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A novel P-I class metalloproteinase with broad substrate-cleaving activity, agkislysin, from *Agkistrodon acutus* venom☆

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Abstract

The venom of *Viperdae* is a rich source of metalloproteinases, which have potential clinical applications for lowering plasma fibrinogen or dissolving thrombi. Recently, we purified a novel proteinase from Formosan *Agkistrodon acutus* venom using DEAE–Sephadex A-50 and Mono-Q HR 5/5 column chromatography. The purified getatinolytic component, agkislysin, is a 22 kDa-monomeric protein without Asn-linked sugar. Functional characterization showed that agkislysin possessed both fibronectin- and type IV collagen-cleaving activities. In addition, agkislysin preferentially cleaved the Aα chain of fibrinogen, followed by the Bβ chain, while the γ chain was finally affected. Furthermore, agkislysin was also capable of cleaving prothrombin into various fragments, as well as suppressing ristocetin-induced platelet aggregation by hydrolyzing von Willebrand factor. However, the proteolytic activity of agkislysin was slightly enhanced by divalent metal ions and completely inhibited by chelating agents, but not serine proteinase inhibitor. These findings suggest that agkislysin is a P-I class metalloproteinase with unique biological properties.

Keywords: Metalloproteinase; Agkislysin; *Agkistrodon acutus*; Proteolytic activity; Platelet aggregation

Snake venom proteins, especially from the *Viperidae* and *Crotalidae* venoms, contain bioactive enzymes affecting hemostasis and thrombosis [1,2]. Currently, these proteinases are used as therapeutic or diagnostic agents for clinical applications, as well as research tools for basic studies [2]. Up to date, the isolated components are generally classified into nucleotidases, phospholipases A_2, serine proteinases, and metalloproteinases [1,2]. Nucleotidases hydrolyze ADP to generate adenosine and behave as ADP scavengers. Phospholipases A_2 act as anticoagulants, cofactors of the prothrombinase complexes, and platelet aggregation inducers. Many serine proteinases have both fibrinogenolytic and/or fibrinolytic activities, but some of them have only fibrinogenolytic activity and are also termed as thrombin-like enzymes [1]. Snake venom metallopoteinases (SVMPs) belong to the metzincin family among a variety of zinc-containing metalloproteinases [1,2]. They are characterized by the presence of a highly conserved zinc-binding motif, HEXXHXXGXXH, for their proteolytic activity. When co-incubated with zinc chelator it deprives their enzymatic activities.

According to the domain structure, SVMPs are classified into four groups, P-I (protein class I) to P-IV [1]. The P-I class (low-molecular mass metalloproteinase) has only a proteinase domain of about 200 residues. The P-II class (disintegrin precursor) has an additional

☆ *Abbreviations: A. acutus*, *Agkistrodon acutus*; *C. atrox*, *Crotalus atrox*; vWf, von Willebrand factor; SVMP(s), snake venom metalloproteinase(s).

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disintegrin domain carboxy to the proteinase domain. The P-III class (high-molecular mass metalloproteinase) has both a disintegrin-like domain and a cysteine-rich domain carboxy to the proteinase domain. Otherwise, the P-IV class (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.

Agkistrodon acutus (formerly hundred-pace snake) is a monotypic pit viper found in Taiwan and Southeastern China [3]. SVMPs of *A. acutus* have already been investigated for over 20 years. Although several small SVMPs have been isolated [4–7], their biological functions were not extensively characterized. Among them, only Ac_1 [5] and acutolysin A [7] have been completely sequenced, while both the full-length sequences were almost identical (>90% sequence identity). Recently, additional six acidic and one alkaline cDNA variants of P-I class SVMPs from *A. acutus* venom have been cloned and sequenced as well [3], but their deduced amino acid sequences were not identical to those of Ac_1 or acutolysin A. Thus, we are interested in isolating and characterizing those components present in *A. acutus* venom for further investigations.

In this report, we purified a low-molecular mass SVMP, agkislysin, from Formosan hundred-pace snake. Our data showed that it belongs to P-I class SVMP and exhibits a broad substrate-cleaving property towards collagen, fibronectin, coagulation factor I and II, as well as vWf. Otherwise, the inhibitory mechanism of agkislysin on platelet aggregation elicited by ristocetin was investigated. To our knowledge, agkislysin is the first small reported SVMP possessing a unique proteolytic activity from *A. acutus* venom.

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 and FPLC/Mono-Q HR 5/5 columns were purchased from Pharmacia (Sweden). Acrylamide, BSA, SDS, Tris, mercaptoethanol, gelatin (bovine skin), prothrombin (human plasma), type VI collagen (human placenta), fibronectin (bovine plasma), EGTA, EDTA, PMSF, azocasein, 1,10-*o*-phenanthroline, and ristocetin were obtained commercially from Sigma Chemical (St. Louis, MO, USA). Molecular-mass standards for electrophoresis were purchased from Bio-Rad. Human vWf and fibrinogen were obtained from Calbiochem–Novabiochem (La Jolla, CA). Recombinant *N*-glycosidase F (*Flavobacterium meningosepticum*) was purchased from Roche Applied Science (Mannheim, Germany). Crotalin, an alkaline P-I class SVMP, was purified from the venom of *Crotalus atrox* as previously described [8].

Isolation and purification of agkislysin. Crude venom of *A. acutus* was applied to a DEAE–Sephadex A-50 column (1.6 × 20 cm) equilibrated with 0.05 N ammonium acetate (pH 8.5). The column was eluted at a flow rate of 20 ml/h 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) 0.05 N ammonium acetate (pH 8.5), 320 ml; (2) 0.05 N ammonium acetate (pH 8.5) vs. 0.25 N ammonium acetate (pH 6.5), 240 ml; and (3) 0.25 N ammonium acetate (pH 6.5) vs. 0.8 N ammonium acetate (pH 5.5), 240 ml, and 2 ml/tube were collected. The fraction containing gelatinolytic activity was collected, dialyzed, and lyophilized for further purification. Second, the active fraction was applied to a FPLC / Mono-Q HR 5/5 column (0.5 × 5 cm) pre-equilibrated with 0.02 N Tris buffer, pH 7.8. Elution was performed with B buffer (1 N NaCl in Tris buffer, pH 7.8) gradient at room temperature and monitored at 280 nm. The active fractions were collected and termed as agkislysin.

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) using bovine serum albumin as a standard.

N-Glycosidase digestion of agkislysin. Agkislysin (5 μg) was preincubated with 25 mM sodium phosphate buffer containing 0.1% SDS, 5 mM EDTA, 0.5% Triton X-100, and 0.5% mercaptoethanol at 95 °C for 2 min and then digested with recombinant *N*-glycosidase F (0.5 U) at 37 °C for 24 h. Samples were then subjected to 15% SDS–PAGE and stained with Coomassie blue.

Zymogram analysis of gelatinolytic activity. Gelatin zymography was performed as described previously [9]. Briefly, venom proteins were diluted in SDS sample buffer without reducing agents and electrophoresed on 12% SDS–polyacrylamide gels co-polymerized with 0.1% gelatin. Following electrophoresis, the gels were washed twice for 30 min in 2.5% Triton X-100 to remove SDS and then incubated in calcium assay buffer (50 mM Tris, 200 mM NaCl, and 10 mM $CaCl_2$, pH 7.8) at 37 °C for 20 h. After incubation, gels were stained with Coomassie blue for 2 h and then destained. The presence of gelatinolytic activity was identified as clear bands on a uniform blue background.

Proteolytic degradation assay on SDS–PAGE. Digestion reactions were carried out for the indicated quantity ratios and time intervals at 37 °C. Reactions were terminated by adding reducing or non-reducing buffer and boiled at 95 °C for 5 min. Samples were then subjected to SDS–PAGE and stained with Coomassie blue.

Preparation of human platelets and aggregation assay. Human blood collected in acid citrate dextrose (9:1, v/v) was centrifuged at 100*g* at room temperature for 10 min to obtain platelet-rich plasma. Platelet suspensions were prepared according to the previously described method [10]. Washed platelets were suspended in Tyrode's solution and the platelet count was adjusted to 3×10^8 platelets/ml. Platelet aggregation was performed using an aggregometer (Payton Scientific) at 37 °C with stirring (900 rpm). The extent of platelet aggregation was continually monitored by turbidimetry and expressed as increase in light transmission.

Caseinolytic activity assay. Caseinolytic activity was measured colorimetrically by the protocol described previously [11]. The reaction mixture, consisting of 85 μl azocasein (5 mg/ml) in 50 mM Tris–HCl buffer (pH 8.0), 10 μl of purified enzyme sample (100 μg/ml) in Tris buffer, and 5 μl Tris buffer or solution (100 mM) of various metal ions, chelators, or protein inhibitors was incubated at 37 °C for 90 min. The reaction was quenched by the addition of 200 μl of 5% trichloroacetic acid at room temperature. After centrifugation at 1000*g* for 5 min, 150 μl of the supernatant was mixed with the equal volume of 0.5 M NaOH and absorbance was determined at 450 nm.

Results

Isolation and purification of agkislysin

The crude venom of *A. acutus* was separated into nine fractions by DEAE–Sephadex A-50 column (Fig. 1A). The last fraction exhibiting gelatinolytic activity was

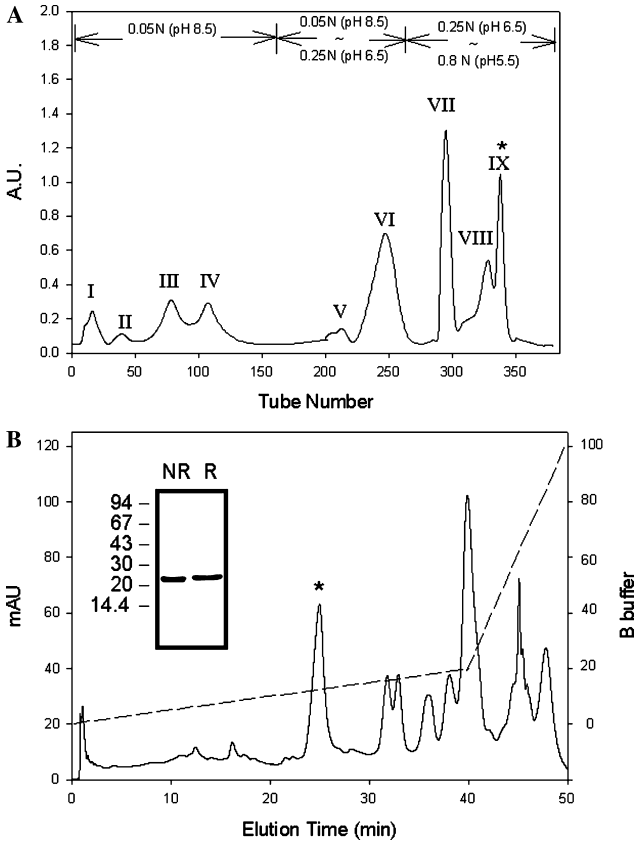

Fig. 1. Purification profile of agkislysin from *A. acutus* venom. (A) The crude venom of *A. acutus* was subjected to a DEAE–Sephadex A-50 column pre-equilibrated with 0.05 N ammonium acetate, pH 8.5. Elution was performed at 0.03 ml/min with a buffer gradient as indicated. Fraction IX (tube number,*335–345) possessing gelatinolytic 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 concentration. (m) AU, (milli) absorbance units. The inset is the SDS–PAGE pattern of the purified agkislysin. SDS–PAGE (15%) was performed under non-reducing (NR) and reducing (R) conditions, and stained with Coomassie blue. The standard proteins used for calibration of the apparent molecular weight were phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa), and α-lactalbumin (14.4 kDa).

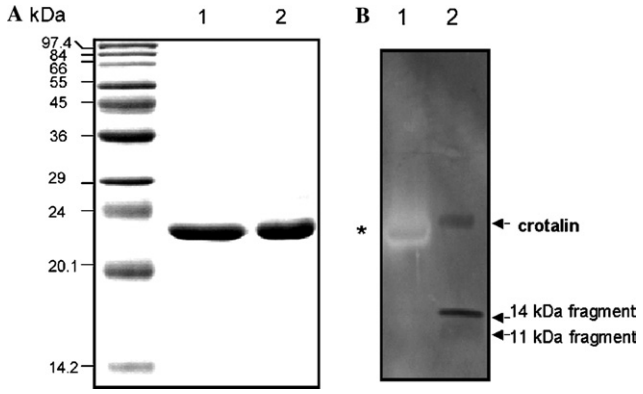

Fig. 2. *N*-Glycosidase digestion and gelatinolytic activity of agkislysin. (A) Agkislysin (5 μg) was incubated with digestion buffer in the absence (lane 1) or presence of *N*-glycosidase F (0.5 U; lane 2) at 37 °C for 24 h. Samples were then subjected to 15% SDS–PAGE under non-reducing conditions. The standard proteins used for calibration of the apparent molecular weight were phosphorylase b (97.4 kDa), fructose-6-phosphate kinase (84 kDa), albumin (66 kDa), glutamic dehydrogenase (55 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), trypsin inhibitor (20.1 kDa), and α-lactoalbumin (14.2 kDa). (B) The gelatinolytic activities of agkislysin (lane 1; *, 5 μg) and a 25-kDa SVMP from *C. atrox* venom, crotalin (lane 2; as indicated, 5 μg), were analyzed by gelatin zymography (12% SDS–PAGE containing 0.1% gelatin). Experimental procedures were performed as described in Materials and methods. The autoproteolytic fragments of crotalin with molecular masses of 14 and 11 kDa were shown as indicated.

collected for further purification. The desalted and lyophilized fraction was refractionated by anion-exchange chromatography on FPLC/Mono-Q column (Fig. 1B). The active fractions were then collected and concentrated. Based on the consideration of species origin (Agkis) and their proteolytic activity, we designated it as agkislysin. As shown in the inset of Fig. 1B, the apparent molecular mass of agkislysin was 22 and 22.1 kDa, respectively, as determined by SDS–PAGE under non-reducing and reducing conditions. Thus, it exists as a single-chain protein.

Several snake venom proteins have been purified and characterized as glycoproteins. Carbohydrate modification is known to affect the physical properties of proteins such as folding, stabilization or quaternary structure [12]. To examine the Asn-linked sugar chain, agkislysin was digested by recombinant *N*-glycosidase F, which specifically cleaves off Asn-linked sugar chains from glycoproteins. By contrast with the native protein, the deglycosylated agkislysin remains exhibiting a single band without a shifted molecular mass on SDS–PAGE (Fig. 2A). Thus, agkislysin is not a glycoprotein and Asn-linked glycosylation is not required for its biological activities. In order to compare the gelatinolytic activity with that of other proteinase, we purified a 25-kDa metalloproteinase from *C. atrox* venom, crotalin [8], as a control. In contrast to crotalin unable to degrade gelatin and unstable in SDS-containing solution leaving additional two autoproteolytic dark bands with apparent molecular masses of 14 and 11 kDa (Fig. 2B, lane 2), agkislysin exhibited a unique effect leaving a lytic zone detected by gelatin zymography (Fig. 2B, lane 1).

Proteolytic activity of agkislysin towards extracellular matrices

To further characterize the proteolytic activity of agkislysin, isolated extracellular matrix proteins were used for investigation. As shown in Fig. 3A, fibronectin

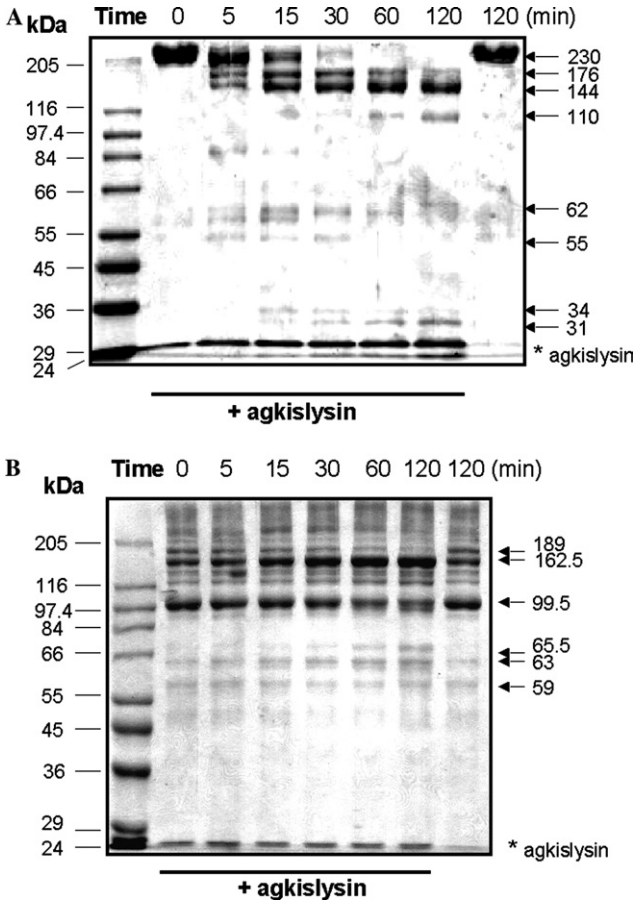

Fig. 3. Degradation products of fibronectin and type IV collagen by agkislysin. Digestion reactions were carried out at a substrate to enzyme ratio of 10:1 (w/w) and aliquots of (A) fibronectin and (B) type IV collagen from bovine plasma and human placenta, respectively, were incubated with agkislysin for the indicated time intervals at 37 °C. The reactions were terminated by adding sample buffer and boiled for 5 min at 95 °C. Samples were then subjected to 8% SDS–PAGE under reducing conditions. In addition to myosin (205 kDa) and galactosidase (116 kDa), the other standard proteins used for calibration of the apparent molecular weight were shown as in Fig. 2A.

digestion by agkislysin was analyzed on SDS–PAGE. When the control fibronectin solution was alone incubated at 37 °C for 2 h, a broad band of ~230 kDa was observed under reducing conditions. After incubation with agkislysin, this broad band disappeared and many degradation products with lower molecular mass were found. The degradation products had apparent molecular masses of 176, 144, 110, 62, 55, 34, and 31 kDa. The 144-kDa fragment seems to be the major product. Additionally, the control type IV collagen solution at 37 °C for 2 h revealed three main protein bands with apparent molecular mass of 189, 162.5, and 99.5 kDa. (Fig. 3B). Following incubation with agkislysin, two bands, 189 and 99.5 kDa, gradually disappeared. However, the band intensity of 162.5 kDa was increased and several new faint bands around 59–65.5 kDa were also observed.

Fibrinogen-degrading activity of agkislysin

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 by thrombin [13]. As indicated previously, proteolytic enzymes of snake venoms have been defined as α- or β-fibrinogenases based strictly on their direct cleaving activity in vitro on fibrinogen molecules [14]. Therefore, we assayed the fibrinogenolytic activity of agkislysin to characterize its proteolytic activity towards this coagulation protein. Human fibrinogen was incubated with agkislysin at 37 °C for various time intervals. The reaction products were then analyzed by SDS–PAGE under reduced conditions. The digestion pattern showed that agkislysin degraded preferentially the Aα-chain of fibrinogen, while the Bβ- and γ-chains were also substantially cleaved with increasing time (Fig. 4). Thus, it belongs to a new member of α-, β-, γ-fibrinogenase subfamily.

Prothrombin-degrading activity of agkislysin

Human coagulation factor II, prothrombin, containing 579 amino acid residues is composed of several distinct domains such as fragment 1 (residues 1–155), fragment 2 (residues 156–271), α-thrombin A-chain (residues 272–320), and α-thrombin B-chain (residues 321–579). In vivo α-thrombin is liberated when prothrombin is activated by Factor Xa, Factor Va, phospholipids, and calcium ions by cleavage of the Arg^{271}–Thr^{272} and Arg^{320}–Ile^{321} peptide bonds, yielding α-thrombin composed of A-chain and B-chain. However, when hydrolysis

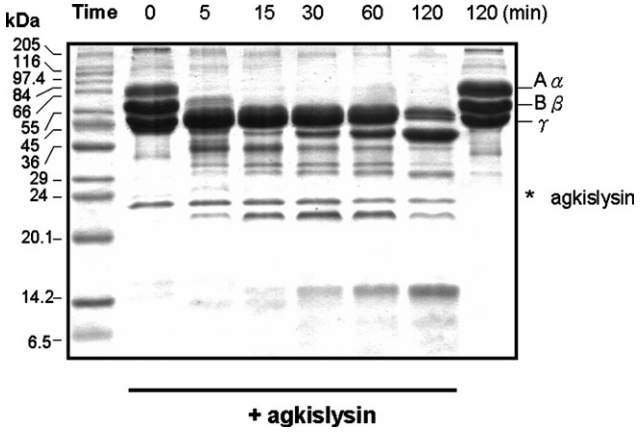

Fig. 4. Digestion pattern of agkislysin-treated human fibrinogen. Human fibrinogen was incubated with agkislyin at a substrate to enzyme ratio of 20:1 (w/w) for the indicated time intervals. The reaction was terminated by adding sample buffer and boiled for 5 min at 95 °C. Samples were subjected to 8% SDS–PAGE under reducing conditions. The molecular masses of three polypeptide chains of fibrinogen (Aα, Bβ, and γ) are shown on the left. The standard proteins used as shown in Fig. 3, as well as aprotinin (6.5 kDa).

of Arg^{320}–Ile^{321} bonds precedes cleavage of the Arg^{271}–Thr^{272} bond, meizothrombin is formed composed of two peptide chains, Ala^{1}–Arg^{320} and Ile^{321}-Glu^{579}, linked by a single disulfide bond [15]. To determine the degradation products of prothrombin, the incubation mixture of human prothrombin with agkislysin was subjected to SDS–PAGE under non-reducing and reducing conditions, respectively (Figs. 5A and B). In the presence of Tris-buffer at 37 °C for 60 min, native prothrombin exhibited an unstable autoproteolytic activity, leading to produce meizothrombin, prethrombin 1, and fragment 1. Then meizothrombin may undergo further autolysis to form one additional degradation fragment, α-thrombin/prethrombin 2. On the other hand, the major degradation products of human prothrombin by agkislysin under non-reducing and reducing conditions yielded major four and five fragments, respectively. Three bands, corresponding to the prothrombin/meizothrombin, prethrombin 1/meizothrombin des-fragment 1, and fragment 1, were intensively hydrolyzed by agkislysin under non-reducing conditions. By contrast, the intensity of a band corresponding to α-thrombin/prethrombin 2 was gradually increased and several new bands around 6.5–24 kDa appeared after 5 min-incubation. Similar to the digestion patterns under non-reducing conditions, an additional band corresponding to a reduced fragment of meizothrombin, consisting of fragment 1, fragment 2, and the A chain of α-thrombin (F1-F2-A), was also intensively hydrolyzed by agkislysin under reducing conditions. In contrast, the intensity of a band corresponding to the B-chain of α-thrombin was gradually increased.

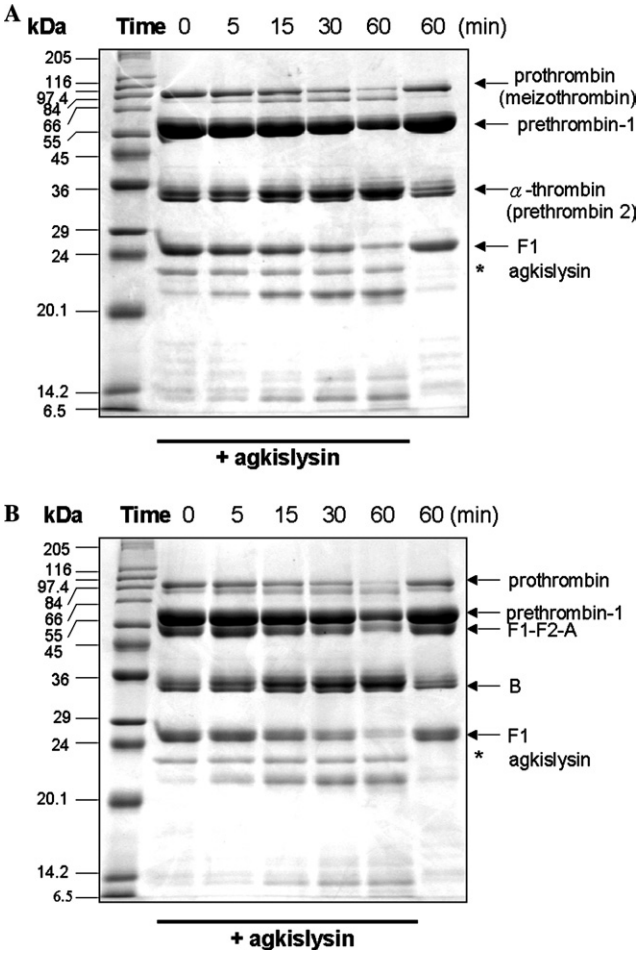

Fig. 5. Digestion pattern of agkislysin-treated human prothrombin. Digestion reactions were performed at a substrate to enzyme ratio of 20:1 (w/w) and aliquots of human prothrombin were incubated with agkislysin for the indicated time intervals at 37 °C. The reactions were terminated by adding sample buffer and boiled for 5 min at 95 °C. Samples were then subjected to 12% SDS–PAGE under (A) non-reducing and (B) reducing conditions. F1, fragment 1; F1-F2-A, the residual fragment consisting of fragment 1, fragment 2, and the A chain of α-thrombin; B, the B chain of α-thrombin.

Effect of agkislysin on platelet aggregation

Multimeric vWf binding to platelet glycoprotein Ib plays a crucial role in hemostasis following vascular injury. This reaction is known as the first event in the formation of hemostatic plugs [13]. The interaction of vWf with glycoprotein Ib on the platelet membrane is dependent upon an activation process or conformation change in vWf molecule. This process can also be mimicked in vitro by specific modulators such as ristocetin and botrocetin that bind to vWf [16]. To examine whether agkislysin affects the vWf–glycoprotein Ib interaction, pretreating vWf with agkislysin (10 μg/ml) for 2 or 10 min deprived of the ristocetin-induced platelet aggregation in a time-dependent manner (Fig. 6A, middle and right panels). However, re-addition of vWf restored the ristocetin-induced platelet aggregation (Fig. 6A, right panel). On the other hand, SDS–PAGE analysis showed that agkislysin 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 162 kDa after 10-min incubation under reducing conditions (Fig. 6B). Therefore, we propose that the vWf-cleaving activity of agkislysin is responsible for its inhibitory action on ristocetin-induced platelet aggregation.

Casinolytic activity of agkislysin

The effect of various reagents on the proteolytic activity of agkislysin (10 μg/ml) was investigated using azocasein as a substrate. As shown in Fig. 7, the relative percentage of enzymatic activity of agkislysin was slightly enhanced by the addition of 5 mM divalent metal ions (Ca^{2+} and Mg^{2+}), and strongly inhibited by 5 mM chelating agents (EGTA, EDTA, and 1,10-*o*-phenanthroline). However, a serine proteinase inhibitor, PMSF, exhibited no inhibitory effect on such reaction. By contrast, crotalin (10 μg/ml), as a positive control, also possessed the same casinolytic activity with agkislysin.

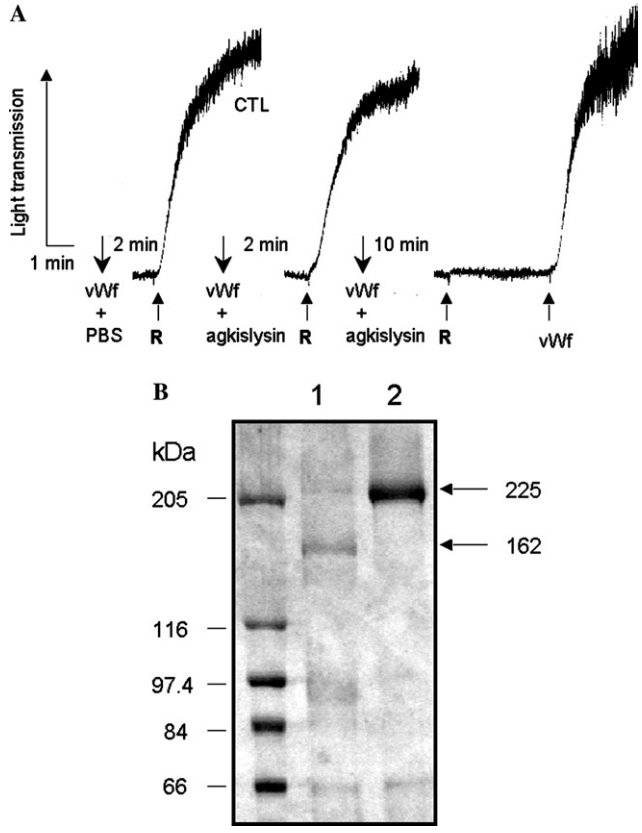

Fig. 6. The inhibitory effect of agkislysin on ristocetin-induced platelet aggregation. (A) Agkislysin (10 μg/ml) was preincubated with human vWf (10 μg/ml) at 37 °C for 2 (middle panel) or 10 min (right panel), and then ristocetin (R, 1 mg/ml) was added to trigger platelet aggregation. The control tracing (CTL) was shown at left panel. (B) Human vWf was incubated with (lane 1) or without (lane 2) agkislysin at a substrate to enzyme ratio of 20:1 (w/w) for 10 min, and then subjected to 12% SDS–PAGE under reducing conditions.

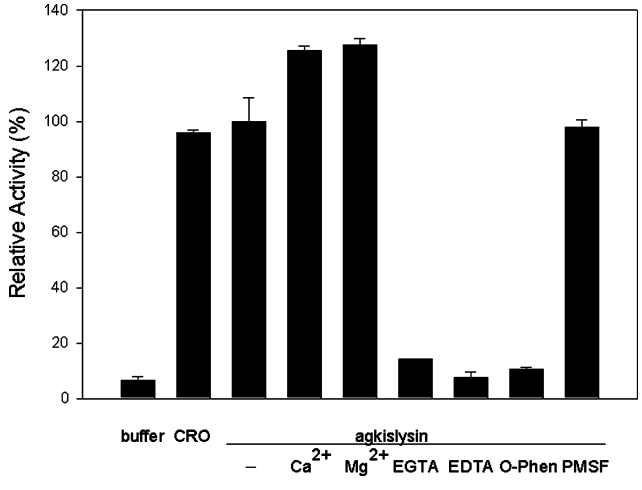

Fig. 7. Caseinolytic activity of agkislysin. Agkislysin in Tris buffer (10 μg/ml) was incubated with azocasein solution (4.25 mg/ml azocasein in Tris buffer, pH 8.0) without or with 5 mM solution of $CaCl_2$, $MgCl_2$, EGTA, EDTA, 1,10-*o*-phenanthroline (*o*-Phen), or PMSF at 37 °C for 90 min. The positive control was measured in the presence of crotalin in Tris buffer (CRO, 10 μg/ml).

Taken together, it was indicated that agkislysin typically is a divalent metal ion-dependent proteinase.

Discussion

Thrombosis is responsible for several cardiovascular diseases such as acute myocardial infarction, stroke and venous thromboembolism. Many available thrombolytic agents have already been used in clinical therapy by promoting rapid dissolution of thrombi. However, these agents have a number of disadvantages, including large therapeutic doses, limited fibrin specificity, short half-life, fast inactivation in vivo, reoccurrence of acute coronary occlusion, and bleeding complications [2,17]. The venoms of *Viperidae* and *Crotalidae* are rich sources of proteinases, which are also capable of directly solubilizing fibrin and selective against various steps in the hemostasis cascade [18,19]. These venom proteins possess a variety of advantages, such as the high stability in vitro or in vivo, high specificity for blood coagulation factors, and the resistance to physical inhibitors, render them highly useful for the design and development of a new generation of anti-thrombotic drugs. Metalloproteinases, particularly, are thought to have more potential clinical use in dissolving thrombi than thrombin-like enzymes, which only diminish fibrinogen but do not affect thrombi [20]. Therefore, metalloproteinases found within *Viperidae* and *Crotalidae* venoms may be of considerable importance because of their potential applications.

In the present study, we isolated a low-molecular mass metalloproteinase from *A. acutus* venom. This component, called agkislysin, was purified by a two-step anion-exchange chromatography. Agkislysin is a 22-kDa monomeric protein without Asn-linked carbohydrate, since it migrated as a single band on SDS–PAGE under non-reducing and reducing conditions (Fig. 1B), as well as the SDS–PAGE pattern of deglycosylated agkislysin without a shifted molecular mass (Fig. 2A). In addition, it should be an acidic proteinase based on its elution from DEAE–Sephadex A-50 column chromatography under a stronger acidic condition (Fig. 1A).

Although the common features of agkislysin (e.g., 22-kDa molecular mass, acidic in nature, and free carbohydrate) are similar to those of other previously reported proteinases found in *A. acutus* venom such as Ac_1 [4], acutolysin A [6], AaH-I [21], or α-fibrinogenase [22,23], its proteolytic activity towards a variety of substrates, especially extracellular matrix fibronectin or basement membrane type IV collagen (Fig. 3), were not yet described in the previous reports [3–7,20–24]. In fact, several lines of evidence listed as follows may suggest that agkilysin is a novel P-I class SVMP with some special characteristics. First, zymogram analysis of gelatinolytic activity showed agkislysin in SDS buffer stably and strongly digested the gelatin-containing gel

leaving a clear band detected, which was not found in the case of crotalin, a basic P-I class SVMP from *C. atrox* venom (Fig. 2B). Second, SDS-PAGE analysis showed agkislysin is an α-, β-, γ-fibrinogenase with Aα and Bβ chains of fibrinogen being rapidly degraded within 5 min followed by a slower degradation of the γ chain (Fig. 4). Third, agkislysin is functionally similar to group A prothrombin activator, which cleaves prothrombin without the need of any cofactors (e.g., Ca^{2+}, phspholipids or Factor Va) [25] (Fig. 5). Fourth, agkislysin time-dependently inhibits platelet aggregation triggered by ristocetin through the mechanism of cleaving vWf (Fig. 6). Until now, these features appear to be unique since there is no reported information about small SVMPs purified from *A. acutus* venom with similar properties.

In conclusion, we have characterized the first member of α-, β-, γ-fibrinogenase subfamily from *A. acutus* venom. Agkislysin is a P-I class SVMP, but it possesses distinct function activities. Further investigation of agkislysin for the determination of full-length amino acid sequence, the search of other active site excluding zinc-binding motif, and/or the cleavage sites within its substrates are necessary to understand the exact proteolytic mechanism of agkislysin. Moreover, the potential clinical applications of agkislysin remain to be explored and evaluated in the near future.

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References

[1] T. Matsui, Y. Fujimura, K. Titani, Snake vemon proteases affecting hemostasis and thrombosis, Biochim. Biophys. Acta 1477 (2000) 146–156.
[2] S. Braud, C. Bon, A. Wisner, Snake venom proteins acting on hemostasis, Biochimie 82 (2000) 851–859.
[3] I.H. Tsai, Y.M. Wang, T.Y. Chiang, Y.L. Chen, R.J. Huang, Purification, cloning and sequence analyses for pro-metalloprotease-disintegrin variants from *Deinagkistrodon acutus* venom and subclassification of the small venom metalloproteases, Eur. J. Biochem. 267 (2000) 1359–1367.
[4] N. Mori, T. Nikai, H. Sugihara, Purification of a proteinase (Ac_5-proteinase) and characterization of hemorrhagic toxins from the venom of the hundred-pace snake (*Agkistrodon acutus*), Toxicon 22 (1984) 451–461.
[5] T. Nikai, C. Kato, Y. Komori, H. Nodani, M. Homma, H. Sugihara, Primary structure of Ac_1-proteinase from the venom of *Deinagkistrodon acutus* (hundred-pace snake) from Taiwan, Biol. Pharm. Bull. 18 (1995) 631–633.
[6] W. Gong, X. Zhu, S. Liu, M. Teng, L. Niu, Crystal structures of acutolysin A, a three-disulfide hemorrhagic zinc metalloproteinase from the snake venom of *Agkistrodon acutus*, J. Mol. Biol. 283 (1998) 657–668.
[7] Q. Liu, W. Xu, W. Cheng, G. Jin, X. Shen, H. Lou, J. Liu, Molecular cloning and sequence analysis of cDNA encoding haemorrhagic toxin acutolysin A from *Agkistrodon acutus*, Toxicon 37 (1999) 1539–1548.
[8] W.B. Wu, H.C. Peng, T.F. Huang, Crotalin a vWF and GPIb cleaving metalloproteinase from venom of *Crotalus atrox*, Thromb. Haemostasis 86 (2001) 1501–1511.
[9] E.A. Hahn-Dantona, R.T. Aimes, J.P. Quigley, The isolation, characterization, and molecular cloning of a 75-kDa gelatinase B-like enzyme, a member of the matrix metalloproteinase (MMP) family. An avian enzyme that is MMP-9-like in its cell expression pattern but diverges from mammalian gelatinase B in sequence and biochemical properties, J. Biol. Chem. 275 (2000) 40827–40838.
[10] J.F. Mustard, D.W. Perry, N.G. Ardlie, M.A. Packham, Preparation of suspension of washed platelet from humans, Br. J. Haematol. 22 (1972) 193–204.
[11] W.J. Wang, T.F. Huang, Purification and characterization of a novel metalloproteinase, acurhagin, from *Agkistrodon acutus* venom, Thromb. Haemostasis 87 (2002) 641–650.
[12] J. Nawarak, S. Phutrakul, S.T. Chen, Analysis of lectin-bound glycoproteins in snake venom from the Elapidae and Viperidae families, J. Proteome Res. 3 (2004) 383–392.
[13] A.S. Kamiguti, C.R.M. Hay, R.D.G. Theakston, M. Zuzel, Insights into the mechanism of haemorrhage caused by snake venom metalloproteinases, Toxicon 34 (1996) 627–642.
[14] F.S. Markland Jr., Snake venom fibrinogenolytic and fibrinolytic enzymes: an updated inventory, Thromb. Haemostasis 79 (1998) 668–674.
[15] B.E. Fischer, U. Schlokat, M. Himmelspach, F. Dorner, Binding of hirudin to meizothrombin, Protein Eng. 11 (1998) 715–721.
[16] M.C. Berndt, C.M. Ward, W.J. Booth, P.A. Castaldi, A.V. Mazurov, R.K. Andrews, Identification of aspartic acid 514 through glutamic acid 542 as a glycoprotein Ib-IX complex receptor recognition sequence in von Willebrand factor. Mechanism of modulation of von Willebrand factor by ristocetin and botrocetin, Biochemistry 31 (1992) 11144–11151.
[17] D. Collen, H.R. Lijnen, Basic and clinical aspects of fibrinolysis and thrombolysis, Blood 78 (1991) 3114–3124.
[18] F.S. Markland, Snake venoms and the hemostatic system, Toxicon 36 (1998) 1749–1800.
[19] T.W. Willis, A.T. Tu, Purification and biochemical characterization of atroxase, a nonhemorrhagic fibrinolytic protease from western diamondback rattlesnake venom, Biochemistry 27 (1988) 4769–4777.
[20] J.B. Bjarnason, J.W. Fox, Hemorrhagic metalloproteinases from snake venoms, Pharmacol. Ther. 62 (1994) 325–372.
[21] X. Xu, C. Wang, J. Liu, Z. Lu, Purification and characterization of hemorrhagic components from *Agkistrodon acutus* (hundred pace snake) venom, Toxicon 19 (1981) 633–644.
[22] C. Ouyang, T.F. Huang, Purification and characterization of the fibrinolytic principle of *Agkistrodon acutus* venom, Biochim. Biophys. Acta 439 (1976) 146–153.
[23] C. Ouyang, T.F. Huang, The properties of the purified fibrinolytic principle from *Agkistrodon acutus* snake venom, Toxicon 15 (1977) 161–167.
[24] R.M. Kini, H.J. Evans, Structural domains in venom proteins: evidence that metalloproteinases and nonenzymatic platelet aggregation inhibitors (disintegrins) from snake venoms are derived by proteolysis from a common precursor, Toxicon 30 (1992) 265–293.
[25] M.B. Silva, M. Schattner, C.R.R. Ramos, I.L.M. Junqueira-de-Azevedo, M.C. Guarnieri, M.A. Lazzari, C.A.M. Sampaio, R.G. Pozner, J.S. Ventura, P.L. Ho, A.M. Chudzinski-Tavassi, A prothrombin activator from *Bothrops erythromelas* (jararaca-da-seca) snake venom: characterization and molecular cloning, Biochem. J. 369 (2003) 129–139.