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Purification, Characterization, and cDNA Cloning of a New Fibrinogenlytic Venom Protein, Agkisacutacin, from *Agkistrodon acutus* Venom

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Agkisacutacin is a new fibrinogenlytic protein from *Agkistrodon acutus* venom. It consists of two heterologous subunits linked by an intersubunit disulfide bond. The cDNAs encoding the two chains of Agkisacutacin were cloned from a λgt11 cDNA library of the snake venom gland and sequenced, including the leader peptides (23/23 amino acid residues) and mature subunits (129/123 amino acid residues). It is structurally related to the family of IX/X-binding protein (IX/X-bp)-like proteins and shows high similarity (α-70%/β-64%) to habu IX/X-bp from *Trimeresurus flavoridis,* but displays distinct biological activity with direct action on fibrinogen. © 1999 Academic Press

Snake venom contains a variety of proteins and polypeptides that affect thrombosis and hemostasis (1, 2). Because of the importance of blood coagulation and platelet aggregation in cardiovascular and cerebrovascular diseases, snake venom proteins which interfere in these processes have received considerable attention in recent years.

Recent studies have revealed the existence of a class of venom anticoagulants that are structurally related as heterodimers linked by an inter-subunit disulfide bond. The molecular weight of the protein is about 30 kDa. The N-terminal amino acid sequences are highly conserved and very similar to the animal C-type lectins (3), but their biological activities and mechanisms of actions are quite different. This class of proteins is called IX/X-bp-like protein and includes the Habu coagulation factor IX factor X-binding protein (IX/X-bp) (4, 5, 6), two chain Botrocetin (7, 8), Alboaggregin-B (9), Bothrojaracin (10), convulxin (11), and mamushigin (12).

In the present study, we purified and characterized a novel protein from *Agkistrodon acutus* venom named Agkisacutacin. It exhibits direct fibrinogenlytic activity. The cDNA clones encoding each chain of Agkisacutacin were accomplished and sequenced. The deduced amino acid sequence reveals that it shares structure homology with IX/X-bp like proteins.

GenBank accession numbers for the cDNA sequence of Agkisacutacin are AF176420 (A-chain) and AF176421 (B-chain).

Abbreviations used: DEAE, diethylaminoethyl; HPLC, high-performance liquid chromatography; SDS/PAGE, sodium dodecyl sulfate/polyacrylamide gel electrophoresis; kPTT, kaolin-induced partial thrombin time; TLCK, tosyl-L-lysinechloromethyl ketone; PVDF, polyvinylidine difluoride.

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MATERIALS AND METHODS

Materials. *Agkistrodon acutus* venom and venom gland were purchased from Qimen Venom Supplies (Southern Anhui, China). DEAE Sepharose Fast Flow and Sephacryl S-200HR were from Pharmacia (Sweden). Protein-PAK DEAE 8HR column was from Waters (U.S.A.). Polyacrylamide, bisacrylamide, mol. wt markers and human fibrinogen were from Sigma Chemical Co. (U.S.A.). Chromogenic substrates S-2238 (H-D-Phe-Pip-Arg-P-nitroanilide hydrochloride) and S-2251 (H-D-Val-Leu-Lys-P-nitroanilide hydrochloride) were from Chromogenix Co. (Sweden). All other chemicals used were of analytical grade.

Isolation and purification of Agkisacutacin. The crude *A. acutus* venom was dissolved in 0.02 M, pH 8.0 Tris-HCl buffer and centrifuged 15 min at 3000 rpm. The supernatant was applied to the DEAE Sepharose Fast Flow column (3 × 80 cm) equilibrated with 0.02 M (pH 8.0) Tris-HCl buffer. Proteins were eluted with a linear NaCl gradient as shown in Fig. 1. The fractions with fibrinogen clotting activity were concentrated and subjected to gel-filtration chromatography on a Sephacryl S-200HR column. The active fractions were collected and separated again on a DEAE 8HR HPLC column equilibrated with 0.02 M, pH 8.0 Tris-HCl buffer.

SDS/PAGE. The molecular weight of Agkisacutacin was estimated by SDS/PAGE on a 12% polyacrylamide gel (13) in the presence and absence of reducing agent, β-mercaptoethanol (4%, v/v). Following electrophoresis, protein bands were stained with Coomassie blue R-250 (0.1%).

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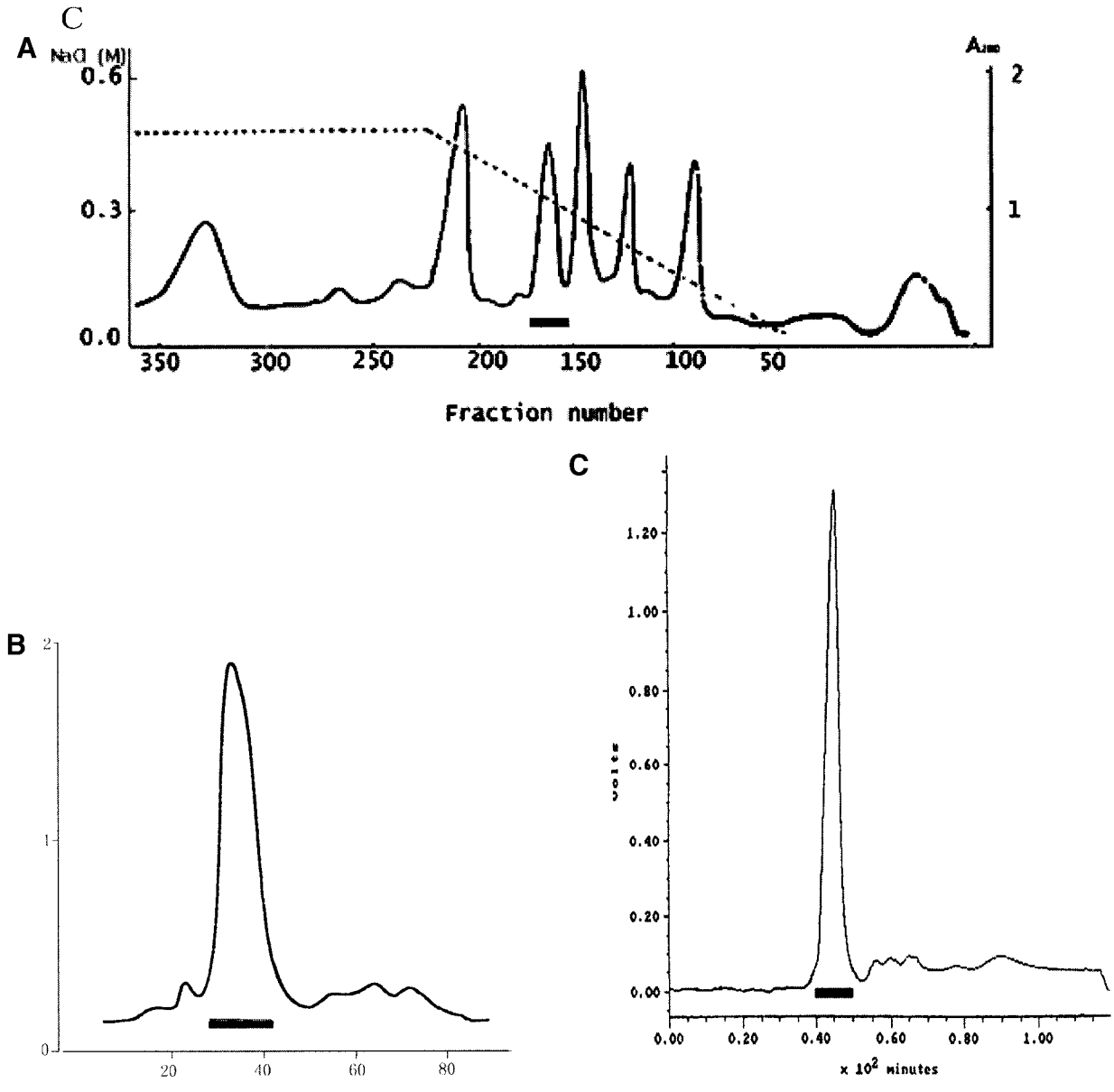

FIG. 1. Purification of Agkisacutacin from *A. acutus* venom. (A) The crude venom of *A. acutus* was subjected to DEAE-Sepharose Fast Flow chromatography on a column (3 × 80 cm) equilibrated with 0.02 M, pH 8.0 Tris-HCl buffer. Proteins were eluted by gradient (dotted line) between the equilibrating buffer and final buffer (0.02 M Tris-HCl, 0.5 M NaCl, pH 8.0) at 4°C. Aliquots of each fraction were tested for fibrinogen clotting activity and the active fractions were collected as shown by a bar. Flow rate: 80 ml/h. (B) The active fractions in A were subjected to gel filtration chromotography on a Sephacryl S-100HR column (1.6 × 80 cm). The column was eluted with 0.1 M NaCl solution. A bar indicates the fractions having fibrinogen clotting activity. (C) Agkisacutacin was finally purified by rechromatography on a DEAE 8HR FPLC column. The fibrinogen clotting activity was eluted at the position with a major protein peak. The active fractions were pooled as shown by a bar and concentrated.

Biological activity. Fibrinogen clotting assay was performed as follows: 100 μl of fibrinogen solution (4 mg/ml) was incubated with 100 μl of Agkisacutacin solution for 2 min at 37°C; then 100 μl of 25 mM $CaCl_2$ solution was added to the mixture and the clotting time was determined. The K_m value of Agkisacutacin to fibrinogen was determined.

Fibrinogenlytic activity was measured as the following: 500 μl of 4 mg/ml fibrinogen solution in 50 mM Tris-HCl, pH 7.4, buffer was incubated with 0.15 nmol Agkisacutacin at 37°C. After the incubation the digestion was stopped by adding 500 μl of SDS/PAGE sample buffer and heated at 90°C for 5 min. The activity was analyzed by SDS/PAGE electrophoresis by observing the cleavage patterns of fibrinogen chains.

The anti-coagulant activity was evaluated by the kaolin-induced PTT assay (14). Thrombin-like amidolytic activity was determined with the chromogenic substrate S-2238 (10). The plasmin activity was determined with the chromogenic substrate S-2251. Briefly, S-2251 (100 μM) in 0.02 M Tris-HCl and 0.15 M NaCl, pH 7.4, was incubated for 1 h at 37°C with Agkisacutacin; absorbance at 405 nm was then read.

Hemorrhagic activity assay was evaluated by a modification of the skin test procedure (15). Agkisacutacin (50–100 μg) is injected sub-

dermally under the clean shaven backs of white mice (approximately 20 g in weight). After 18 h the animals were sacrificed. The skin was removed, and the area of hemorrhage on the underside of the skin was measured.

Isolation and determination of partial amino acid sequences of Agkisacutacin A and B subunits. Purified Agkisacutacin was first submitted to SDS/PAGE under reducing conditions and then electrotransferred to a PVDF membrane (16). The protein bands corresponding to α and β chains were sequenced for the N-terminal sequence. The bands were also cut out and digested with TLCK-trypsin. The trypic peptide fragments were separated by HPLC on a reversed-phase column (5 × 250 mm) with acetonitrile/water/trifluoroacetic acid. The isolated peptide fragments were sequenced by means of Edman degradation with an Applied Biosystems 120-A gas-phase sequencer.

Construction of venom gland cDNA library. RNA was isolated from a total of 1 g tissue from Agkisacutacin venom glands as previously described (17). Poly(A)$^+$ mRNA was purified using an oligo(dT)-cellulose column (Pharmacia Biotech, Piscatawa, NJ). cDNA was synthesized using the TimeSave cDNA Synthesis Kit. *Eco*RI/*Not*I adapters were ligated on. The cDNA was purified by gel filtration chromotography using SizeSep 400 Spun columns. This cDNA was ligated into λgt11 vector (Promega Co.). The packaged library was amplified on *E. coli* Y1090. The insert and titre were measured.

cDNA library screening and DNA sequencing. A partial Agkisacutacin cDNA fragment was obtained by PCR using the *A. acutus* venom gland cDNA library total λDNA as template. The primers were designed based on the N-terminal amino acid sequences of the Agkisacutacin A chain and C-terminal poly(A) tail. The PCR product was ^{32}P-labeled as a probe to screen the *A. acutus* cDNA library. Several positive plaques were isolated. The phage inserts DNA were PCR-generated or excised from λgt11 vector, and were subcloned into pBluescriptSK(+) plasmids.

DNA sequencing was performed on an ABI 373A automated DNA sequencer. Both the DNA and deduced protein sequences were compared with sequences in GenBank.

RESULTS

Purification and Characterization of Agkisacutacin

Chromatographic profiles and protein yields in the purification steps of Agkisacutacin are shown in Fig. 1. Fractions with fibrinogen clotting activity are indicated by the black bar. The purified protein showed a molecular mass of 29 kDa before reduction, and two distinct bands with a molecular mass of 15 kDa (α-subunit) and 14 kDa (β-subunit) after reduction (Fig. 2), indicating that Agkisacutacin is a disulfide-linked heterodimer. The N-terminal sequences of the Agkisacutacin subunits separated by SDS/PAGE were determined to be DCSSGWSSYEGHCYKVFKQSKTWTDAESFC (α-subunit, 31 residues) and DCPSDWSSYEGHCYKPFDEPKTWADAE (β-subunit, 27 residues). Other sequences of peptide fragments from the subunit are shown in Fig. 3.

Agkisacutacin exhibits direct action on fibrinogen (Fig. 4). The K_m value of fibrinogen clotting activity is 26.2 mg/ml. Agkisacutacin made an initial cleavage on the α-chain of fibrinogen and got the first digesting peptide of 43 kDa. It cannot degrade β and γ-chain at all. The major fragments cleaved are polypeptides of molecular weights 43, 40 and 23 kDa. In contrast, Agkisacutacin did not show any thrombin-like amidolytic or plasmin activity. It exhibited neither effect on kPTT time, nor possess any hemorrhagic activity (data not shown).

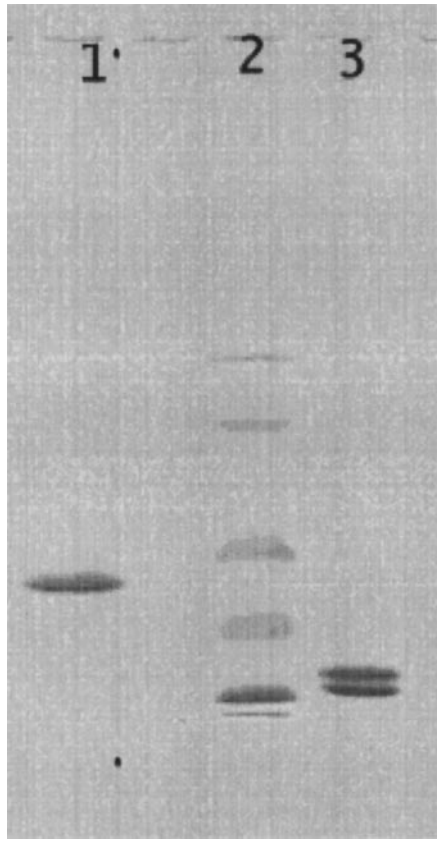

FIG. 2. SDS/PAGE electrophoresis of Agkisacutacin under denaturing conditions in the absence (lane 1) and presence (lane 3) of Agkisacutacin. Lane 2: standard proteins used as MW. Markers (14.4, 24.7, 34, 45, and 66 kDa). The gel was stained with Coomassie blue. Note: Agkisacutacin shows a pure single band with an estimated molecular mass of 29.5 kDa, consisting of two subunits of 14 kDa and 15 kDa upon reduction.

cDNA Cloning and Sequencing of Agkisacutacin Subunits

For screening of cDNAs for Agkisacutacin subunits, a 361 bp cDNA fragment was obtained by PCR using total λcDNA as template and sequenced. The sequence of this fragment is similar to IX/X-bp from *T. flavoviridis*. The DNA of this fragment was ^{32}P-labeled and used to screen the cDNA library. A large number of positive clones were obtained and several clones of DNA were sequenced. Clone PAλ133 encoding the total A chain and Clone PB213 encoding the open reading frame of B chain were chosen and sequenced again. For A chain, 733 bp nucleotide contained a 5′-untranslated region of 88 bp, an open reading frame of 459 bp, a stop

α-subunit

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tgagcagacttgctacctgtggaggccgagggacaggtgagtgcggctctcccacctgtg      60
cgcacctgacaggaaaggaaggaagaccATGGGGCGATTCATCTTCGTGAGCTTCGGCTT     120
                            M  G  R  F  I  F  V  S  F  G  L
GCTGGTCGTGTTCCTCTCCCTGAGTGGAACTGCAGCTGATTGTTCCTCTGGTTGGTCCTC     180
 L  V  V  F  L  S  L  S  G  T  A  A  D  C  S  S  G  W  S  S
CTATGAAGGGCATTGCTACAAGGTCTTCAAACAATCTAAGACCTGGGCAGATGCAGAGAG     240
 Y  E  G  H  C  Y  K  V  F  K  Q  S  K  T  W  A  D  A  E  S
CTTCTGCACGAAGCAGGTGAACGGGGGGCATCTGGTCTCTATCGAAAGCTCGGGAGAAGC     300
 F  C  T  K  Q  V  N  G  G  H  L  V  S  I  E  S  S  G  E  A
AGACTTTGTGGCCCATCTGATTGCTCAGAAGATAAAGTCAGCCAAAATCCATGTCTGGAT     360
 D  F  V  A  H  L  I  A  Q  K  I  K  S  A  K  I  H  V  W  I
CGGACTGAGGGCTCAAAACAAAGAAAAGCAATGCAGCATAGAGTGGAGCGATGGCTCCAG     420
 G  L  R  A  Q  N  K  E  K  Q  C  S  I  E  W  S  D  G  S  S
CATCAGTTATGAGAATTGGATTGAAGAAGAATCCAAAAAGTGTCTTGGGGTGCACATAGA     480
 I  S  Y  E  N  W  I  E  E  E  S  K  K  C  L  G  V  H  I  E
GACAGGGTTTCATAAGTGGGAGAATTTTTACTGTGAACAACAAGATCCTTTTGTCTGCGA     540
 T  G  F  H  K  W  E  N  F  Y  C  E  Q  Q  D  P  F  V  C  E
GGCATAGtctgaagatccagctgagtgaagtctggagaagcaaggaagccccccacccca     600
 A  *
tccccaaccctgcctgccacaatctctgctctgcaccctcccctcaacggatgctgcgt     660
gtagctggatctggtgttgctgctcctgatgggccggaaggtccaataaattctgcctag     720
cctgaaaaaaaaaa                                                  733
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β-subunit

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ccATGGGGCGATTCATCTTCGTGAGCTTCGGCTTGCTGGTCGTGTTCCTCTCCCTGAGTG      60
  M  G  R  F  I  F  V  S  F  G  L  L  V  V  F  L  S  L  S  G
GAACTGCAGCTGATTGTCCCTCTGAGTGGTCCTCCTATGAAGGGCATTGCTACAAGCCCT     120
 T  A  A  D  C  P  S  E  W  S  S  Y  E  G  H  C  Y  K  P  F
TCGATGAACCTAAGACCTGGGCAGATGCAGAGAAATTCTGCACACAACAACACAAAGGCA     180
 D  E  P  K  T  W  A  D  A  E  K  F  C  T  Q  Q  H  K  G  S
GCCATCTGGCCTCCTTTCACAGCAGTGAAGAAGCAGATTTTGTGGTCACGTTGACCACAC     240
 H  L  A  S  F  H  S  S  E  E  A  D  F  V  V  T  L  T  T  P
CAAGTTTGAAAACTGATTTAGTCTGGATTGGACTGAAGAACATCTGGAACGGATGCTACT     300
 S  L  K  T  D  L  V  W  I  G  L  K  N  I  W  N  G  C  Y  W
GGAAGTGGAGCGATGGCACCAAGCTCGACTACAAAGACTGGCGTGAACAATTTGAATGTC     360
 K  W  S  D  G  T  K  L  D  Y  K  D  W  R  E  Q  F  E  C  L
TCGTATCCAGGACAGTTAATAACGAATGGCTAAGTATGGACTGCGGCACTACTTGCTCTT     420
 V  S  R  T  V  N  N  E  W  L  S  M  D  C  G  T  T  C  S  F
TCGTCTGCAAGTTCCAGGCATAGtctgaagatgcagct                          458
 V  C  K  F  Q  A  *
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FIG. 3. Nucleotide sequences of cDNAs encoding Agkisacutacin A chain (α-subunit) and B chain (β-subunit) and the deduced polypeptide sequences. 5′-UTR and 3′-UTR sequences are shown in lowercase letters. The translation stop codon is indicated by asterisk. The leader sequences are denoted in boldface. The peptide fragments sequences are underlined.

codon, and a 3′-end noncoding region of 186 bp that included a polyadenylation signal (AATAAA) and a poly(A)+ tail (Fig. 5). For B chain, clone with 458 bp was obtained.

The deduced amino acid sequences of the A- and B-subunits contained a leader peptide of 23 amino acid residues, followed by a mature subunit of 129 and 123 residues, respectively. They were identical to those obtained by N-terminal protein sequencing and partial peptide fragment sequencing.

Comparison of the Sequences between Agkisacutacin and Other IX/X-bp-like Proteins

The deduced amino acid sequences of the Agkisacutacin subunits possess most major characteristics defined by Spiess (18) for the carbohydrate-recognition domain (CRD) of C-type lections. The identity for the A-chain sequences of Agkisacutacin is as follows: IX/X-bp α-chain, 70%; Botrocetin α-chain, 58%; Convulxin α-Chain, 57%; mamushigin α-chain, 55%; AL-B α-chain, 55%; echicetin α-chain, 48%, rattlesnake lectin, 36%. The identity for the β-chain sequence of Agkisacutacin is as follows: IX/X-bp β-chain, 64%; Botrocetin β-chain, 52%; Convulxin β-Chain, 68%; mamushigin β-chain, 65%; AL-B β-chain, 60%; echicetin β-chain, 51%; rattlesnake lectin, 34%. A comparison of the leader peptide sequences between Agkisacutacin and the habu IX/X-bp revealed the higher similarity: a 78% identity for the A-chain, and a 91% identity for the B-chain. The cysteine residues in each subunit of Agkisacutacin are well conserved and located at the positions corresponding to CRDs.

DISCUSSION

A fibrinogenlytic protein in *Agkistrodon acutus* snake venom was purified and the cDNAs encoding the α/β chains of Agkisacutacin were cloned and sequenced. The functional characteristics were elementary analyzed and compared with those IX/X-BP like proteins.

Structurally, Agkisacutacin is a heterologous protein with α/β subunits linked by a disulfund bond. The deduced amino acid sequence contains 23 residues of leader peptide of both α/β chain, 129 residues for the mature protein of α-chain, and 123 residues for the

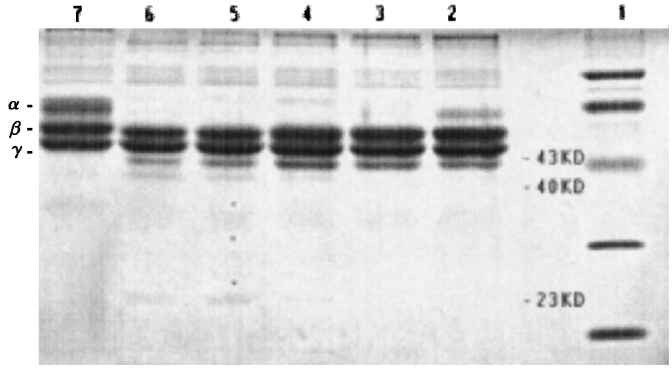
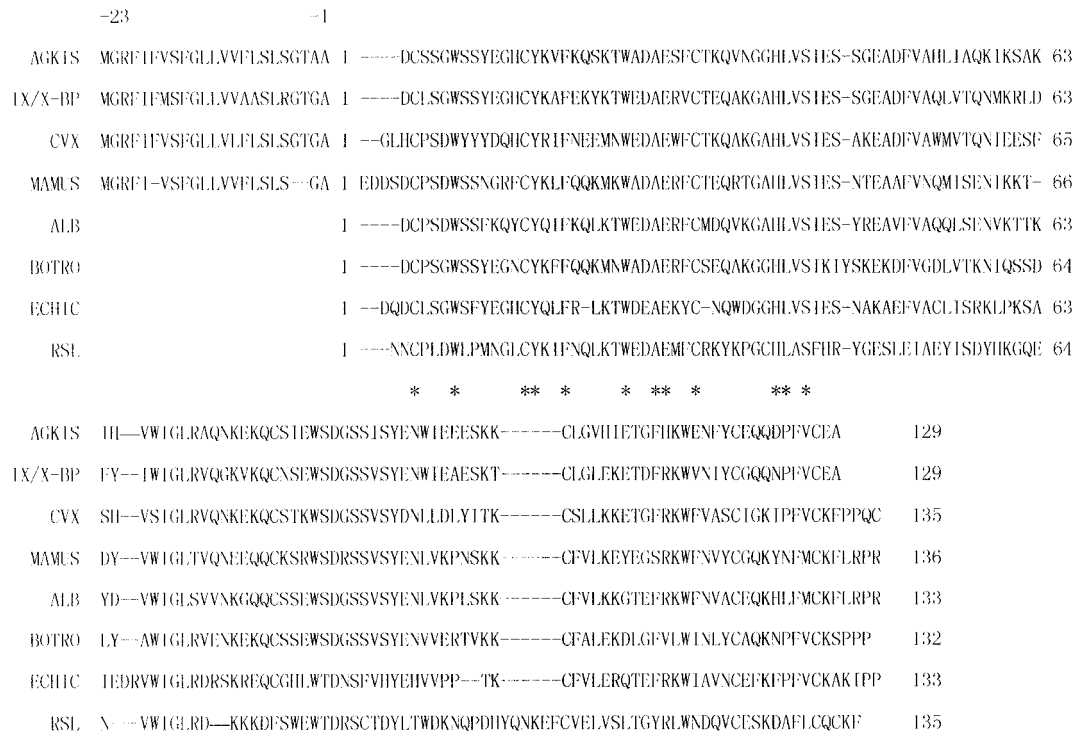


FIG. 4. Effect of Agkisacutacin on fibrinogen: time-course analysis by SDS/PAGE (10% acrylamide gels). Digestions were performed as described under Materials and Methods. Staining was with Coomassie blue R250. MW of marker proteins were phosphorylase B (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa) and soybean tripsin inhibitor (20 kDa). Lane 1: MW markers. Lanes 2–6: Sample incubated with fibrinogen solution for 1, 2, 4, 12, 24 h. Lane 7: fibrinogen solution for 24 h.

A chain:



B chain:

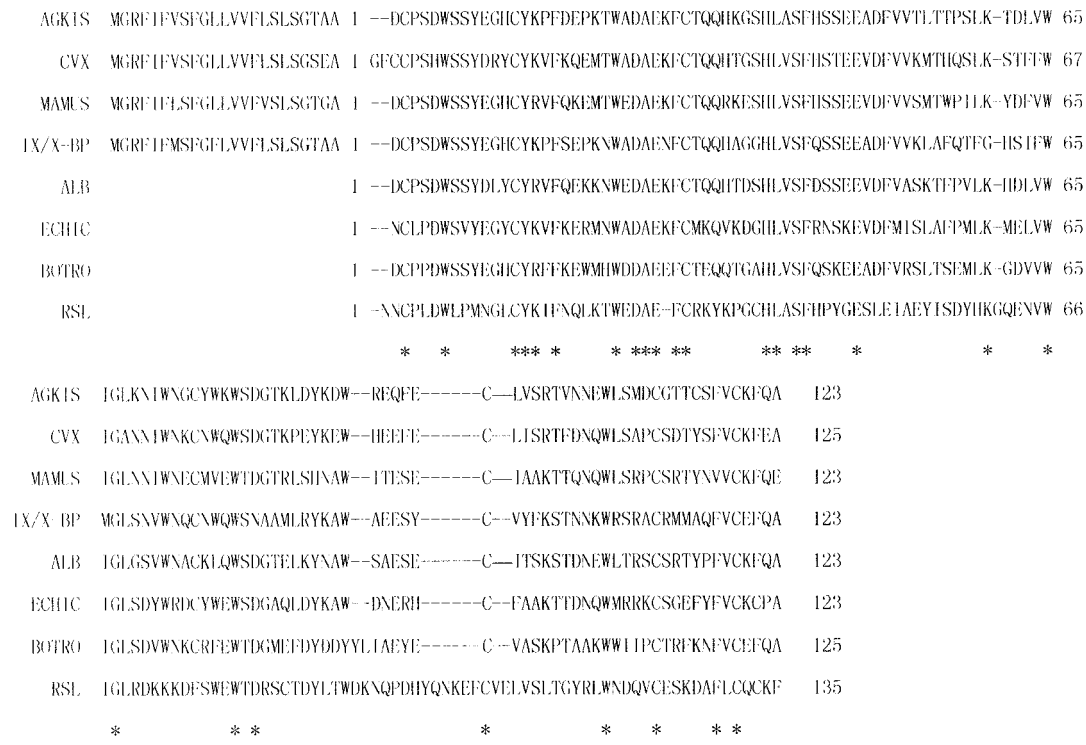


FIG. 5. Comparison of the amino acid sequences deduced from Agkisacutacin cDNAs with IX/X-BP like proteins, including IX/X-BP from *Trimerresurus flavoviridis* (6) (IX/X-BP), convulxin from *Crotalus clurissus terrificus* (11) (CVX), mamushigin from *Agkistrodon halys blomhoffii* (12) (MAMUS), Alboagreggin-B from *Trimeresurus albolabris* (9) (ALB), Botrocetin from *Bothrops jararaca* (8) (BOTRO), echicetin from *Echi cariratus leucogaster* (19) (ECHIS) and the single chain of RSL from *Crotalus atrox* (18). Gaps have been inserted to maximize homology. Asterisks indicated similarity between residues.

mature protein of β-chain. The sequences between the α/β subunits of Agkisacutacin show only 37% similarity with each other and 36/34% similarity with rattle snake lectin (C-type lectins). But they show a high degree of similarity with those IX/X-bp like proteins from snake venoms as described in *Comparison of the sequences between Agkisacutacin and other IX/X-bp like proteins*. It is possible that the snake contained a variety of the kinds of venom proteins with different degrees of structural similarity.

Agkisacutacin displays distinct biological activity from other IX/X-BP like proteins. It can directly hydrolyse the α-chain of fibrinogen, which results in the fibrinogen clotting activity in vitro. The initial cleavage on fibrinogen obtained a polypeptide of 43 kDa; the degrading peptides of 43, 40 and 23 kDa finally resulted. Agkisacutacin shows no effect on S-2238 and S-2251, meaning the cleavage site on fibrinogen of Agkisacutacin is not the same site of thrombin and plasmin. Unlike those fibrinolytic venom metalloproteins (20) from snake venoms, Agkisacutacin hydrolyses fibrinogen on a specific site and possess virtually no hemorrhagic and anti-coagulant activity.

In the IX/X-BP like protein family, the binding properties and biological activities of some proteins have been studied. For example, IX/X-BP is an anticoagulant protein binds to the Gla-domain of factor IX and Factor X in a calcium-dependent fashion; mamushigin is a platelet glycoprotein Ib-binding protein that directly induces platelet aggregation. Batrocetin is another platelet GPIb binding protein inhibits platelet aggregation, whereas Agkisacutacin directly degrades the α-chain of fibrinogen. As fibrinogen plays an important role in fibrin formation and platelet aggregation, the interaction between Agkisacutacin and fibrinogen should be elucidated. For this purpose, the fibrinogen cleavage sites should be determined, and whether the action on fibrinogen will interfere with the platelet aggregation or not should be further evaluated.

From the alignment of the sequences of IX/X-BP like proteins, the proteins seem to have evolved from a very old common ancestor, which has diverged into proteins having a variety of binding properties and functions and acts on the different stage of the coagulation cascade. Agkisacutacin is the first protein with direct fibrinogenlytic activity reported in the family and makes the family more interesting.

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