at 254 nm by alkaline sucrose sedimentation of V79 hamster cell DNA. They obtained values of about 2.5 dimers per J/m^2 per 10^8 daltons. Determination of dimer yields at 254 nm by the gel method gave a value of 2.3 dimers per J/m^2 per 10^8 daltons, in good agreement with those obtained by Ahmed and Setlow.

We thus conclude that for simple systems (homogeneous populations of small DNAs), this agarose gel technique and analysis procedure can give an accurate measure of pyrimidine dimer formation in nonradioactive DNAs. However, many DNAs of biological interest (e.g., eucaryotic cells or tissues) pose additional problems: (1) they are higher in molecular weight and would not enter easily the 0.4% gels used for T7 DNA; (2) because of their high molecular weight, they are easily sheared during extraction and thus are heterogeneous in size even without irradiation or endonuclease treatment. Of course, agarose gels as low as 0.1% gels are useful for DNAs of higher molecular weight, and it seems likely that a theoretical treatment similar to that used in analyzing the T7 DNA would also be applicable (we note that even unirradiated T7 DNAs suffered ~1–2 nicks per molecule during the heat liberation from the viral capsid, so indeed the unirradiated DNA was heterogeneous). In addition, molecular weight markers spanning the range of molecular sizes of the untreated and cleared molecules would be required. Of course, some tissues may also contain nondividing or necrotic cells with degraded DNA which would interfere with dimer determination.

The simple system we have used does not address these problems of highly heterogeneous, high molecular weight DNA populations. It does show that the gel technique and our analysis procedure give good measures of pyrimidine dimer content under carefully chosen experimental conditions. It seems likely that with appropriate modification of experimental conditions for other DNA populations, the same methodology and analysis can provide the foundation for a wide range of DNAs not amenable to radioactive labeling.

Acknowledgments

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Registry No. *Micrococcus luteus* UV-endonuclease, 9073-83-0.

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Isolation and Characterization of a Hemorrhagic Proteinase from Timber Rattlesnake Venom†

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ABSTRACT: A protein isolated from timber rattlesnake (*Crotalus horridus horridus*) venom by ion-exchange and high-pressure liquid chromatography is hemorrhage inducing and lethal to mice (LD_{50} of 10 μg/g of body weight). It is a Ca^{2+}- and Zn^{2+}-containing proteinase and has the ability to hydrolyze hide powder azure. Atomic absorption spectroscopy shows 2.5 Ca^{2+} and 1 Zn^{2+} per protein monomer. The proteinase activity is destroyed by incubation with disulfide-reducing agents and by dialysis against ethylenediaminetetraacetate. Coincident with the loss of proteinase activity is a corresponding loss of lethal and hemorrhagic activities, suggesting that all three are related. Attempts to replace the metals and restore activity have been unsuccessful. Amino acid analysis and isoelectric focusing reveal that this component is an acidic protein (p*I* = 5.1) containing about 20 disulfide bonds and 507 residues. Reduction of one disulfide bond per molecule decreases proteinase activity by 50% while reduction of eight disulfide bonds decreases activity by 80%. Loss of hemorrhagic activity parallels the decrease in proteinase activity.

Rattlesnake bites in the United States result in considerable damage to the tissues adjacent to the site of envenomation. Hemorrhage is one of the most common manifestations of snakebite and is of clinical concern since antivenom treatments may not prevent hemorrhage (Minton, 1954). A number of hemorrhage-inducing components have been isolated from North American rattlesnake venoms. An even larger number of proteases have been isolated from these venoms, but very few of the hemorrhagins have been shown to contain proteolytic activity.

Fabiano & Tu (1981) have reported the isolation of a tissue-damaging toxin from prairie rattlesnake (*Crotalus viridis viridis*) venom which was lethal and hemorrhagic in mice and catalyzes limited hydrolysis of dimethylcasein. Five hemor-

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rhagic toxins have been isolated from western diamondback rattlesnake (*Crotalus atrox*) venom (Bjarnason & Tu, 1978), all of which exhibit proteolytic activity. Dialysis against *o*-phenanthroline destroyed both the hemorrhagic and proteolytic activities of all five toxins. Twenty percent recovery of both activities was obtained upon dialysis against zinc and calcium ions.

The venom of the timber rattlesnake, *Crotalus horridus horridus*, has only recently been fractionated. Kocholaty et al. (1971) and Moran & Geren (1979) have reported the presence of a number of enzyme activities in this venom, and Bonilla (1975) has described the isolation of a thrombin-like enzyme from it. Bonilla & Fiero (1971) have also described the isolation of a low molecular weight basic protein from timber rattlesnake venom that had a peripheral neurotoxin activity. Crotalocytin, a platelet-aggregating protein from timber rattlesnake venom, has also been purified (Schmaier et al., 1980) and characterized (Schmaier & Colman, 1980). Sullivan et al. (1979) have recently reported separation of this venom into a number of distinct, reproducible fractions by ion-exchange chromatography. Four of these fractions are lethal to mice, and one of them has been shown to be a systemic effector (Sullivan & Geren, 1979).

This paper presents a characterization of another of the lethal fractions of timber rattlesnake venom, the fraction (fraction IV) which is most strongly retained on DEAE-cellulose.[1] Sullivan et al. (1979) have reported that fraction IV migrates essentially as one major component of M_r 56 000 on $NaDodSO_4$/polyacrylamide gel electrophoresis. Fraction IV was reported to be both lethal and hemorrhagic in mice and to contain all of the hide powder azure hydrolytic activity observed in the whole venom. Even though hide powder azure is not a well-defined substrate, its use in this work was dictated by the hydrolytic specificity of fraction IV. This will be described in detail in the following paper (Civello et al., 1983).

Fraction IV has been found in all commercial preparations of timber rattlesnake venom examined to date and comprises approximately 25% of the total venom protein. In this present report, the first of two, we describe the further isolation of fraction IV's toxic component and its biochemical properties. The next paper will deal with this component's substrate specificity.

Materials and Methods

Timber rattlesnake venom was obtained in lyophilized form from the Miami Serpentarium. Hide powder azure and Pronase (EC 3.4.24.4) were purchased from Calbiochem-Behring Co. Ion-exchange cellulose was from Whatman; molecular sieve resins, electrophoretic reagents, and isoelectric ampholytes were from Bio-Rad; acetylated cytochrome *c* standards for isoelectric focusing were from United States Biochemical Corp.; dialysis tubing and Schiff reagent were from Fisher Scientific Co.; all other reagents were from Sigma Chemical Co.

Isolation. Fraction IV was obtained by ion-exchange chromatography of timber rattlesnake venom on DEAE-cellulose by a continuous sodium acetate gradient as described by Sullivan et al. (1979).

High-Pressure Liquid Chromatography. Preparative and analytical separations were performed on Waters Associates I-125 protein analysis columns (0.78 × 30 cm, two in series) with a Beckman Model 110 A isocratic pump and monitored at 280 nm by a Spectra-Physics Model 770 detector. Routinely, 400 μg of protein was separated at a flow rate of 2 mL/min with a pressure of 2000 psi at room temperature. Elution buffer was 100 mM Tris, pH 7.4. The hemorrhage-inducing protein purified by this method from fraction IV was used for all experiments and assays. It will be called HP-IV, which stands for the hemorrhagic proteinase isolated from fraction IV, throughout this report.

Extinction Coefficient. Purified HP-IV was dialyzed extensively against deionized distilled water and lyophilized in an acid-cleaned and tared glass vial. The vial was then weighed, and exactly 1 mL of deionized distilled water was added to the lyophilized protein and the absorbance at 280 nm determined. A value of $E_{280}^{1\%} = 13.0$ was determined and used to estimate actual amounts of HP-IV used in all assays and experiments.

Hide Powder Azure Assay. The action of HP-IV upon hide powder azure was accomplished by a modification of the method of Rinderknecht et al. (1968). Ten milligrams of the substrate was incubated with HP-IV for a measured length of time at 37 °C followed by centrifugation in an International clinical centrifuge and recovery of the supernatant. Amounts of solubilized hide powder azure were estimated by absorbance at 595 nm. The action of Pronase on hide powder azure was performed under identical conditions. Spectrophotometric determinations were accomplished with a Gilford Model 252 updated Beckman DU.

Gel Electrophoresis. $NaDodSO_4$/polyacrylamide gel electrophoresis was by the method of Weber & Osborn (1969). Visualization of proteins and glycoproteins was accomplished with Coomassie brilliant blue and Schiff stain (Zacharias et al., 1969). Discontinuous gel electrophoresis was by the method of Ornstein (1964) and Davis (1964). A Hoefer DE 101 electrophoresis unit and a Buchler power supply were used for all electrophoresis.

Isoelectric Focusing. Estimation of isoelectric point was performed over the pH range 3–10 in polyacrylamide gels. Gels were 7.5% acrylamide according to Davis (1964) with dimensions of 0.5 × 9.0 cm. The upper reservoir buffer was 20 mM H_3PO_4, and the lower reservoir buffer was 1 N NaOH. The gels contained 5% ampholytes and were focused for 3 h at 200 V. The gels were stained with Coomassie brilliant blue G-250 in 7% acetic acid/40% methanol for 4 h and destained with 7% acetic acid. The isoelectric point was calculated relative to the R_f values of acetylated cytochrome *c* standards.

Toxicity Studies. The lethal dose 50% (LD_{50}) value was determined by intraperitoneal injection in Charles Rivers CD-1 mice by using the graphical technique of Reed & Muench (1938). Fifty 20-g mice were injected with HP-IV in concentrations of 80–300 μg. The carrier was 20 mM Tris-buffered saline, pH 7.4, and a constant injection volume of 0.5 mL was maintained. Mice were scored 24 h postinjection. Local effects were determined by subcutaneous injections between the shoulders in 25-g mice, with sacrifice by diethyl ether and autopsy 4 h after injection. Injection volume was 0.4 mL, and the carrier was 20 mM Tris-buffered saline, pH 7.4. Hemorrhage was estimated on a scale from 0 to 4 where 0, 1, 2, 3, and 4 correspond to hemorrhage sizes of 0, 1, 2, 3, and 4 cm in diameter. Minimum hemorrhagic dose was defined as the amount of HP-IV in micrograms needed to produce a hemorrhage of 1 cm in diameter.

Atomic Absorption Spectroscopy. Analysis for Zn^{2+}, Ca^{2+}, and Mg^{2+} was accomplished with a Perkin-Elmer Model 303

[1] Abbreviations: DEAE, diethylaminoethyl; $NaDodSO_4$, sodium dodecyl sulfate; HP-IV, hemorrhagic proteinase IV; DTT, dithiothreitol; HPLC, high-pressure liquid chromatography; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetate; MHD, minimum hemorrhagic dose.

with an air/acetylene flame. Solutions of HP-IV (mg/mL) were used directly, and metal content was estimated by comparison with standard curves. The Ca^{2+} standard solution was prepared with primary standard $CaCO_3$ while the Mg^{2+} and Zn^{2+} solutions were prepared by the direct action of HCl on the pure metals. The presence of milligram per milliliter protein did not quench the contribution of known amounts of added standards.

Removal of Metals from HP-IV. Two milliliters of a 1 mg/mL solution of HP-IV was dialyzed against 1.5 L of 0.1 M Tris, pH 7.4, containing 20 mM EDTA at 4 °C for 24 h. Quantitative removal of metals was confirmed by atomic absorption spectroscopy.

Protein Modification. Fifty micrograms of HP-IV was incubated with either 50 mM *N*-ethylmaleimide or 20 mM iodoacetamide for 1 h at room temperature in 0.1 M Tris, pH 7.4, in order to test for the presence of sulfhydryl groups in the active center. Ten microliters of a 1 mg/mL solution of HP-IV in 0.1 M Tris, pH 7.4, was mixed by vortexing for 2 min with 50 μL of 0.21 M phenylmethanesulfonyl fluoride in acetonitrile to test for the participation of serine residues in the active center. In addition, the effect of several trypsin inhibitors was tested. Ten micrograms of HP-IV was incubated with either 50 μg of Sigma type IV-0 chicken egg white trypsin inhibitor, 50 μg of type 1-S soybean trypsin inhibitor, or 50 μg of Sigma α_1-antitrypsin for 15 min at 37 °C in 0.1 M Tris, pH 7.4. Since all preparations of HP-IV were shown to be homogeneous, and if no effect upon the hide powder azure hydrolytic activity was observed after various modifications, the effect on toxicity was not tested. Usually if toxicity was tested, only hemorrhage was used as death was assumed to be secondary to hemorrhage.

Amino Acid Analysis. The amino acid composition of HP-IV was determined by using a microcomputer-controlled microbore amino acid analyzer with ninhydrin detection as described by Durham & Geren (1981). Ninhydrin detection was monitored at 546 nm except for proline which was monitored at 405 nm. Hydrolysis of samples was done in duplicate with constant boiling HCl at 110 °C for 24, 48, and 72 h (Spackman et al., 1958), and all values were extrapolated to zero time by using a least-squares approximation. Tryptophan was determined spectrophotometrically by the method of Edelhoch (1967). Total cysteine content was accomplished with DTT and iodoacetamide as described by Gurd (1967). Reactions were done in 50 mM Tris, pH 8.3, containing 8 M recrystallized urea. One milliliter of a 1 mg/mL solution of HP-IV was incubated with 0.1 M DTT for 1 h at room temperature, then sufficient recrystallized iodoacetamide was added to make the solution 0.25 M, and incubation was continued for 2 h at room temperature. The carboxymethylated protein was then dialyzed against deionized distilled water for 24 h at 4 °C and subsequently hydrolyzed and analyzed. Alkylation without reduction was performed in the same way, except for the omission of DTT. In this manner, the amount of free cysteine residues was determined. The presence of carbohydrate in the form of neutral hexose was tested by the phenol–sulfuric acid procedure of Dubois et al. (1956).

Results

Chemical Properties. On the basis of separations from six different preparations of timber rattlesnake venom, fraction IV comprises approximately 25% of the protein (based on 280-nm absorbance) of the whole venom. Fraction IV as obtained by DEAE-cellulose ion-exchange chromatography is in 0.35 M sodium acetate, pH 7.0, but its lethal, hemorrhagic, and proteolytic activities are stable under a variety of conditions. The activities are stable for at least 3 years at −20 °C when stored in either a lyophilized or frozen state.

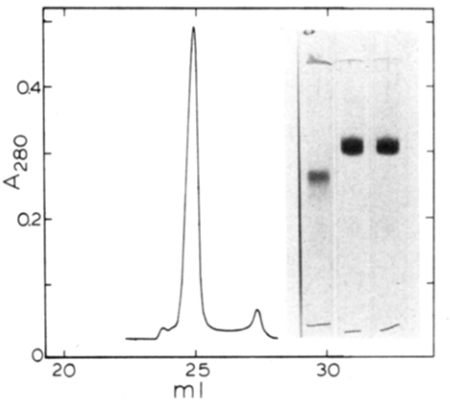

FIGURE 1: HPLC purification of HP-IV. Four hundred micrograms was separated by passage through two Waters I-125 protein analysis columns (0.78 × 30 cm) placed in series. The elution buffer was 0.1 M Tris, pH 7.4, and separations were performed at a flow rate of 2 mL/min with a pressure of 1800 psi at room temperature. The gels shown are discontinuous and $NaDodSO_4$ gels of HP-IV purified by HPLC. From left to right, a discontinuous separation of HP-IV, an $NaDodSO_4$ separation of nonreduced HP-IV, and an $NaDodSO_4$ separation of reduced HP-IV. Electrophoresis procedures are described under Materials and Methods. Each gel contained 15 μg of HP-IV.

$NaDodSO_4$ and discontinuous polyacrylamide gel electrophoresis of various preparations of fraction IV reveal the presence of a major band with varying numbers of other minor protein components. Reduction of samples prior to $NaDodSO_4$ electrophoresis caused no change in the migration of the major band. Removal of minor contaminants by HPLC using Waters I-125 protein analysis columns has been accomplished (Figure 1). Also shown in Figure 1 are $NaDodSO_4$ and discontinuous electrophoretic gels of HP-IV purified by HPLC. HP-IV migrates with an apparent molecular weight of 56 000 ± 3000 as compared to cross-linked bovine hemoglobin standards. HP-IV had an apparent molecular weight of 57 000 ± 8000 on $NaDodSO_4$ gels when compared to the migration of phosphorylase *a*, human serum albumin, pyruvate kinase, ovalbumin, soybean trypsin inhibitor, and lysozyme. An apparent molecular weight of 52 000 was obtained by molecular sieve chromatography on a 1 × 90 cm Bio-Gel A 0.5 m column. The molecular weight of the purified component was determined relative to the elution of blue dextran, bovine serum albumin, ovalbumin, bovine hemoglobin subunit dimer, and horse heart myoglobin. HP-IV's specific proteolytic activity was constant across the profile. Isoelectric focusing of HP-IV is shown in Figure 2. Carbohydrates were not detected by $NaDodSO_4$ electrophoresis of 75 μg of HP-IV and treatment with Schiff stain (Zacharias et al., 1969). Thirty micrograms of fetuin was used as a positive control. The phenol–sulfuric acid method of carbohydrate determination (Dubois et al., 1956) was also negative with 50 μg of HP-IV. Glucose was used as the standard for neutral hexoses.

Toxicity Studies. An LD_{50} value of 10.0 μg/g of body weight in mice has been consistently demonstrated in all preparations of HP-IV as compared to 2.84 μg/g for whole timber rattlesnake venom (Kocholaty et al., 1971). A minimum hemorrhagic dose of 4.0 μg was determined for HP-IV. Subcutaneous injection of 10 μg of HP-IV will consistently produce a size 2 (2-cm diameter) hemorrhage and 20 μg a size 3 (3-cm diameter) in C3H mice.

Proteolytic Activity. Several substrates including hemoglobin, casein, elastin, and keratin were tested under a variety of pH and buffer conditions as possible substrates for HP-IV. Hide powder azure was the only substrate which was rapidly

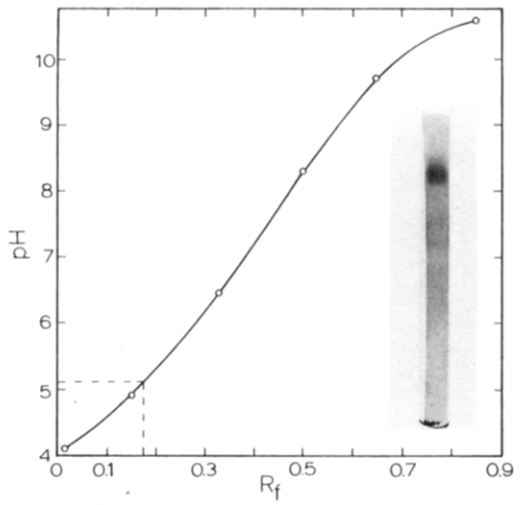

FIGURE 2: Isoelectric focusing of HP-IV. Ten micrograms of HP-IV was focused over the pH range 3–10. An isoelectric point of 5.1 for HP-IV was computed relative to the migration of acetylated cytochrome *c* standards. The isoelectric focusing procedure is described under Materials and Methods.

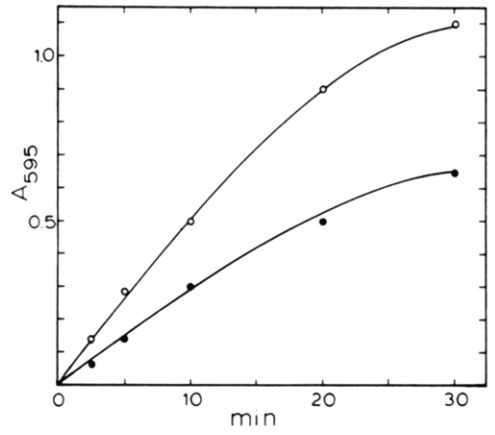

FIGURE 3: Activity of HP-IV vs. Pronase with hide powder azure. Ten micrograms of each enzyme was used to assay proteolytic activity. The assay was performed under optimal conditions for HP-IV's hide powder azure hydrolytic activity. (○) HP-IV; (●) Pronase.

hydrolyzed by HP-IV and whose hydrolysis could be easily determined (Civello et al., 1983). The conditions for optimal hide powder azure hydrolytic activity have been determined. These include 0.2 M Tris, pH 7.5, assay buffer and a temperature optimum of 50 °C; however, all assays were routinely performed at 37 °C. The hide powder hydrolytic activity showed no dependence upon added Ca^{2+} and Zn^{2+} ions in concentrations up to 20 mM. The assay was expected to be nonlinear due to the insoluble substrate. Therefore, when quantitation was desired, the assay was stopped when the supernatant absorbance at 595 nm approached 0.2. Usually this was achieved after a 10-min incubation with 5 μg of HP-IV. HP-IV's hide powder hydrolytic activity was 50% as potent per mg as that of Pronase under conditions for maximal proteolytic activity of HP-IV (Figure 3). Incubation of HP-IV at increasing temperatures for 15 min caused a decrease in proteolytic activity and a corresponding loss of hemorrhage-inducing activity (Figure 4). Also, acidification to pH 3.0 and neutralization to pH 7.4 destroyed both activities. Figure 5 provides further proof that the hide powder hydrolytic and hemorrhagic activities are the result of the same protein.

Effects of Proteinase Inhibitors and Inactivators. Incubation with 50 μg of chicken egg white or soybean trypsin inhibitor had no effect on the hide powder hydrolytic or

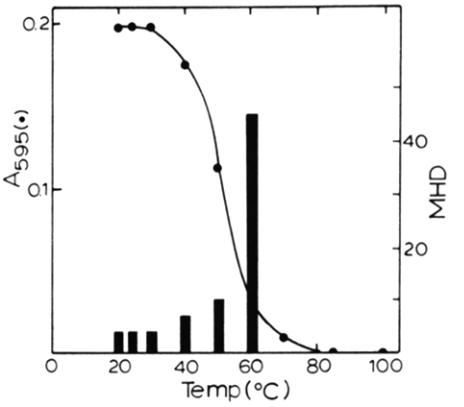

FIGURE 4: Heat inactivation of HP-IV. Milligram per milliliter HP-IV in 20 mM Tris, pH 7.4, was incubated at the indicated temperatures for 15 min, and then 5-μg samples were assayed for proteolytic activity by using hide powder azure. The assay was performed under optimal conditions for HP-IV's hide powder azure hydrolytic activity. The increase in MHD is represented by the bar graph for each temperature tested. At temperatures above 60 °C HP-IV's hemorrhagic activity was totally destroyed. The MHD is defined as the amount of HP-IV (in micrograms) required to produce a hemorrhage of 1-cm diameter in mice.

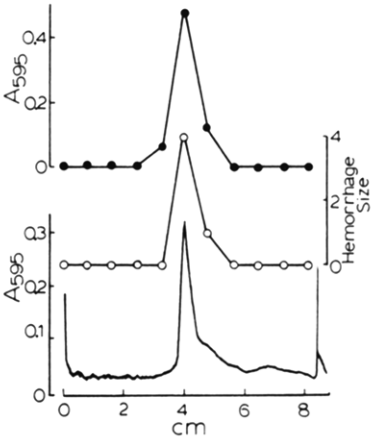

FIGURE 5: Extraction of HP-IV from discontinuous gels. Sixty micrograms of HP-IV/gel was separated by discontinuous electrophoresis. A control gel was stained with Coomassie blue, and an absorbance scan at 595 nm was made. All other gels were cut into 5-mm slices and extracted with 0.5 mL of 20 mM Tris-buffered saline, pH 7.4. The protein concentration of each extract was estimated from the scan. Approximately 5 μg of each fraction was tested for hide powder azure hydrolytic activity under optimal conditions for HP-IV, and 30 μg was tested for hemorrhagic activity in mice. The top graph (●) represents hide powder azure hydrolytic activity while the middle one (○) describes hemorrhagic activity. The bottom graph is the scan of the stained gel. This gel expanded during the staining process. The other two sets of data were corrected to this final length.

hemorrhagic activity of HP-IV. Phenylmethanesulfonyl fluoride (0.21 M), α_1-antitrypsin (50 μg), *N*-ethylmaleimide (50 mM), and iodoacetamide (20 mM) also failed to affect either of the activities.

Analysis of Metal Content. HP-IV dialyzed against EDTA was inactive toward hide powder while a control dialyzed against 0.2 M Tris, pH 7.5, retained 90% of the original activity. Table I shows the hemorrhagic activity of the EDTA- and Tris-dialyzed HP-IV solutions. Lethality was also destroyed by dialysis against EDTA.

By use of HP-IV which had been extensively dialyzed against deionized distilled water and lyophilized, values of 0.98 zinc atom, 0.14 magnesium atom, and 2.4 calcium atoms per M_r 56 000 molecule were determined by atomic absorption spectroscopy. When the three preparations of HP-IV in Table

Table I: Hemorrhagic Effect of HP-IV Proteinase Apoenzyme[a]

dose (μg)	hemorrhagic size: control[b]	dialyzed control[c]	apoenzyme[d]
10	2	2	0
20	3	2	0
30	3	3	0

[a] Data were based upon results from injection of nine mice at each dosage. Hemorrhage size was scored on a scale from 0 to 4. [b] Milligram per milliliter HP-IV in 0.1 M Tris, pH 7.4. [c] Milligram per milliliter HP-IV dialyzed against 0.1 M Tris, pH 7.4, for 24 h at 4 °C. [d] Milligram per milliliter HP-IV dialyzed against 0.1 M Tris, pH 7.4, containing 20 mM EDTA for 24 h at 4 °C.

Table II: Amino Acid Composition of HP-IV

amino acid	hydrolysis[a] 24 h	48 h	72 h	extr value[b]	nearest integer	wt
Asx	67.90	68.61	67.78	68.22	68	7820
Thr	20.47	20.10	20.19	20.53	21	2121
Ser	28.41	25.08	25.44	29.28	29	2523
Glx	48.77	49.54	47.47	49.23	49	6321
Pro	30.33			30.33	30	2910
Gly	42.41	40.65	41.27	42.58	43	2451
Ala	27.08	25.29	25.63	27.40	27	1917
Val	26.00	25.20	24.53	26.71	27	2673
Met	13.54	13.05	13.63	13.32	13	1703
Ile	20.31	19.25	19.68	20.38	20	2260
Leu	26.26	26.10	24.77	27.20	27	3051
Tyr	25.97	24.24	21.58	28.32	28	4564
Phe	14.00	13.82	13.44	14.31	14	2058
His	17.88	16.99	17.38	17.92	18	2466
Lys	30.99	30.24	30.00	31.40	31	3968
Arg	12.93	12.21	11.62	13.56	14	2184
½-Cys	41.00			41.00	41	4223
Trp	7.00			7.00	7	1302
total					507	56 515

[a] The number of residues per molecule was based on an assumed molecular weight of 56 000. [b] All values are a zero time extrapolation using a least-squares approximation. Proline was monitored at 405 nm. Trp was determined by the method of Edelhoch (1967), and Cys was determined by the reduction-carboxymethylation method of Gurd (1967).

I were used, the following results were obtained: nontreated control, 1.08 Zn:4.50 Ca; Tris-dialyzed control, 0.91 Zn:2.90 Ca; EDTA-dialyzed control, 0.05 Zn:0.85 Ca. Attempts to restore the proteolytic activity were unsuccessful. Incubation of 40 mM $ZnCl_2$, $CaCl_2$, or $MgCl_2$ and combinations thereof with 20 μg of apoenzyme for 48 h at room temperature failed to reconstitute any activity. The elaborate procedure of Bjarnason & Tu (1978) for reactivation of the Zn-dependent hemorrhagins from western diamondback rattlesnake venom was unsuccessful when performed with the HP-IV apoenzyme. Dialysis of HP-IV (mg/mL) against 10 mM *o*-phenanthroline in 20 mM Tris, pH 7.4, for 24 h at 4 °C destroyed both the proteolytic and hemorrhagic activities of HP-IV. The procedure for deactivation/reactivation of leucostoma peptidase from western cottonmouth venom using *o*-phenanthroline (Spiekerman et al., 1973) was tested but resulted in no reconstitution of activity with HP-IV apoenzyme.

Amino Acid Analysis. The amino acid composition of HP-IV is given in Table II. The values presented are the nearest integer to the extrapolated values multiplied by a constant which assumed a molecular weight of 56 000. The extrapolated value represents a zero time composition based upon duplicate assays of 24-, 48-, and 72-h hydrolysates. The most noticeable aspect of the amino acid composition is the high content of acidic amino acids. Determination of the amount of ammonia released during hydrolysis as compared to the amount of ammonia contained in the analysis buffers allowed the determination of zero time values for the total amount of glutamine plus asparagine. Of the 117 residues of Glx and Asx, 46 residues were glutamine plus asparagine; therefore, 71 residues were glutamic acid plus aspartic acid (14% of total protein composition). The acidic nature of HP-IV is confirmed by its retention on DEAE-cellulose (Sullivan et al., 1979) and with its isoelectric point of 5.1 which was determined from the gel shown in Figure 2. Amino acid analysis performed after carboxymethylation of HP-IV revealed the presence of one cysteine residue while analysis of HP-IV which had been reduced with DTT prior to carboxymethylation revealed the presence of 41 cysteine residues. Neither *N*-acetylglucosamine nor *N*-acetylgalactosamine was detected during amino acid analysis.

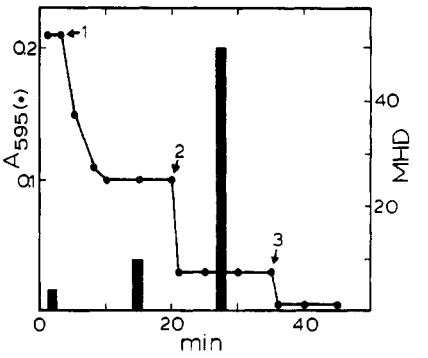

FIGURE 6: Inactivation of HP-IV with dithiothreitol. Milligram per milliliter HP-IV in 20 mM Tris, Ph 7.5, was incubated with final concentrations of 2, 20, and 50 mM DTT (arrows 1, 2, and 3, respectively) for the indicated time at room temperature. Five-microgram aliquots were incubated with 10 mg of hide powder azure for 10 min at 37 °C, and the color released was determined by absorbance at 595 nm. The bar graph represents the MHD for each concentration of DTT added. At 50 mM DTT, HP-IV's hemorrhagic activity was totally destroyed.

Disulfide Bonds and Proteolytic Activity. Since sulfhydryl-containing reducing agents destroyed all three activities of HP-IV, the concentrations of reducing agent necessary to partially inactivate the proteinase were determined. Incubation of milligrams per milliliter HP-IV with 2 mM DTT apparently reduced one disulfide bond, resulting in a 50% decrease in hide powder azure hydrolytic activity. Incubation of HP-IV with 20 mM DTT reduced eight disulfide bonds and decreased hydrolytic activity by 80%. Total inactivation was accomplished with 50 mM DTT. All samples of DTT-treated HP-IV were incubated with iodoacetamide prior to being assayed. Quantitation of disulfide reduction was confirmed by amino acid analysis. The time course of the inactivation and the effect upon hemorrhagic activity are shown by Figure 6.

Discussion

Timber rattlesnake venom contains an acidic protein component which is capable of inducing hemorrhage and death in mice. This venom component also has proteolytic activity, and this activity cannot be destroyed without causing loss of the component's toxic properties.

Unless HP-IV was assayed under the conditions for optimal hydrolytic activity, it would be assumed to be nonproteolytic. This will be described in more detail in the following paper (Civello et al., 1983). HP-IV is unlikely to be a carboxylic group active center proteinase as it has a neutral pH optimum, pH 7.5. Neither is it a sulfhydryl active center enzyme as *N*-ethylmaleimide and iodoacetamide had no effect. It is not a serine active center proteinase as phenylmethanesulfonyl

Table III: Comparative Amino Acid Composition of Proteins from Rattlesnake Venoms

	Crotalus horridus horridus HP-IV[a]	*Crotalus atrox* toxin[b]					*Crotalus viridis viridis*		*Crotalus durissus terrificus*	*Crotalus scutulatus*	*Crotalus adamanteus*
		a	b	c	d	e	viriditoxin[c]	myotoxin a[d]	crotamine[e]	Mojave toxin[f]	proteinase I[g]
Asx	68	85	26	33	33	30	110	2	2	7	30
Thr	21	36	7	11	10	10	54	0	0	3	8
Ser	29	35	14	16	16	14	91	3	3	3	18
Glx	49	55	16	24	25	26	118	2	2	6	21
Pro	30	27	6	6	6	8	34	3	3	2	77
Gly	43	43	13	9	9	14	68	5	5	6	14
Ala	27	36	8	10	9	7	57	0	0	3	9
Val	27	35	11	11	11	12	57	0	0	1	13
Met	13	11	6	5	5	8	18	1	1	1	6
Ile	20	36	12	17	18	21	39	2	1	3	15
Leu	27	49	19	25	25	14	62	1	1	3	21
Tyr	28	21	7	7	7	11	36	1	1	5	8
Phe	14	17	7	7	7	6	55	1	2	3	7
His	18	18	8	8	8	9	25	2	2	1	6
Lys	31	27	10	7	7	9	84	10	9	4	9
Arg	14	33	12	10	11	8	36	1	2	3	15
½-Cys	41	66	4	2	2	8	53	4	6	8	4
Trp	7	6	4	5	5	4	21	1	2		3
total	507	635	200	213	214	209	1018	39	42	62	214

[a] Present investigation. [b] Bjarnason & Tu (1978). [c] Fabiano & Tu (1981). [d] Cameron & Tu (1977). [e] Cameron & Tu (1978). [f] Bieber et al. (1975). [g] Kurecki et al. (1978).

fluoride and, as expected, various protein trypsin inhibitors had no effect. HP-IV is a metalloproteinase containing Zn^{2+} and Ca^{2+}. Like other metalloproteinases, it is inactivated by sulfhydryl-containing reducing agents and by dialysis against chelators (Barrett, 1970).

Even though HP-IV is similar in certain respects to the five hemorrhagic proteinases of Bjarnason & Tu (1978), important differences exist. HP-IV will not elute from DEAE-cellulose under the conditions stated by these workers. The molecular weight of HP-IV (M_r 56 000) is in the neighborhood of Bjarnason and Tu's toxin a (M_r 68 000), but nevertheless it is different. Table III compares the amino acid composition of HP-IV with components isolated from different rattlesnake venoms. HP-IV's composition appears distinctly different from the others. Of the components listed in Table III, only HP-IV, toxins a–e, and viriditoxin were reported to contain both proteolytic and hemorrhagic activity. Since 1978, when the first hemorrhagins from North American rattlesnakes were characterized (Bjarnason & Tu, 1978), only one additional North American rattlesnake hemorrhagic proteinase has been reported (viriditoxin; Fabiano & Tu, 1981). The current report describes a hemorrhage-inducing protein that comprises a major portion of the protein in whole timber rattlesnake venom. It also establishes that this component catalyzes the hydrolysis of cow hide powder azure. A positive correlation between hide powder hydrolytic and hemorrhagic activities was established.

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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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