Characterization of Three Endogenous Peptide Inhibitors for Multiple Metalloproteinases with Fibrinogenolytic Activity from the Venom of Taiwan Habu (*Trimeresurus mucrosquamatus*)

Kai-Fa Huang,* Chin-Chun Hung,* Shih-Hsiung Wu,*,† and Shyh-Horng Chiou*,†,[1]

†*Institute of Biochemical Sciences, National Taiwan University, Taipei; and *Institute of Biological Chemistry,
P.O. Box 23-106, Academia Sinica, Taipei, Taiwan*

   **Three small peptide components were isolated and purified from the venom of Taiwan habu (*Trimeresurus mucrosquamatus*), which show specific activity to inhibit the strong proteolytic activity of multiple metalloproteinases present in the crude venom. Using multiple chromatographies coupled with successive ultrafiltrations, three inhibitors, *i.e.* pyroglutamate-lysine-tryptophan (pyroGlu-Lys-Trp), pyroglutamate-asparagine-tryptophan (pyroGlu-Asn-Trp) and pyroglutamate-glutamine-tryptophan (pyroGlu-Gln-Trp) were obtained in good yields and high homogeneity. The yields of these peptide fractions were estimated to be about 0.65 mg, 0.55 mg and 0.42 mg from 250 mg total lyophilized crude venom, which corresponded to the approximate concentrations of 8.4 mM, 7.3 mM and 5.4 mM respectively in venom secretion. Detailed and unambiguous structural determination was established by amino acid analyses, mass spectrometry and microsequencing of purified peptides. Further functional characterization of these three tripeptides showed that they could weakly inhibit three metalloproteinases previously isolated from the same venom. The inhibitory activities were similar among these tripeptides and their IC$_{50}$ (concentration for 50% inhibition) were estimated in a range of 0.20-0.95 mM, which is much more effective than citrate, another venom protease inhibitor of low molecular-weight component. Since these tripeptides are the endogenous peptide inhibitors present in the lumen of venom glands, it is conceivable that they may act as a self-defensive mechanism against the auto-digestive deleterious effect of the strong metalloproteinases *in vivo,* particularly several zinc-dependent metalloproteinases present in crotalid and viperid venoms.
© 1998 Academic Press**

   Venoms of the snake families of *Viperidae* and *Crotalidae* cause shock, intravascular clotting, systemic and local hemorrhage, edema and necrosis upon victimized prey. It has also been well known that snake venoms contain complex mixtures of pharmacologically active peptides and proteins including some potent proteolytic enzymes (1-3). The common symptom of hemorrhage caused by snakebites is usually the result of structural destruction of capillary basement membranes via proteolytic degradation by these proteases (4). Death is usually the result of the combined effects of several components in the venom. In order to avoid the auto-digestion of venom gland by its own degradative proteases present in gland secretions, snake venoms of various species have been reported to contain some self-defensive enzyme inhibitors such as serine protease inhibitors (5-7), citrate and small peptides (8), that can selectively bind to these proteases thereby partially inhibit and minimize their proteolytic activities in crude venoms. Venom proteinases so far characterized have been shown 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 (9-11).

   Early studies have indicated that, a few snake species of *Viperidae* and *Crotalidae* families contained endogenous small peptides present as a form of pyroglutamate(pyroGlu)-containing tripeptides, *e.g.* pyroGlu-Asn-Trp and pyroGlu-Gln-Trp (12,13). These small N-terminally blocked peptides were later found in venoms of various species of snakes, and shown to be relatively good inhibitors of zinc-dependent metalloproteinases (14,15). On the other hand, the high citrate

   [1] Corresponding address: Institute of Biological Chemistry, Academia, P.O. Box 23-106, Taipei, Taiwan. Fax: (886)-2-3635038. E-mail: shchiou@bc.ntu.edu.tw.

concentration present in various snake venoms of *Crotalidae* and *Viperidae* families (16), has also been identified as a low molecular-weight component in crude venom to play a role as an endogenous inhibitor of venom enzymes and proteinases (17). Therefore it would be of interest to compare the inhibitory activities of these small endogenous components in snake venoms on the proteinases isolated from the same venom in order to provide some insights into the protracted issue on how snakes develop the self-defensive mechanism in protecting the venom gland itself from the damaging effect of its secreted metalloproteinases. Previously we have identified three metalloproteinases with strong fibrinogenolytic activity from the Taiwan habu (*Trimeresurus mucrosquamatus*), designated as TM-1, TM-2, and TM-3 (18,19). In this report we have focused on the isolation and characterization of three pyroglutamate tripeptides and compare their inhibitory activities with citrate ion on these three metalloproteinases. One novel tripeptide, pyroGlu-Lys-Trp, has for the first time been identified and shown to be a genuine inhibitor for these metalloproteinases.

MATERIALS AND METHODS

Materials. Lyophilized venom powder was obtained from the local snake farm. The substrate FTC-casein was purchased from Sigma Chemical Company (St. Louis, MO). Gel suspensions of TSK DEAE-650(M) anion-exchange resin were purchased from Merck (Darmstadt, Germany).

Isolation and purification of small peptides from crude venom. An anion-exchange chromatography on an open column (2.5 × 46 cm) packed with TSK DEAE-650(M) gel suspension was employed to separate small peptide components. Venom powder dissolved in 0.025 M ammonium bicarbonate, pH 7.8 starting buffer (total 5 ml) was applied to the column 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 buffer, similar to those described previously (18). Two fractions rich in small peptides were obtained from the above chromatography and ultrafiltered through membranes (YM-10, Amicon) and the flow-throughs were collected and lyophilized. Reverse-phase chromatography was performed on a Hitachi's liquid chromatograph using C$_{18}$ column (0.46 × 25 cm, Vydac). Elution was carried out in a linear gradient of 5-95% acetonitrile in 0.1% TFA and the eluates were monitored at either 214 nm or 280 nm. Major fractions eluted from the column were collected and lyophilized.

SDS–polyacrylamide gel electrophoresis and amino acid analyses. The purity of purified polypeptides was checked by SDS-polyacrylamide slab gel electrophoresis (5% stacking/15% resolving gel) as described (20) with some modifications (*i.e.,* 5% cross-linking N, N′-methylenebisacrylamide in the gel solution). The amino acid compositions were determined with a Beckman 6300 amino acid analyzer using a single-column system based on a conventional ion-exchange chromatography system.

Mass spectrometry analyses. Fast atom bombardment (FAB) mass spectra were obtained on an Autospec mass spectrometer (Micromass, UK) fitted with a cesium ion gun operated at 25 kV. Samples were dissolved in 5% acetic acid for loading onto the probe tip coated with monothioglycerol as matrix. Multiple scans at 6 seconds per decay were acquired and summed for data processing using the Opus software.

Cleavage of the N-terminal pyroglutamate from peptides and sequence analysis. Cleavage of the N-terminal pyroglutamate from peptides was accomplished using sequencing grade pyroglutamate aminopeptidase (Boehringer Mannheim). About 5-10 μg peptides were dissolved in 45 μl digestion buffer (100 mM sodium phosphate, 10 mM EDTA, 5 mM dithiothreitol and 5% glycerol, pH 8.0). After addition of 5 μl pyroglutamate aminopeptidase (0.25 μg/μl) and incubation for 18 hours at 4 °C first and then 4 hours at 25 °C, peptides in reaction mixture were separated by reverse-phase high performance liquid chromatography. Sequencing of deblocked peptides was carried out by automated Edman degradation with a microsequencing sequenator (Model 477A, Applied Biosystems) as described (21).

Determination of protein and peptide concentration. Protein concentration was determined by dye-binding assays (22) or estimated from the absorbance of the purified protein at 280 nm, using an extinction coefficient (E$^{1\%}$) of 10. Concentrations of tryptophan-containing tripeptides were measured by absorbance at 280 nm using a molar extinction coefficient of 5600 M^{-1}cm^{-1}.

FTC-caseinolytic activity assay of metalloproteinases and its inhibition by peptides and citrate. Caseinolytic activity of metalloproteinases was measured using a fluorescence substrate, FTC-casein as reported previously (23), with or without the addition of small peptide inhibitors or sodium citrate. Reaction mixtures, containing 5 μl enzyme (0.2-0.4 μg/μl) and 5 μl of the indicated concentrations of inhibitor fractions (or replacing this with the assay buffer, 100 mM Tris.HCl/10 mM CaCl$_2$, pH 8.0, as positive control), was incubated at room temperature for 15 min, followed by adding 5 μl FTC-casein (10 mg/ml in 50 mM Tris.HCl, pH 7.2) and 35 μl of the assay buffer for a prolonged incubation at 37°C for 70 min. Proteolysis was terminated by adding 120 μl of 5% TCA and mixing extensively. The reaction mixture was allowed to stand at room temperature for 1 h and the TCA-insoluble protein was sedimented by centrifugation at 13,000 rpm for 2.5 min. A 90-μl aliquot of the supernatant fraction was diluted with 0.5 M Tris buffer, pH 8.5, with vigorous mixing to ensure the entire sample was at the proper pH. Fluorescence was measured using an excitation wavelength at 490 nm and an emission wavelength at 522 nm on a Hitachi's F-4010 fluorescence spectrophotometer. Percent inhibition for the caseinolytic activity of metalloproteinases was estimated from the fluorescence yields of assayed samples as compared with that of control.

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* (24-26). Currently several proteolytic enzymes of snake origin have been used clinically as potential antithrombotic agents (27-30) with the special concern on how to modulate and mitigate unwanted toxic effects of local and systemic hemorrhage or bleeding caused by these proteinases. Most venom proteases with such hemorrhagic side effects consist mainly of metalloproteinases (31). In this study we have made an effort to look for natural proteinase inhibitors present in the crude venom of Taiwan habu and their effects on three strong hemorrhagic metalloproteinases isolated previously from the same venom (18,19). Successful identification and characterization of three pyroglutamate tripeptides could account for the resistance of snake venom components to these metalloproteinases *in vivo*.
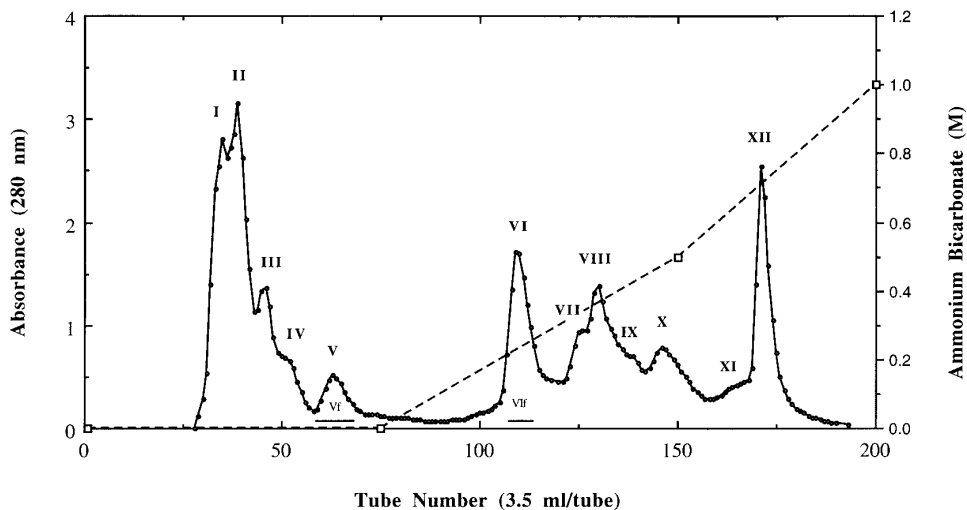
FIG. 1.   Isolation of small peptides from the crude venom of Taiwan habu (*Trimeresurus mucrosquamatus*) on TSK DEAE-650(M) column. About 250 mg of lyophilized venom powder was dissolved in the starting buffer of 0.025 M ammonium bicarbonate, pH 7.8, and applied to the column equilibrated with the same buffer. Elution was carried out in three steps similar to that described in the previous report (18,19) with some modifications. The column eluates (3.5 ml/tube) were monitored for absorbance at 280 nm. Fractions I-XII, as indicated, were collected and ultrafiltered through YM-10 Amicon membrane, the flowthroughs were collected, and lyophilized for further analysis and purification. The bars marked with Vf and VIf represent the flowthrough fractions obtained after ultrafiltrations through membrane.

Isolation and purification of peptides from crude venom of Taiwan habu.   Fractionation of *T. mucrosquamatus* venom on a TSK DEAE-650 anion-exchange column (Fig. 1) using ammonium bicarbonate as elution buffer yielded an elution pattern superior to that reported previously (19). It is to be noted that fractions I-V, eluted at the initial buffer, contained multiple proteolytic enzymes with molecular weights of about 24,000 as judged by SDS-gel electrophoresis (data not shown), corresponding to those of metalloproteinases reported by our group (19). Fractions VI-X, eluted at the first-step gradient, possessed also multiple proteinases with molecular weights in a range of 27,000 to 32,000, corresponding to a family of serine proteases described in the previous report (32). We collected twelve fractions and ultrafiltered through membranes with a molecular weight cut-off of 10,000. The flowthroughs from ultrafiltration were collected, concentrated and examined for their UV and fluorescence emission spectra. Flow-through fractions V_f and VI_f, obtained from fractions V and VI in Fig. 1, which were eluted by the initial buffer and the first-step gradient respectively, were shown to have a high absorbance at 280 nm and an emission maximum of about 358 nm detected on a Hitachi F-4010 fluorescence spectrophotometer when excited at 295 nm.

High performance liquid chromatographies (HPLC) of these two flow-throughs were further carried out on a Hitachi liquid chromatograph (Fig. 2) in order to purify these fractions in a homogeneous form. HPLC showed

TABLE 1

Amino Acid Compositions, Sequences, and Fluorescence Emission Wavelengths of Small Peptide Fractions

	Composition[a]				λemi[c] (nm)	Sequence residue[d]	
	Asx	Glx	Lys	Trp		2nd	3rd
V_f	—	44.6%	45.0%	n.d.[b]	358.2	Lys	Trp
VI_f-a	46.8%	50.2%	—	n.d.	358.0	Asn	Trp
VI_f-b	—	95.5%	—	n.d.	358.0	Gln	Trp
L-Trp					354.4		

[a] Data of amino acid analyses are expressed as mol%, and V_f, VI_f-a and VI_f-b are fractions collected from reverse-phase HPLC.

[b] n.d., not determined.

[c] λemi, the emission wavelength with the highest fluorescence yield using excitation at 295 nm. L-Trp is used as the standard for wavelength comparison.

[d] The N-terminal first residue was blocked and the second and third residues were determined by the treatment of peptides with pyroglutamate aminopeptidase and then sequenced accordingly.
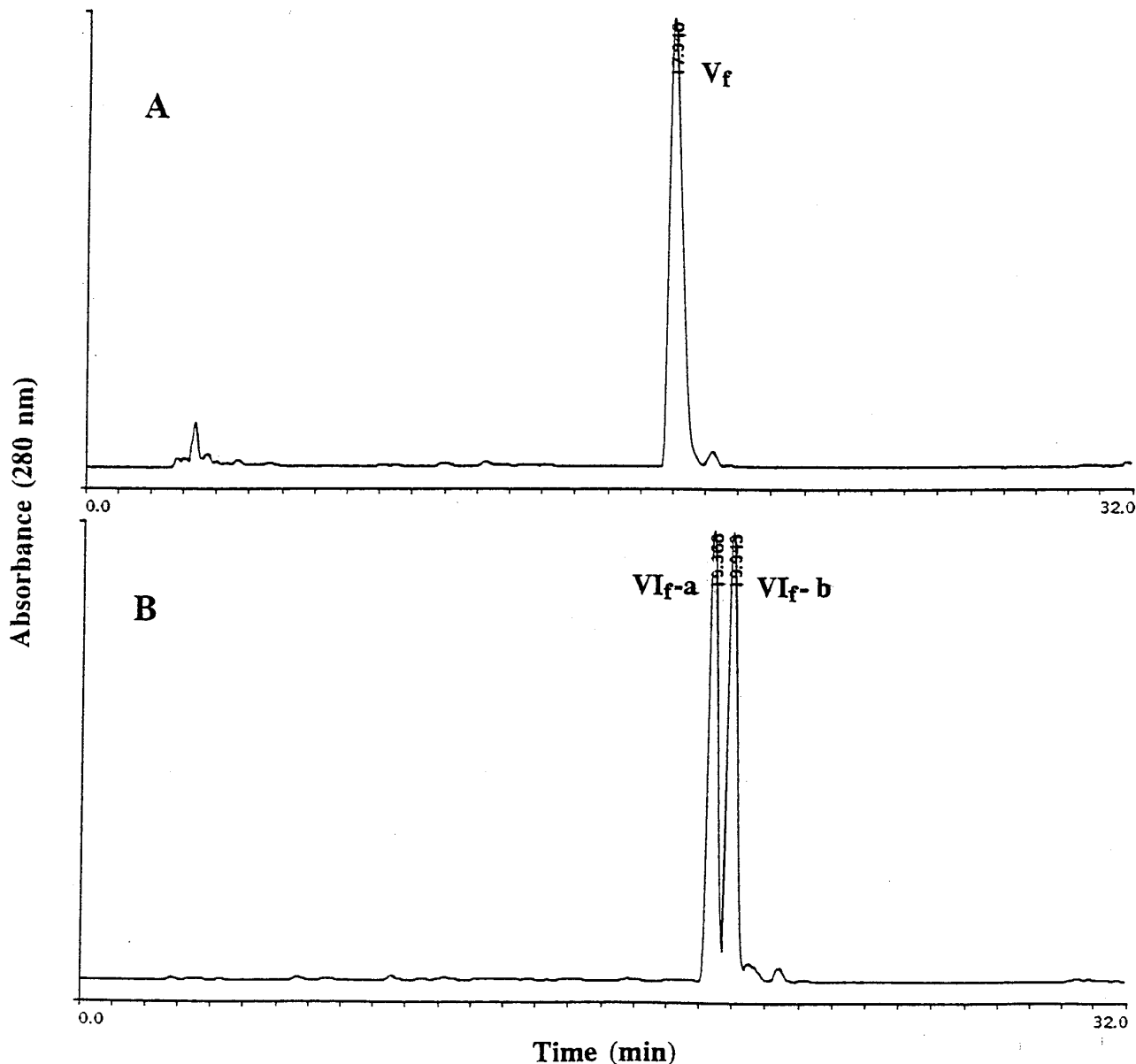
FIG. 2. High-performance liquid chromatographies (HPLC) on a reverse-phase C_{18} column of fractions Vf and VIf obtained from TSK DEAE-650(M) column after ultrafiltrations. About 50 μl (2 mg/ml) of fraction Vf (A) or fraction VIf (B) was injected into a C_{18} column equilibrated with 5% acetonitrile in 0.1% TFA, and the bound materials were eluted with a linear gradient of 5-95% acetonitrile in 0.1% TFA for 30 min. After HPLC the fraction VIf eluted as two peaks of equal amounts, denoted as VIf-a and VIf-b respectively as indicated in (B), whereas fraction Vf eluted as one major homogeneous peak (A).

that fraction VI_f can be split into two peaks at elution times of 19.4 and 19.9 min with equal yields, denoted as VI_f-a and VI_f-b respectively (Fig. 2B). Fraction V_f was eluted as one major peak (Fig. 2A).

Amino acid compositions and mass spectrometry. Amino acid analyses showed that peak V_f contained equal amounts of Glx (Glu or Gln) and Lys. Likewise, peak VI_f-a contained Glx and Asx (Asp or Asn), however only Glx was identified in peak VI_f-b (Table 1). Furthermore, mass spectra of the de-salted and dry materials

in peak VI_f-a and VI_f-b produced molecular ions with a mass/charge ratio of about 430 and 444 (Fig. 3), corresponding to those molecular masses of pyroGlu-Asn-Trp and pyroGlu-Gln-Trp respectively. These two pyroglutamate tripeptides are identical to those isolated previously from snakes of *A. halys blomhoffii, B. jararaca, C. adamanteus* and *T. flavoviridis* (12). On the other hand, the molecular ion emitted from the sample of peak V_f showed a mass/charge ratio of about 444 (Fig. 3), corresponding to a peptide of pyroGlu-Lys-Trp, a novel pyroglutamate tripeptide which has not been
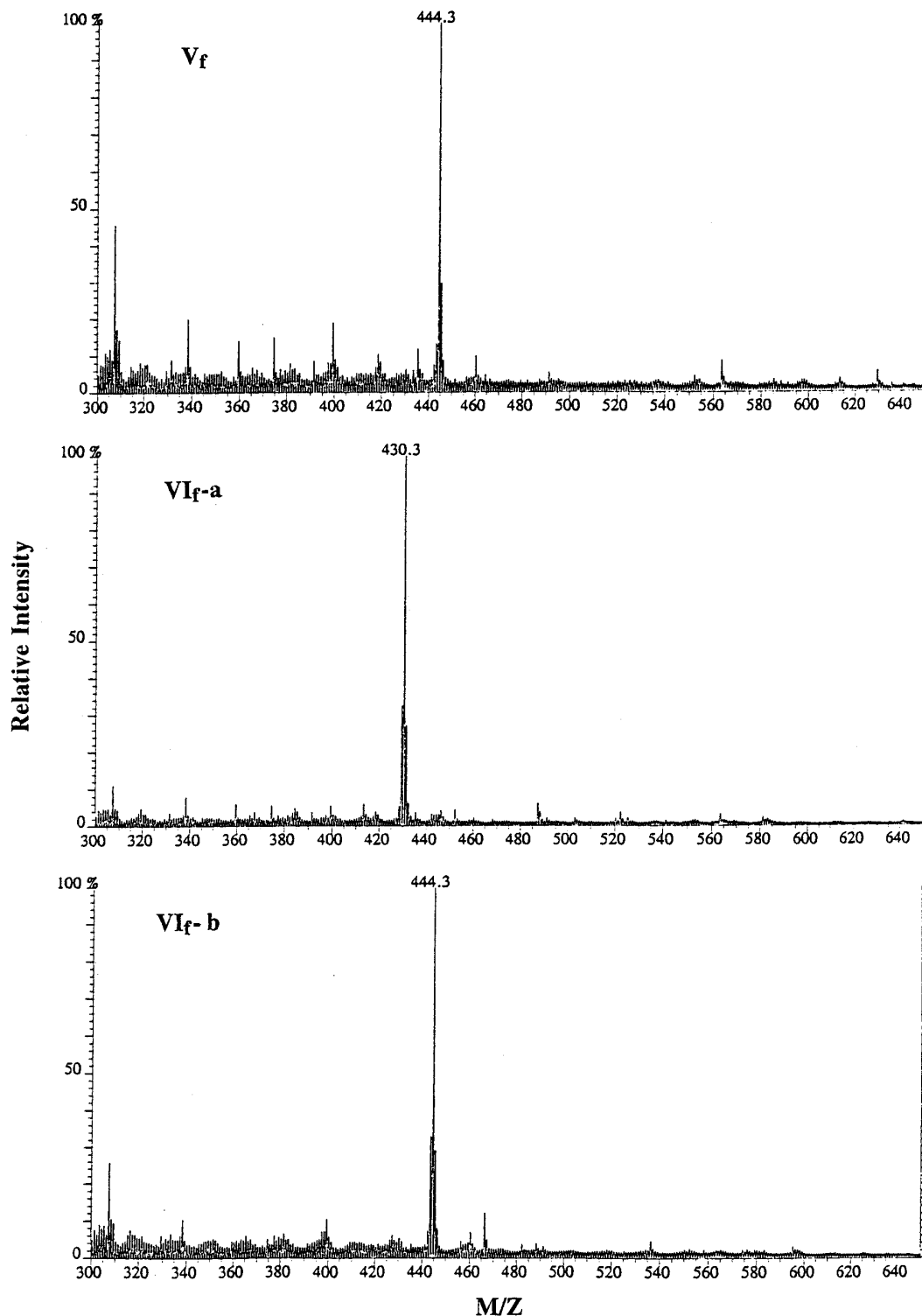
FIG. 3. Mass spectrometry of small peptides in peaks Vf, VIf-a and VIf-b from HPLC. Approximately 1 μg of each peptide sample dissolved in 5% acetic acid was analyzed by mass spectrometry (FAB-MS). The X-axis designates the mass/charge ratio, which is representative of the molecular weights of these peptides. The number above the major peak in each spectrum shows the measured molecular weight of major molecular ion peak by FAB-MS.

reported before. It is concluded that these small pep-tides are all composed of three amino acids and trypto-phan residue is certainly included in each peptide as

judged by the fluorescence spectra and microsequenc-ing after deblocking of the N-terminal residue (Table 1). The yields of these peptides in fractions VI_f-a,
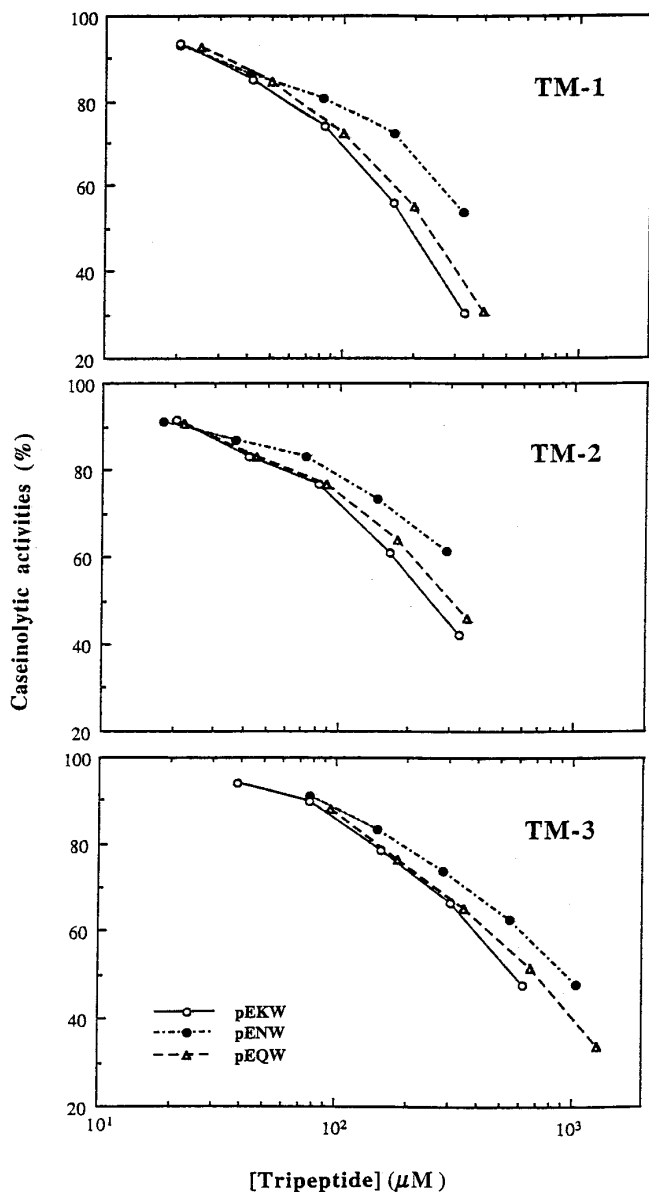
FIG. 4. Dose-dependent inhibition of *T. mucrosquamatus* metalloproteinase activities by three pyroglutamate tripeptides. The percentage inhibition of FTC-caseinolytic activity (1 mg/ml) by tripeptides on TM-1, TM-2 and TM-3 metalloproteinases isolated from *T. mucrosquamatus* venom (18,19), was measured and estimated according to that as described in Materials and Methods. Incubation of FTC-casein substrates with each metalloproteinase isoform in the absence of tripeptides was used as control for 100% FTC-caseinolytic activity and each data point was the average of two experiments.

VI_f-b and V_f from 250 mg crude venom were estimated to be about 0.65 mg, 0.55 mg and 0.42 mg, respectively, using a molar extinction coefficient for tryptophan of 5600 $M^{-1}cm^{-1}$ at 280 nm.

Inhibition of metalloproteinase activities by tripeptide inhibitors. Three metalloproteinase isoforms, TM-1, TM-2 and TM-3 isolated previously from the same venom of Taiwan habu, were employed to investigate the inhibitory activities of these tripeptide samples using a fluorescence substrate, fluorescein thiocarbamoyl (FTC)-casein. In the absence of tripeptides, FTC-casein was almost completely hydrolyzed after incubation with metalloproteinases at 37 °C for 2 h, whereas the FTC-caseinolytic activity of metalloproteinases was dose-dependently inhibited by increasing the tripeptide inhibitors. As shown in Fig. 4, there are no much activity difference among three pyroglutamate tripeptides to inhibit each metalloproteinase within the concentration range used in this study. The IC_{50} (concentration for 50% inhibition) of these tripeptides for TM-1, TM-2 and TM-3 were estimated to be 0.20 mM, 0.25 mM and 0.58 mM for pyroGlu-Lys-Trp, respectively; 0.37 mM, 0.46 mM and 0.95 mM for pyroGlu-Asn-Trp, respectively; and 0.24 mM, 0.31 mM and 0.71 mM for pyroGlu-Gln-Trp, respectively. These IC_{50} values are generally weaker than most conventional protease inhibitors of larger sizes such as trypsin or chymotrypsin inhibitors. However they still exhibit the high specificity for these venom metalloproteinases and not the thrombin-like serine proteases present in the same venom (32, data not shown). Moreover when comparing IC_{50} of these tripeptides with that of citrate ion (17), another endogenous proteinase inhibitor present in snake venoms, these tripeptides are actually 100-fold more effective than citrate. It is also noteworthy that TM-1 and TM-2 appear to be slightly more susceptible to tripeptide inhibitors than TM-3, which corroborates our previous conclusion that TM-1 and TM-2 are more closely related to each other than to TM-3, and both of them are distinguishable from TM-3 in their partial protein sequences (19).

In conclusion we have isolated and characterized three endogenous tripeptides which show specific inhibition against the strong metalloproteinases present in the same venom. Characterization of these pyroglutamate tripeptides may provide a molecular basis to account for the resistance of snake venom components to these metalloproteinases *in vivo*. Further elucidation of the mechanistic aspects of these tripeptides in relation to the inhibition of venom metalloproteinases could open avenues for exploring the potential of using these peptide inhibitors as modulators in alleviating the toxic side effect of bleeding or hemorrhage associated with several venom protease-based antithrombotic agents.

REFERENCES

1. Jimenez-Porras, J. M. (1968) *Ann. 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. Hokama, Y., Iwanaga, S., Tatsuki, T., and Suzuki, T. (1976) *J. Biochem.* **79,** 559–578.

6. Strydom, D. J. (1977) *Biochim. Biophys. Acta* **491,** 361–369.

7. Ritonja, A., Meloun, B., and Gubensek, F. (1983) *Biochim. Biophys. Acta* **746,** 138–145.

8. Bieber, A. L. (1979) *in* Handbook of Experimental Pharmacology (Lee, C. Y., Ed.), Vol. 52, pp. 295–306, Springer, Berlin.

9. Pirkle, H., and Stocker, K. (1991) *Thromb. Haemostas.* **65,** 444–450.

10. Markland, F. S. (1991) *Thromb. Haemostas.* **65,** 438–443.

11. Kini, R. M., and Evans, H. J. (1992) *Toxicon* **30,** 265–293.

12. Kato, H., Iwanaga, S., and Suzuki, T. (1966) *Experientia* **22,** 49–50.

13. Lo, T. B. (1972) *J. Chin. Biochem. Soc.* **1,** 39–46.

14. Robeva, A., Politi, V., Shannon, J. D., Bjarnason, J. B., and Fox, J. W. (1991) *Biomed. Biochim. Acta* **50,** 769–773.

15. Francis, B., and Kaiser, I. I. (1993) *Toxicon* **31,** 889–899.

16. Freitas, M. A., Geno, P. W., Sumner, L. W., Cooke, M. E., Hudiburg, S., Ownby, C. L., Kaiser, I. I., and Odell, G. V. (1992) *Toxicon* **30,** 461–464.

17. Francis, B., Seebart, C., and Kaiser, I. I. (1992) *Toxicon* **30,** 1239–1246.

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

19. 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.

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

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

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

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

24. Ouyang, C. (1957) *J. Formosan Med. Assoc.* **56,** 435–448.

25. Meaume, J. (1966) *Toxicon* **4,** 25–58.

26. Jimenez-Porras, J. M. (1970) *Clin. Toxicol.* **3,** 389–431.

27. Esnouf, M. P., and Tunnah, G. W. (1967) *Brit. J. Haematol.* **13,** 581–590.

28. Bell, W. R., Pitney, W. R., and Goodwin, J. F. (1968) *Lancet* **i,** 490–493.

29. Cercek, B., Lew, A. S., Hod, H., Jano, J., Lewis, B., Reddy, K. N. N., and Ganz, W. (1987) *Thromb. Res.* **47,** 417–426.

30. Pollak, V. E., Glas-Greenwalt, P., Olinger, C. P., Wadhwa, N. K., and Myre, S. A. (1990) *Am. J. Med. Sci.* **299,** 319–325.

31. Bjarnason, J. B., and Fox, J. W. (1994) *Pharmacol. Ther.* **62,** 325–372.

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