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 C₄ 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 IC₅₀ of about 0.2–1.1 μM. Its IC₅₀ 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

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 prey 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. Japanease 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 α₂-HS (15) and α₁B glycoprotein (16).
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. 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 showing 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. 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.](image-url)
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 x 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 x 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 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 x 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 (C4 column, 0.46 x 25 cm, Vydac) on a Hitachi’s liquid chromatograph. The column was pre-equilibrated with 20% B buffer (60% acetonitrile in 0.1% TFA) in 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 0–100% 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 ml of each fraction was mixed with 3 ml TM-2 (0.25 mg/ml) and incubated at room temperature for 20 min, followed by adding 5 ml FTC-casein (1%) and 30 ml of the assay buffer (100 mM Tris·HCl/10 mM CaCl2, 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). Proteinase 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 mg protein samples or 8–10 mg crude venoms were loaded on a 5% stacking/15% resolving gel. 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.
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 detection on gels of metalloproteinases in crude venoms of various snake species, the gels were subjected to electroblotting 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-purified 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/ml) or crude venom (about 1 μg/ml) and 5 μl of different concentrations of TMI inhibitor, were incubated at room temperature for 20 min, followed by added 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 carried out and the caseinolytic activity was measured using the substrates FTC-casein for metalloproteinases and crude venoms by proteinase inhibitor TMI.

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 spectrophotometric 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-deactivating 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 E²⁸⁰ = 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

<table>
<thead>
<tr>
<th>Proteinases or crude snake venoms</th>
<th>IC₅₀ (μM) *</th>
<th>pEKW*</th>
<th>pENW*</th>
<th>pEQW*</th>
<th>TMI</th>
</tr>
</thead>
<tbody>
<tr>
<td>TM-1</td>
<td>198</td>
<td>373</td>
<td>235</td>
<td>0.229</td>
<td></td>
</tr>
<tr>
<td>TM-2</td>
<td>254</td>
<td>458</td>
<td>312</td>
<td>0.222</td>
<td></td>
</tr>
<tr>
<td>TM-3</td>
<td>578</td>
<td>946</td>
<td>710</td>
<td>1.053</td>
<td></td>
</tr>
<tr>
<td>TM-VIG (27)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>No inhibition</td>
<td></td>
</tr>
<tr>
<td><em>T. mucrosquamatus</em></td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>0.187</td>
<td></td>
</tr>
<tr>
<td><em>T. flavoviridis</em></td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>0.425</td>
<td></td>
</tr>
<tr>
<td><em>T. stejnegeri</em></td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>0.663</td>
<td></td>
</tr>
<tr>
<td>A. piscivorus leukostoma</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>&gt;1.4</td>
<td></td>
</tr>
<tr>
<td>A. piscivorus piscivorus</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>&gt;1.5</td>
<td></td>
</tr>
<tr>
<td>B. atrox</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>&gt;10</td>
<td></td>
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</table>

* Concentrations causing 50% inhibition.
* Pyroglutamyl-containing tripeptides purified from *T. mucrosquamatus* venom (22).
* xx Not determined.
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_18 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 larger 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),
and serine protease TM-VIG (25) from T. murosquamous 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, IC₅₀ 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 IC₅₀ 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 to TM-3 in crude venoms from Taiwan habu. 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.

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