves vWF and alters the conformation of the essential binding motif of vWF molecule, diminishing the binding capacity of vWF to platelet GPⅡb.

Our data showed that acurhagin did not abolish ADP-induced platelet aggregation under a 5 min-incubation with PRP in agreement with jararhagin and catrocollastatin (39, 41). Nevertheless, a longer incubation than 30 min significantly impaired ADP-induced platelet aggregation. On the other hand, acurhagin did not affect collagen-induced platelet aggregation in PRP except a prior co-incubation with collagen, indicating that collagen-cleaving activity is essential for its inhibitory effect on collagen-induced platelet aggregation. Crovidisin and catrocollastatin have been reported to inhibit collagen-induced platelet aggregation through their specific binding to collagen (12, 38). Furthermore, jararhagin was reported to exert proteolytic activity on α2β1 integrin (GPⅡb/Ⅲa) (41), however acurhagin and crovidisin apparently did not affect α2β1 as evidenced by the observation that preincubation of platelets with acurhagin did not decrease the binding capacity of the specific mAb (6F1) against α2β1 (Fig. 9). Although we could not explain the apparent discrepancy among them, the study of the structure-function activity among those metalloproteinases may be rewarding in future.

In conclusion, we have identified a novel high-molecular mass hemorrhagic metalloproteinase, acurhagin, a new member of P-III class metalloproteinases. Acurhagin is an effective enzyme in hydrolyzing many extracellular matrices, such as collagen, fibrinogen and vWF. Considering the importance of these extracellular matrix proteins in maintaining the structural and functional integrity of the extracellular matrix, one can readily understand that enzymatic degradation of the extracellular matrices surrounding capillary endothelial cells by acurhagin could be one of the principal mechanisms for the bleeding associated with Viperidae envenomation. However, if an optimal dosage of acurhagin is intravenously given to animal, one would expect that its enzymatic activity on plasma soluble matrices, such as fibrinogen and vWF, results in the impairment of platelet aggregation by interfering with the interaction of fibrinogen-GPⅡb/Ⅲa, and vWF-GPⅡb. Therefore, whether acurhagin and its derivatives can be utilized as an antithrombotic agent without causing hemorrhage remains to be investigated.

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