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CHARACTERIZATION OF MULTIPLE METALLOPROTEINASES WITH FIBRINOGENOLYTIC ACTIVITY FROM THE VENOM OF TAIWAN HABU (*TRIMERESURUS MUCROSQUAMATUS*): PROTEIN MICROSEQUENCING COUPLED WITH cDNA SEQUENCE ANALYSIS[1]

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Three fibrinogenolytic proteases were isolated and purified from the venom of Taiwan habu (*Trimeresurus mucrosquamatus*) using anion-exchange and gel-filtration chromatographies followed by cation-exchange HPLC. Further characterization of these purified fractions with fibrinogenase activity indicated that they are single-chain proteases of approximately 24 kDa, possessing strong cleaving activity mainly on the Aα and less on Bβ and γ chains of fibrinogen subunit chains. Enzyme activities were strongly inhibited by EDTA or 1,10-phenanthroline and not by phenylmethanesulfonyl fluoride, suggesting that these fibrinogenases belong to the family of metalloproteinases and not thrombin-like serine proteases. N-Terminal sequence analysis of these proteases failed to show any free amino-terminal residues, thus hampering the sequence determination by conventional sequencing strategy. Microsequencing on the electroblotted fragments of CNBr-treated proteases separated on SDS-PAGE was then used to determine the partial sequences. Sequence comparison of the determined partial sequences of these proteins with published sequences of the protein data bank revealed that they showed sequence homology with H_2-protease, HR2a and protrigramin, which were all shown to belong to metalloproteinases present in various snake venoms. Polymerase chain reaction (PCR) was employed to amplify cDNAs constructed from the poly(A)$^+$RNA of fresh venom glands of the same snake species to facilitate cloning and sequencing of these proteases. Sequencing several positive clones containing amplified cDNAs revealed the existence of one fibrinogenase in the Taiwan habu, which was contained within one complete cDNA encoding the preproproteinase precursor of hemorrhagic metalloproteinases.

Venoms of the *Viperidae* and *Crotalidae* families cause shock, intravascular clotting, systemic and local hemorrhage, edema and necrosis upon victimized prey. Death is usually the result of the combined effects of several components in the venom. It has also been well known that snake venoms contain complex mixtures of pharmacologically active peptides and proteins

[1]The sequence data of *T. mucrosquamatus* mRNA for fibrinogenolytic metalloproteinase have been deposited in the EMBL, GenBank and DDBJ nucleotide sequence databases under the accession number **X91190**.

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including some potent proteolytic enzymes. Venom proteinases have been characterized to be a heterologous group of proteins, generally belonging to two major classes of proteases, *i.e.* thrombin-like enzymes and metalloproteinases with a wide range of molecular masses [1-4].

Recently we have focused on the study of blood fibrinogenolytic enzymes isolated from venoms of crotalid snakes. Previous characterization on proteases from the American-Western diamondback rattlesnake (*Crotalus atrox*) revealed that all fractions isolated from the anion-exchange chromatography showed varying extents of specific proteolytic activity against α- or β-chains of fibrinogen molecules [5,6]. On the other hand, studies on the protease components from the Taiwan habu [7,8], a major crotalid species in this island, also indicated several kinds of fibrinogenases present in this phylogenetically more remote species of the *Crotalidae* family. It is deemed essential to isolate and compare these venom proteases to shed some light on their possible structural relatedness. In this study we applied multiple-step chromatographies for the isolation and purification of several novel proteases with strong activity upon α-chains of fibrinogen, which showed distinct physical and enzymological properties from some reported thrombin-like or metalloproteinases isolated from venoms of closely-related snake species.

In this report we have circumvented the difficulty of sequencing analysis on N-terminally blocked proteases by employing microsequencing technique to solve partial sequences of the CNBr-cleaved fragments of purified proteins separated on SDS-containing polyacrylamide gel. The partial sequences obtained were then compared with known venom sequences in the protein data bank to search for possible structural homology. We have designed primers based on the homology comparison and amplified cDNAs constructed from the venom glands of Taiwan habu using newer PCR methodology to aid in the structural analysis of one large and relatively complex protein molecule with blocked N-terminus. A complete and unambiguous cDNA coding for the intact pre-proproteinase which encompasses the sequence corresponding to the purified fibrinogenase isolated from the crude venom is shown herein.

MATERIALS AND METHODS

Protein isolation and purification from crude venom

Venom glands of Taiwan habu were donated from the National Institute of Preventive Medicine, Taipei, Taiwan. The lyophilized venom powder was obtained from the local snake farm. The substrate fibrinogen and various protease inhibitors were obtained from Sigma Chemical Company (St. Louis, MO). Gel suspensions of TSK DEAE-650 anion-exchanger resin were purchased from Merck (Darmstadt, Germany).

Proteolytic components were separated by anion-exchange chromatography on an open column (2.5 x 18 cm) packed with TSK DEAE-650(M) gel suspension. Dissolved venom powder in 0.025 M ammonium bicarbonate, pH 7.8 starting buffer was applied to an open column (total 8 ml) and then eluted in a linear gradient of 0.025-0.5 M ammonium bicarbonate, followed by 0.5-1.0 M ammonium bicarbonate, pH 8.0 buffers, similar to those described previously [8,9]. Two gel-filtration chromatographic steps were carried out on Sephadex G-75(S) (2.5 x 48 cm) and G-50(S) (1.6 x 95 cm) using 0.025 M ammonium bicarbonate, pH 7.8 as the elution buffer. A cation-exchange column (7.8 x 50 mm, Bio-Rad MA7C) on a Hitachi's liquid chromatograph with a linear gradient of 0.025-0.8 M ammonium acetate, pH 6.0 buffer was carried out to obtain three chromatographically pure protease components from the fraction isolated from the above gel-filtration chromatographies.

SDS-PAGE assays for fibrinogenolytic activity

The fibrinogenolytic activity was assayed on SDS-polyacrylamide slab gel (5 % stacking/ 14 % resolving gel) as described [10]. Small vials containing about 5 μg purified fibrinogen in

50 mM Tris-HCl pH 7.7 buffer were incubated with 1-10 μl of various fractions (containing about 0.1-1.0 μg purified protein) at 37 °C for 1 h. After the incubation the digestion was stopped by adding 0.1% SDS/1% β-mercaptoethanol and heated at 90 °C for 3 min. The proteolytic activity was monitored on the Coomassie blue-stained gels after electrophoresis by observing the cleavage patterns of purified fibrinogen chains.

N-Terminal sequence analysis of blotted CNBr-treated purified proteases

Purified proteins from the above multiple-step chromatographies were treated with CNBr at 37 °C for 24 h and electrophoresed on an SDS-PAGE system as described. The generated protein fragments on the gels were then electroblotted on to polyvinylidene difluoride (PVDF) membranes (Fluortrans , Pall Ultrafine Filtration Co., Glen Cove, NY) according to the published protocol [11] with a semi-dry-blotting system (Nihon-Eido, Tokyo, Japan) at 1 mA/cm^2 for 4 h at room temperature. The stained protein bands were cut out and transferred to a microsequencing sequencer (Model 477A, Perkin Elmer/Applied Biosystems).

PCR amplification and cloning

Snake venom glands from Taiwan habu were removed and stored in liquid-nitrogen container immediately after they were sacrificed and before the processing for mRNA isolation. Two deep-frozen venom glands from one snake were homogenized and RNA was extracted according to the standard procedures [12]. To obtain a full-length venom cDNA coding for the desired fibrinogenase, poly(A)$^+$RNA was purified using QuickPrep mRNA preparation kit (Pharmacia, Uppsala, Sweden) and then subjected to the synthesis of cDNA mixture by cDNA Synthesis System/Plus kit (Amersham, England).

Two oligonucleotide primers of sense and antisense orientations based on the highly conserved 5'- and 3'-noncoding regions of cDNA coding for trigramin, rhodostomin and Ht-e [13-15] with the forward sequence, 5'-CCAAATCCAGCCTCCAAAATG-3' and the reverse one 5'-TTCCATCTCCATTGTTGTTTA-3' were synthesized. The reactions were subjected to 35 cycles of heat denaturation at 94 °C for 1.5 min, annealing the primers to the DNAs at 48 °C for 2 min, and DNA chain extension with *Taq* polymerase at 72 °C for 2.5 min, followed by a final extension at 72 °C for 10 min. The PCR products were subcloned into pGEM-T vector, and then transformed into *E. coli* strain JM109. Plasmids purified from positive clones were prepared for nucleotide sequencing by dideoxynucleotide chain-termination method [16].

Homology search and sequence comparison

In the sequence comparison and analysis of amino-acid sequences for the characterized fibrinogenase and various metalloproteinases deposited in the protein data bank, a multiple-sequence alignment software program (DNASTAR Inc., Madison, WI) was used for the optimal alignment of various related sequences and estimation of sequence homology.

RESULTS AND DISCUSSION

The venoms of various snakes have been shown to possess fibrinolytic or fibrinogenolytic activity, notably in the snake families of *Crotalidae* and *Viperidae* [17-19]. It is intriguing that venoms of the crotalid family contain many proteases which are related to the blood and platelet coagulation [20]. Currently several proteolytic enzymes have been used clinically as potential antithrombotic agents [21-24] in addition to their use as basic biochemical tools in the study of structural and functional relationships of these enzymes to the important process of fibrin polymerization and clotting process. More than 30 proteolytic enzymes have been isolated from crotalid and viperid snake venoms. Most venom proteases characterized so far consist of two major classes, *i.e.* thrombin-like enzymes [25] and metalloproteinases with hemorrhagic activity [26]. In this study we have made an effort to complement the previous structural characterization of several proteolytic components with strong fibrinogenolytic activity from an endemic crotalid species, *i.e.* Taiwan habu [8]. Successful identification and characterization of the complete precursor for one fibrinogenolytic metalloproteinase have been accomplished by means of sensitive microsequencing on electroblotted protein bands coupled with cloning and sequencing of its corresponding cDNA by PCR technology.

Protein isolation and purification from crude venom

Fig. 1A shows an elution pattern of the crude venom of Taiwan habu on TSK DEAE-650 anion-exchange column, which is different from that of venom separation of the American-Western rattlesnake reported previously [27]. The first peak fraction (indicated by arrow) was analyzed to possess the greatest fibrinogenolytic activity among all fractions studied, as revealed in the fibrinogenolytic activity assay on SDS-gel electrophoresis. There are more than

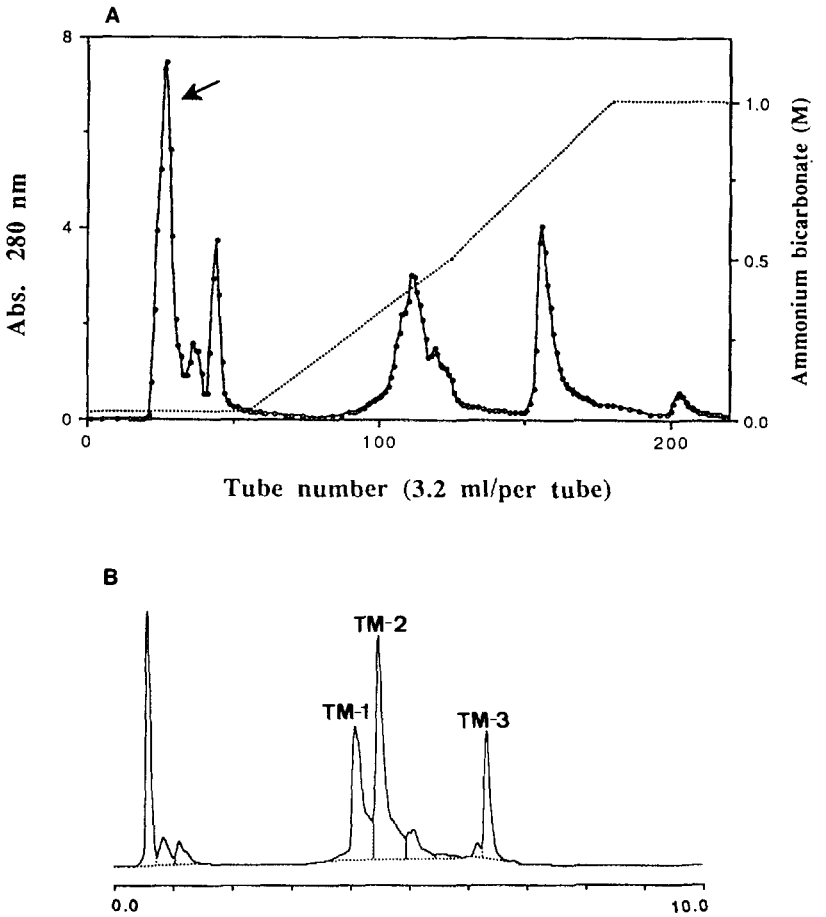

Fig. 1. (A) Anion-exchange chromatography on TSK DEAE-650 (M) column of crude venom from Taiwan habu, *Trimeresurus mucrosquamatus*. About 300 mg of lyophilized crude venom dissolved in the starting buffer of 0.025 M ammonium bicarbonate, pH 7.8, was applied to the column equilibrated with the same buffer. Elution was carried out in four steps similar to that described in the previous report [8]. The column eluates (3.2 ml/tube) were monitored for absorbance at 280 nm. The first peak (arrow) was collected, lyophilized and used for further purification on Sephadex G-75(S) and G-50(S) columns (figures not shown) to collect the major fraction eluting in a range of 20-35 kDa. (B) Cation-exchange HPLC on a MA7C column of the major fraction collected from Sephadex G-50(S). About 50 µl (1.5 mg/ml) of protein sample was injected to the column each time and the elution was run with a linear gradient of 20-100% buffer B (0.8 M ammonium acetate, pH 5.7) in the starting buffer A (0.025 M ammonium acetate, pH 6.0) for 10 min. Three major peaks denoted as TM-1, TM-2 and TM-3,respectively, were collected for further sequence analysis.

three components present in this unretarded fraction of DEAE chromatography [8]. Therefore further purification of this fraction was performed in order to obtain purified components responsible for its strong proteolytic activity. A second purification step of the lyophilized powder from this fraction was carried out on two consecutive gel-filtration chromatographies, first on Sephadex G-75 and then on Sephadex G-50 columns (data not shown), resulting in removal of the main single peak from most contaminating components of small molecular weights (< 15,000). It is noteworthy that the purified fraction of G-50 column with increased fibrinogenolytic activity showed a single peak of about 27 kDa as estimated from the calibration curve based on native molecular-weight standards [8]. A cation-exchange column (Bio-Rad MA7C) further resolved this peak into 3 components designated as TM-1, TM-2 and TM-3 (**Fig. 1B**), possessing different extents of fibrinogenolytic activities against fibrinogen molecules. They were all shown to be single bands of 24 kDa on SDS-PAGE under reduced and non-reduced conditions (**Fig. 2**). Since the molecular-mass estimation of the combined fraction on G-50 is about 27 kDa, it is concluded that TM-1, TM-2 and TM-3 are all single-chain polypeptides without extra subunits. It is noted that the molecular masses of these three components were estimated to be about 27 kDa previously [8], a higher value of molecular mass arising probably from using different calibration curves for molecular weight estimation.

Fibrinogenolytic activity and effects of protease inhibitors

Proteolytic enzymes of snake venoms have been defined operationally as α- or β-chain degrading fibrinogenases based strictly on their direct-acting digestive activity *in vitro* on the fibrinogen molecules according to the guidelines of the *International Committee on Thrombosis and Haemostasis* [25]. It is of interest to note that purified fractions of TM 1-3 isolated from the venom of Taiwan habu possess mixed types of fibrinogenolytic activity when assayed using purified fibrinogen, with TM-1 showing proteolytic activity against both Aα and Bβ chains of

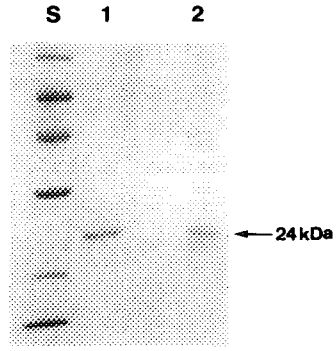

Fig. 2. SDS-gel electrophoresis (SDS-PAGE) of purified TM-3 fraction under denaturing conditions in the presence (lane 1) and absence (lane 2) of 5% β-mercaptoethanol. Lane S: relative electrophoretic mobilities of standard proteins used as molecular mass markers (in kDa): phosphorylase b (94), bovine serum albumin (67), ovalbumin (43), carbonic anhydrase (30), soybean trypsin inhibitor (20), and lysozyme (14). The gel was stained with Coomassie blue. Note that the other two fractions (TM-1 and TM-2) also show pure single bands on the gel with an estimated molecular mass of about 24 kDa similar to TM-3 (arrow).

fibrinogens and TM-2 and TM-3 mainly on Aα chain and slightly on Bβ chain under identical digestion conditions [8]. TM-2 is the weakest fibrinogenolytic enzyme among the three.

The effects of various protease inhibitors on these three proteases were also studied using fibrinogenolytic activity assays on SDS-PAGE. It was found that all three enzymes were inhibited by EDTA and 1,10-phenanthroline, attesting to their being metalloproteinases. The serine protease inhibitors, phenylmethanesulfonyl fluoride (PMSF) and aprotinin, were shown to have no effects on the fibrinogenolytic activity of TM-1, TM-2 and TM-3, indicating that these proteases do not belong to the serine-proteases family as commonly shown for some venom fibrinogenases and thrombin-like proteases [20,28]. Similar to crotalase isolated from *C. adamanteus* [28], the first thrombin-like serine protease found in the American-Eastern rattlesnake of *Crotalidae* family, TM-1, TM-2 and TM-3 lost their fibrinogenolytic activity in the presence of β-mercaptoethanol. Thus these enzymes with fibrinogenolytic activities should be classified in the metalloproteinase family of venom proteases and not that of thrombin-type serine proteases in spite of some similar inhibitory properties shared between these two major types of venom proteases. All these mentioned enzymological properties attest to the unique nature of these special mixed-type α/β fibrinogenase as compared to other characterized venom fibrinogenases. It is noteworthy that the present study has indeed revealed the existence of these multiple proteases with defined proteolytic activities in the venom of Taiwan habu, which are distinct from those characterized from venoms of varied crotalid species [25].

Sequence analysis of purified fibrinogenases (TM-1, TM-2 and TM-3)

The amino acid compositions (data not shown) of purified TM-1 to TM-3 were determined in order to establish the identity of these novel α/β fibrinogenases. They were found to be somewhat similar between each other, indicative of their being closely related in their primary sequences [8]. In general the pair-wise composition comparison of TM proteases and published fibrinogenases showed distinct differences regarding sulfhydryl contents of our proteases and those other fibrinogenases. Our purified enzymes were shown to possess 3-4 pairs in contrast to about 6-8 pairs of disulfides present in hemorrhagic factors from the Chinese habu [29].

N-Terminal sequence analysis of these three proteases indicated blocked amino-terminal residues in all three fractions. We have therefore resorted to the determination of the partial sequences of some proteolytic fragments generated from CNBr cleavage of purified proteins. The generated protein fragments were separated on SDS-PAGE and then electroblotted on to polyvinylidene difluoride (PVDF) membranes. The stained protein bands were cut out and transferred to a microsequencing sequencer for partial sequence determination using Edman degradation methodology. The unambiguous sequence assignments were achieved for the first 15 residues on one of the CNBr fragments (about 6 kDa) obtained from chemical cleavage of each purified protease (**Fig. 3**). Based on these partial sequences, a homology search in the protein data bank was carried out to identify and compare with the homologous sequences of venom proteinases published previously. Five homologous sequences with high similarity scores were found and listed in Fig. 3 for sequence comparison. TM-1 and TM-2 are shown to possess the identical sequence in this peptide fragment and they exhibit only about 80% sequence identity with TM-3, in accord with our previous suggestion that TM-1 and TM-2 are

TM-1 or TM-2	Y K P L N I A T T L S L L X I
TM-3	P L N I A I T L A L L D V W S
H_2-Proteinase (*T. flavoviridis*) 44-60	Y R P L N I A I S L N R L Q I W S
HR2a (*T. flavoviridis*) 45-61	Y R P L N I A I T L S L L D V W S
pro-Trigramin (*T. gramineus*) 46-62	C R A L N I V T T L S V L E I W S
pro-Rhodostomin (*C. rhodostoma*) 44-60	Y K Y M H F G I S L V N L E T W C
Ht-e (*C. atrox*) 44-60	Y T Y M Y I D I L L A G I E I W S

Fig. 3. Comparison of the partial sequences for cyanogen bromide (CNBr)-cleaved fragments from purified TM-1, TM-2 and TM-3 proteases. The partial sequences listed for H_2-proteinase [33] and HR2a [30] were determined from protein sequencing, whereas those for pro-trigramin [13], pro-rhodostomin [14] and Ht-e [15] were derived from cDNA sequences. Segment numbering shown after each listed protease is the corresponding amino-acid residue numbering for each sequence segment based on the original sequences. Amino acid residues are denoted by one-letter symbols with X indicating unidentified residue in the sequencer. Note that TM-1 and TM-2 show identical partial sequence in the compared CNBr-cleaved fragments.

very similar in primary structures and probably mutually related [8]. The sequence-homology comparison revealed that about 53-93% partial sequence identity exists among these eight venom proteases (Fig. 3). TM-3 is found most closely related to HR2a of *Trimeresurus flavoviridis* [30], a closely related species of Taiwan habu. Judging from the partial sequences determined for TM-1 to TM-3 in this report and those published sequences, fibrinogenases reported here seem to belong to members of some hemorrhagic proteinases. We have therefore designed primers based on the highly conserved 5'- and 3'-noncoding regions of cDNA coding for trigramin, rhodostomin and Ht-e [13-15]. A PCR cloning/sequencing methodology was used to amplify cDNAs constructed from the venom glands of Taiwan habu in order to aid in the structural analysis of these large and relatively complex protein molecules with blocked N-terminus in each of the mature proteases (shown below).

cDNA amplification by PCR and sequence determination

PCR amplification of cDNA mixtures constructed from total mRNA of venom glands with the designed primers corresponding to the conserved noncoding regions of the reported venom proteinase precursors was employed to achieve the isolation of one 1.5 kb PCR fragment encoding supposedly venom proteases of Taiwan habu. There were 24 positive clones being identified from the transformed *E.coli* strain JM109 and most of them were shown to possess identical cDNA sequence after automatic fluorescence-based nucleotide sequencing. This seems to indicate the existence of multiple cDNA copies encoding this group of venom proteinases. We have determined three distinct cDNA clones in their entirety, each possessing one complete sequence corresponding to the expected 5' and 3' primer sequences and the partial sequence of TM-3 (Fig. 3) determined by protein microsequencing. One of the determined clones (Mpts-8) together with its deduced protein sequence for this novel fibrinogenase is shown in **Fig. 4**.

It is worth noting that the deduced total protein sequence consists of an 18 amino-acid signal peptide, a zymogen propeptide of 171 amino acids and a mature protein of 203 amino acids, and a disintegrin-like carboxy-terminal segment of 89 amino acids. This uninterrupted precursor sequence corresponding to the purified fibrinogenase of Taiwan habu is found to be very similar to several characterized proteinase precursors of hemorrhagic proteases such as trigramin [13] and hemorrhagic toxin e (Ht-e) [15] regarding the overall organization of the

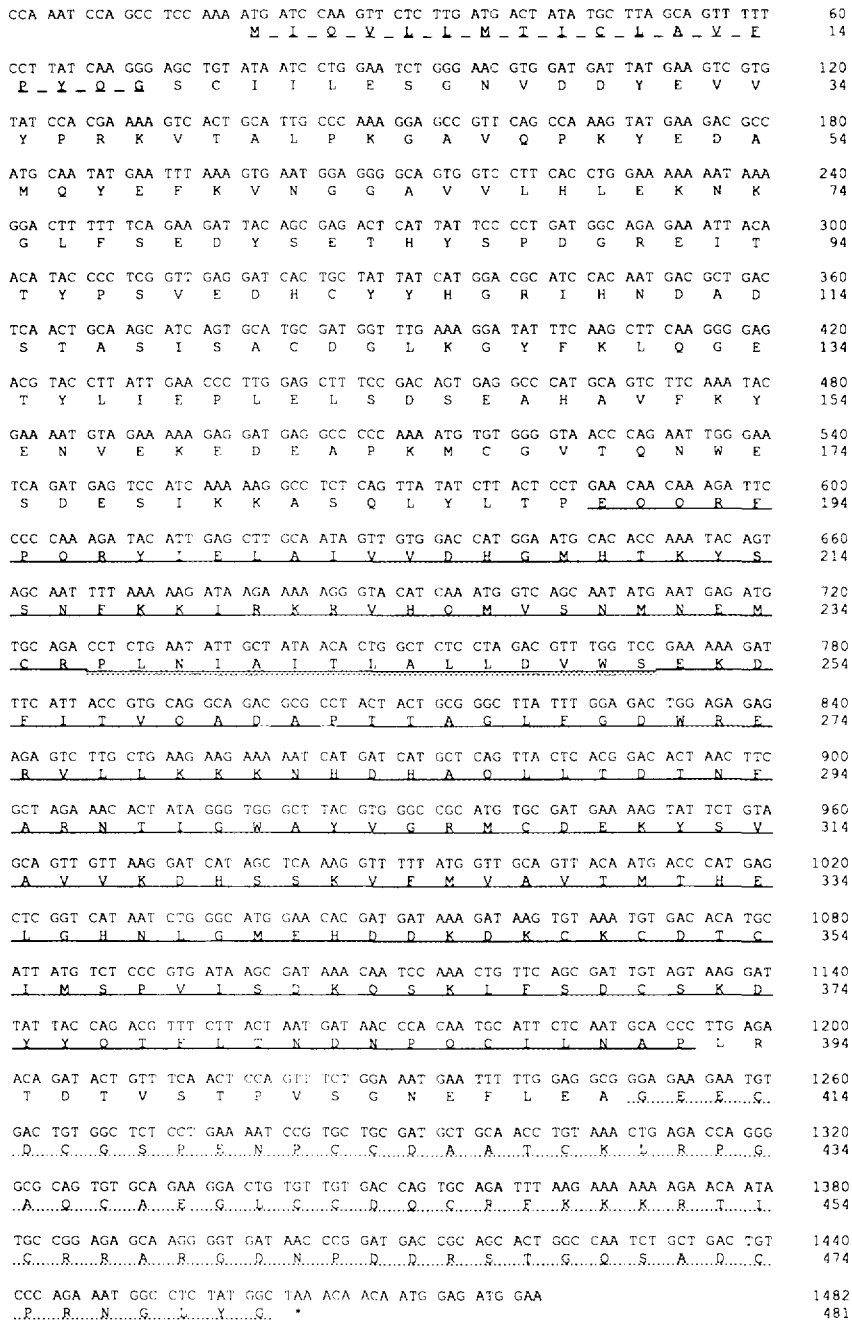

Fig. 4. Nucleotide and deduced protein sequences of the precursor for fibrinogenolytic protease (TM-3) from one PCR-amplified cDNA clone (Mpts-8). The nucleotide sequence of 1482-base pairs including the designed primer sequences is shown above the amino-acid sequence of 481 residues, which includes an 18 amino-acid signal peptide plus an 171 amino-acid segment of zymogen-like propeptide and a mature protein of 203 amino acids starting with Glu-Gln-GLn as the first three N-terminal amino acids. Nucleotide and amino-acid sequences are numbered in every 60 nucleotide or 20 amino-acid segment for easy tracing of sequence contents. Amino acids are denoted by one-letter symbols. Note that the putative signal peptide, mature metalloproteinase and disintegrin peptide are indicated by dash line, solid line and dotted line, respectively. Amino-acid fragment (doubly underlined) in the metalloproteinase represents the sequence determined for the CNBr fragment by protein microsequencing.

above mentioned protein subdomains endowed with different functions. The protein sequence homology between the complete precursor of TM-3 (Mpts-8) and precursors of trigramin and Ht-e is 77 and 71% respectively. We have recently learned of the report of another cDNA sequence encoding the precursor of platelet aggregation inhibitor from Taiwan habu [31] also contains a hemorrhagic metalloproteinase domain similar to trigramin and Ht-e. However our characterized mature fibrinogenase is not identical to this domain, corroborating our suggestion of the existence of multiple fibrinogenase isoforms in this local habu. We believe that the deduced TM-3 sequence coupled with the sequence determination of several undetermined cDNA clones coding for homologous isoforms of the same fibrinogenolytic metalloproteinase (unpublished results) would constitute a major multigene family of venom fibrinogenases similar to that of hemorrhagic zinc-containing metalloproteinases [32].

Structural comparison of TM-3 fibrinogenase and homologous sequences by multiple-sequence alignment

Fig. 5 shows the optimal alignment by introducing a minimum number of gaps along the entire lengths of mature TM-3 fibrinogenase and six known homologous sequences from different snake species. It is found that TM-3 fibrinogenase of the Taiwan habu shows 70, 84, 50, 55, 57 and 69% sequence homology to the respective homologous sequences of H_2-proteinase [33], HR2a [30], HT-2 [34], Ht-e [15], LHF-II [35] and pro-trigramin [13] from venoms of different crotalid snakes. The sequence homology is not very high as judged by the medial % homology in a range of only 50-70% for most proteases except HR2a from one closely related species of *Trimeresurus flavoviridis* [30]. However, the critical segment (residues #144-155 indicated by asterisks in Fig. 5) with multiple histidine residues, which is supposedly the consensus zinc-binding domain is well conserved for all sequences. Therefore it is to be expected that this common structural feature coupled with some more pronounced sequence homology in various domains and similar pharmacological properties reported in the literature [26] should lay a firm basis in grouping these venom fibrinogenases as members of zinc-containing hemorrhagic proteases from various snake venoms.

CONCLUSION

It is well known that fibrinogenases are the major venom principles from the crotalid family of snakes. α-Fibrinogenases are usually metalloproteases which are markedly inhibited by EDTA and β-mercaptoethanol, while those of β-fibrinogenases are mostly serine proteases which are inhibited by PMSF. Judging on these criteria, TM-1, TM-2 and TM-3 characterized here should be classified as α-type fibrinogenases. Based on pharmacological properties, α-fibrinogenases possess hemorrhagic activity while β-fibrinogenases lack this activity. Detailed sequence analysis based on microsequencing and PCR cloning/sequencing has established purified TM-3 in this study as one of the major domains present in an intact precursor encoding pre-proproteinase for zinc-containing metalloproteinase with α-fibrinogenase activity. TM-3 is supposedly the mature metalloprotease processed from a much bigger pre-proproteinase which also encompasses its carboxy-terminal region as a RGD-containing disintegrin peptide with inhibitory activity for platelet aggregation. Further characterization of TM-1 and TM-2 at the

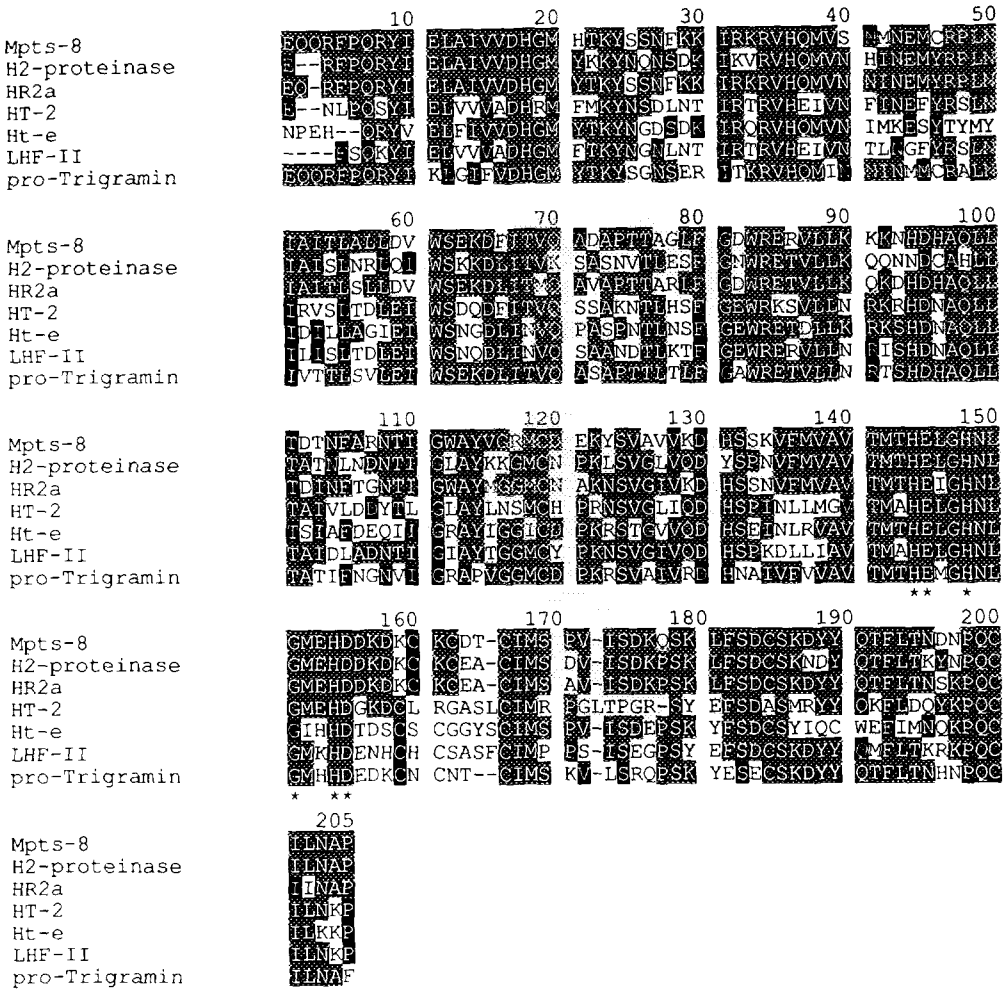

Fig. 5. Multiple sequence alignment and sequence comparison of mature metalloproteinase (TM-3) deduced from Mpts-8 clone and six venom metalloproteinases from varied snake species. Sequence alignment was carried out in the DNASTAR program using published sequences of H_2-proteinase [33], HR2a [30], HT-2 [34], Ht-e [15], LHF-II [35] and pro-Trigramin [13]. The highly conserved and consensus amino-acid residues involved in zinc binding are indicated by asterisks. The gaps are introduced for optimal alignment and maximum homology for the sequences. The key amino-acid residues located in the bold regions (highlighted) are conserved for most of the compared sequences. Amino acid residues are denoted by one-letter symbols.

protein and gene levels is currently in progress, which should provide more information regarding the structure/function relationship between these venom fibrinogenases.

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