Isolation and Characterization of a Novel Proteinase Inhibitor from the Snake Serum of Taiwan Habu (*Trimeresurus mucrosquamatus*)

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A proteinase inhibitor (designated as TMI) was isolated and purified from the snake serum of Taiwan habu (*Trimeresurus mucrosquamatus*) by using successive chromatographies which included Sephadex G-100, DEAE-Sephacel chromatographies, and C_4 reverse-phase HPLC. The purified inhibitor was shown to be a homogeneous protein with a molecular mass of about 47 or 36 kDa in the presence or absence of a reducing agent, β-mercaptoethanol. The inhibitor decreases in molecular mass by about 23% with N-linked neuraminidase treatment, suggesting that it is a glycoprotein. Further enzymatic analyses indicated that this inhibitor possesses strong inhibitory activities toward three zinc-dependent metalloproteinases and not fibrinogenolytic serine proteases previously isolated from the venom of the same snake species with an IC_{50} of about 0.2–1.1 μM. Its IC_{50} value was approximately three orders of magnitude more effective than those of the tripeptide inhibitors we previously purified from the crude venom of the same snake (*Biochem. Biophys. Res. Commun.* 248, 562–568 (1998)). The purified inhibitor showed stronger inhibitory action against caseinolytic activities of crude venoms from closely related species of Taiwan habu than those from unrelated species. *N*-terminal sequence analysis showed that its sequence is distinctly different from sequences of those serum inhibitors reported for other snake species in the literature. Based on inhibition susceptibility and primary structures of various snake protease inhibitors, it is suggested that this novel inhibitor isolated from the serum of Taiwan habu may be a unique self-defense protein factor mainly for protection against envenomation from snakes of the same genus. © 1999 Academic Press

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Venoms of the snake families of *Viperidae* and *Crotalidae* have been known to contain complex mixtures of many toxins and enzymes (1–3), which cause victimized preys shock, intravascular clotting, systemic and local hemorrhage, edema and necrosis after envenomation by snakebites. The major complication arising from snake envenomation is hemorrhagic effects, which have been widely studied for almost a century and are thought to be the result of structural destruction of capillary basement membranes via proteolytic degradation by venom proteases (4–6). The principal components responsible for this effect are hemorrhagic protein factors, mainly being characterized as zinc-dependent metalloproteinases (7).

One intriguing issue related to venom toxin research is the observation that a few snakes and certain warm-blooded animals possessing remarkable resistance to envenomation of snakes (8–11). This natural immunity was ascribed to some factors present in sera of resistant animals, which may play a role of neutralizing the hemorrhagic factors or toxins in snake venoms (12).

Many hemorrhagin neutralizing factors have been purified thus far from snake sera (13), e.g. Japanese habu's (*T. flavoviridis*) HSF (14,15), and from mammalian sera such as Virginia opossum's (*D. virginiana*) oprin (16,17). The physical and chemical characteristics of these antihemorrhagins are distinguishable from immunoglobulins because of the fact that no cross-reacting precipitate was formed between these neutralizing factors and crude venoms or purified hemorrhagic factors in various immunodiffusion tests (18,19). It has been suggested that they may be various plasma proteinase inhibitors which act by noncovalently binding to hemorrhagic metalloproteinases (14,16). Some of these factors are homologous in sequence to those of plasma glycoproteins such as human α_2-HS (15) and α1B glycoprotein (16).
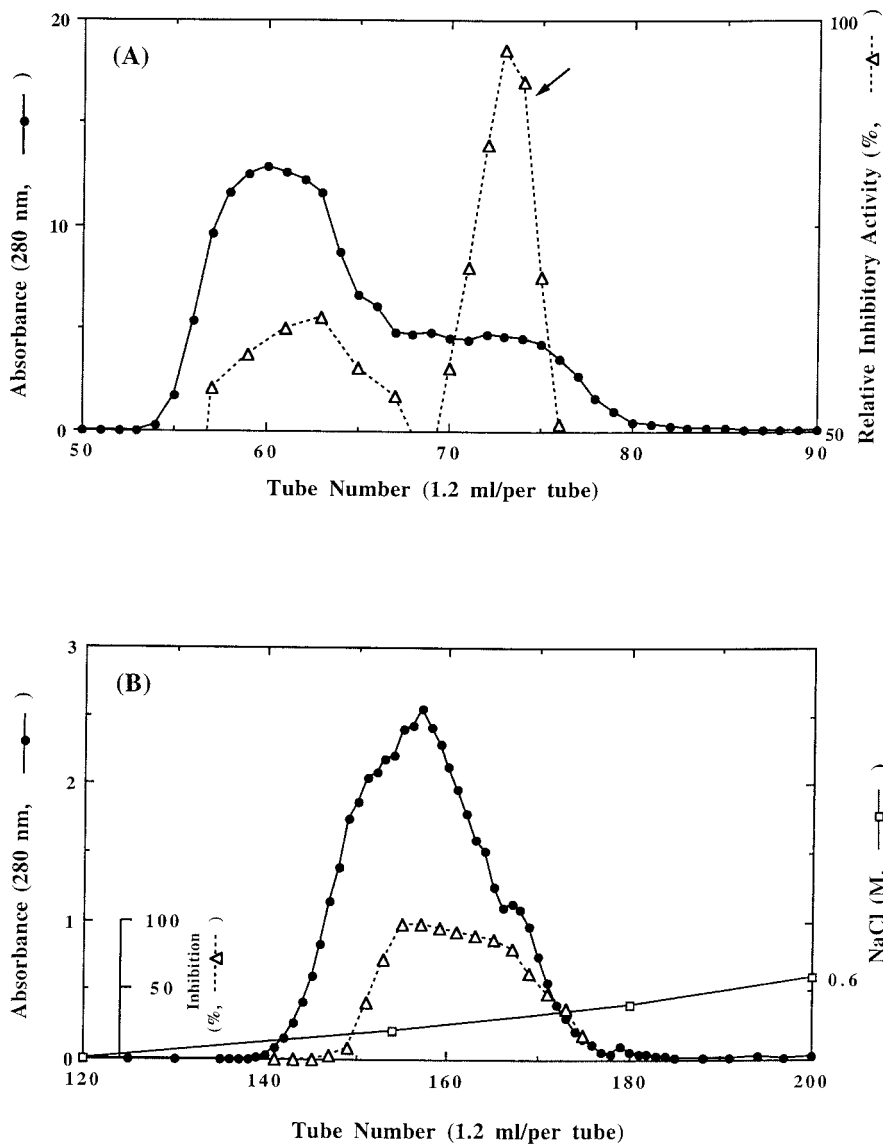
FIG. 1. Isolation of proteinase inhibitor (designated as TMI) from the blood serum of Taiwan habu (*T. mucrosquamatus*) on Sephadex G-100 (A) and DEAE-Sephacel (B) columns. Lyophilized serum powder dissolved in the elution buffer (1 ml at 200 mg/ml) was fractionated by Sephadex G-100 gel permeation chromatography (A). Elution was carried out at a flow-rate of 0.12 ml/min and each 1.2 ml fraction was collected for absorbance measurements at 280 nm. Proteinase inhibitory activity was estimated by using a venom metalloproteinase, TM-2, and the substrate FTC-casein as stated under Materials and Methods. The activity of each fraction was represented as percent inhibition on caseinolytic activity of TM-2. Fractions which possess strong activity of proteinase inhibitor (indicated with arrow) were pooled, lyophilized and redissolved in the starting buffer (3 ml at 18.3 mg/ml), followed by subjecting to anion-exchange chromatography on DEAE-Sephacel column (B). In (B) the column was initially washed with the starting buffer followed by a three-step linear gradient using sodium chloride in the same buffer as in (A). Fractions with activity as denoted by triangle symbols were also pooled for further purification.

In the present study, a novel proteinase inhibitor from the snake serum of Taiwan habu was isolated and shown to possess strong activity against metalloproteinases from the snake venom of the same species (20,21). This inhibitor was purified from the serum by employing multiple chromatographies, and compared for its inhibitory actions against the caseinolytic activity of crude venoms from different snake species. *N*-terminal sequence determination of the purified protein exhibited a unique partial protein sequence show-ing no structural homology with proteinase inhibitors reported previously in the literature.

MATERIALS AND METHODS

Materials and chemicals. Lyophilized venom powder of Taiwan habu was obtained from the local snake farm. The substrate FTC (fluorescein isothiocyanate)-labeled casein (FTC-casein, 38 μg FTC/mg protein) was purchased from Sigma Chemical Company (St. Louis, MO). Gel suspensions of Sephadex G-100 and DEAE-Sephacel were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden).
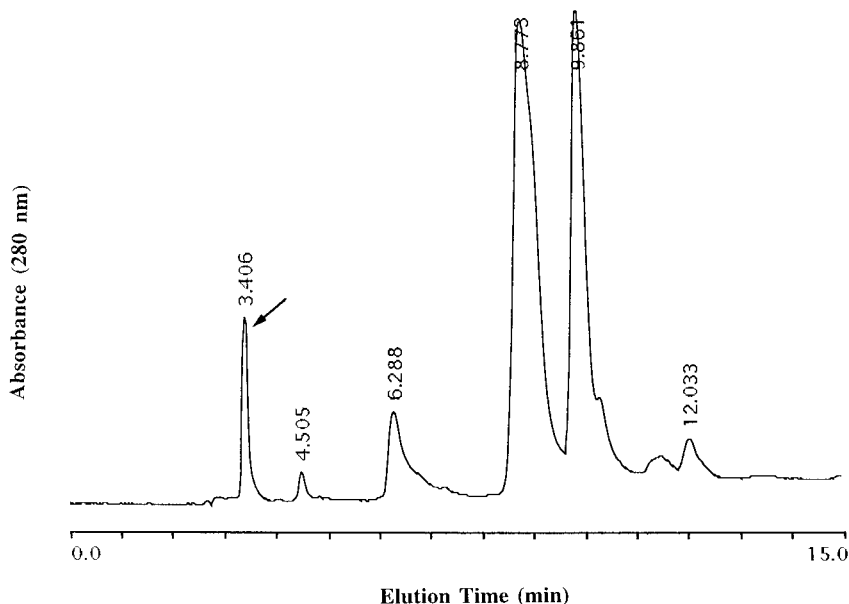
FIG. 2. High-performance liquid chromatography (HPLC) on a reverse-phase C_4 column of fractions with proteinase inhibitory activity obtained from DEAE-Sephacel column. About 50 μl (6–7 mg/ml) of the active fraction was injected into the C_4 column equilibrated with 20% B buffer (60% acetonitrile in 0.1% TFA) in A buffer (30% acetonitrile in 0.1% TFA), and the bound materials were eluted with a linear gradient of 20–100% B in A buffer for 15 min at a flow-rate of 1 ml/min. A peak with a retention time of 3.406 min, as indicated with an arrow, was shown to possess high activity of proteinase inhibition as analyzed by FTC-caseinolytic activity assay using the metalloproteinase, TM-2.

N-Benzoyl-Pro-Phe-Arg-p-nitroanilide and neuraminidase (type V) were also purchased from Sigma.

Isolation of the proteinase inhibitor from the serum. Snake blood of Taiwan habu was collected by decapitation and pooled from four specimens, of which the serum was separated and lyophilized. About 200 mg of serum powder dissolved in 50 mM Tris · HCl, pH 8.0 (total 1 ml) was fractionated by gel permeation chromatography on an open column (1.6 × 95 cm) packed with Sephadex G-100 gel suspension. Elution was carried out at 0.12 ml/min using the same buffer and 1.2 ml fraction each was collected for further analysis. Fractions with proteinase inhibition activity were pooled, lyophilized and applied for the second anion-exchange chromatography. About 55 mg of the lyophilized powder were dissolved in starting buffer (total 3 ml), 50 mM Tris · HCl, pH 8.0, and applied to a pre-equilibrated open column (2.5 × 18 cm) filled with DEAE-Sephacel gel suspension. Unbound materials were eluted with 140 ml starting buffer, followed by a three-step linear gradient of 0–0.2 M, 0.2–0.4 M and 0.4–0.6 M NaCl in starting buffers. The flow-rate was 0.3 ml/min and 1.2 ml fraction each was collected. Purification of proteinase inhibitor was performed by using reverse-phase HPLC (C_4 column, 0.46 × 25 cm, Vydac) on a Hitachi's liquid chromatograph. The column was pre-equilibrated with 20% B buffer (60% acetonitrile in 0.1% TFA) and 80% A buffer (30% acetonitrile in 0.1% TFA). After injecting fractions with activity collected from anion-exchange chromatography, elution was carried out in a linear gradient of 20–100% B in A buffer at a flow-rate of 1.0 ml/min. The eluates were monitored at 280 nm.

The proteinase inhibitory activity of each fraction was assayed using a metalloproteinase isolated from the same venom, i.e., TM-2 (21), and the substrate, FTC-casein. 12 μl of each fraction was mixed with 3 μl TM-2 (0.25 μg/μl) and incubated at room temperature for 20 min, followed by adding 5 μl FTC-casein (1%) and 30 μl of the assay buffer (100 mM Tris · HCl/10 mM CaCl$_2$, pH 8.0) for a further incubation at 37°C for 90 min. Termination of proteolysis and measurement of the residual FTC-caseinolytic activity for each reaction mixture were accomplished according to previous report (22). Pro-

teinase inhibitory activity of each fraction was represented as percent reduction of the caseinolytic activity for TM-2.

Gel electrophoresis, carbohydrate detection, and immunoblotting. The purity of purified proteinase inhibitor was checked by SDS-polyacrylamide slab gel electrophoresis (SDS-PAGE, 5% stacking/15% resolving gel) as described (23) with some modifications. About 2.0–2.5 μg protein samples or 8–10 μg crude venoms were loaded on
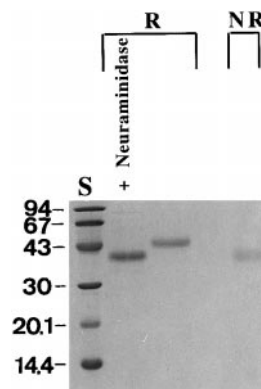

FIG. 3. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of the purified proteinase inhibitor under denaturing conditions in the presence (R) and absence (NR) of 5% β-mercaptoethanol. Lane S showed the relative electrophoretic mobilities of standard proteins used as molecular mass markers (in kDa), and the gel was stained with Coomassie blue. The proteinase inhibitor in each lane was shown to be homogeneous with molecular masses of 47 and 36 kDa under reduced and nonreduced conditions, respectively. After the incubation with neuraminidase, the inhibitor was found to move with a molecular mass of about 36 kDa.
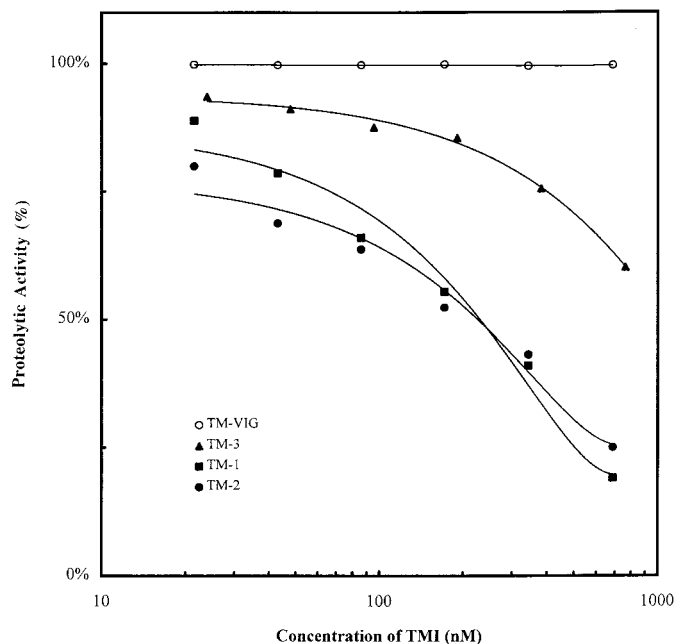
FIG. 4. Dose-dependent inhibition of *T. mucrosquamatus* venom proteinase activities by the proteinase inhibitor, TMI. The percent inhibitions of various proteinase activities by TMI were measured and estimated using the substrates FTC-casein for metalloproteinases (TM-1, TM-2, and TM-3) and *N*-benzoyl-Pro-Phe-Arg-*p*-nitroanilide for serine protease (TM-VIG) as described under Materials and Methods. Replacement of the proteinase inhibitor in each reaction mixture with assay buffers was used as the control for 100% proteolytic activity, and each data point was the average of two experiments.

each well for analyses. For assessing the presence of carbohydrate, protein sample was pre-incubated with neuraminidase (about 0.01 unit) at 37°C for 90 min and then heated to 90°C for 5 min to stop the reaction, followed by gel electrophoresis. The carbohydrate content (weight %) was estimated according to mobility differences in gel between proteins with and without treatments of neuraminidase. For immunoblotting on gels of metalloproteinases in crude venoms of various snake species, the gels were subjected to electro-blotting onto a nitrocellulose membrane after SDS-PAGE followed by immunological analysis using rabbit antiserum against the venom metalloproteinase (21) of *T. mucrosquamatus,* TM-3, and reacted with peroxidase-conjugated goat anti-rabbit IgG (whole molecule, affinity-pure grade, Sigma). A color development reaction was performed using diaminobenzidine and hydrogen peroxide.

Inhibition of FTC-caseinolytic activities of metalloproteinases and crude venoms by proteinase inhibitor TMI. Caseinolytic activity of proteinases and crude venoms was measured using FTC-casein (24) with or without the addition of purified TMI. Reaction mixtures, each containing 5 μl of proteinase (about 0.25 μg/μl) or crude venom (about 1 μg/μl) and 5 μl of different concentrations of TMI inhibitor, were incubated at room temperature for 20 min, followed by adding 5 μl FTC-casein (1%) and 35 μl of the assay buffer. Proteolysis of the mixture was carried out and the caseinolytic activity was measured as reported previously (22). A positive control which contained the assay buffer in place of proteinase inhibitor in each reaction mixture was used to estimate the percent inhibition of TMI on metallopro-teinases and crude venoms.

Effect of TMI on fibrinogenolytic serine protease from Taiwan habu. Activity of a novel serine protease (TM-VIG) isolated from the same habu venom as TMI (25) was assayed by spectrophotomet-

ric method using a chromogenic substrate for kallikrein-like proteases, i.e., *N*-benzoyl-Pro-Phe-Arg-*p*-nitroanilide. 5 μl TM-VIG (containing about 0.5 μg) mixing with 5 μl of various concentrations of proteinase inhibitor TMI were incubated at room temperature for 20 min, followed by adding 5 μl substrate (about 2.5 mM) and the assay buffer (100 mM Tris · HCl, 175 mM NaCl, pH 8.0) to a final volume of 800 μl. Substrate-cleaving activity of the reaction mixture was continuously monitored on a spectrophotometer by measuring absorbance at 405 nm. Concentration of the *p*-nitroanilide containing substrate was estimated using a molar extinction coefficient of 9936 cm^{-1}M^{-1}.

N-terminal protein sequence analysis and protein concentration determination. Protein concentrations of crude venoms, proteinases and inhibitor fractions were mainly determined by dye-binding assays (26), or estimated from absorbances of protein solutions at 280 nm using an extinction coefficient of $E^{4\%} = 10$. *N*-terminal sequence analysis of the purified proteinase inhibitor was carried out by automated Edman degradation with a microsequencing sequenator (Model 477A, Perkin Elmer/Applied Biosystems) as described (27).

RESULTS AND DISCUSSION

The venoms of various snakes have been shown to possess very strong and stable proteolytic enzymes with fibrinolytic or fibrinogenolytic activity, notably in the snake families of *Crotalidae* and *Viperidae* (1,2). It is also well known that snake venoms may be the abundant and stable sources of metalloproteinases which are similar to some medically important tissue matrix metalloproteinases. Some snake-resistant animals are suggested to possess blood or tissue inhibitors against hemorrhagic metalloproteinases (13). Therefore we have made an endeavour to identify these proteinase inhibitors which may prove to be of value for the development of anti-hemorrhagic therapeutic drugs.

TABLE 1

Comparison of Inhibitory Activities of the Proteinase Inhibitor (TMI) and Tripeptide Inhibitors on Metalloproteinases, Serine Protease, and Various Venoms

Proteinases or crude snake venoms	IC$_{50}$ (μM)[a]			
	pEKW[b]	pENW[b]	pEQW[b]	TMI
TM-1	198	373	235	0.229
TM-2	254	458	312	0.222
TM-3	578	946	710	1.053
TM-VIG	—[c]	—	—	No inhibition
T. mucrosquamatus	—	—	—	0.187
T. flavoviridis	—	—	—	0.425
T. stejnegeri	—	—	—	0.663
A. piscivorus leukostoma	—	—	—	>1.4
A. piscivorus piscivorus	—	—	—	>1.5
B. atrox	—	—	—	>10

[a] Concentrations causing 50% inhibition.

[b] Pyroglutamyl-containing tripeptides purified from *T. mucrosqua-matus* venom (22).
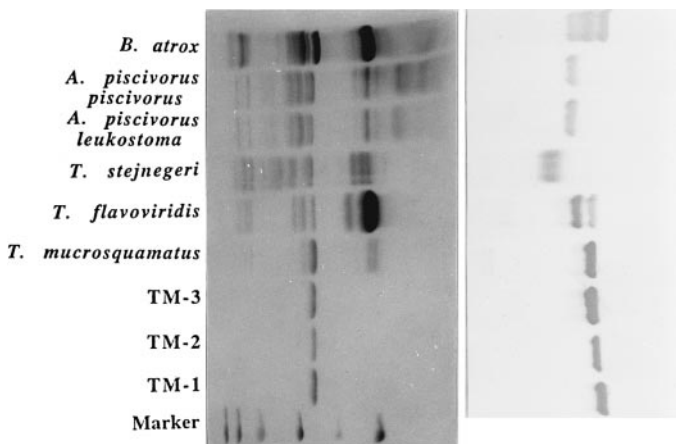
[c] Not determined.

FIG. 5. Immunoblotting detection of metalloproteinases in crude venoms of various snake species. The gel was loaded with metalloproteinase isoforms (2–2.5 μg/each well) and various crude venoms (8–10 μg/each well) and electrophoresed under reduced conditions. After SDS-PAGE the gel was stained with Coomassie blue (Top), or subjected to electroblotting onto a nitrocellulose membrane followed by immunological analysis using rabbit antiserum against TM-3 (as first antibody) and commercial peroxidase-conjugated goat anti-rabbit IgG (as second antibody) (Bottom). Marker: Standard proteins are the same as those in Fig. 3. Lanes TM-1, TM-2 and TM-3: Metalloproteinase isoforms from Taiwan habu (21) are used as controls for comparison of homologous proteinases present in different venoms. Venom sources of various snake species are indicated above each gel lane.

Isolation and purification of proteinase inhibitor from the blood serum of Taiwan habu. Blood of Taiwan habu was obtained from four snake specimens by decapitation in a local snake shop. The serum was then separated from whole blood, lyophilized and subjected to fractionation by gel permeation chromatography on a Sephadex G-100 column. A broad peak with a shoulder (Fig. 1A) was obtained from the column and shown to possess varied extents of inhibitory activity against a metalloproteinase from Taiwan habu (21), designated as TM-2. The shoulder peak showed about three-time inhibitory activity when compared with the first main broad peak. These fractions of higher activity was then pooled, lyophilized and estimated to constitute about 28% of total serum proteins. The lyophilized powder of about 55 mg was applied to an anion-exchange column packed with DEAE-Sephacel gel suspension. By elution using linear gradients of sodium chloride (Fig. 1B) about 60–65% of total fractions showed relatively strong inhibitory activity. The active fractions were eluted at 0.2–0.4 M sodium chloride gradient. Upon concentration of 20 ml pooled fractions, 30–35 mg proteins were collected for further purification. These concentrated proteins were further separated into at least six peaks by separation on a reverse-phase C_4 column using a Hitachi liquid chromatograph (Fig. 2). The major components eluted at 8.5–10 min retention times appeared to contain proteins with molecular masses large than 65 kDa (data not shown),

showing no inhibitory activity against TM-2. They may correspond to serum albumins and/or other glycoproteins. However, a minor and more hydrophilic fraction eluted at a retention time of 3.406 min was shown to possess the strongest inhibition against TM-2 metalloproteinase (indicated with an arrow in Fig. 2). The net yield of this proteinase inhibitor was estimated to be about 1.6 mg from an initial lyophilized serum powder of 200 mg. We designated this purified proteinase inhibitor as TMI since it was obtained from serum of *T. mucrosquamatus*.

SDS-polyacrylamide gel electrophoresis of the purified proteinase inhibitor, TMI. The purity of purified TMI was checked by SDS-PAGE as shown in Fig. 3. The inhibitor appeared to be a homogeneous protein with molecular mass of about 47 and 36 kDa under reduced and nonreduced conditions, respectively. TMI inhibitor migrated to a molecular mass of about 36 kDa after incubation with neuraminidase to remove *N*-linked carbohydrate moieties, which is 76.6% in molecular size as compared with the original untreated protein, suggesting that it is a glycoprotein with a single polypeptide chain.

Inhibition of metalloproteinases and serine protease activity by TMI. We have previously purified some metalloproteinases such as TM-1, TM-2 and TM-3 (21),
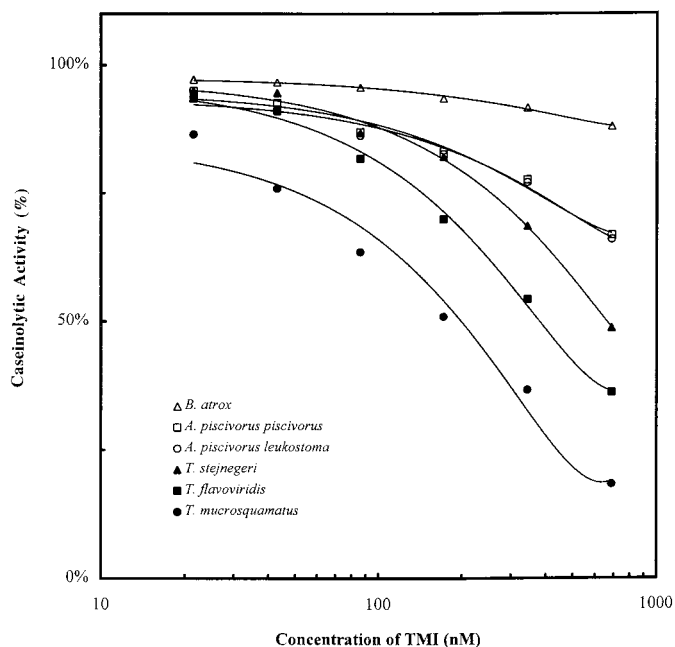

FIG. 6. Comparison of the caseinolytic activity of various crude venoms in the presence of proteinase inhibitor, TMI. About 5 μg crude venom of each species was assayed for FTC-caseinolytic activity in the presence or absence (as the positive control) of the indicated concentrations of TMI. The inhibitory activity of TMI on each venom was estimated according to those described under Materials and Methods. The venom of Taiwan habu (*T. mucrosquamatus*) seemed to be most sensitive to TMI inhibition as compared with other venoms of different snake species. Each data point is the average of two experiments.

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                                              1          5         10        15
Taiwan habu      TMI (this report)    L K ? Q K ? V A P I K V   E Y   S L T G
Japan habu       HSF                   D Q V R G D L E C D D K E A K N W A
Opossum          Oprin                L K A M D T T P R L W I E T E S P S
Human            α1B-Glycoprotein     A I F Y E T Q P S L W A E S E S L L
Mongoose         AHF1, AHF2           T M V A L D P P N L W I E A E S L P
Mongoose         AHF3                 A L V F E T Q P N L W I E A E S L T
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FIG. 7. Comparison of the N-terminal partial sequences for proteinase inhibitors reported from various species. All the partial sequences listed for HSF (15), oprin (16), human α1B-glycoprotein (28) and AHF1, 2 and 3 (29) were determined from protein sequencing. Residue numbering was according to that of human α1B-glycoprotein. Amino acid residues were denoted by one-letter symbols with "?" indicating unidentified residue in protein sequencing. Note that most homologous residues were marked with asterisks below the sequences.

and serine protease TM-VIG (25) from *T. mucrosquamatus* venom, of which all are strong fibrinogenolytic proteinases. In Fig. 4 we examined the inhibitory activity of TMI on these proteinases. It is of interest to find that TM-1, TM-2 and TM-3 were all sensitive to TMI and TM-VIG was relatively resistant. The sensitivities of TM-1 and TM-2 to TMI inhibition were closely similar to each other and both can be distinguished from that of TM-3. This is consistent with our previous conclusion that TM-1 and TM-2 are structurally more related to each other than to TM-3 (21). As shown in Table 1, IC_{50} values of TMI on metalloproteinases were in the high nanomolar range, which is approximately three orders of magnitude more effective than the tripeptide inhibitors (22) with IC_{50} being near the millimolar range.

Immunoblotting detection of metalloproteinases in varied venoms and their inhibition by TMI. In order to detect homologous metalloproteinases of Taiwan habu in various crude venoms of 12 snake species encompassing species from different snake families, crude venoms from these species were subjected to immunoblotting analysis using rabbit antiserum against TM-3 (Fig. 5). Six of them appeared to contain abundant metalloproteinases which could strongly cross-react with TM-3 antibodies. Not surprisingly, a few of these six species have actually been reported to possess metalloproteinases in their venom, e.g. HR2a, HR2b and H$_2$-proteinase from *T. flavoviridis* (30,31), piscivorase I and II from *A. piscivorus piscivorus* (32). It is noteworthy that TM-1 and TM-2 shared similar antigenicities with TM-3, and there appear to be no other major metalloproteinases with different molecular masses from that observed for TM-1 and TM-2 and TM-3 in *T. mucrosquamatus* venom.

Based on the observation that immunologically related metalloproteinases of TM-3 are widely distributed in many snake venoms of different species, we have also examined the susceptibilies of these venoms to TMI inhibition. As shown in Fig. 6 and Table 1, crude venoms of more remotely related species seemed to be poorly sensitive to TMI. For instance in genus *Trimeresurus,* the IC_{50} values of crude venoms against TMI are still in the high nanomolar range as those of purified metalloproteinases (Table 1). However in the remotely related genus, *Bothrops,* the IC_{50} values of crude venoms against TMI are estimated to be more than 10 μM, that is 15–50 times more resistant to TMI as compared with *Trimeresurus.* Two species belonged to the genus of *Agkistrodon* appeared to be equally sensitive to TMI. It is therefore suggested that TMI may be an important defensive factor mainly for evolutionarily related species, especially for the same snake genus of *Trimeresurus.*

Alignment and comparison of N-terminal sequences. When N-terminal 18 amino-acid sequence determined for TMI was directly aligned with those of previously reported inhibitors, no significant homology was found except the presence of a conserved residue, Glu_{13} (according to the numbering system of human α1B-glycoprotein (28). Four homologous residues Pro_8, Glu_{13}, Ser_{16} and Leu_{17} (indicated with asterisks in Fig. 7) are identified if we make an optimal alignment by introducing gaps. Nevertheless, in spite of these conserved residues identified in the same snake genus, the partial sequence of TMI shows no homology to that of HSF isolated from *T. flavoviridis* serum. In conclusion we have isolated and purified a novel proteinase inhibitor from the serum of Taiwan habu, which showed a distinct partial sequence from those serum proteinase inhibitors reported previously. It should prove fruitful in the future if we could clone and sequence the complete gene encoding this novel protein.

ACKNOWLEDGMENT

REFERENCES

1. Jimenez-Porras, J. M. (1968) *Annu. Rev. Pharmacol.* **8,** 299–318.
2. Markland, F. S., Jr. (1988) *in* Hemostasis and Animal Venoms (Pirkle, H. and Markland, F. S., Eds.), pp. 149–172, Dekker, New York.
3. Tu, A. T. (1982) *in* Rattlesnake Venoms: Their Actions and Treatment (Tu, A. T., Ed.), pp. 247–312, Dekker, New York.

4. Ownby, C. L., Bjarnason, J., and Tu, A. T. (1978) *Am. J. Pathol.* **93,** 201–218.

5. Baramova, E. N., Shannon, J. D., Bjanason, J. B., and Fox, J. W. (1989) *Arch. Biochem. Biophys.* **275,** 63–71.

6. Maruyama, M., Sugiki, M., Yoshida, E., Shimaya, K., and Mihara, H. (1992) *Toxicon* **30,** 1387–1397.

7. Markland, F. S., Jr. (1998) *Thromb. Haemost.* **79,** 668–674.

8. Kellaway, C. H. (1931) *Med. J. of Australia* **2,** 35–52.

9. Nichol, A. A., Douglas, V., and Peck, L. (1933) *Copeia* **4,** 211–217.

10. Kilmon, J. A. (1976) *Toxicon* **14,** 337–340.

11. Werner, R. M., and Vick, J. A. (1977) *Toxicon* **15,** 29–33.

12. Thwin, M. M., and Gopalakrishnakone, P. (1998) *Toxicon* **36,** 1471–1482.

13. Perez, J. C., and Sanchez, E. E. (1999) *Toxicon* **37,** 703–728.

14. Yamakawa, Y., and Omori-Satoh, T. (1991) *J. Biochem.* **110,** 767–769.

15. Yamakawa, Y., and Omori-Satoh, T. (1992) *J. Biochem.* **112,** 583–589.

16. Catanese, J. J., and Kress, L. F. (1992) *Biochemistry* **31,** 410–418.

17. Catanese, J. J., and Kress, L. F. (1993) *Biochemistry* **32,** 509–515.

18. Clark, W. C., and Voris, A. K. (1969) *Science* **164,** 1402–1404.

19. Omori-Satoh, T., Sadahiro, S., Ohsaka, A., and Murata, R. (1972) *Biochim. Biophys. Acta* **285,** 414–426.

20. Huang, K.-F., Hung, C.-C., and Chiou, S.-H. (1993) *Biochem. Mol. Biol. Int.* **31,** 1041–1050.

21. Huang, K.-F., Hung, C.-C., Pan, F.-M., Chow, L.-P., Tsugita, A., and Chiou, S.-H. (1995) *Biochem. Biophys. Res. Commun.* **216,** 223–233.

22. Huang, K. F., Hung, C. C., Wu, S. H., and Chiou, S. H. (1998) *Biochem. Biophys. Res. Commun.* **248,** 562–568.

23. Laemmli, U. K. (1970) *Nature* **227,** 680–685.

24. Twining, S. S. (1984) *Anal. Biochem.* **143,** 30–34.

25. Hung, C. C., Huang, K. F., and Chiou, S. H. (1994) *Biochem. Biophys. Res. Commun.* **205,** 1707–1715.

26. Bradford, M. (1976) *Anal. Biochem.* **72,** 248–254.

27. Chiou, S. H., Hung, C. C., and Huang, K. F. (1992) *Biochem. Biophys. Res. Commun.* **187,** 389–396.

28. Ishioka, N., Takahashi, N., and Putnam, F. W. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 2363–2367.

29. Qi, Z. Q., Yonaha, K., Tomihara, Y., and Toyama, S. (1994) *Toxicon* **32,** 1459–1469.

30. Miyata, T., Takeya, H., Ozeki, Y., Arakawa, M., Tokunaga, F., Iwanaga, S., and Omori-Satoh, T. (1989) *J. Biochem.* **105,** 847–853.

31. Takeya, H., Arakawa, M., Miyata, T., Iwanaga, S., and Omori-Satoh, T. (1989) *J. Biochem.* **106,** 151–157.

32. Hahn, B. S., Chang, I. M., and Kim, Y. S. (1995) *Toxicon* **33,** 929–941.