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A new protein structure of P-II class snake venom metalloproteinases: it comprises metalloproteinase and disintegrin domains

Run-Qiang Chen,[a,b] Yang Jin,[a] Jian-Bo Wu,[a,b] Xing-Ding Zhou,[a,b] Qiu-Min Lu,[a] Wan-Yu Wang,[a] and Yu-Liang Xiong[a,*]

[a] *Department of Animal Toxinology, Kunming Institute of Zoology, The Chinese Academy of Sciences, Kunming 650223, PR China*
[b] *Graduate School of The Chinese Academy of Sciences, Beijing 100080, PR China*

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Abstract

A new metalloproteinase–disintegrin, named Jerdonitin, was purified from *Trimeresurus jerdonii* venom with a molecular weight of 36 kDa on SDS–PAGE. It dose-dependently inhibited ADP-induced human platelet aggregation with IC_{50} of 120 nM. cDNA cloning and sequencing revealed that Jerdonitin belonged to the class II of snake venom metalloproteinases (SVMPs) (P-II class). Different from other P-II class SVMPs, metalloproteinase and disintegrin domains of its natural protein were not separated, confirmed by internal peptide sequencing. Compared to other P-II class SVMPs, Jerdonitin has two additional cysteines (Cys219 and Cys238) located in the spacer domain and disintegrin domain, respectively. They probably form a disulfide bond and therefore the metalloproteinase and disintegrin domains cannot be separated by posttranslationally processing. In summary, comparison of the amino acid sequences of Jerdonitin with those of other P-II class SVMPs by sequence alignment and phylogenetic analysis, in conjunction with natural protein structure data, suggested that it was a new type of P-II class SVMPs.

Keywords: SVMP; Disintegrin; Platelet aggregation; Phylogenetic analysis; *Trimeresurus jerdonii*

Crotalid and viperid venoms contain a large number of hemorrhagic proteins. Hemorrhage is the result of the synergistic action of certain metalloproteinases which degrade the extracellular matrix (ECM) surrounding blood vessels and proteins that interfere with hemostasis [1]. According to their domain structures, the venom metalloproteinases have been classified into four major groups [2,3]. The P-I class is composed of a single metalloproteinase domain. The P-II class consists of a metalloproteinase domain and a disintegrin domain. These two domains will be separated by posttranslationally processing. The P-III class consists of metalloproteinase, disintegrin-like, which contains a disulfide-binded XXCD (mostly SECD) in place of RGD, and cysteine-rich domains. The P-IV class contains an additional disulfide-linked C-type lectin-like domain compared to P-III class.

Disintegrins are low molecular weight, cysteine-rich, and Arg–Gly–Asp (RGD)-containing peptides which inhibit platelet aggregation by antagonizing fibrinogen binding to platelet glycoprotein IIb/IIIa [4,5]. Disintegrins also inhibit the adhesion of human umbilical vein endothelial cell (HUVEC) to fibrin or to immobilized ECM through the blockade of $\alpha_v\beta_3$ integrin [4]. Because the endothelial $\alpha_v\beta_3$ integrin plays a major role in angiogenesis, the antiadhesive function of disintegrins between endothelial cells and ECM may be a key factor in inhibiting angiogenesis [6].

Here, we report the purification and cloning of a new metalloproteinase–disintegrin from the venom of *Trimeresurus jerdonii*, Jerdonitin, which comprises metalloproteinase, spacer, and RGD-containing disintegrin domains. To our knowledge, it is the first reported metalloproteinase–disintegrin protein.

Materials and methods

Materials. T. jerdonii crude venom was from the stock of the Kunming Institute of Zoology, The Chinese Academy of Sciences. Sephadex G-100 and Resource Q column (1 ml) were from LKB

* Corresponding author. Fax: +86-871-519-1823.
E-mail address: chenrunqiang@hotmail.com (Y.-L. Xiong).

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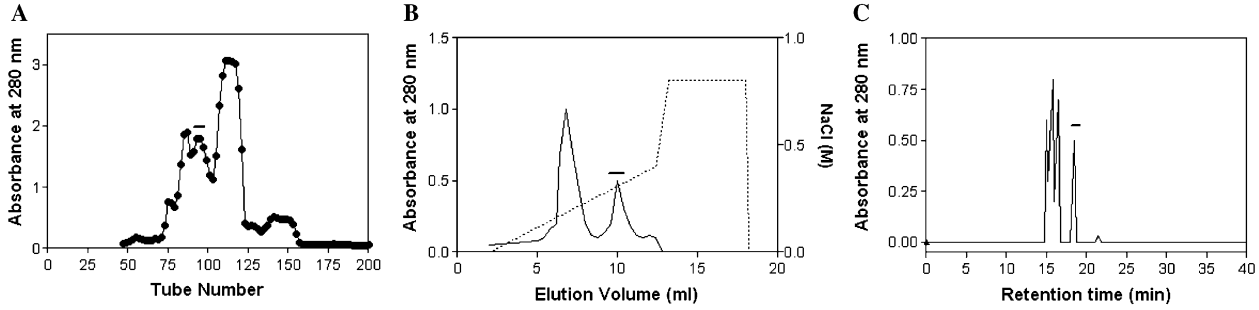

Fig. 1. Purification of Jerdonitin. (A) Gel filtration on Sephadex G-100. Lyophilized *Trimeresurus jerdonii* venom (500 mg) was loaded onto a Sephadex G-100 (2.6 × 120 cm) which was equilibrated with 50 mM Tris–HCl buffer, pH 7.2, containing 0.15 M NaCl. (B) Ion exchange chromatography on FPLC Resource Q column (1 ml). Active fractions after Sephadex G-100 column were further separated by FPLC Resource Q column (1 ml) with 25 mM Tris–HCl buffer, pH 8.0, at a flow rate of 0.5 ml/min. (C) Hydrophobic chromatography on RP-HPLC C_4 column (4.6 × 250 mm) equilibrated with solvent A (0.1% TFA in water) at 1 ml/min flow rate. Bars represent fractions containing Jerdonitin.

Pharmacia (Uppsala, Sweden). Reverse-phase C_4 and C_{18} columns for HPLC were from Waters (USA). Low molecular weight markers and reagents for SDS–PAGE were purchased from Sigma (St. Louis, MO, USA). Other reagents used were of analytic grade.

Purification of Jerdonitin. Lyophilized crude venom of *T. jerdonii* (500 mg) was dissolved in 5 ml of 50 mM Tris–HCl buffer, pH 7.2, containing 0.15 M NaCl and applied to a Sephadex G-100 (2.6 × 120 cm) equilibrated with the same buffer. Fractions containing hemorrhagic activity were pooled. The sample was dialyzed against 25 mM Tris–HCl buffer, pH 8.0, and applied to FPLC Resource Q column (1 ml) equilibrated with the starting buffer (25 mM Tris–HCl buffer, pH 8.0). Elution was achieved with a linear NaCl gradient from 0 to 0.8 M in the same buffer at a flow rate of 0.5 ml/min on Pharmacia FPLC system. Fractions containing hemorrhagic activity were pooled and submitted to a reverse-phase HPLC (RP-HPLC), employing a C_4 column (4.6 × 250 mm). The solvents were 0.1% trifluoroacetic acid (TFA) in water (solvent A) and 0.1% TFA/90% acetonitrile (solvent B).

Electrophoresis. According to the method of Laemmli [7], non-reducing and reducing (in the presence of 5% β-mercaptoethanol) SDS–PAGE were performed in 12.5% polyacrylamide gel. Protein was stained with Coomassie brilliant blue G-250.

Platelet aggregation assay. Human blood was obtained from healthy donors who denied having taken any medication for two weeks. Platelet aggregation was carried out on a LBY-NJ aggregometer (Precil group, Beijing, China) at 37 °C with stirring (1100 rpm). Platelet-rich plasma (PRP) was prepared from whole blood anticoagulant with 3.8% sodium citrate in a 9:1 ratio. The mixture was transferred to a clean tube. The concentration of platelet used in each assay was adjusted to 250,000 cell/μl in a final volume of 0.25 ml.

Amino acid sequence determination. Reduction and S-carboxymethylation of Jerdonitin was carried out as described by Chang et al. [8]. Reduced and S-carboxymethylated (RCM) protein was subjected to auto Edman degradation to obtain the N-terminal sequence of intact protein. Moreover, RCM-protein was hydrolyzed with trypsin in 50 mM NH_4CO_3, pH 7.8, at a molar ratio of 50:1 (RCM-protein:proteinase) at 37 °C for 3 h. The peptides in the digest were separated by HPLC on a reverse-phase C_{18} column (5 × 300 mm) equilibrated with 0.1% TFA and eluted with a linear gradient of 5–70% acetonitrile for 50 min. Amino terminal sequencing of trypsin-generated peptides was carried out by Edman degradation with an Applied Biosystems Model 476A sequencer.

cDNA cloning of Jerdonitin. Isolation of mRNA and reverse transcription were conducted using polyA Tract System 1000 kit and Reverse Transcription System kit (Promega Biotech), respectively, according to the manufacturer's protocols. DNAs were amplified by PCR using total RT-PCR products as template and two oligonucleotide primers. The two primers were designed according to the highly conserved 5′- and 3′-non-coding regions of cDNA encoding for elegantin-2a from *Trimeresurus elegans* (GenBank Accession No. AB059572), elegantin-1a from *T. elegans* (GenBank Accession No. AB059571), and HR2a from *Trimeresurus flavoviridis* [9], primer 1 was 5′-CCAAATCCAG(C/T)CTCCAAAATG-3′ and primer 2 was 5′-TTCCA(G/T)CTCCATTGTTG(G/T)TTA-3′. The recovered PCR products were cloned into PMD18-T vector (TaKaRa, Dalian) and then transformed into *Escherichia coli* strain JM109.

DNA sequencing and analysis. DNA sequencing was performed on DNA sequencing System Model 377 (PE Applied Biosystems). The DNA sequence and deduced amino acid sequence were compared with sequences in the GenBank database using the Blast program [10].

Construction of phylogenetic tree. The sequences of P-II class SVMPs were searched with the non-redundant protein sequence database using FASTA program [11]. Alignment was made by

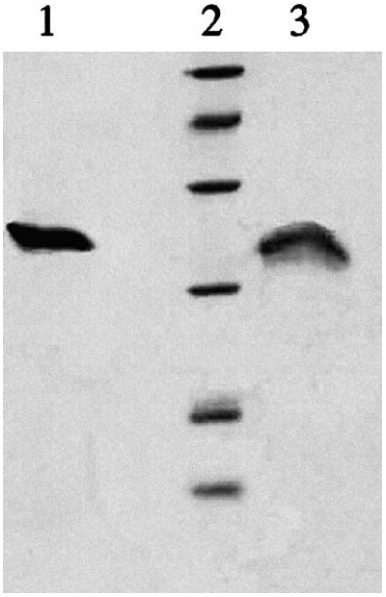

Fig. 2. SDS–PAGE analysis of Jerdonitin. Lane 1, purified Jerdonitin under non-reducing condition; lane 2, molecular weight markers; rabbit phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (43.2 kDa), bovine carbonic anhydrase (30.0 kDa), trypsin inhibitor (20.1 kDa), and hen egg white lysozyme (14.4 kDa). Lane 3, Jerdonitin under reducing condition. The molecular weight was estimated to be 36 kDa.

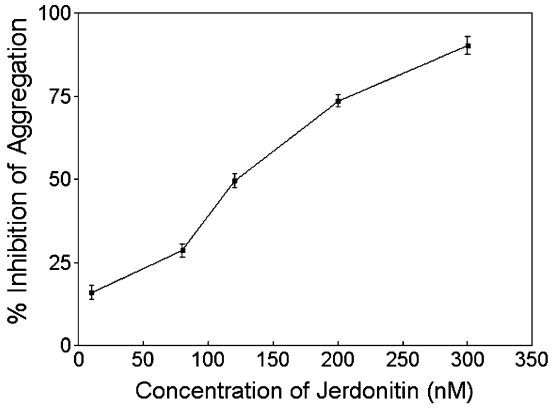

Fig. 3. Concentration dependence of ADP-induced platelet aggregation inhibition by Jerdonitin. The extent of the inhibition of platelet aggregation was assessed by comparison with the maximal aggregation induced by the control dose of ADP (20 μM). The IC_{50} value determined from the curve is 120 nM. The data are shown as means ± SE. All experiments were performed in triplicate.

CLUSTAL W program [12]. Phylogenetic tree of P-II class SVMPs was constructed using PHYLIP package software. The distance matrix for amino acid substitutions was analyzed by Kimura's method [13]. Tree was conducted by the neighbor-joining algorithm [14] based on evolutionary distance matrix. A P-III class SVMP, berythractivase [15], was used as outgroup. The degree of confidence for internal lineages in phylogenetic tree was determined by the bootstrap confidence using Kimura's method to compute the distance matrix with 1000 replicates.

Results

Purification of Jerdonitin

Following gel filtration on Sephadex G-100 (Fig. 1A), FPLC Resource Q column (1 ml) (Fig. 1B), and RP-HPLC C_4 column (Fig. 1C), a homogeneous Jerdonitin, as ascertained by SDS–PAGE, was obtained. From SDS–PAGE, its molecular weight was 36 kDa under reducing and non-reducing conditions (Fig. 2).

Biochemical characterization of Jerdonitin

Jerdonitin was capable of dose-dependently inhibiting ADP-induced human platelet aggregation. The IC_{50} was determined to be 120 nM (Fig. 3).

```
1     ATGATCCAGGTTCTCTTGGTAACTATATGCTTAGCAGTCTTTCCTTATCAAGGGAGCTCTATAATCTTGGAATCTGGGAACATTGATGAT
       M  I  Q  V  L  L  V  T  I  C  L  A  V  F  P  Y  Q  G  S  S  I  I  L  E  S  G  N  I  D  D
91    TATGAAGTCGTGTACCCACGAAAAGTCACTGCATTGCCCAAAGGAGCAGTTCAGCAAAAGTATGAAGACACCATGCAATATGAATTTAAA
       Y  E  V  V  Y  P  R  K  V  T  A  L  P  K  G  A  V  Q  Q  K  Y  E  D  T  M  Q  Y  E  F  K
181   GTGAATGAAGAGCCAGTGGTCCTTCACCTGGAAAAAAATAAAGGACTTTTTTCAAAAGATTACAGCGAGACTCATTATTCCCCTGATGGC
       V  N  E  E  P  V  V  L  H  L  E  K  N  K  G  L  F  S  K  D  Y  S  E  T  H  Y  S  P  D  G
271   AGAGAAATTACAACATACCCCCCGGTTGAGGATCACTGCTATTATCATGGACGCATCCAGAATGATGCTGACTCAACTGCAAGCATCAGT
       R  E  I  T  T  Y  P  P  V  E  D  H  C  Y  Y  H  G  R  I  Q  N  D  A  D  S  T  A  S  I  S
361   GCATGCAATGGTTTGAAAGGACATTTCAAGCTTCAAGGGGAGACGTACTTTATTGAACCCTTGAAGCTTCCTGACAGTGAAGCCCATGCA
       A  C  N  G  L  K  G  H  F  K  L  Q  G  E  T  Y  F  I  E  P  L  K  L  P  D  S  E  A  H  A
451   GTCTTCAAATATGAAAATGTAGAAAAGGAGGATGAGGCCCCCAAAATGTGTGGGGTAACCGAGACTAATTGGGAATCAGATGAGCCCATC
       V  F  K  Y  E  N  V  E  K  E  D  E  A  P  K  M  C  G  V  T  E  T  N  W  E  S  D  E  P  I
541   AAAAAGCTTTCTCAGATAATGATTCCTCCTGAACAACAAAGATACATTGAGCTTGTCATAGTTGCGGACCACAGAATGTACACAAAATAT
       K  K  L  S  Q  I  M  I  P  P  E  Q  Q  R  Y  I  E  L  V  I  V  A  D  H  R  M  Y  T  K  Y
631   GACGGTGATAAAACTGAGATAAGTTCAAAAATATATGAAACTGCCAATAATTTAAATGAGATTTACAGACATTTGAAAATTCATGTAGTT
       D  G  D  K  T  E  I  S  S  K  I  Y  E  T  A  N  N  L  N  E  I  Y  R  H  L  K  I  H  V  V
721   CTGATTGGCCTAGAAATGTGGTCCAGCGGAGAATTGAGTAAAGTCACATTATCAGCAGATGAGACTTTGGACTCATTTGGAGAATGGAGA
       L  I  G  L  E  M  W  S  S  G  E  L  S  K  V  T  L  S  A  D  E  T  L  D  S  F  G  E  W  R
811   GAGAGAGATTTGTTGCAGCGCAAAAGACATGATAATGCTCAGTTACTCACGGGCATGATCTTCAATGAAAAAATTGAAGGAAGGGCTTAC
       E  R  D  L  L  Q  R  K  R  H  D  N  A  Q  L  L  T  G  M  I  F  N  E  K  I  E  G  R  A  Y
901   AAAGAGAGCATGTGCGACCCGAAGCGTTCTGTAGGAATTGTTCGAGATCATAGAACTAGACCTCATTTGGTTGCAAATAGAATGGCCCAT
       K  E  S  M  C  D  P  K  R  S  V  G  I  V  R  D  H  R  T  R  P  H  L  V  A  N  R  M  A  H
991   GAGCTGGGTCATAATCTGGGCTTTCATCATGATGGAGATTCATGTACTTGCGGTGCTAACTCATGCATTATGTCTGCGACAGTAAGCAAC
       E  L  G  H  N  L  G  F  H  H  D  G  D  S  C  T  C  G  A  N  S  C  I  M  S  A  T  V  S  N
1081  GAACCTTCCAGTCGGTTCAGCGATTGTAGTCTTTTTCAATATTCGAGTGATATTATTCATAATCCTTTTACATCACGGTGCCTTTACAAT
       E  P  S  S  R  F  S  D  C  S  L  F  Q  Y  S  S  D  I  I  H  N  P  F  T  S  R  C  L  Y  N
1171  GAACCCTCGAAGACAGATATTGTTTCACCTTCAGTTTGTGGCAATTACTATATGGAGGTGGGAGAAGATTGTGACTGTGGCCCTCCTGCA
       E  P  S  K  T  D  I  V  S  P  S  V  C  G  N  Y  Y  M  E  V  G  E  D  C  D  C  G  P  P  A
1261  AATTGTCAAAATCCATGCTGTGATGCTGCAACGTGTAGACTGACACCAGGGTCACAGTGTGCAGATGGACTGTGTTGTGACCAGTGCAGA
       N  C  Q  N  P  C  C  D  A  A  T  C  R  L  T  P  G  S  Q  C  A  D  G  L  C  C  D  Q  C  R
1351  TTTATGAAAAAAGGAACAATATGCCGGATAGCAAGGGGTGATGACCTGGATGATTACTGCAATGGCATATCTGCTGGCTGTCCCAGAAAT
       F  M  K  K  G  T  I  C  R  I  A  R  G  D  D  L  D  D  Y  C  N  G  I  S  A  G  C  P  R  N
1441  CCCTTCCATGCC
       P  F  H  A
```

Fig. 4. cDNA and deduced amino acid sequences of Jerdonitin: the RGD motif is shaded. The peptide fragments obtained by digestion of Jerdonitin and Edman reaction are underlined. The cDNA sequence was submitted to GenBank and the Accession No. is AY364231.

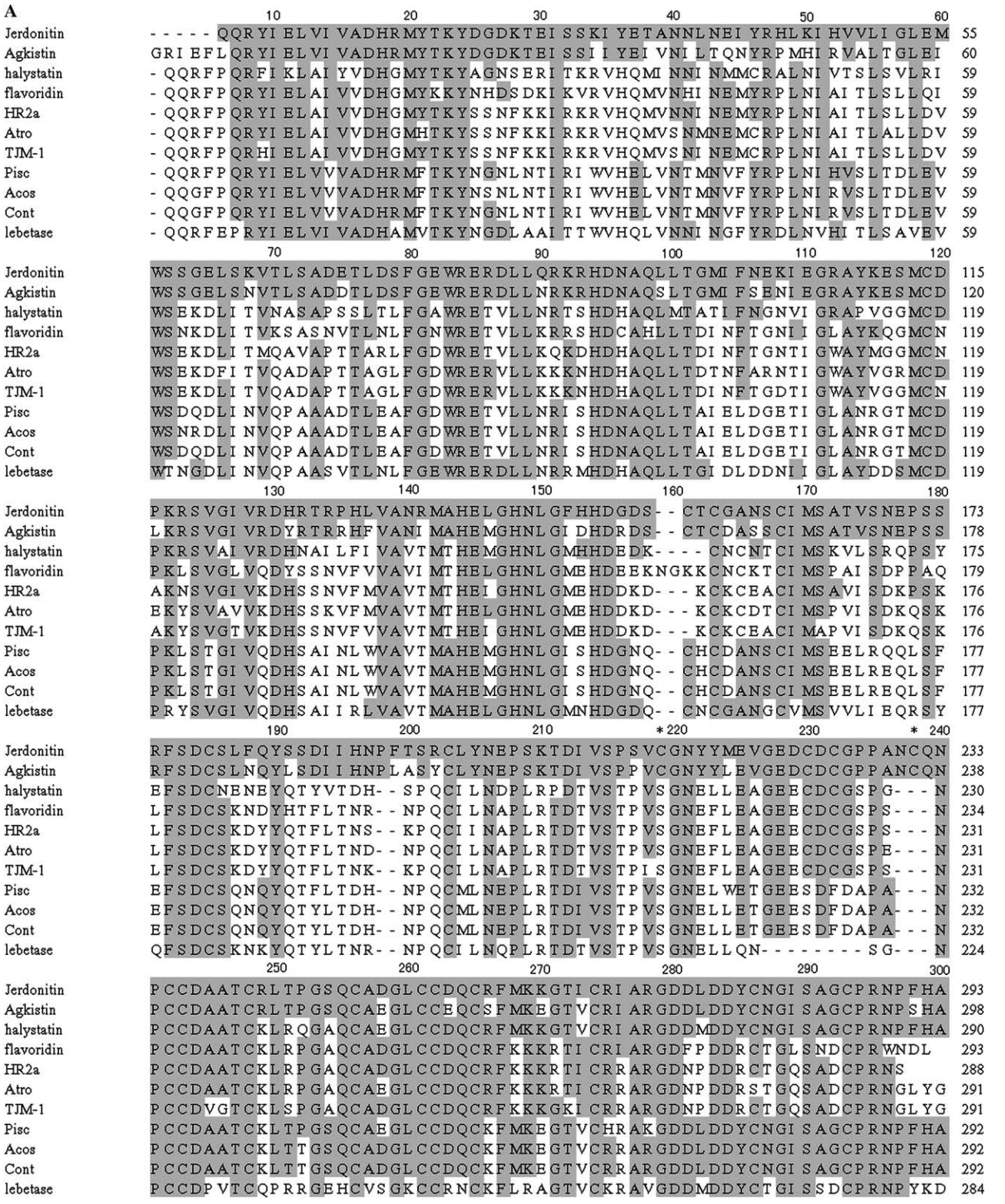

Fig. 5. Amino acid sequence alignment and the phylogenetic relationships of P-II class SVMPs. (A) Agkistin (GenBank Accession No. AY071905); halystatin (GenBank Accession No. BAA06025); flavoridin (GenBank Accession No. BAC00515); HR2a (GenBank Accession No. AAK68850); Atro, atrolysin e (GenBank Accession No. CAA62600); TJM-1 (GenBank Accession No. AY267902); Pisc, piscivostatin beta chain (GenBank Accession No. BAC55947); Acos, acostatin beta chain (GenBank Accession No. BAC55945); Cont, contortrostatin (GenBank Accession No. AAF65171); and lebetase (GenBank Accession No. X97894). The symbols (*) indicate the additional cysteines of Jerdonitin compared to other P-II class SVMPs. (B) Phylogenetic relationships of P-II class SVMPs based on amino acid sequences with Bery, berythractivase (GenBank Accession No. AAL47169), as outgroup. The numbers at the nodes represent the bootstrap probability.

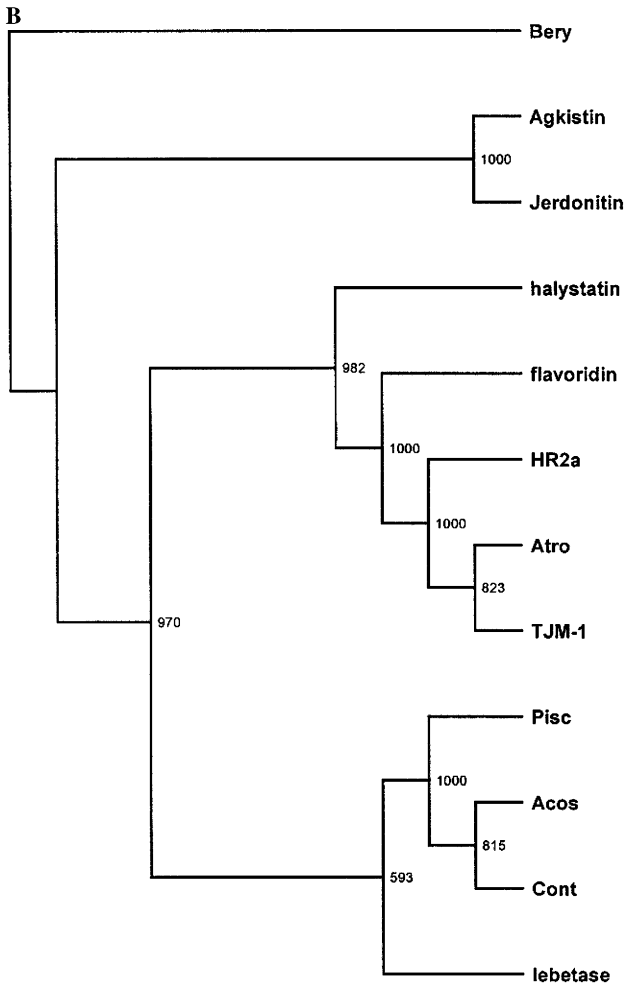

Fig. 5. (*continued*)

Amino acid sequence determination

The N-terminus of Jerdonitin was blocked. Internal peptide sequences were obtained and gave an amino acid sequence covering a total of 95 residues (shown as underlined in Fig. 4), which comprises 32.3% of the mature protein. These internal peptides locate in the metalloproteinase domain and disintegrin domain, respectively.

Cloning and sequence determination of Jerdonitin

PCR amplification of total cDNAs with the designed primers achieved a DNA fragment of about 1500 bp. The PCR product was then cloned into pMD18-T vector and sequenced (Fig. 4). The mature Jerdonitin covered an open reading frame of 882 nucleotides which encode 294 amino acid residues.

Construction of phylogenetic tree of P-II class SVMPs

Based on the comparison of amino acid sequence similarities of Jerdonitin with those of other P-II class SVMPs (Fig. 5A), a phylogenetic tree of P-II class SVMPs (Fig. 5B) was constructed. Jerdonitin and Agkistin [16] formed a group and separated from other P-II class SVMPs.

Discussion

Snake venom metalloproteinases (SVMPs) can cause hemorrhage by degrading the ECM surrounding blood vessels and proteins that interfere with homeostasis [1]. The presence of RGD-containing disintegrin contributed to this effect by inhibiting platelet aggregation [17–21]. According to their domain structure, SVMPs were classified into four groups [2,3]. In the present study, we purified and cloned a new metalloproteinase–disintegrin, named Jerdonitin, from the venom of *T. jerdonii*. Jerdonitin comprised metalloproteinase, spacer, and disintegrin domains and belonged to P-II class SVMPs. Comparison of the cDNA deduced protein sequence of Jerdonitin with those of other SVMPs, it shares low similarity with other P-II class SVMPs such as contortrostatin (59%) [22], acostatin beta chain (59%) [23], and piscivostatin beta chain (57%) [23], except Agkistin (84%) [16]. Interestingly, Jerdonitin has two additional cysteines (Cys219 and Cys238) located in spacer domain and disintegrin domain, respectively (Fig. 5A, indicated by asterisk). There is a serine at the position 219 (numbering in this paper *, Fig. 5A) of the other P-II class SVMPs. The change of Ser219 to Cys219 is a characteristic feature of all members of the known mammalian metalloprotease/disintegrin/cysteine-rich (MDC) proteins. This position was critical for the maintenance of the protein structure. If there was a cysteine residue in this position, it would form a disulfide bond with free cysteine in the succeeding disintegrin-like domain, processing a medium size protein, and therefore the spacer and disintegrin domains cannot be processed away from the metalloproteinase [24–26]. In our case, there is a cysteine in this position and it would form a disulfide bond with free Cys238 in the succeeding disintegrin domain. The additional disulfide bond possibly hinders the release of the disintegrin domain. Unlike other P-II class SVMPs, the mature protein of Jerdonitin will still comprise metalloproteinase, spacer, and disintegrin domains after posttranlationally processing. Internal peptide sequence determination confirmed that Jerdonitin comprised metalloproteinase, spacer, and disintegrin domains (Fig. 4). Jerdonitin dose-dependently inhibited ADP-induced human platelet aggregation with IC_{50} of 120 nM.

Phylogenetic analysis of P-II class SVMPs revealed that Jerdonitin and Agkistin [16] formed a group and separated from other P-II class SVMPs (Fig. 5B) which will be separated into metalloproteinases and disintegrins by posttranslationally processing. But

metalloproteinase and disintegrin domains of Jerdonitin are not separated. Although the natural protein of Agkistin has not been reported until now, sequence alignment indicated that Agkistin had also two additional cysteines, we speculate that those two domains of Agkistin will not be separated away.

In conclusion, we purified and cloned a new metalloproteinase–disintegrin, Jerdonitin, from *T. jerdonii* venom and studied its effect on platelet aggregation. Comparison of the amino acid sequences of Jerdonitin with those of other P-II class SVMPs by sequence alignment and phylogenetic analysis, in conjunction with natural protein structure data, suggested that Jerdonitin was a new type of P-II class SVMPs. The relation of its structure and function will be further studied in future.

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