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Substrate Specificity of a Hemorrhagic Proteinase from Timber Rattlesnake Venom†

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ABSTRACT: The substrate specificity of hemorrhagic proteinase IV (HP-IV) from timber rattlesnake (*Crotalus horridus horridus*) venom has been investigated. HP-IV exhibited little activity toward most protein substrates but totally solubilized cow hide powder azure. HP-IV also catalyzed the hydrolysis of cow hide powder that did not contain covalently bound dye. Dansylation of the hydrolysis fragments of cow hide showed the formation of six new N-terminal residues. Only one peptide bond was cleaved in each of the oxidized A and B chains of insulin. Bee venom melittin was cleaved at the Ile_2–Gly_3, Pro_{14}–Ala_{15}, and Ser_{18}–Trp_{19} bonds. Various unblocked dipeptides and the doubly blocked dipeptides *N*-Cbz-Ser-Leu-NH_2, *N*-Cbz-Ala-Leu-NH_2 and *N*-Cbz-Ile-Gly-NH_2 were not cleaved. The peptides used corresponded to known cleavage sites in the insulin chains and melittin. HP-IV also had no esterase, elastase, or phospholipase activity under our assay conditions but did exhibit a weak collagenase activity. HP-IV catalyzed the complete hydrolysis of glomerular basement membrane in the presence of 10 mM Ca^{2+} at a rate 60% as fast as an equal concentration (by weight) of bacterial collagenase. When incubated with fibrinogen solutions, HP-IV caused a 50% decrease in soluble protein. Coincident with the decrease in soluble protein was the formation of a precipitate in which the α and β chains of fibrinogen had been degraded. Sodium dodecyl sulfate/polyacrylamide gel electrophoresis revealed that fibrinogen with degraded α and β chains was present in the supernatant after the formation of the precipitate. High-pressure liquid chromatography analysis of HP-IV-treated fibrinogen revealed the release of a peptide similar in composition to thrombin-induced fibrinopeptide A, but no peptide corresponding to fibrinopeptide B was detected. Incubation of HP-IV with thrombin-induced fibrin clots caused an increase in soluble protein with electrophoretic patterns showing degradation of the α chain. Results obtained from the hydrolysis of the various substrates by HP-IV suggest that cleavage points are determined by the size and conformation of the substrate, not just by recognition of the amino acids comprising the cleaved peptide bond.

Recently a hemorrhagic protein with proteolytic activity (HP-IV)[1] was isolated from timber rattlesnake (*Crotalus horridus horridus*) venom (Civello et al., 1983). The conditions for optimal proteolytic activity have been determined by using hide power azure as the substrate (Civello et al., 1983). As will be shown in this report, ordinary cow hide powder is also solubilized by HP-IV, but at a decreased rate. The action of HP-IV upon other substrates has been investigated, and its proteolytic specificity was examined by using the oxidized A and B chains of insulin, bee venom melittin, and cow hide powder. The action of HP-IV on blocked and unblocked dipeptides corresponding to the observed cleavage sites in the insulin and melittin polypeptides was also examined.

Sullivan et al. (1979) have reported that HP-IV does not catalyze the hydrolysis of tosyl-L-arginine methyl ester, benzoyl-L-arginine ethyl ester, or benzoyl-L-tyrosine ethyl ester, that it lacks collagenase, elastase, and neuraminidase activities, and that it is not a procoagulant with fibrinogen solutions. However, HP-IV has the ability to catalyze the complete hydrolysis of hide powder azure and ordinary cow hide powder. This proteinase activity has been characterized in relation to its hemorrhagic activity (Civello et al., 1983).

Since cow hide powder was the only substrate of those listed above to be hydrolyzed by HP-IV, other substrates were tested. Hemoglobin and casein as well as the N,N-dimethylated derivatives of these proteins were not hydrolyzed by HP-IV. In view of the fact that hide powder is rapidly hydrolyzed by

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[1] Abbreviations: HP-IV, hemorrhagic proteinase IV from timber rattlesnake venom; dansyl, 5-(dimethylamino)naphthalene-1-sulfonyl; *N*-Cbz, *N*-carbobenzoxy; $NaDodSO_4$, sodium dodecyl sulfate; HPLC, high-pressure liquid chromatography.

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HP-IV, collagen was tested because of the high content of collagen in cow hide (Rao et al., 1967). Basement membrane and elastin were also tested as substrates because of their similarity to collagen and structural role. Fibrinogen was tested as a substrate to determine if HP-IV possessed any anticoagulant activity. By analysis of the effect of HP-IV upon structural proteins and fibrinogen, it was hoped that some insight into HP-IV's hemorrhagic activity could be gained.

Materials and Methods

Timber rattlesnake venom was obtained in lyophilized form from the Miami Serpentarium. Hide powder, hide powder azure, Pronase (EC 3.4.24.4), human thrombin (3200 NIH units/mg) and bovine fibrinogen, 98% clottable protein, were obtained from Calbiochem-Behring Corp., molecular sieve materials and electrophoresis reagents from Bio-Rad, Schleicher & Schuell F 1700 Micro Polyamide TLC sheets from Pierce Chemical Co., blocked dipeptides from Vega Biochemicals, and type I bovine fibrinogen, 75% clottable protein, and all other reagents and substrates from Sigma Chemical Co. Type IV collagenase (210 units/mg) from *Clostridium histolyticum* (EC 3.4.24.3) obtained from Sigma was further purified by the method of Mandl et al. (1964). Bovine glomerular basement membrane was generously donated by Dr. Billy Hudson of the University of Kansas Medical Center. Performic acid oxidation and fractionation of the A and B chains of bovine insulin were accomplished by the method of Sanger (1949). Amino acid analyses were used to confirm the purity of the A (97% pure) and B (99% pure) chains.

Isolation. HP-IV was obtained by ion-exchange and high-pressure liquid chromatography of timber rattlesnake venom (Civello et al., 1983).

Phospholipase Activity. Phospholipase A_2 activity was estimated by titration of acid produced during the hydrolysis of an egg yolk phosphatidylcholine (type I-EH) suspension with 10 mM NaOH. The substrate suspension was prepared by sonicating a suspension of 20 mg of lecithin/mL in 150 mM sodium chloride/5 mM $CaCl_2$/3 mM sodium deoxycholate for 1 min at 4 °C (Moran et al., 1981). The titrimetric assay was performed with a Radiometer automatic titrator set at an end point of pH 8.5 and at a temperature of 40 °C.

Proteolytic Assays. The action of HP-IV upon hide powder, keratin azure, hide power azure, elastin/congo red, and elastin/orcein was determined by a modification of the method of Rinderknecht et al. (1968). Ten milligrams of each insoluble subtrate was incubated with HP-IV under conditions for optimal activity in the hide power azure assay (Civello et al., 1983). The supernatant was clarified by centrifugation in a clinical centrifuge for 5 min at maximum speed, and amounts of solubilized keratin azure, hide powder, hide powder azure, elastin/congo red, and elastin/orcein were estimated by absorbance at 595, 280, 595, 500, and 550 nm, respectively. The action of HP-IV on type I collagen from bovine achilles tendon was determined in a similar manner except that solubilized peptides were measured by the ninhydrin reaction (Rosen, 1957). A unit of collagenase activity was defined as the amount which would catalyze the release of amino acids from collagen equivalent in ninhydrin color to that of 1.0 μmol of leucine in 5 h at pH 7.4 and 37 °C. The hydrolysis of various soluble proteins was monitored by the acid-soluble method of Kunitz (1946). N,N-Dimethylated casein and hemoglobin were prepared by the method of Lin et al. (1969), and determination of the hydrolysis of these substrates was performed as described by these same authors. All spectrophotometric determinations were accomplished with a Gilford Model 252 updated Beckman DU.

Percent Soluble Protein. Fibrinogen (75% clottable protein, 5 mg/mL in 20 mM Tris-buffered saline, pH 7.4) was incubated with 100 μg/mL HP-IV at room temperature for various time intervals. After each time interval, the precipitate was removed by centrifugation in a clinical centrifuge, and the protein concentratioin of the supernatant was determined by its absorbance at 280 nm.

Lysis of Fibrin Clots. Enough thrombin was added to a fibrinogen solution (98% clottable protein, 5 mg/mL in 20 mM Tris-buffered saline, pH 7.4) to cause clotting in 2 min. Thirty seconds after the addition of thrombin (but before fibrin strands were apparent) 50 μg/mL HP-IV was added, and incubation was continued for 24 h at room temperature. The resulting clots were crushed and centrifuged for 5 min in a clinical centrifuge. The absorbance at 280 nm (A_{280}) of the supernatant was compared to supernatants from fibrinogen clots formed in the absence of HP-IV. The fibrin plate assay of Astrup & Mullertz (1952) and a modification of the fibrin plate assay which substituted plasma for fibrin were employed. In the fibrin plate assay, 10 mL of a 2 mg/mL fibrinogen solution (98% clottable protein in 20 mM Tris-buffered saline, pH 7.4) was poured into a 100 × 15 mm Petri dish, and sufficient thrombin solution to cause clotting in 2 min was added. After 15 min, wells were cut in the fibrin with a Pasteur pipet. Various concentrations of HP-IV were added to the wells, and the Petri dish was incubated for 20 h at room temperature. In the plasma clot method, 3.5 mL of fresh citrated human plasma was placed in a 100 × 15 mm Petri dish and clotted by the addition of 0.35 mL of 5% $CaCl_2$. Wells were cut in the plasma clot, and the assay was performed the same as with the fibrin clot.

Gel Electrophoresis. $NaDodSO_4$/polyacrylamide gel electrophoresis was performed by the method of Weber & Osborn (1969), except that gels contained 7.5% acrylamide when fibrinogen was examined. Fibrinogen (98% clottable protein, 5 mg/mL in 20 mM Tris-buffered saline, pH 7.4) was incubated with 50 μg/mL HP-IV at room temperature, and aliquots were removed at various time intervals. Each aliquot was incubated for 20 h at room temperature with an equal volume of 4% $NaDodSO_4$/4% 2-mercaptoethanol/10 M urea (Ouyang & Huang, 1979) prior to electrophoresis.

High-Pressure Liquid Chromatography. Fibrinogen (98% clottable protein) was dissolved in 0.2 M NH_4HCO_3, pH 7.8, at a concentration of 5 mg/mL to analyze for fibrinopeptide production. HP-IV (100 μg/mL) was added and the solution incubated for 6 h at room temperature. The supernatant was lyophilized and the residue dissolved in 0.3 mL of 10% acetonitrile/83 mM sodium phosphate, pH 3.1. Samples were applied to a 0.46 × 25 cm Altex Ultrasphere ODS C-18 column equilibrated with 14% acetonitrile/83 mM sodium phosphate, pH 3.1. After an initial wash with starting buffer (~7 mL), elution with 19% acetonitrile/83 mM sodium phosphate, pH 3.1, was initiated (Higgins & Shafer, 1981). All separations were performed with a Spectra-Physics SP8700 solvent delivery system by using a flow rate of 1 mL/min and monitored at 205 nm with a Tracor 970 A variable wavelength detector. Thrombin-induced clots and untreated fibrinogen were used as controls.

Proteolytic Specificity. The oxidized A and B chains of bovine insulin, bee venom melittin, and various dipeptides were used in the determination of cleavage sites by HP-IV. The insulin chains and melittin were tested for proteolytic susceptibility by incubating 1 mg of each with 30 μg of HP-IV, added in 10-μg aliquots at 3-h intervals to ensure complete

Table I: Hydrolytic Activity of HP-IV Compared to That of Pronase under Two Different Conditions[a]

	A_{280}							
	casein[b]				hemoglobin[c]			
	Pronase		HP-IV		Pronase		HP-IV	
buffer	15[d]	30[d]	15[d]	30[d]	15[d]	30[d]	15[d]	30[d]
20 mM NaOAc, pH 6.5	0.128	0.207	0.024	0.056	0.505	0.582	0.012	0.004
200 mM Tris, pH 7.5	0.133	0.224	0.037	0.055	0.477	0.566	0.022	0.004

[a] Casein concentration was 6.2 mg/mL, and hemoglobin concentration was 20 mg/mL. The Pronase concentration was 10 μg/mL assay, and that of HP-IV was 30 μg/mL with incubation at 37 °C. Aliquots were withdrawn from the assay mixture at the indicated times, and equal volumes of 0.44 M trichloroacetic acid were added. The resulting mixtures were incubated 30 min at room temperature, and then the insoluble protein was removed by centrifugation. Absorbance at 280 nm (A_{280}) was used to estimate the amount of acid-soluble protein. All values shown have been corrected for the appropriate blanks. [b] The casein solutions were prepared by dissolving 0.125 g of casein in 20 mL of buffer with gentle warming and then boiling the sclutions for 15 min followed by filtering. [c] Type I bovine hemoglobin (Sigma) was dissolved in the indicated buffer and used with no further treatment. [d] Time of incubation (min).

hydrolysis of the peptide, at 37 °C for 20 h. Incubation was also continued up to 72 h in order to analyze for further degradation of the peptides. Hydrolysis of the A chain of insulin and bee venom melittin was performed in 50 mM Tris, pH 7.5. The B chain of insulin was hydrolyzed in 50 mM Tris, pH 8.5, due to the insolubility of the B chain at pH 7.5. The fragments were separated on a 1 × 80 cm Bio-Gel P-4 molecular sieve column, eluted with 50 mM NH_4HCO_3, pH 8.5, and subjected to amino acid analysis. The nonblocked dipeptides were tested for proteolytic susceptibility by incubating 10 μg of each with 5 μg of proteinase for 5 h at 37 °C in 0.7 mL of 20 mM Tris, pH 7.5. Blocked dipeptides were first dissolved in 50% ethanol (1 mg/mL), and then 10 μL was assayed the same as the nonblocked dipeptides. The added ethanol in the assay did not affect HP-IV's proteolytic activity on hide powder azure. Cleavage of the dipeptides was monitored by the increase in ninhydrin-positive material (Rosen, 1957).

Amino Acid Analysis. Compositions of peptide fragments were determined on a microcomputer-controlled microbore amino acid analyzer (Durham & Geren, 1981). Samples were hydrolyzed in duplicate with 6 N HCl for 24 h at 110 °C. Detection was by the ninhydrin reaction monitored at 405 nm.

Dansylation and Identification of Fragments from Hydrolyzed Hide Powder. Ten milligrams of cow hide powder was incubated with 10 μg of HP-IV in 1 mL of 50 mM Tris, pH 7.5, for 20 h at 37 °C. The nonsolubilized hide was discarded after centrifugation. (This nonsolubilized hide was not different from that solubilized by HP-IV as determined by amino acid composition. Also, it could be hydrolyzed by further incubation with HP-IV). The supernatant was lyophilized, and the N-terminal amino acids were reacted with dansyl chloride according to the procedure of Tapuhi et al. (1981). The labeled fragments were then lyophilized and hydrolyzed in 6 N HCl for 24 h at 110 °C. The dansylated amino acids were separated by two-dimensional thin-layer chromatography (Schulze & Neuhoff, 1976) with visualization of labeled amino acids using a Model UVS-11 lamp from Ultra-Violet Products, Inc. Identification of amino acids was accomplished by comparison to previously published results (Schulze & Neuhoff, 1976).

Results

Proteolytic Assays. As previously reported (Sullivan et al., 1979), HP-IV hydrolyzes hide powder azure. Unmodified cow hide powder was hydrolyzed at only 20% of the rate at which hide powder azure was hydrolyzed by HP-IV (Figure 1) but was a much better substrate than several other proteins. HP-IV displayed little activity toward hemoglobin or denatured casein (Table I) when tested with various buffers and at

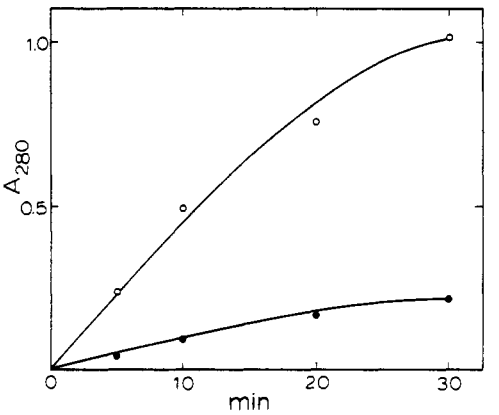


FIGURE 1: Activity of HP-IV with hide powder as compared to hide powder azure. The substrates were 5 mg/mL in 0.2 M Tris, pH 7.5, and 10 μg/mL HP-IV was used. Samples were incubated at 37 °C. At the times indicated, the solutions were centrifuged for 5 min in a clinical centrifuge, and the absorbance at 280 nm was measured. After 40 h all of the hide powder azure was solubilized while 50% of the hide powder remained.

various pHs including the optimal conditions for hide powder azure hydrolysis (Civello et al., 1983). HP-IV had no apparent hydrolytic activity with *N,N*-dimethylhemoglobin and *N,N*-dimethylcasein. Incubation of elastin/orcein, elastin/congo red, and keratin azure (Sigma) with 100 μg of HP-IV/mL under optimal conditions for hide powder azure hydrolysis of 27 h resulted in no dye solubilization. Twenty micrograms of Pronase was used as a positive control. At 120 μg/mL, HP-IV hydrolyzes collagen but exhibits only 3.4 units/mg of collagenase-like activity while purified bacterial collagenase displayed 309 units/mg of activity in the presence of 0.4 mM Ca^{2+}. The addition of Ca^{2+} did not enhance HP-IV's collagenase activity. Only a small amount of the collagen appeared susceptible to hydrolysis by the proteinase. An examination of the time course revealed that the total amount of ninhydrin-positive material released by different concentrations of HP-IV approached the same maximum (Figure 2). HP-IV contained gelatinase activity as evidenced by the observed clearing of a 1.5 × 1.5 cm piece of exposed X-ray film when incubated with 50 μg of HP-IV/mL in 0.1 M Tris, pH 7.4 for 2 h at 37 °C. Bacterial collagenase displayed a 40% greater rate of hydrolysis per mg against hide powder azure than did HP-IV. The proteinase (20 μg/mL) did hydrolyze glomerular basement membrane (1.5 mg/mL assay volume) under optimal conditions for hide powder hydrolytic activity. The addition of 1 mM Ca^{2+} enhanced the rate of hydrolysis by 100% with maximal activity obtained with 10 mM Ca^{2+} (500% increase in rate of hydrolysis over that obtained in the absence of added Ca^{2+}). Figure 3 illustrates the time course of

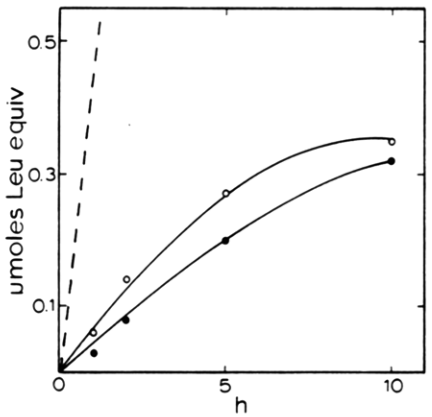


FIGURE 2: Collagenase activity of HP-IV. Collagen (10 mg/mL) was incubated with 50 and 100 μg/mL HP-IV in 0.2 M Tris, pH 7.4, at 37 °C. At the indicated times, 0.2-mL aliquots were withdrawn and assayed for increases in ninhydrin-positive material. Purified bacterial collagenase (15 μg/mL) was used as a control. (●) 50 μg/mL HP-IV; (○) 100 μg/mL HP-IV; (---) 15 μg/mL collagenase.

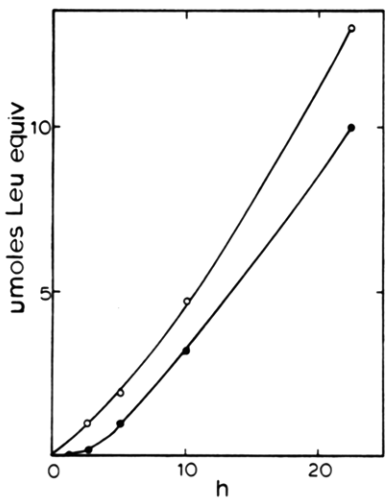


FIGURE 3: Hydrolysis of bovine glomerular basement membrane by HP-IV. HP-IV (20 μg/mL) was incubated with 1.5 mg/mL basement membrane in the presence of 10 mM Ca^{2+} for 23 h at 37 °C. Aliquots were removed at various time intervals, and the ninhydrin assay was performed. (●) Hydrolysis of basement membrane by HP-IV; (○) hydrolysis of basement membrane by 20 μg/mL purified collagenase.

basement membrane hydrolysis by HP-IV in the presence of 10 mM Ca^{2+}. Forty percent more basement membrane was hydrolyzed by an equivalent weight of bacterial collagenase after incubation for 23 h at 37 °C. Both of these proteolytic preparations completely solubilized the basement membrane upon prolonged incubation. HP-IV had no phospholipase A_2 activity in concentrations up to 100 μg/mL. Twenty micrograms of a phospholipase isolated from northern copperhead (*Agkistrodon contortrix mokasen*) venom (Moran et al., 1981) was used as a positive control.

Effect on Fibrinogen. HP-IV (50 μg/mL) produced a precipitate in fibrinogen solutions when incubated at room temperature for 20–40 min. The precipitate accounted for 50% of the total A_{280} protein in solution. The precipitate was easily removed from solution by centrifugation. No firm clot formed even with incubation up to 20 h, nor would thrombin induce a clot with HP-IV treated fibrinogen. $NaDodSO_4$ gel electrophoresis of the fibrinogen precipitate present after 2 h of incubation of fibrinogen with HP-IV revealed the appearance of a M_r 31 000 component coincident with the disappearance of the α chains. Incubation up to 24 h resulted in the partial degradation of the β chain with the formation of a M_r 54 000

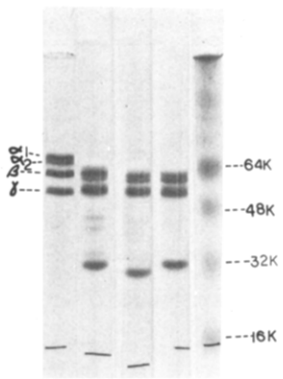


FIGURE 4: $NaDodSO_4$/polyacrylamide gel electrophoresis of fibrinogen precipitate produced by HP-IV. HP-IV (50 μg/mL) was incubated with fibrinogen (98% clottable protein, 5 mg/mL in 20 mM Tris-buffered saline, pH 7.4) at room temperature for various time intervals. The precipitate was recovered after centrifugation in a clinical centrifuge and prepared for electrophoresis as described under Materials and Methods. From left to right, the gels represent incubation times of 0, 2, 6, and 24 h. Each gel contained 15 μg of protein. The last gel contained cross-linked hemoglobin standards.

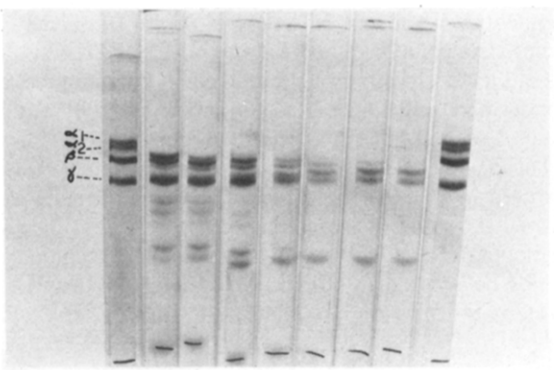


FIGURE 5: Time-dependent effect of HP-IV on fibrinogen as revealed by $NaDodSO_4$ by gel electrophoresis. Bovine fibrinogen (98% clottable protein, 5 mg/mL in 20 mM Tris-buffered saline, pH 7.4) was incubated with 50 μg/mL HP-IV for 24 h at room temperature. Aliquots of the supernatant were removed from the incubation mixture and prepared for electrophoresis. From left to right, the incubation times were 0, 0.25, 0.5, 1, 2, 6, 12, and 24 h. The last gel was fibrinogen incubated without HP-IV for 24 h. Each gel contained 18 μg of protein.

fragment (Figure 4). Figure 5 shows the time course of HP-IV's action on the fibrinogen supernatant. The molecular weights of the α_1, α_2, β, and γ chains were 66 500, 63 000, 57 000, and 51 000, respectively, as compared to cross-linked bovine hemoglobin standards. The molecular weights of the components at 24 h were 54 000 (a suspected fragment of the β chain), 51 000 (the intact γ chain), and 32 500. HP-IV (50 μg/mL) completely degraded the α chain within 15 min, and this occurred several minutes before visible precipitate formation. Prolonged incubation of HP-IV with fibrinogen resulted in the disappearance of the β chain from the supernatant while incubation up to 24 h failed to affect the γ chain. HPLC of the supernatant collected after removal of HP-IV-induced precipitate by centrifugation revealed that one of the hydrolysis fragments coeluted with thrombin-produced fibrinopeptide A (Figure 6). Amino acid analysis revealed that this peptide has a composition similar to fibrinopeptide A (Table II). Several other peptides were released by HP-IV which were not apparent when thrombin acted upon fibrinogen. None of the new peptides appeared to correspond to thrombin-produced fibrinopeptide B.

Effect on Fibrin Subunits. Fibrin clots formed by thrombin in the presence of HP-IV (50 μg/mL) had a supernatant

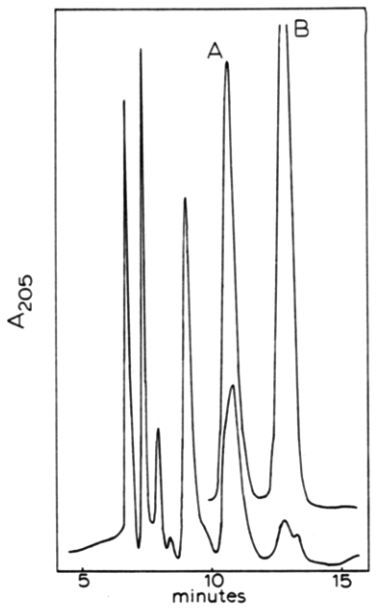


FIGURE 6: HPLC elution profiles of supernatants from thrombin-induced clots (top tracing) and HP-IV precipitate formation (bottom tracing). Fibrinogen (98% clottable protein, 5 mg/mL in 0.2 M NH_4HCO_3, pH 7.8) was incubated with 50 μg/mL HP-IV for 6 h at room temperature. Thrombin was added to the control fibrinogen in concentrations which would cause the control to clot in 2–3 min. The supernatants from both samples were recovered by centrifugation, lyophilized, and prepared for HPLC as described under Materials and Methods. The letters A and B refer to the elution of fibrinopeptides A and B in the supernatant from the thrombin-induced clots.

Table II: Amino Acid Composition of a Peptide Produced by the Action of HP-IV on Fibrinogen[a]

amino acid	HP-IV peptide	thrombin-induced fibrinopeptide A
Asx	3	3
Thr	2	1
Ser	3	2
Glx	2	2
Pro	2	2
Gly	5	5
Ala	2	1 (0)[c]
Cys	0	0
Val	1	1
Met	0	0
Ile	0	0
Leu	1	1
Tyr	0	0
Phe	1	1
His	0	0
Lys	1	0
Arg	–[b]	2 (1)[c]

[a] A 5 mg/mL solution of 98% clottable fibrinogen in 0.2 M NH_4HCO_3, pH 7.8, was incubated with 100 μg/mL HP-IV for 6 h at room temperature. Thrombin was added to a fibrinogen solution and allowed to clot for 24 h. The supernatants from both samples were analyzed by HPLC. Thrombin-induced fibrinopeptide A and the peptide produced by HP-IV which had the same retention time as fibrinopeptide A were pooled and subjected to amino acid analysis. [b] Arg was not quantitated because a large concentration of ammonia was in the sample. [c] Numbers in parentheses refer to values previously reported (Laki, 1968).

absorbance at 280 nm of 1.71 as compared to 1.10 for clots formed in the absence of HP-IV. This corresponds to a 55% increase in soluble protein. $NaDodSO_4$ gels showed that HP-IV degraded the α chain of fibrin with the appearance of a M_r 32 500 fragment (Figure 7). If thrombin and HP-IV were added simultaneously to a fibrinogen solution, no clot formed. However, in this case it is not known if HP-IV was degrading both the thrombin and the fibrinogen, or just the

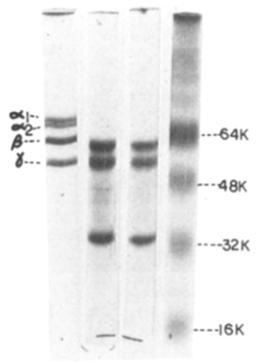


FIGURE 7: Effect of HP-IV on fibrin subunits. Thrombin was added to fibrinogen (98% clottable protein, 5 mg/mL in 20 mM Tris-buffered saline, pH 7.4) in concentrations which would cause clotting in 2 min. After 30 s, 50 μg/mL HP-IV was added and incubation continued at room temperature for 24 h. The clots were concentrated by centrifugation and prepared for electrophoresis. From left to right, the gels were thrombin-induced clot, thrombin-induced clot with HP-IV after 2 h of incubation, thrombin-induced clot with HP-IV after 24 h of incubation, and cross-linked hemoglobin standards.

Table III: Amino Acid Composition of Fragments from Peptides Hydrolyzed by HP-IV[a]

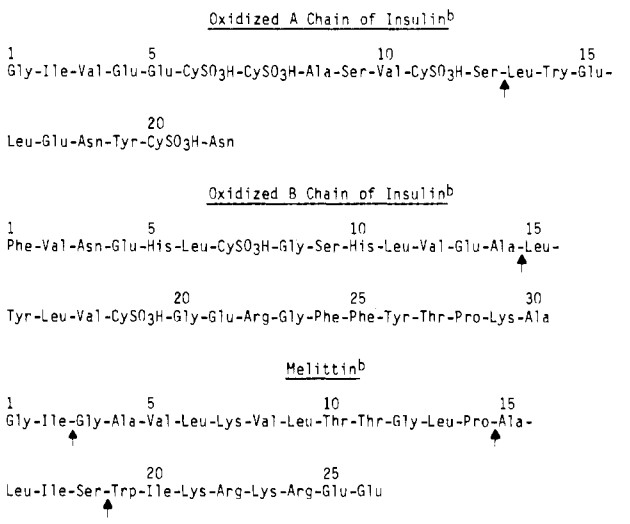
	insulin					
	A chain		B chain		melittin[c]	
peak	I	II	I	II	I	II
Asx	0	2	0	1	–	–
Thr	–[b]	–	1	0	2	0
Ser	2	0	0	1	0	1
Glx	2	2	1	2	0	0
Pro	–	–	1	0	1	0
Gly	1	0	2	1	2	0
Ala	1	0	1	1	1	1
$CySO_3H$	3	1	1	1	–	–
Val	2	0	1	2	2	0
Met	–	–	–	–	–	–
Ile	1	0	–	–	0	1
Leu	0	2	2	2	3	1
Tyr	0	2	2	0	–	–
Phe	–	–	2	1	–	–
His	–	–	0	2	–	–
Lys	–	–	1	0	1	0
Arg	–	–	1	0	0	0

[a] Each peptide was incubated with HP-IV as described under Materials and Methods and then subjected to chromatography on a 1 × 80 cm Bio-Gel P-4 column eluted with 50 mM NH_4HCO_3, pH 8.5. Two fragments were obtained from each of the three peptides, and these are labeled I and II in the order of their elution. Amino acid analyses of the fragments were as described under Materials and Methods. [b] Not present in the intact, parent peptide. [c] All fragments from hydrolyzed melittin were not recovered.

fibrinogen. HP-IV likewise prevented citrated whole plasma from clotting when excess Ca^{2+} was added. When monitored by the fibrin plate and plasma clot assay, HP-IV caused significant lysis within 20 h and completely dissolved each clot within 72 h. After a 20-h incubation, 30 μg of HP-IV had lysed an area of approximately 60 mm^2 in the fibrin clot and 240 mm^2 in the plasma clot.

Proteolytic Specificity. The ability of HP-IV to hydrolyze peptide bonds in oxidized A and B chains of insulin and bee venom melittin was examined. In all cases, the integrity of the native polypeptides was confirmed by Bio-Gel P-4 molecular sieve chromatography prior to their incubation with HP-IV. Comparison of the fragments obtained by incubation of HP-IV with the three peptides for 20 h vs. 72 h did not reveal any additional cleavage sites. Amino acid composition of each fragment (Table III) separated by P-4 molecular sieve

Table IV: Cleavage Sites of Oxidized A and B Chains of Insulin and Bee Venom Melittin by HP-IV[a]



[a] Cleavage sites were determined as described under Materials and Methods. The arrows indicate the cleavage sites. [b] Sequence of each peptide obtained from Tu (1977).

chromatography of the A and B chains of insulin incubated with HP-IV indicated a specific peptide bond being attacked. On the basis of the amount of 220-nm absorbing material collected from the molecular sieve, protein recoveries of 97% and 98% were obtained for the fragments of the A and B chains of insulin, respectively. However, only a 51% recovery of melittin polypeptide was obtained from the molecular sieve. The N-terminal dipeptide Gly_1-Ile_2 was not recovered which may either have been degraded further to individual amino acids or have escaped detection. The C-terminal octapeptide Trp_{19}-Glu_{26} was also not recovered, but it is postulated that this fragment had been retained by the polyacrylamide resin due to the presence of four basic amino acids (two Arg, two Lys). The addition of 1.0 M NaCl to the elution buffer failed to elute this octapeptide. Raising the pH of the elution buffer was abandoned because above pH 10.0, depolymerization of the resin occurred. The bonds known to be hydrolyzed by HP-IV in these three peptides are illustrated in Table IV.

Thin-layer chromatography of the dansylated cow hide powder, before and after hydrolysis catalyzed by HP-IV, revealed the appearance of Tyr, Leu, Met, Trp, Pro, and hydroxylysine as new N-terminal residues. $NaDodSO_4$ electrophoresis (10% acrylamide) of solubilized cow hide powder failed to reveal any protein bands. This suggests that HP-IV cleaves hide powder into small fragments which cannot be retained within the acrylamide matrix of the gel.

A variety of dipeptides which contained the amino acids comprising the new N-terminal residues from the hydrolysis of hide powder by HP-IV were also tested. Ten micrograms each of Pro-Ile, Ser-Met, Pro-Val, Ser-Gly, Pro-Leu, Ser-Leu, Ser-Phe, Pro-Ala, Pro-Trp, and Ser-Ala was not cleaved by 5 μg of HP-IV. Ten micrograms of the doubly blocked dipeptides *N*-Cbz-Ser-Leu-NH_2, *N*-Cbz-Ala-Leu-NH_2, and *N*-Cbz-Ile-Gly-NH_2, which correspond to known cleavage sites in the peptides described in Table IV, was not hydrolyzed by 5 μg of HP-IV.

Discussion

Timber rattlesnake venom contains a hemorrhagic protein component which also has the ability to hydrolyze cow hide but is not active with elastin and exhibits little activity against collagen. The apparent lack of activity toward hemoglobin and the low activity with casein may be due to the assay used; i.e., the production of large fragments would not be detected by the Kunitz assay (1946). It was thought that if dimethylated casein and hemoglobin were used, more hydrolysis would be detected (Lin et al., 1969), but these also proved to be poor substrates.

Results from the cleavage of the A and B chains of insulin, bee venom melittin, and hide powder indicate no obvious specificity for the peptide bonds hydrolyzed by HP-IV. The variation in cleavage sites may be related to the tertiary structure of each substrate. As expected, none of the nonblocked or blocked dipeptides were cleaved, since this proteinase has been shown to lack esterase activity (Sullivan et al., 1979). This is not to imply that an extensive survey with all available esters has been completed as Sullivan et al. (1979) only examined the hydrolysis of three. That this proteinase has hemorrhagic activity has been definitely established (Civello et al., 1983). As compared to other snake venom proteases, HP-IV hydrolyzes fewer bonds in the B chain of insulin (Table V). This coupled with the apparent lack of much action of HP-IV with casein and hemoglobin implies a fairly specific protease. The choice of melittin as a test substrate was dictated not only by its small size and known sequence but also because of its ability to interact with membranes. It is interesting to note that HP-IV hydrolyzed more bonds in melittin than either the A or B chains of insulin.

HP-IV's action on cow hide but not on collagen or elastin is somewhat perplexing, but explanations are possible. First, the cow hide was denatured during its preparation in such a way as to make it a more suitable substrate, while the highly ordered structures of collagen and elastin resist HP-IV's hydrolytic action. We attempted to determine how the cow hide powder had been prepared from the supplier, Calbiochem-Behring, but they did not have that information. In this report, it was shown that the cow hide powder azure was more susceptible to hydrolysis than cow hide powder. These data would tend to support this first explanation. A second explanation would be that cow hide contains elastin, collagen, and a third substance that is a good substrate for HP-IV. The small amount of activity seen with collagen could indicate that this preparation is slightly contaminated with the HP-IV substrate. The lack of large fragments of cow hide after hydrolysis by HP-IV would, however, argue against this hypothesis. The fact that HP-IV causes rapid clearing of exposed X-ray film would further suggest that HP-IV's action on the hide powder was due to its denatured form.

HP-IV will hydrolyze glomerular basement membrane at an appreciable rate when compared to collagenase. This observation is consistent with the results of Ohsaka et al. (1973) describing three hemorrhagins isolated from Habu (*Trimeresurus flavoviridis*) venom. These investigators have suggested that the destruction of the basement membrane which surrounds capillaries could cause hemorrhage by weakening the capillaries. One cannot, however, rule out that the basement membrane was not made susceptible to degradation by HP-IV during its isolation.

In addition to its hydrolytic action upon basement membrane, HP-IV also inhibits the clotting of fibrinogen solutions by thrombin. HP-IV does exhibit hydrolytic action upon fibrinogen with the α and β chains being degraded. One of the hydrolysis fragments is a peptide which resembles fibrinopeptide A in composition. This cleavage probably initiates formation of the fibrinogen precipitate. The appearance of additional hydrolysis products suggests that HP-IV cleaves

Table V: Comparison of Cleavage Sites of the Oxidized B Chain of Insulin by Snake Venom Proteases

	1 Phe-Val-Asn-Glu-His-Leu-$CySO_3$H-Gly-Ser-His-Leu-Val-Glu-Ala-Leu-Tyr-Leu-Val-$CySO_3$H-Gly-Glu-Arg-Gly-Phe-Phe-Tyr-Thr-Pro-Lys-Ala[a] 30	reference
HP-IV (*C. h. horridus*)	↑ ↑ ↑ ↑	present investigation
proteinase C (*A. halys blomhoffii*)	↑ ↑ ↑	Satake et al. (1963)
α-protease (*C. atrox*)	↑ ↑ ↑ ↑ ↑	Pfleiderer & Krauss (1965)
protease A (*B. jararaca*)	↑ ↑ ↑	Mandelbaum et al. (1967)
hemorrhagic toxin a (*C. atrox*)	↑ ↑ ↑	Tu et al. (1981)
leucostoma peptidase A (*A. piscivorus leucostoma*)	↑ ↑ ↑ ↑ ↑ ↑ ↑	Spiekerman et al. (1973)

[a] Sequence from Tu (1977), p 112.

multiple sites in fibrinogen which renders it unclottable by thrombin. The difference in amino acid composition between the HP-IV-produced firbinopeptide A and that of thrombin-induced fibrinopeptide A is probably due to the former not being a pure preparation. Figure 6 shows that this peak is not symmetrical. HP-IV could even be degrading fibrinopeptide A further. Obviously HP-IV is different from other reported snake venom components that cause only the release of fibrinopeptide A and result in clot formation (Holleman & Coen, 1970; Stocker & Barlow, 1976; Markland & Pirkle, 1977). If HP-IV's specificity is directed by conformation, then the Arg–Gly bond of fibrinogen to liberate fibrinopeptide A would have to be considered a potentially susceptible bond, and HP-IV did cleave an Ile–Gly bond in melittin (Table IV). The α-chain doublet observed in Figures 4, 5, and 7 corresponds to the α_1 and α_2 chains of fibrinogen reported by Mills & Karpatkin (1970). The fibrinogen used in all experiments contained 95% clottable protein by our calculations which indicates that it was not damaged during isolation by the supplier (Calbiochem-Behring). Cottrell & Doolittle (1976) have shown that the molecular weight difference between the α_1 and α_2 chains is a result of the in vivo plasmin cleavage of a 27-residue peptide from the C-terminal end of the α chain.

The observation that HP-IV rapidly hydrolyzes basement membrane coupled with the anticoagulant properties presented provides a plausible mechanism for HP-IV's hemorrhagic activity.

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Registry No. *Crotalus horridus horridus* venom hemorrhagic proteinase, 84056-81-5; collagenase, 9001-12-1; insulin, 9004-10-8; melittin, 37231-28-0.

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Purification and Characterization of a Cysteine Dioxygenase from the Yeast Phase of *Histoplasma capsulatum*†

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ABSTRACT: A cysteine dioxygenase, cysteine oxidase (EC 1.13.11.20), has been purified from the cytosolic fraction of yeast phase cells of the dimorphic fungus *Histoplasma capsulatum*. The cysteine oxidase is an iron-containing dioxygenase with a molecular weight of 10 500 (±1500) and is present only in the yeast phase of the fungus. The enzyme is highly specific for L-cysteine, with a K_m of 2×10^{-5} M in vitro. The product of cysteine oxidation is cysteinesulfinic acid, as analyzed by thin-layer chromatography and mass spectroscopy. To our knowledge, this is the first cysteine oxidase isolated from a fungus, and it probably plays an important role in the mycelial to yeast phase transition of *H. capsulatum* during which redox potential and cysteine levels are crucial factors.

Histoplasma capsulatum is a dimorphic pathogenic fungus which exists in a multicellular form in nature and a unicellular yeast in infected tissue. In axenic culture, the organism grows as a mycelium at 25 °C and a yeast at 37 °C. Phase transitions can be induced by raising or lowering the temperature.

We have prviously identified unique features of each phase of *H. capsulatum* which may be importnat in the morphologic transition. One of these is a cysteine oxidase or cysteine dioxygenase (L-cysteine:oxygen oxireductase, EC 1.13.11.20) activity found in the cytosol of yeast phase cells (Maresca et al., 1981). As a first step in trying to understand the regulation of its activity and its role in the transition, we have purified the cysteine oxidase from yeast cells and characterized the enzyme and its product.

Experimental Procedures

Materials

DEAE-cellulose was purchased from Whatman Chemical Co. (Clifton, NJ). Cysteine, potassium cyanide, glutathione, SHAM,[1] cysteinesulfinic acid, cysteic acid, *S*-methylcysteine, glutamine, cysteine, cyanogen bromide activated Sepharose, β-mercaptoethanol, and PMSF were all obtained from Sigma Chemical Co. (St. Louis, MO). Acrylamide, bis(acrylamide), ammonium persulfate, crystalline $NaDodSO_4$, and TEMED were obtained from Bio-Rad Laboratories (Richmond, CA). Ampholines were supplied by LKB Instrument, Inc. (Rockville, MD). Glass beads were obtained from B. Braun Melsungen AG (Fernruf, West Germany). TPCK-trypsin was supplied by Millipore Corp. (Freehold, NJ). The molecular weight standards phosphorylase *b* (M_r 94 000), bovine serum albumin

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[1] Abbreviations: DEAE, diethylaminoethyl; SHAM, salicylhydroxamic acid; PMSF, phenylmethanesulfonyl fluoride; $NaDodSO_4$, sodium dodecyl sulfate; TEMED, tetramethylethylenediamine; KCN, potassium cyanide; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

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