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Complete Primary Structure and Biochemical Properties of Gilatoxin, a Serine Protease with Kallikrein-like and Angiotensin-degrading Activities*

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The activity and the complete primary structure of gilatoxin, a glycoprotein component from the venom of the Mexican beaded lizard (*Heloderma horridum horridum*) has been elucidated. Gilatoxin, a serine protease, showed kallikrein-like activity, releasing bradykinin from kininogen; toxin-treated kininogen also produced lowered blood pressure in rats and contraction of isolated rat uterus smooth muscle. Gilatoxin catalyzed the hydrolysis of various arginine ester substrates for trypsin and thrombin and degraded both angiotensin I and II by cleavage of the dipeptide Asp-Arg from the NH_2-terminal end. Fibrinogen was degraded but a fibrin clot was not produced, indicating that gilatoxin has specificities different from thrombin and snake venom thrombin-like proteases.

The complete amino acid sequence of gilatoxin (245 residues) was deduced from NH_2-terminal sequencing of overlapping peptide fragments cleaved from the reduced and alkylated toxin by enzymatic and chemical methods. The toxin is extensively glycosylated, containing approximately 8 mol of monosaccharide/mol of toxin, but appears to lack *O*-glycosylation sites. Amino acid sequence alignment of gilatoxin with batroxobin, crotalase, kallikrein, thrombin, trypsin, and several partial sequences of other *Heloderma* toxins reveals that there is considerable homology between these enzymes, particularly in the regions of the presumed catalytic site. Gilatoxin contains an additional 7 residues in the highly conserved catalytic region of serine proteases (including Asp-96, in the basic specificity pocket of thrombin) which may contribute to the unusual substrate specificity of the toxin.

The Mexican beaded lizard (*Heloderma horridum horridum*) is one of only two known species of venomous lizards. *Heloderma* venoms, like snake venoms, contain a variety of different proteins with diverse biological activities (Tu, 1991). Nonenzymatic polypeptides found in the venoms include the helodermins, which stimulate pancreatic enzyme secretion (Raufman *et al.*, 1982; Vandermeers *et al.*, 1987), exendin-3, which interacts with vasoactive intestinal peptide receptors (Raufman *et al.*, 1991), helospectins, which are vasoactive peptides (Parker *et al.*, 1984), a lethal toxin (Komori *et al.*, 1988a, 1988b) and nerve growth factor (Levi-Montalcini and Angletti, 1968). Several enzyme activities have been detected in *Heloderma* venoms including phospholipase A_2 (Sosa *et al.*, 1986; Gomez *et al.*, 1989), hyaluronidase (Tu and Hendon, 1983), proteolytic enzymes (Tu and Murdock, 1967; Alagon *et al.*, 1986; Nikai *et al.*, 1988), phosphomonoesterase (Murphy *et al.*, 1976), and phosphodiesterase (Murphy *et al.*, 1976). Little is known of most enzymes at the molecular level, and only partial sequence data (up to 33 residues) are available for the proteolytic enzymes.

Enzymes which interfere with hemostasis in vertebrates are common components of snake and *Heloderma* venoms. Thrombin-like and kallikrein-like serine proteases are prevalent among crotalid and viperid snake venoms and these activities may reside in different proteins (see Pirkle and Markland, 1988) or may be found as multiple activities of a single enzyme (such as crotalase; Markland *et al.*, 1982). All appear to be serine proteases and those which have been sequenced show considerable amino acid sequence similarity, particularly in the regions surrounding the presumed catalytic site. Sequence comparisons of gilatoxin with snake toxins and vertebrate serum enzymes are of interest from an evolutionary perspective and may also shed light on structure-function relations among the serine proteases, since preferred substrates for the various enzymes share several features, such as the preference of an arginine residue at the P1 site. In spite of this, the serine proteases cleave unique sites on native substrates, and analysis of the increasing number of known primary structures may assist in determining structural features which confer specificity.

Gilatoxin was isolated previously from venoms of both *H. suspectum suspectum* (Gila monster) and *H. horridum horridum* (Hendon and Tu, 1981); however, the mode of action of this toxin was unclear. In the present report, we describe some of the unique properties of gilatoxin isolated from *H. horridum horridum* venom and present the complete amino acid sequence of the toxin.

EXPERIMENTAL PROCEDURES

Materials

Crude *H. horridum horridum* venom was purchased from Miami Serpentarium (Salt Lake City, UT). Human fibrinogen (grade L) was obtained from Kabi Diagnostica (Franklin, OH). HMW[1] kininogen

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[1] The abbreviations used are: HMW kininogen, high molecular weight kininogen; PAGE, sulfate-polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; BNPS-skatole, 3-bromo-3-methyl-2-(2-nitrophenylmercapto)-3H-indole; RP-HPLC, reverse phase high performance liquid chromatography; Arg C endopeptidase, arginine C endopeptidase; DFP, diisopropylfluorophos-

was obtained from Enzyme Research Laboratories, Inc. (South Bend, IN). Bradykinin was purchased from Calbiochem (La Jolla, CA). Acrylamide Tricine gels (16%) and buffers were purchased from Novel Experimental Technology Co. (NOVEX: Encinitas, CA). Immobilon-P was purchased from Milligen (Burlington, MA). Cyanogen bromide was obtained from Aldrich. Immobilized trypsin was obtained from Pierce Chemical Co. Arginine C endopeptidase and carboxypeptidase Y were purchased from Boehringer Mannheim. All other reagents were purchased from Sigma. Phast Gel isoelectric focusing media (pH 3–9) were purchased from Pharmacia LKB Biotechnology Inc.

Purification

Purification of gilatoxin from *H. horridum horridum* followed a procedure modified from Hendon and Tu (1981). The homogeneity and relative molecular weight of toxin were demonstrated by SDS-PAGE (10% gels, Laemmli, 1970). The isoelectric point was determined using the Phast system and isoelectric focusing gels, pH 3–9 (Pharmacia).

Amino Acid Sequence

Gilatoxin was deglycosylated by incubating 300 μg of gilatoxin with 100 μl of trifluoromethanesulfonic acid (TFMS, Edge *et al.*, 1981). The sample was dialyzed against 0.01% ammonium bicarbonate at pH 7.0 and lyophilyzed.

Gilatoxin was reduced by dissolving 0.3 mg of protein in 1 ml of 0.1 M Tris-HCl, pH 7.5, containing 1% SDS and 2.46 mg (15.9 μmol) of dithiothreitol and incubating at 37 °C for 3 h. The sample was alkylated by adding 6.6 mg (63.6 μmol) of 4-vinylpyridine, incubating at 37 °C for 3 h, and dialyzing against 50 mM ammonium bicarbonate, pH 7.5, containing 0.001% SDS for 24 h. NH_2-terminal analysis was performed by Edman degradation with an ABI 473A Sequencer.

Chemical Cleavage

Cyanogen Bromide—The reduced and alkylated toxin was dissolved in 300 μl of solution containing 70% trifluoroacetic acid and 30 mg/ml CNBr (Chen *et al.*, 1982). CNBr-cleaved toxin was electrophoresed on 16% Tricine SDS-PAGE and electrotransferred to an Immobilon-P membrane as described by Aebersold *et al.* (1986). The membrane was stained with 0.1% Coomassie Blue, 10% acetic acid, and 50% methanol for 1 min before destaining with 50% methanol for 5 min. The stained bands were excised for NH_2-terminal amino acid sequencing.

3-Bromo-3-methyl-2-(2-nitrophenylmercapto)-3H-indole (BNPS) Skatole—Reduced and alkylated toxin (300 μg) was extracted in a solution containing anhydrous acetone/acetic acid/triethylamine/$_2$O (85:5:5:1, v/v). The solution was centrifuged for 10 min at 5000 revolutions/min, and the supernatant removed. The sample was lyophilyzed and dissolved in 150 μl of 80% acetic acid containing 2.4 mg of BNPS-skatole (Fontana, 1972). The ampule was sealed, wrapped with aluminum foil, and kept at room temperature for 72 h. The BNPS-skatole-cleaved toxin was separated by SDS-PAGE and electrotransferred to Immobilon-P as described above. Skatole fragments 5 and 6 (<3000 daltons) could not be resolved by SDS-PAGE on 16% acrylamide tricine gels. These fragments were electroeluted from SDS-PAGE and loaded onto a Vydac 4.5 × 25-cm C_{18} RP column (Beckman System Gold HPLC) eluted with a 0–90% acetonitrile linear gradient (1%/min) at a flow rate of 1 ml/min, monitored at 214 nm. The HPLC-isolated fragments were then analyzed for amino acid sequence.

Enzymatic Cleavage

Trypsin—Three-hundred μg of reduced and alkylated toxin was dissolved in 100 μl of 0.1 M ammonium bicarbonate, pH 8.2, and incubated with immobilized trypsin (1:100 (w/w)) for 18 h at 37 °C. Immobilized trypsin was pelleted by centrifugation at 5000 revolutions/min for 15 min. The supernatant peptide fragments were separated by HPLC as above. The major peptide peaks were collected and analyzed for NH_2-terminal amino acid sequence.

Arginine C Endopeptidase—The reduced and alkylated sample (300 μg) was suspended in 100 μl of 0.1 M ammonium bicarbonate, pH 7.6, containing 0.01 M $CaCl_2$, 50 mM dithiothreitol, 5 mM EDTA, and was incubated with 5 μg of Arg-C endopeptidase (1:60 by weight) at 37 °C for 18–24 h. Fragments were isolated via HPLC as above.

Glu-C Endopeptidase—Reduced and alkylated toxin (300 μg) was suspended in 100 μl of 50 mM ammonium bicarbonate, pH 7.8, and incubated with 8.3 μg of Glu-C endopeptidase enzyme (1:100 by weight) at 37 °C for 18–24 h. Fragments were isolated via HPLC as above.

COOH Terminus Determination

One μg of reduced and alkylated gilatoxin was dissolved in 100 μl of sodium citrate, pH 5.6, and incubated with 20 μl of carboxypeptidase Y (ratio 1:100) at 37 °C. Thirty μl aliquots were taken at time 0, 10, 15, and 30 min. The reactions were stopped by precipitating the protein with 6 M acetic acid. The supernatant, removed from the precipitant by centrifugation at 5000 revolutions/min for 15 min, was analyzed for amino acid content.

Carbohydrate Composition

Gilatoxin (1.0 mg) was analyzed for monosaccharide composition (Oxford Glycosystems, Oxford, United Kingdom). Oligosaccharides were removed by hydrazinolysis, derivatized by anhydrous methanolic HCl to 1-*O*-methyl monosaccharides and then converted into per-*O*-trimethylsilyl methyl glycosides. The per-*O*-trimethylsilyl glycosides were analyzed using gas chromatograph-mass spectrometry with a flame ionization detector. Scyllo-inositol was used as an internal standard to calculate the absolute monosaccharide content/milligram of gilatoxin.

Enzyme Assays

Kallikrein-like Activity—Degradation of high molecular weight kininogen was measured by incubating 50 μl of HMW kininogen (2 mg/ml) in 0.1 M Tris-HCl, pH 8.0, with 20 μg of toxin (in 10 μl) at 37 °C. At 0, 10, 30, 60, and 120 min, 12 μl of the incubation mixture was withdrawn and added to 12 μl of denaturing solution (0.125 M Tris-HCl, pH 6.8, containing 4% SDS, 20% glycerol, and 10% β-mercaptoethanol). The samples were boiled for 5 min before being electrophoresed on SDS-PAGE (10% gels).

Bradykinin release was measured by incubating 30 μl of HMW kininogen (2 mg/ml) in 0.1 M Tris-HCl, pH 8.0, with 5 μl of toxin (2 mg/ml) at 37 °C for 2 h. The reaction mixture was diluted to 200 μl with 0.1 M Tris-HCl, pH 8.0, before microcentrifuge filtration (molecular weight cut off of 10,000). The filtrate was analyzed by HPLC on a Vydac 4.5 × 25 cm C_{18} RP column and eluted with a linear 0–50% acetonitrile gradient (1%/min) at a flow rate of 1 ml/min, monitored at 214 nm. The released peptide (B_{40}) was collected for NH_2-terminal analysis and smooth muscle contraction assays.

Arginine and Lysine Ester Hydrolysis—Arginine ester hydrolase activity was assayed using benzoyl-L-arginine ethyl ester (BAEE), tosyl-L-arginine methyl ester (TAME), and *N*-acetyl-L-tyrosine ethyl ester (ATEE) as substrates as described by the method of Roberts (1958). The effect of the serine protease inhibitor diisopropylfluorophosphate (DFP) and metalloprotease inhibitor EDTA on gilatoxin-catalyzed hydrolysis of BAEE was also determined.

Paranitroanilide (pNA) peptide substrates (*N*-Bz-Ile-Phe-Lys-pNA, *N*-Bz-Phe-Val-Arg-pNA, *N*-Bz-Val-Leu-Lys-pNA, *N*-Bz-Ile-Glu-Gly-Arg-pNA, and *N*-Bz-Pro-Phe-Arg-pNA) were assayed under the following conditions. The substrates (1 mg) were dissolved in 20 μl of dimethyl sulfoxide and brought to 2 ml with 0.1 M HEPES, pH 8.0, containing 0.1 M NaCl. The reaction mixture containing 600 μl of 0.1 M HEPES, pH 8.0, 0.1 M NaCl, 150 μl of substrate, and 50 μl of toxin (2 mg/ml) was incubated at 37 °C for 15 min. The reaction was terminated by adding 75 μl of 50% acetic acid before measuring the absorbance of the samples at 405 nm.

Degradation of Fibrinogen and Fibrin—Fibrinogenolytic activity was measured by incubating 2% human fibrinogen in 5 mM imidazole-saline (1:9), pH 7.4, with 50 μg of toxin. At various time intervals, 80 μl of the incubation mixture was withdrawn and added to 80 μl of denaturing solution (10 M urea, 4% SDS, 4% β-mercaptoethanol). The samples were reduced and denatured overnight at room temperature and were analyzed by SDS-PAGE (10% acrylamide). The ability of toxin to dissolve fibrin clot was observed by the disappearance of the clot and by SDS-PAGE as described by Willis and Tu (1988).

Cleavage of Angiotensin I and II—Cleavage of angiotensin I and II was measured by incubating 100 μl of angiotensin I or II (1 mg/ml) in 0.1 M Tris-HCl, pH 7.5, with 10 μl of toxin (2 mg/ml) at 37 °C. At intervals of 0, 1, 3, and 6 h, 25 μl of the reaction mixture was

phate; AChR *Torpedo californica*, nicotinic acetylcholine receptor; BAEE, benzoyl-L-arginine ethyl ester; TAME, tosyl-L-arginine methyl ester; ATEE, *N*-acetyl-L-tyrosine ethyl ester; pNA, paranitroanilide; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; Bzl, benzyl.

withdrawn, diluted to 200 μl with 0.1 M Tris-HCl, pH 7.5, and filtered with a microcentrifugation filter (molecular mass cutoff = 10,000 daltons). The filtrate was analyzed by HPLC on a Beckman 4.5 × 25-cm C_8 RP column eluted with a 0–50% acetonitrile linear gradient (1%/min, 1 ml/min) and monitored at 214 and 280 nm. Peptide peaks were collected and sequenced.

Biological Assays

Hemorrhagic activity was assayed by the method of Bjarnasson and Tu (1978). All experiments with rodents were conducted in accordance with the guidelines established by the National Institutes of Health and the Colorado State University Institutional Animal Care and Use Committee (IACUC).

To monitor toxin effects on blood pressure, male Sprague-Dawley rats (275 g) were anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneally). The trachea was isolated via a midline incision and was cannulated with a polyethylene endotracheal tube (PE 205 intramedic tubing, 1.57 mm inner diameter, Clay Adams). The rat was connected to a rodent ventilator (Harvard Apparatus, model 681) and ventilated with air at 65 breaths/min. The right carotid artery and left jugular vein were isolated and cannulated with polyethylene catheters (PE 50 intramedic tubing, 0.58 inner diameter, Clay Adams). For measurement of arterial blood pressure, the carotid catheter was attached to a pressure transducer (Gould P23ID) connected to a Gilson Duograph recorder. The jugular catheter was used for infusion of gilatoxin or saline. Once arterial blood pressure stabilized, baseline pressure (systolic, diastolic, and mean) and heart rate measurements were obtained. The sample (210 μg of gilatoxin in saline or saline alone, 100 μl) was then administered intravenously.

Smooth muscle contraction produced by the B_{40} peptide (0.01 or 0.02 μg) was assayed according to Trautschold's method (1970). Bradykinin (0.02 μg) was used as a positive control.

Various dosages (up to 2 mg/kg) of gilatoxin were injected intravenously into female Swiss Webster mice (18–22 g). After death selected tissues were dissected (brain, eye, heart, liver, kidney, adrenal glands, spleen, lung, and small intestine) and preserved in Z-fix (zinc containing formalin solution). The tissues were embedded in paraffin, and thick sections were stained with hematoxylin/eosin. Sections were examined under light microscopy for histopathological changes by comparison with normal controls.

Acetylcholine Receptor Binding Assay

Acetylcholine receptor (AChR) was isolated from *Torpedo californica* electroplax organ by the methods of Brockes and Hall (1975) and Lindstrom *et al.* (1980). Homogeneity of AChR was established by SDS-PAGE (10% gel). Gilatoxin (10 μg) and α-bungarotoxin (10 μg) were labeled with Na-^{125}I by the lactoperoxidase method (Morrison and Bayse, 1970). Toxin binding to AChR was determined using the methods of Schmidt and Raftery (1973) and Vazquez *et al.* (1989).

RESULTS

Purification of Gilatoxin—Gilatoxin was isolated by a three-step method of gel filtration and ion-exchange chromatography (Fig. 1, *A-C*). Analysis on SDS-PAGE gave a single band, indicating the homogeneity of gilatoxin (Fig. 1*D*); HPLC analysis also showed a single peak (data not shown). The relative molecular weight of gilatoxin, 33,000, is slightly higher than horridum toxin, a hemorrhagic toxin present in the same venom.

Primary Structure—The NH_2-terminal amino acid sequence of gilatoxin (after carbohydrate removal) was first determined on the whole toxin by Edman degradation before and after reduction and alkylation with 4-vinylpyridine, providing the first 49 residues (Fig. 2). The complete sequence was obtained from overlapping peptide fragments generated by chemical cleavages and enzymatic digestions. Gilatoxin consists of 245 residues, and the entire sequence and the sequences of peptide fragments are presented in Fig. 2.

Four peptide fragments were obtained from the reduced and alkylated toxin after CNBr treatment, designated as CNBr-1, CNBr-2, CNBr-3, and CNBr-4. Seven fragments were obtained from the reduced and alkylated toxin using BNPS-skatole. Three peptide fragments were obtained from

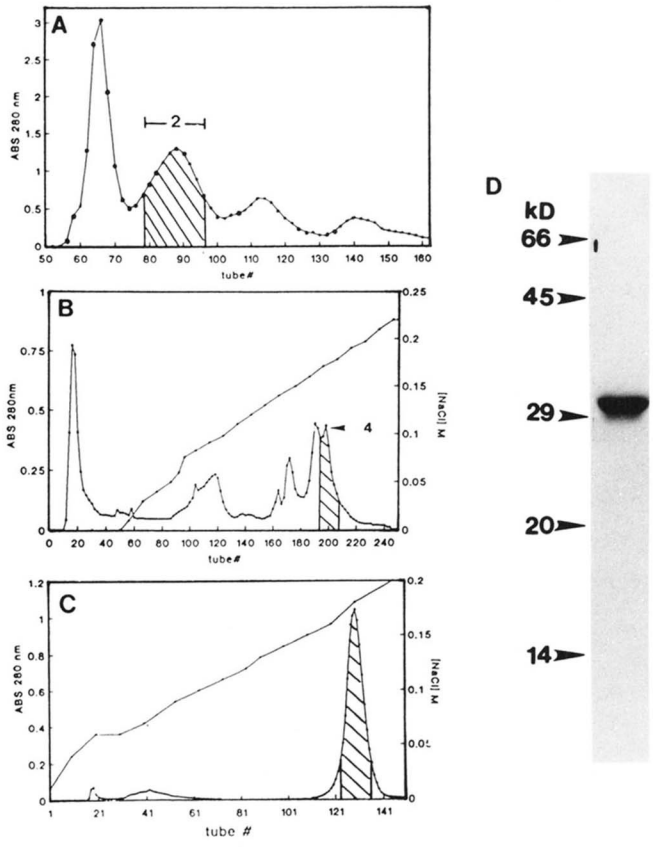

FIG. 1. **Isolation of gilatoxin.** *A*, molecular sieve chromatography (G-75). *B*, ion-exchange chromatography (DEAE-Sephacryl). *C*, ion-exchange chromatography (diethyl[2-hydroxy-propyl]aminoethyl-Sephadex). *D*, homogeneity in SDS-PAGE. See "Experimental Procedures" for details.

reduced and alkylated toxin using arginine C endopeptidase. Eight major fragments, obtained from the reduced and alkylated toxin after digestion with trypsin, were isolated by HPLC. Six bands were observed upon SDS-PAGE of the glutamine C endopeptidase digest of gilatoxin. Amino acid sequences of peptides from four bands were determined.

To determine the COOH-terminal amino acid, reduced and alkylated gilatoxin was incubated with carboxypeptidase Y for varying time intervals. The released amino acids were isolated by RP-HPLC and identified by amino acid analysis. The order of amino acid release was proline, cysteine, and threonine. The amino acid sequence of the fragments identified as skatole-7 and Trypsin-5 have the sequence Ile-Gln-Asn-Ile-Ile-Gln-Gly-Gly-Thr-thr-Cys-Pro and Phe-Asn-Phe-Trp-Ile-Gln-Asn-Ile-Ile-Gln-Gly-Glythr-Thr-Cys-Pro, respectively, and represent the COOH-terminal end of gilatoxin.

Comparison of the primary structure of gilatoxin with the partial sequences of two other *Heloderma* toxins (Fig. 3) revealed significant homology between gilatoxin and horridum toxin (Nikai *et al.*, 1988), a hemorrhagic toxin. However, gilatoxin and horridum toxin showed close elution profiles after DEAE-Sephacryl ion-exchange chromatography (*third peak* in Fig. 1*B*; identity confirmed by sequence analysis and hemorrhagic assay), and the preparation of Nikai *et al.* (1988) are likely a mixture of both gilatoxin and horridum toxin. Gilatoxin also showed sequence homology with helodermatine (Alagon *et al.*, 1986), a hypotensive enzyme, but helodermatine has a relative molecular weight (63,000) approximately twice that of gilatoxin.

Kallikrein-like Activity—When kininogen was incubated with gilatoxin, the disappearance of kininogen and the ap-

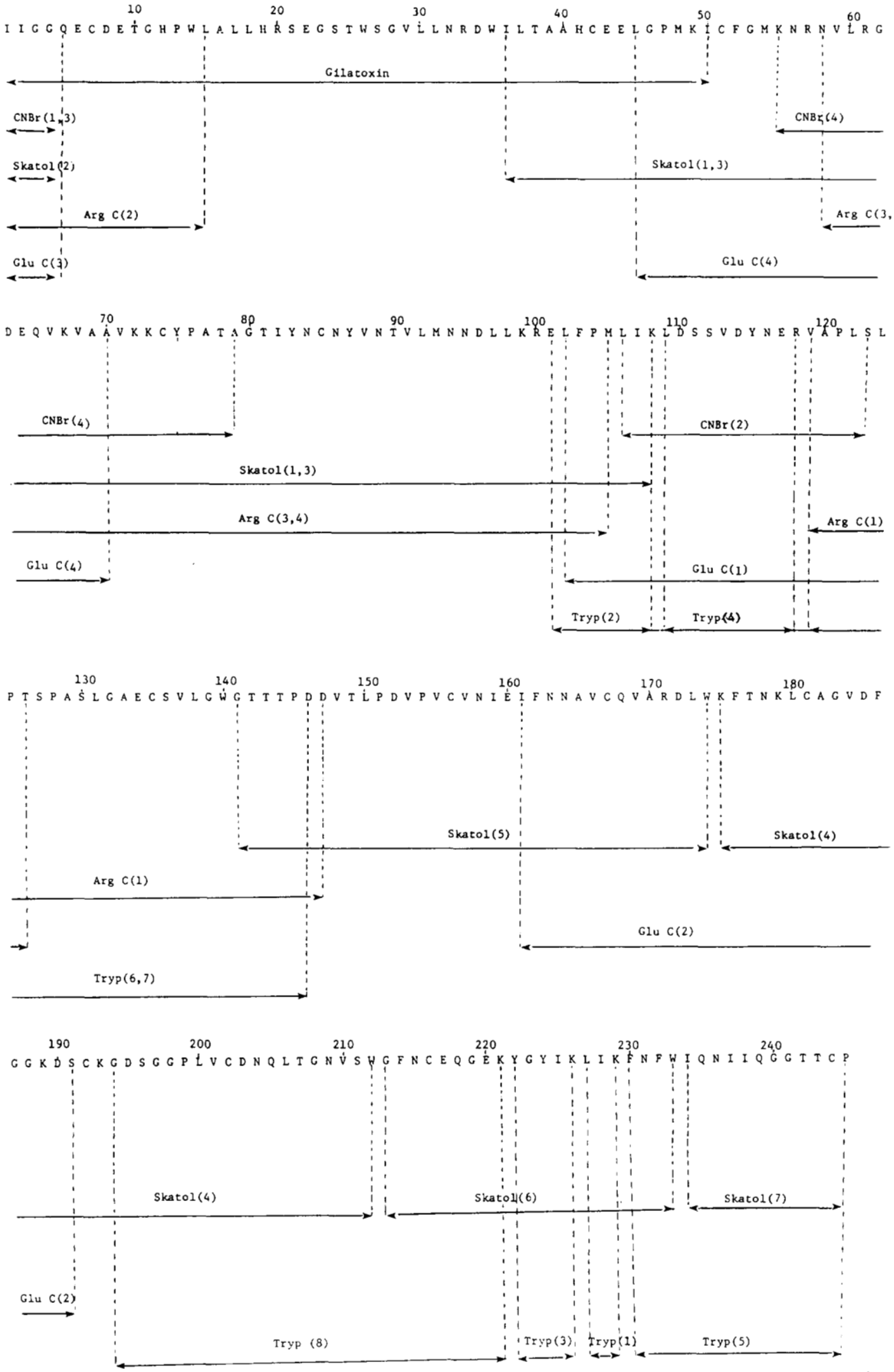

FIG. 2. **Alignment of amino acid sequences of overlapping peptide fragments generated by chemical or enzymatic cleavage of gilatoxin.** Residues 1–49 were determined by automated Edman degradation of the intact toxin. *CNBr*, cyanogen bromide fragments; *Skatole*, skatole-generated fragments; *Tryp*, immobilized trypsin-generated fragments; *Arg C*, arginine C endopeptidase-generated fragments; *Glu C*, glutamine C-generated fragments.

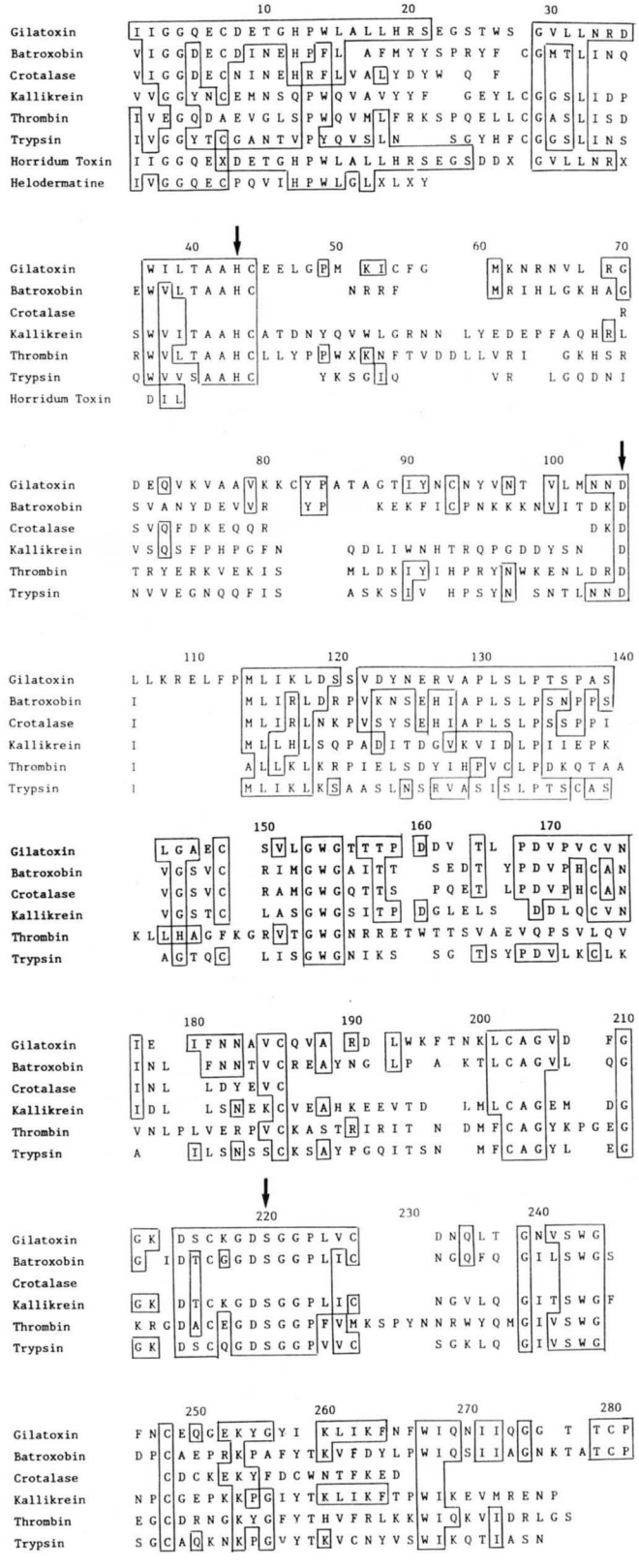

FIG. 3. **Alignment of amino acid residues for gilatoxin, batroxobin from *B. moojeni* venom (Itoh *et al.*, 1987), crotalase from *C. adamanteus* venom (Pirkle *et al.*, 1981), kallikrein from rat pancreas (Swift *et al.*, 1982), bovine thrombin (Magnusson *et al.*, 1975), dogfish trypsin (Titani *et al.*, 1975), and partial sequences for horridum toxin (Nikai *et al.*, 1988) and helodermatine (Alagon *et al.*, 1986).** The putative active site residues His^{43}, Asp^{105}, and Ser^{220} of gilatoxin are marked with *arrowheads* and are based on homology with the known active sites of thrombin (Elion *et al.*, 1977). Note the extensive sequence homology

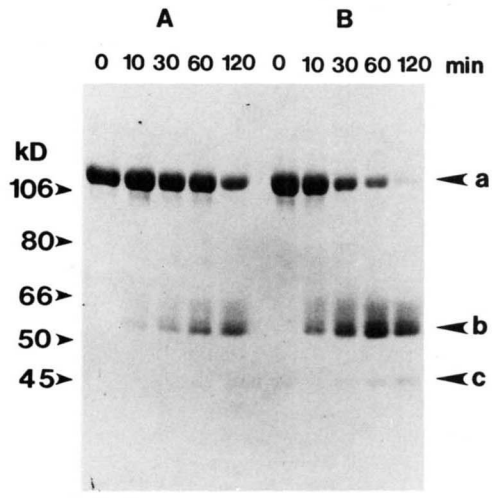

FIG. 4. **SDS-PAGE analysis of degraded HMW kininogen after digestion by plasma kallikrein or gilatoxin.** HMW kininogen was incubated with plasma kallikrein and gilatoxin as described under "Experimental Procedures." *A*, HMW kininogen after incubation with plasma kallikrein for specified times. *B*, HMW kininogen after incubation with gilatoxin for specified times; *a*, indicates original HMW kininogen (114 kDa); *b*, indicates light chain (58 kDa); *c*, indicates modified light chain (45 kDa).

pearance of degradation products (light chain (58 kDa) and modified light chain (45 kDa)) were apparent (Fig. 4). Human kallikrein was used as a positive control, and the kininogen degradation products resulting from incubation with gilatoxin and human kallikrein appeared to be identical. A separate aliquot of the incubation mixture of kininogen and gilatoxin described above was filtered in order to remove high molecular sized proteins, and subsequent HPLC analysis of the filtrate revealed a peak (B_{40}) with a retention time of 40 min (data not shown). Bradykinin, as a positive control, had a retention time of 40 min. In addition, the amino acid sequence of B_{40}, Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg, is identical to that of bradykinin. The B_{40} peptide did not appear when incubating HMW kininogen in the absence of toxin. Therefore, contamination of HMW kininogen with bradykinin can be ruled out.

The B_{40} fraction produced contractions of rat uterus smooth muscle in a concentration-dependent manner (Fig. 5, *A* and *B*), indicating that the B_{40} peptide is bradykinin. The positive control (bradykinin) showed a typical contraction curve (Fig. 5*C*). As a negative control, HMW kininogen incubated without gilatoxin was filtered and the filtrate produced no contraction (Fig. 5*D*).

The hypotensive action of gilatoxin after injection was investigated in cannulated rats. A significant blood pressure drop is shown in Fig. 6, and this decrease is likely due to the release of bradykinin from endogenous HMW kininogen.

Gilatoxin hydrolyzed BAEE and TAME, trypsin substrates, but did not hydrolyze the chymotrypsin substrate ATEE (Table I). In spite of the kallikrein-like action on native substrate (HMW kininogen), gilatoxin did not hydrolyze the chromogenic substrate for urinary kallikrein (Bz-Pro-Phe-Arg-pNA).

Enzyme Assays Related to Blood Coagulation Factors—Gilatoxin did not produce a fibrin clot when incubated with

in the immediate vicinity of His^{43} and Ser^{220}; the region immediately adjacent to Asp^{105} is highly conserved in most serine proteases, but in gilatoxin it is interrupted by an intervening sequence of 7 residues. Alignments were made to maximize homology and spaces indicate residues absent. Residues which are identical to gilatoxin are *boxed*, and × indicates an unidentified residue.

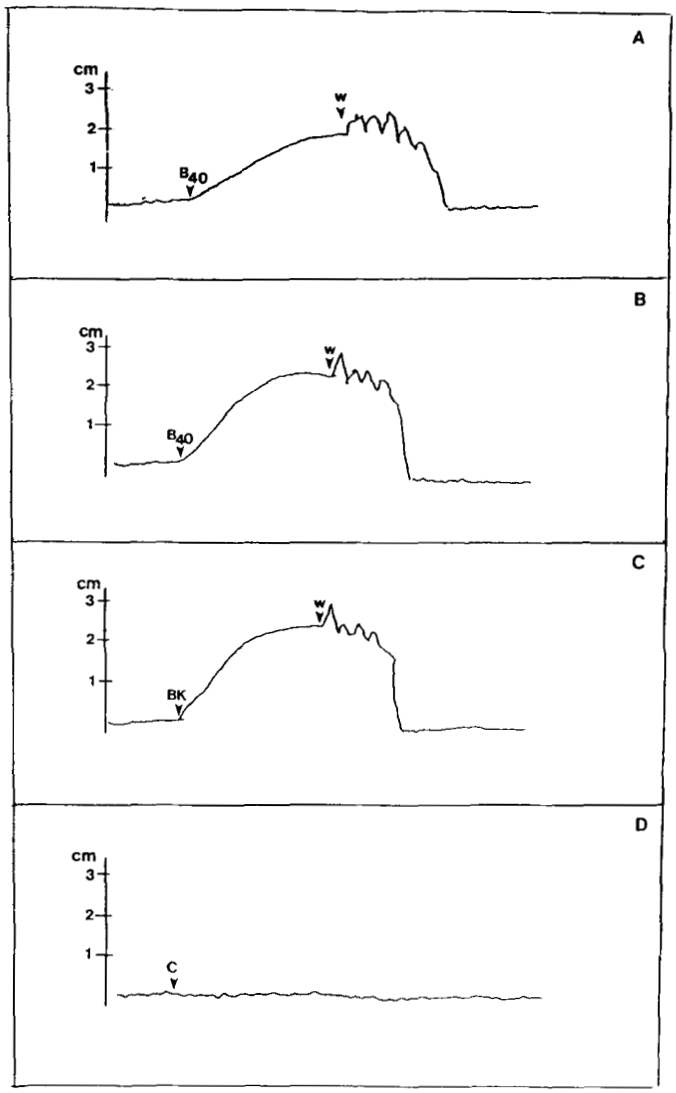

FIG. 5. **Stimulated contraction of isolated rat uterus smooth muscle.** The assay for rat uterus smooth muscle contraction is described under "Experimental Procedures." *A*, effect of 0.01 μg of B_{40}. B_{40} is the purified product released from HMW kininogen by the action of gilatoxin. *W* is the point of washing of muscle with buffer. *B*, effect of 0.02 μg of B_{40}. *C*, effect of 0.02 μg of bradykinin (positive control). *D*, effect of filtrate (potential spontaneously released product) from incubation of HMW kininogen alone (negative control).

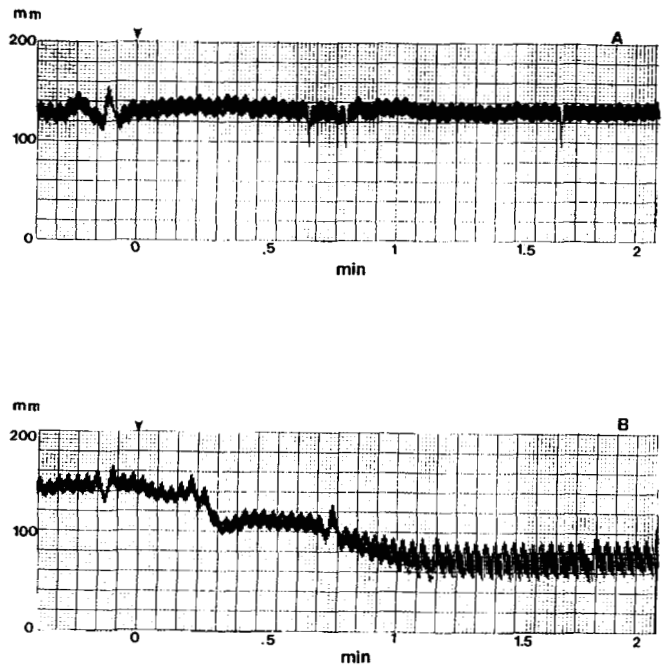

FIG. 6. **The effect of gilatoxin on rat blood pressure.** *A*, blood pressure after injection with normal saline. *B*, blood pressure after intravenous injection of gilatoxin (0.5 μg/g; 20% of LD_{50}). *Arrow* indicates the point of sample injection.

TABLE I

Substrate specificity and inhibition of gilatoxin

One unit = μmol of substrate hydrolyzed/min/mg toxin (based on ϵ_{405nm} = 10,300 for free paranitroaniline).

Substrate	Gilatoxin
BAEE	245 units
TAME	209 units
ATEE	0
N-Benzoyl-Phe-Val-Arg-pNA (thrombin-like)	51.6 units
N-Benzoyl-Pro-Phe-Arg-pNA (kallikrein-like)	0
N-Benzoyl-Ile-Glu-Gly-Arg-pNA (Factor X A)	0
N-Benzoyl-Val-Leu-Lys-pNA (human plasmin)	0
N-Benzoyl-Ile-Phe-Lys-pNA (plasmin-like)	0
Inhibited by DFP	Yes
Inhibited by EDTA	No

fibrinogen. However, gilatoxin did hydrolyze the Aα, Bβ, and γ chains of fibrinogen. The Aα chain was hydrolyzed completely within 6 h, and the Bβ chain was completely degraded within 12 h. The γ chain was most resistant to gilatoxin hydrolysis and required at least 18 h of incubation for complete hydrolysis (Fig. 7). In comparison, the fibrinolytic protease atroxase (isolated from *Crotalus atrox* venom) hydrolyzed Aα and Bβ chains but did not hydrolyze the γ chain (Fig. 7).

A chromogenic substrate for thrombin, *N*-Bz-Phe-Val-Arg-pNA, was also readily hydrolyzed by gilatoxin (Table I). Gilatoxin's proteolytic activity was inhibited by DFP, using BAEE as substrate, indicating that gilatoxin is a serine-type protease. The same activity was not inhibited by EDTA, indicating that gilatoxin is not a metalloenzyme. From these results it is clear that gilatoxin has some properties similar to other serine proteases.

Two synthetic peptide substrates for plasmin, *N*-Bz-Val-Leu-Lys-pNA and Ile-Phe-Lys-pNA, were not hydrolyzed by gilatoxin (Table I). Incubation of gilatoxin with fibrin clot also showed that the toxin did not dissolve fibrin clot or hydrolyze α, β, and γ-γ chains of fibrin (data not shown). The lack of activity toward both native and model substrates indicated that gilatoxin lacks plasmin-like activity. Gilatoxin also did not hydrolyze chromogenic substrates for factor Xa, indicating that the toxin does not have factor Xa activity (Table I).

Cleavage of Angiotensin I and II by Gilatoxin—Incubation of angiotensin I with gilatoxin resulted in the degradation of angiotensin I, a hypertensive peptide originating from angiotensinogen. At zero time, only angiotensin I was visible (as *peak a*, Fig. 8*A*). As incubation continued, digestion of angiotensin I is demonstrated by the appearance of a new peak, peak b (Fig. 8, *B–D*). The amino acid sequence of peak b was determined and found to be Val-Tyr-Ile-His-Pro-Phe-His-Leu, demonstrating that the arginylvaline bond of angiotensin I was cleaved by gilatoxin. Gilatoxin also hydrolyzed angiotensin II and released a dipeptide from the NH_2-terminal end (data not shown). The cleavage of angiotensin I may be a contributing factor for the prolonged hypotensive action of

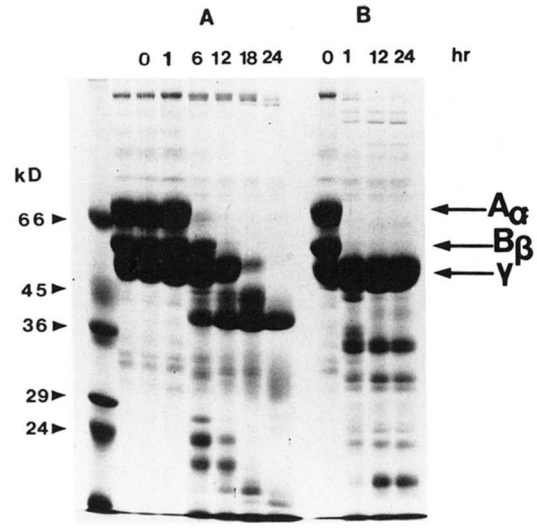

FIG. 7. **SDS-PAGE analysis of reduced human fibrinogen after digestion with gilatoxin or atroxase, a fibrinolytic enzyme from *C. atrox* venom.** Fibrinogen was incubated with gilatoxin and atroxase for specified times as described under "Experimental Procedures." *A*, fibrinogen samples after incubation with 50 μg of gilatoxin for specified times. *B*, fibrinogen samples after incubation with 50 μg of atroxase for specified times. Note that the fragments produced are dissimilar, indicating different cleavage sites on human fibrinogen for gilatoxin and atroxase.

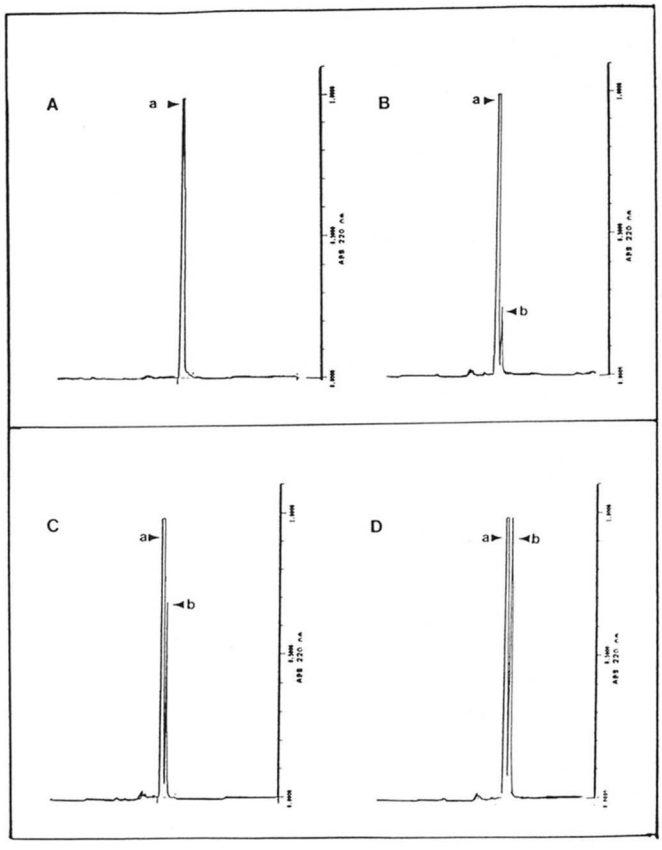

FIG. 8. **HPLC chromatograms of angiotensin I cleavage by gilatoxin.** Each chromatogram represents angiotensin I (*a*) and degradation products (*b*) after 0-, 1-, 3-, and 6-h incubation times (*A–D*, respectively). Chromatography was performed by using a linear gradient of 0–50% acetonitrile in water containing 0.1% trifluoroacetic acid for 50 min at a flow rate of 1 ml/min as described under "Experimental Procedures."

gilatoxin.

Other Biochemical Characterizations—Gilatoxin is a single polypeptide chain, as only one band was resolved on SDS-PAGE in the presence or absence of β-mercaptoethanol. The relative molecular weight is approximately 33,000. It is an acidic glycoprotein with a pI of 4.0.

The toxin is glycosylated, and the carbohydrate content was determined by gas chromatograph-mass spectrometry after removal of the carbohydrate moieties from gilatoxin. The result is summarized in Table II. Gilatoxin contains approximately 5% total carbohydrate. No fucose, xylose, or *N*-acetylgalactosamine were detected; the absence of a detectable amount of *N*-acetylgalactosamine suggests a lack of any *O*-glycosylation in gilatoxin.

Biological Activities—Gilatoxin was nonhemorrhagic at doses of up to 50 μg and did not produce hemopericardium. Unlike horridum toxin, which is present in the same venom, gilatoxin did not produce exophthalmia. Gilatoxin did produce a toxic effect in mice, as evidenced by the loss of equilibrium in the animal (mice moved around the cage in gyrations) and producing hind limb paralysis.

Thick sections of brain, eye, heart, lung, spleen, small intestine, liver, kidney, and adrenal glands were examined under light microscopy in an attempt to locate any pathological effects of gilatoxin. No detectable damage, including hemorrhagic damage to any of these tissues, was noted. Gilatoxin does not appear to produce gross histopathological effects in mouse tissues.

Toxicity experiments indicated an LD_{50} of 2.5 μg/g (intravenous, mice) for both the crude venom and for gilatoxin.

Examination of AChR Binding Capacity—Earlier reports suggested a neurotoxic effect of gilatoxin when injected in mice. ^{125}I-bungarotoxin bound to acetylcholine receptor isolated from electroplax tissue, demonstrating that an active preparation of receptor was obtained. However, gilatoxin did not show AChR binding activity, indicating that gilatoxin is not a postsynaptic neurotoxin.

DISCUSSION

Gilatoxin was first isolated by Hendon and Tu (1981). The nature of this toxin was not fully determined, but since it appeared to be the major lethal component of the venom, the name gilatoxin was assigned to this protein. In the present report, we have shown that gilatoxin is a glycosylated serine protease (inhibited by DFP) with a rather broad specificity for arginyl-*X* bonds. Like thrombin, gilatoxin catalyzed the hydrolysis of the chromogenic substrate *N*-Bz-Phe-Val-Arg-pNA and the hydrolysis of the Aα and Bβ subunits of human fibrinogen (but without clot production); furthermore, it is gylcosylated, with a carbohydrate content of approximately

TABLE II
Carbohydrate composition of gilatoxin

Monosaccharide	Absolute molar content	Molar ratio	Nearest integer molar ratio
	nmol/mg protein		
Fucose	0	0	0
Xylose	0	0	0
Mannose	58.8	1.94	2
Galactose	52.8	1.74	2
Glucose	33.6	1.11	1
N-Acetylgalactosamine	0	0	0
N-Acetylglucosamine	58.8	1.94	2
Sialic acid	39.6	1.30	1
Total monosaccharide content	243.6	8.03	8

5%. In addition, similar to the thrombin-like proteases from snake venoms (Alexander *et al.*, 1988), gilatoxin produced axial gyrations upon intravenous injection of mice, perhaps due to the liberation of neuroactive peptides. Gilatoxin shows some activities similar to trypsin, and chromogenic arginyl substrates for trypsin were readily hydrolyzed. However, it is more specific than trypsin as demonstrated by the specific cleavage sites on HMW kininogen and, to a lesser extent, on human fibrinogen. Gilatoxin did not hydrolyze *X*-*X*-Lys-pNA substrates, suggesting that arginine but not lysine is required at the P_1 site for substrate recognition.

The activity of gilatoxin is also similar in many respects to plasma kallikrein, as demonstrated by the specific cleavage of kininogen, the release of bradykinin from kininogen, the stimulation of rat uterus contraction, and the hypotensive effect of the filtrate obtained from HMW kininogen digestion with gilatoxin. Gilatoxin showed moderate toxicity toward Swiss-Webster mice (IV LD_{50} = 2.5 μg/g body weight); lethality values for other reptile venom kallikrein-like enzymes are generally lacking (*e.g.* Alagon *et al.*, 1986; Komori *et al.*, 1988b; Yabuki *et al.*, 1991). However, unlike the kallikrein-like enzymes from snake venoms (*e.g.* Mackessy, 1989), gilatoxin did not hydrolyze the kallikrein substrate *N*-Bz-Pro-Phe-Arg-pNA. This result is particularly surprising since this substrate is a model of the NH_2 terminus of bradykinin, which is released from the native substrate. The various activities exhibited by gilatoxin and their similarities to other well-characterized serine proteases prompted our work on the primary structure in an attempt to clarify structure-function relations of gilatoxin to other members of the trypsin-kallikrein family of proteases.

Gilatoxin showed considerable sequence similarity with serine proteases from both snake venoms and mammalian tissues (Fig. 3). When aligned for maximum sequence homology, gilatoxin showed approximately 40% sequence identity with batroxobin (Itoh *et al.*, 1987), a thrombin-like enzyme from the venom of *Bothrops moojeni*. Flavoxobin, from *Trimeresurus flavoviridis* venom, is the only other venom serine protease whose complete primary structure is known, and it shows a high degree of sequence identity with batroxobin (Shieh *et al.*, 1988; Pirkle and Theodor, 1991). *Trimeresurus* and *Bothrops* are phylogenetically more closely related to each other than either is to *Heloderma*, and the primary structures of these toxins reflect this relation as well.

There is significant sequence similarity between gilatoxin and other members of the trypsin-kallikrein family of serine proteases. Based on a single homologous alignment of amino acid sequences (Fig. 3), gilatoxin shows 32, 29, and 26% sequence identity with trypsin (Titani *et al.*, 1975), kallikrein (Swift *et al.*, 1982), and thrombin (Magnusson *et al.*, 1975), respectively. Regions of greatest sequence homology include the NH_2 terminus, residues flanking and including the presumed catalytic groups, and cysteines involved in disulfide bonds. Catalytic groups and disulfide positions have not been determined experimentally for gilatoxin, but based on similarity with the known structure of trypsin, tentative assignments can be made. In the following discussion, all numerical assignments of amino acid residues are based on those given in Fig. 3.

Trypsin contains six disulfides which help define active site topography and stabilize higher order structure (Cys^7-Cys^{173}, Cys^{28}-Cys^{44}, Cys^{138}-Cys^{262}, Cys^{149}-Cys^{226}, Cys^{186}-Cys^{202}, and Cys^{216}-Cys^{248}). At least four of these disulfides (Cys^7-Cys^{173}, Cys^{149}-Cys^{226}, Cys^{186}-Cys^{202}, and Cys^{216}-Cys^{248}) are likely identical in gilatoxin, since cysteines are present in identical positions with a high degree of homology among flanking residues. Gilatoxin lacks Cys^{28}, an invariant residue in all other serine proteases (including batroxobin and flavoxobin); however, Cys^{44} is present and may participate in disulfide formation (with Cys^{54}?) to provide positional restraints on the catalytic residue His^{43}. The sixth disulfide of trypsin (Cys^{216}-Cys^{248}) is completely absent in gilatoxin, but several other cysteine residues (82, 93, 280) may participate in disulfide formation.

The presumed catalytic residues of serine proteases, His^{43}, Asp^{105}, and Ser^{220}, are also present in gilatoxin, and the sequences of flanking residues are highly conserved, suggesting that these residues are necessary for the spatial constraint of the active site residues. Perhaps significant, an obvious exception is the insertion of an additional 7 residues on the carboxyl side of Asp^{105} in gilatoxin (Fig. 3). This intervening sequence (Leu-Lys-Arg-Glu-Leuy-Phe-Pro) is seen only in gilatoxin and may explain some of the differences in substrate specificity between gilatoxin and other serine proteases. Three ionizable residues (Lys-Arg-Glu) are within this sequence and perhaps modulate the effect of Asp^{105} on His^{43}. Since Cys^{29} is absent from gilatoxin, the positional constraint on the catalytic histidine is likely shifted, producing a change in the active site and/or specificity pocket topography.

Despite the ability of gilatoxin to catalyze the hydrolysis of several types of peptide bonds, its action toward HMW kininogen appears identical to that of plasma kallikrein. Examination of sequence homology between gilatoxin and kallikrein reveals only one region of the toxin which shares sequence identity with kallikrein and not with the above mentioned serine proteases. Residues 260–264 (Fig. 3) comprise the sequence Lys-Leu-Ile-Lys-Phe which is found only in gilatoxin and kallikrein and may represent a domain of the molecule involved in binding to HMW kininogen. Other than the active site and disulfide regions mentioned above which are common to all members of the trypsin-kallikrein family of serine proteases, gilatoxin contains no other regions (of >1 residue) homologous with kallikrein.

Gilatoxin showed significant sequence homology with the partial sequences of two other enzymes isolated from *Heloderma* venom; however, horridum toxin (Nikai *et al.*, 1988) is strongly hemorrhagic, and gilatoxin lacked hemorrhagic activity. Highly purified gilatoxin and horridum toxin showed an LD_{50} of 2.5 μg/g intravenous injection in mice; when combined, the LD_{50} was 0.3 μg/g intravenous injection in mice (data not shown), similar to that reported by Nikai *et al.* (1988) for horridum toxin. We therefore conclude that this earlier preparation contained both horridum toxin and gilatoxin, and the apparent sequence homology may simply reflect this.

Gilatoxin also showed sequence homology with helodermatine, a hypotensive enzyme (Alagon *et al.*, 1986). Activity of helodermatine was quite different from that of gilatoxin, and tripeptide substrates for kallikrein as well as native plasmin were hydrolyzed by only helodermatine, indicating that gilatoxin and helodermatine are different enzyme components of the same venom.

The *in vivo* effects of gilatoxin are likely several, but hypotensive effects are dominant, have a rapid onset, and may lead to death. An additional activity of gilatoxin, the degradation of the hypertensive peptides angiotensins I and II via the release of the dipeptide Asp-Arg, may potentiate this hypotensive effect. Removal of the Asp-Arg dipeptide from angiotensins I and II inactivates these peptides (Kosla *et al.*, 1974), and this action of gilatoxin contributes to the prolonged hypotensive effect seen in rats. The biological role of gilatoxin in the effect of *Heloderma* venom on prey also may include a

potentiating effect on potent hemorrhagic toxins present in the venom.

In conclusion, gilatoxin shares some structural and catalytic properties with other members of the trypsin/kallikrein family of serine proteases, including a high degree of sequence homology and identity among functionally important residues. The bradykinin-releasing and hypotensive action of gilatoxin results from the structural similarity to kallikrein. Structure-function studies utilizing synthetic peptide models of this region of sequence identity with kallikrein (residues 260–264) and native HMW kininogen substrate may help elucidate the functional significance of this region of sequence identity.

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