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Proteolytic Specificity of Hemorrhagic Toxin *a* Isolated from Western Diamondback Rattlesnake (*Crotalus atrox*) Venom†

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ABSTRACT: The proteolytic specificity of hemorrhagic toxin *a* from the venom of *Crotalus atrox* (western diamondback rattlesnake) has been investigated by using the oxidized B chain of bovine insulin and other peptides as substrates. The toxin appears highly specific for X-Leu bonds (cleaving the His_{10}-Leu_{11}, Ala_{14}-Leu_{15}, and Tyr_{16}-Leu_{17} bonds), with no detectable activity against the Gly-Phe, Phe-Phe, Phe-Tyr, and Leu-Tyr bonds also present in the insulin B chain. The X-Leu bond of the peptides Tyr-Gly-Gly-Phe-Leu, Phe-Ala-Leu, and Ala-Leu was also cleaved. The toxin seems to be a strict endopeptidase, in that the cleavage of the two most susceptible bonds, Ala_{14}-Leu_{15} and Tyr_{16}-Leu_{17}, are mutually exclusive; i.e., cleavage of either bond results in the other being too close to either the amino- or carboxyl-terminal of its respective fragment for the enzyme to be effective against it. The X-Met bond of Tyr-Gly-Gly-Phe-Met was cleaved, although a dipeptide Gly-Met was not hydrolyzed after 16 h of incubation. The substrates not hydrolyzed are furylacryloylglycyl-L-leucinamide, carbobenzoxy-L-glutamylglycine, carbobenzoxyglycyl-L-glutamic acid, benzoyl-L-arginine-*p*-nitroanilide, L-lysine-*p*-nitroanilide, $(L\text{-}Ala)_3$-*p*-nitroanilide, Gly-Met, Gly-Phe-Phe, Gly-Gly-Ala, TAME, and ATEE. The absence of hydrolytic activity against the last two substrates indicates that hemorrhagic toxin *a* does not possess trypsin- or chymotrypsin-like activity.

Hemorrhage is commonly induced by rattlesnake envenomation. Recently five hemorrhagic toxins with proteolytic enzyme activities were isolated from the venom of the western diamondback rattlesnake (*Crotalus atrox*) (Bjarnason & Tu, 1978). In the course of physical and chemical characterization of these toxins, all five were shown to be zinc-containing proteolytic enzymes. Light and electron microscopic studies by Ownby et al. (1978) have shown that exposure of endothelial cells to hemorrhagic toxin *a* results in lysis of the cells; these observations raise the possibility that cell membrane proteins may be the site of attack by the toxin. Proteolytic activity of each of the hemorrhagic toxins against the general protease substrate, dimethylcasein, has been measured by titration with TNBS[1] of the newly generated free amino groups (Bjarnason & Tu, 1978); the identities of the peptide bonds attacked had not, however, been determined prior to the present study. Determination of the bond specificity of the toxins is the logical next step in understanding the relationship of proteolytic and hemorrhagic activities. Since hemorrhagic toxin *a* (HT-*a*) is by far the most potent hemorrhage-producing agent of the five, it was considered the most interesting subject for specificity studies. The performic acid oxidized B chain of bovine insulin and other peptides were employed as substrates, and the fragments resulting from proteolysis of the oxidized B chain were purified by preparative thin-layer chromatography. From the amino acid compositions of the isolated fragments, we were able to identify the principal sites of attack of hemorrhagic toxin *a* on this substrate. Fragments of other peptides were identified by thin-layer chromatography.

Materials and Methods

Crude *C. atrox* venom was purchased as the lyophilized powder from Miami Serpentarium, Miami, FL. Hemorrhagic

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[1] Abbreviations used: HT-*a*, hemorrhagic toxin *a*; TNBS, trinitrobenzenesulfonic acid; Cbz-, carbobenzoxy-; FAGLA, furylacryloylglycyl-L-leucinamide; TAME, *p*-tolylsulfonyl-L-arginine methyl ester; ATEE, acetyl-L-tyrosine ethyl ester; Tris, tris(hydroxymethyl)aminomethane.

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toxin *a* was isolated from the crude venom by the previously published procedure (Bjarnason & Tu, 1978), with Tris–glycinate disc gel electrophoresis used as the criterion of purity. The oxidized B chain of bovine insulin, H_2N-Tyr-Gly-Gly-Phe-Leu-OH, and H_2N-Tyr-Gly-Gly-Phe-Met-OH were from Boehringer Mannheim, furylacryloylglycyl-L-leucinamide, *N*-Cbz-glycyl-L-glutamic acid, and *N*-Cbz-glutamylglycine were from Vega, benzoyl-DL-arginine-*p*-nitroanilide, glycyl-L-methionine, glycyl-L-phenylalanyl-L-phenylalanine, glycyl-glycyl-L-alanine, and L-alanyl-L-leucine were from Mann, and L-lysine-*p*-nitroanilide was from Nutritional Biochemicals. *N*-Succinyl-(L-alanyl)$_3$-*p*-nitroanilide, L-tyrosyl-L-leucine, L-phenylalanyl-L-leucine, TAME (*p*-tolylsulfonyl-L-arginine methyl ester), ATEE (acetyl-L-tyrosine ethyl ester), and L-leucyl-L-tyrosine were from Sigma.

Isotachophoretic Studies. Analytical isotachophoresis was performed on a LKB (Uppsala, Sweden) Tachophor Model 2127 by using an anionic system, with 0.01 M chloride as the leading anion at pH 9.0 and 0.01 M ε-aminocaproic acid as the trailing ion at pH 10.5. Oxidized insulin B chain (1.0 mg/mL) was incubated at 25 °C with hemorrhagic toxin *a* (12.5 μg/mL) in 0.01 M ammonium acetate buffer, pH 9.1. At various intervals during the incubation, 0.2-mL aliquots were withdrawn from the incubation mixture and immersed for 1.5 min in a boiling water bath to inactivate the enzyme. A 6.0-μL portion of the aliquot was then injected into the capillary of the isotachophoresis apparatus, along with 2.0 μL of a 1:40 dilution of pH 8–9.5 Ampholine (LKB, Uppsala, Sweden) to provide mobility spacers.

Kinetic Studies. Oxidized insulin B chain (1.0 mg/mL) was incubated with HT-*a* (50 μg/mL) at 25 °C in 0.01 M borate, pH 9.0. At intervals, 0.2-mL aliquots were withdrawn from the digestion mixture, and the digestion was stopped by immersing the aliquot in a boiling water bath. The method used for quantitation of newly generated free α-amino groups is a modification of that of Fields (Fields, 1972). To each aliquot of the reaction mixture were added 0.3 mL of glass-distilled water and 0.5 mL of 0.2 M borate, pH 10.15. The addition of 0.02 mL of 1.1 M trinitrobenzenesulfonic acid (TNBS) in H_2O with immediate vortex mixing changed the pH of the borate buffer to 9.0. After a 10-min period to allow the TNBS to react with free amino groups at this pH, the addition of 5.0 mL of 0.2 M NaH_2PO_4, pH 6.87, containing 1.5 mM Na_2SO_3 lowered the pH to 7.0. The extinction of the solution at 420 nm was then read vs. a blank prepared by applying the color development procedure described above to 0.2 mL of an enzyme–substrate mixture identical with the reaction mixture except for the fact that the enzyme had been denatured by immersion in a boiling water bath *before* it was mixed with substrate. An extinction coefficient of 22000 M^{-1} cm^{-1}, based on values determined (Fields, 1971) for sulfite complexes with trinitrophenyl amino acids and peptides, was used to estimate the concentration of proteolytically generated free amino groups present in the original assay mixture at the time the aliquot was withdrawn.

Thin-Layer Chromatography. Oxidized insulin B chain (1.0 mg/mL) was incubated with HT-*a* (50 μg/mL) at pH 9.0 in 0.01 M ammonium acetate buffer containing no additional salts. At various times during the incubation, 0.2-mL aliquots were withdrawn and immersed for 1.5 min in a boiling water bath. The aliquots were then evaporated to dryness at 45 °C (Lab-Line Temp-Blok module heater) under a stream of nitrogen, then twice resuspended in 0.1 mL of distilled water, and reevaporated to dryness. After this treatment, intended to remove all traces of the ammonium acetate buffer, each aliquot was redissolved in 0.1 mL of 25% acetic acid, and 20 μL of the resulting solution was spotted 1.5 cm from the bottom of a 10 × 20 cm silica gel TLC plate (Eastman 13181 chromatogram sheet). The plate was developed for approximately 4.5 h by ascending chromatography with 1-butanol/acetic acid/water (4:1:1). (The solvent front moved approximately 12 cm beyond origin.) The plates were air-dried and peptide spots visualized by dipping in 1% ninhydrin in acetone, followed by a 10-min development in a 110 °C oven.

Peptide Isolation. Digestion fragments were isolated by preparative thin-layer chromatography. Digestion was carried out at pH 9.0 in 10 mM ammonium acetate buffer, pH 9.0. After a 7-h incubation at 25 °C, the reaction mixture (3.6 mL in volume and containing 2.0 mg/mL insulin B chain and 50 μg/mL HT-*a*) was immersed for 6 min in a boiling water bath to stop the enzymatic reaction. The mixture was evaporated to dryness, twice resuspended in 0.5 mL of distilled water, and reevaporated to dryness. The dried peptide and protein mixture was then redissolved in 0.25 mL of 25% HOAc and applied as a 7.0-cm streak, some 3-mm wide, 1.5 cm above the bottom of a 10 × 20 cm silica gel TLC plate. The plate was developed with butanol/acetic acid/water (4:1:1) for approximately 4.5 h. After the plate was air-dried, vertical strips were cut from the right and left edges of the chromatograms, and a 0.5-cm-wide vertical strip was cut from the center of the plate. These strips were dipped in 1% acetone–ninhydrin and heated at 110 °C for 10 min. With the position of the peptide bands thus revealed, the silica gel was scraped off the indicated areas of the remaining portions of the TLC plate and collected in 1.5-mL centrifuge tubes. Each peptide was eluted by washing the silica gel with a 0.9-mL portion followed by a 0.6-mL portion of 25% acetic acid in 95% ethanol, with the silica gel being removed by centrifugation (Eppendorf 5412 microcentrifuge). The acetic acid solution of each peptide was then evaporated to dryness at 45 °C under nitrogen.

Assays for Activity against Small Substrates. Assays for cleavage of dipeptides were carried out by measuring newly generated amino groups by titration with TNBS (Fields, 1972) or by observing the appearance of new ninhydrin-positive spots upon thin-layer chromatography of incubation mixtures on silica gel in butanol/acetic acid/water (4:1:1). Cleavage of the Gly–Leu bond of furylacryloylglycyl-L-leucinamide was measured by the absorbance change of 345 nm (Feder, 1968). Activity against chromogenic *p*-nitroanilide substrates was measured by the change in absorbance at 405 nm (Tuppy et al., 1962).

For Ala-Leu, Phe-Ala-Leu, Tyr-Gly-Gly-Phe-Leu, Tyr-Gly-Gly-Phe-Met, Gly-Met, and Gly-Phe-Phe, 1 mg of each was incubated with 100 μg of hemorrhagic toxin *a* for 16 h at 25 °C. Cleavage products were identified by thin-layer chromatography after spraying with ninhydrin.

Use of Trypsin and Chymotrypsin Substrates. So that the trypsin and chymotrypsin activities could be tested, *p*-tolylsulfonyl-L-arginine methyl ester (TAME) and acetyl-L-tyrosine ethyl ester (ATEE) were used and the hydrolyses carried out at 37 °C. A mixture of enzyme in 0.9 mL of 0.4 M Tris-HCl buffer, pH 8.5, was incubated for 15 min, and then 0.01 mL each of 0.1 M substrates was added. After 30 min, the amount of substrate hydrolyzed was determined by the hydroxamate method of Roberts (1958).

Results

Isotachophoretic and Kinetic Studies. Qualitative indications of the cleavage of the oxidized insulin B chain are given by the isotachophoretic patterns shown in Figure 1. In a

Table I: Amino Acid Compositions of Primary Cleavage Fragments

amino acid	peptide 1 theor	peptide 1 exptl	peptide 2 theor	peptide 2 exptl	peptide 3 theor (100% peptide 3)	peptide 3 theor (50% peptide 3 + 50% peptide 4)	peptide 3 exptl	peptide 4 theor	peptide 4 exptl
lysine	0	0	1	0.85	0	0.5	0.28	1	0.81
histidine	2	1.44	0	0.09	2	1	1.55	0	0.13
arginine	0	0.08	1	0.89	0	0.5	0.38	1	0.80
cysteic acid	1	1.02	1	0.95	1	1	1.17	1	0.94
aspartic acid	1	1.13	0	0.12	1	0.5	0.96	0	0.20
threonine	0	0.05	1	0.41	0	0	0.28	0	0
serine	1	0.27	0	0	1	0.5	0.48	0	0
glutamic acid	2	1.71	1	0.99	2	1.5	2.08	1	0.98
proline	0	0	1	0.93	0	0.5	0.52	1	0.88
glycine	1	1.10	2	1.89	1	1.5	1.56	2	1.79
alanine	1	0.86	1	0.97	1	1	1.04	1	1.01
half-cystine	0	0	0	0	0	0	0	0	0
valine	2	1.72	1	1.04	2	1.5	1.61	1	1.01
methionine	0	0	0	0	0	0	0	0	0
isoleucine	0	0	0	0	0	0	0	0	0
leucine	2	1.79	2	1.71	3	2.0	2.27	1	0.99
tyrosine	0	0.23	2	1.28	1	1.0	0.44	1	0.74
phenylalanine	1	0.92	2	1.62	1	1.5	1.40	2	1.61

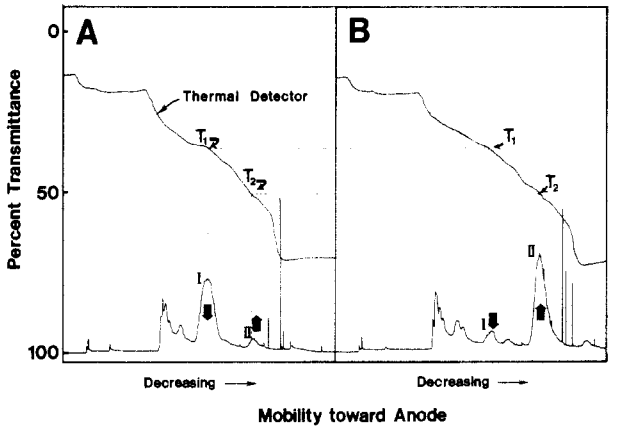

FIGURE 1: Isotachophoretic evidence of proteolytic cleavage of insulin B chain by HT-*a*. (Peak I) original peptide; (peak II) major tyrosine-containing digestion fragment.

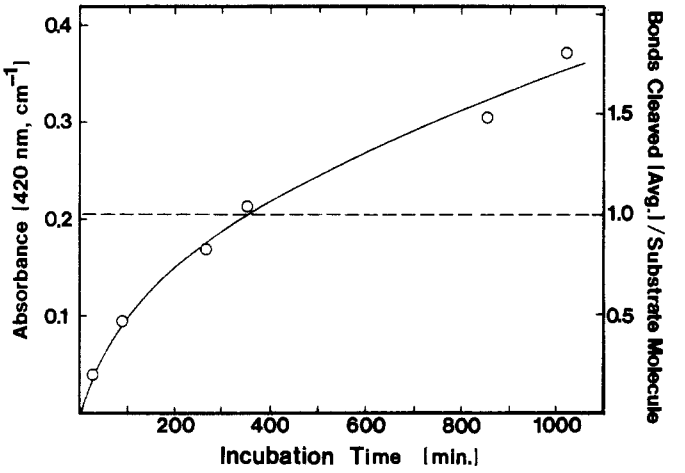

FIGURE 2: Kinetics of degradation of insulin B chain by HT-*a*. Proteolysis results in liberation of new α-amino groups, which can be monitored by TNBS titration.

pattern produced by an aliquot withdrawn from the reaction mixtures quite early, after only a 2-min incubation (Figure 1A), the large ultraviolet absorption peak (I) appearing at T_1 on the thermal scale apparently represents B chain as yet uncleaved, while the small peak (II) at T_2 on the thermal scale represents one of the tyrosine-containing fragments produced from the B chain by proteolytic cleavage. In the second pattern, taken after a 360-min incubation, peak II has become quite prominent, and peak I (substrate) is greatly diminished. Peak II remains the most prominent peak in patterns taken after longer incubation times (up to 20 h), indicating that peak II represents a fragment that is relatively resistant to further degradation by the hemorrhagic toxin.

More quantitative data are given in Figure 2, which illustrates the appearance of new TNBS-reactive α-amino groups due to proteolytic cleavage of the insulin B chain. During the initial stage of incubation, free amino groups (TNBS reactive) are rapidly released, followed by a period of slow release.

Thin-Layer Chromatography of Cleavage Products. The production of specific proteolytic fragments can be followed in the thin-layer patterns (Figure 3) produced by incubation of insulin B chain with hemorrhagic toxin *a* for periods ranging from 1 min to more than 20 h. By 30-min incubation time, there is clear evidence of cleavage at more than one point in the insulin B chain. At 120-min incubation, there is indication of another cleavage, resulting in the fragment with an R_f value near 0.48; this fast migrating fragment becomes clearly evident by 282-min incubation.

Analysis of Cleavage Products. After a 420-min incubation, the enzyme reaction was stopped (by immersion of the sample in a boiling water bath) in the major portion of the reaction mixture from which the aliquots chromatographed in Figure 3 were taken. Preparative thin-layer chromatography was carried out on this mixture, with the amino acid compositions of the separated peptides reported in Tables I and II. These compositions are keyed to the spots observed at 415 min in Figure 3 by means of the numbers appearing on the right-hand side of the chromatogram. The observed amino acid compositions have been compared with the compositions of certain segments of the sequence of oxidized insulin B chain ("theoretical" values in Tables I and II), and on the basis of the observed correspondences, the isolated peptides have been assigned the identities indicated in Figure 4.

Some of the peptides show fairly high background values of amino acids that are not included in the sequence segment to which the composition has been matched. This is probably due to the distribution of minor cleavage products across the chromatogram along with the major products reported here. The consistently low values for serine and threonine are ap-

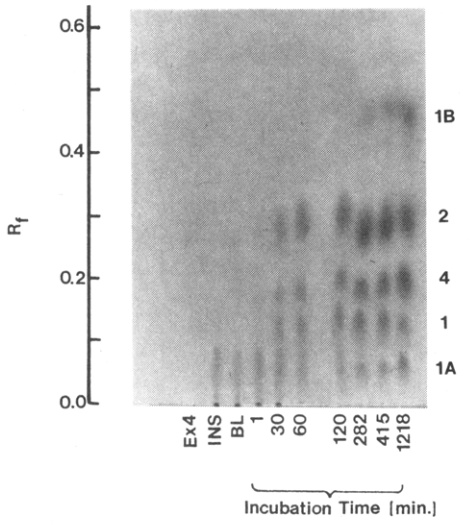

FIGURE 3: Progress of cleavage monitored by thin-layer chromatography. Ex4, aliquot containing only buffer and enzyme, with enzyme at 4 times the concentration contained in the digestion aliquots; INS, oxidized insulin B chain alone; BL, blank prepared by mixing insulin B chain with enzyme previously denatured by heating.

parently an artifact of the peptide isolation procedure. When samples of the original, uncleaved oxidized insulin B chains were streaked on, and eluted from, a previously developed thin-layer plate at positions corresponding to the range of R_f values observed for the proteolytic fragments, these samples showed similar losses of serine and threonine upon acid hydrolysis. Samples of the B chain which were analyzed according to the procedure of this study, but without first being eluted from the TLC plates, showed values close to those expected for all amino acids, including threonine and serine.

Sites of Cleavage. Peptide 2 (R_f 0.29) apparently represents the carboxyl-terminal fragment resulting from cleavage of insulin B chain between Ala_{14} and Leu_{15} and differs from peptide 4 (which has an R_f of 0.20 and apparently represents the carboxyl-terminal fragment produced by cleavage of Tyr_{16}-Leu_{17}) only by including an additional residue each of tyrosine and leucine (Leu_{15} and Tyr_{16}). The additional hydrophobicity imparted by these two residues is apparently responsible for the greater solubility of peptide 2 (as compared with peptide 4) in the relatively hydrophobic mobile phase of the butanol/acetic acid/water system.

The composition of peptide 1 matches very closely to the composition of the sequence Phe_1–Ala_{14}; peptide 1 evidently arises from Ala_{14}–Leu_{15} cleavage and is complementary to peptide 2. No peptide corresponding to peptide 3 in Figure 4 was found on the chromatogram of the 420-min reaction mixture. In consideration of the possibility that this fragment

Table II: Amino Acid Composition of Secondary Cleavage Fragments

	peptide 1A		peptide 1B	
amino acid	theor	exptl	theor	exptl
lysine	0		0	
histidine	2	1.25	0	
arginine	0	0.05	0	
cysteic acid	1	0.96	0	
aspartic acid	1	0.96	0	0.04
threonine	0	0.05	0	0.01
serine	1	0.36	0	0.01
glutamic acid	1	0.10	1	0.93
proline	0		0	0.00
glycine	1	1.02	0	0.08
alanine	0	0.25	1	0.88
half-cystine			0	
valine	1	1.07	1	1.005
methionine	0		0	0
isoleucine	0		0	0
leucine	1	1.11	1	1.19
tyrosine	0	0.09	0	0.03
phenylalanine	1	0.74	0	0.03

may have been degraded by that point in the incubation, a shorter preparative incubation (60 min) was carried out. The band reported as peptide 3 was isolated from that mixture. The observed amino acid composition does not correspond exactly to the theoretical value of peptide 3. Due to the closeness of this spot to the spot which contains peptide 4, it is reasonable to assume that the spot may contain both peptides 3 and 4. By the somewhat arbitrary assumption of an equimolar mixture of peptides 3 and 4, the experimental value of amino acids can be made to correspond much more closely to the observed values than can be accounted for by peptide 3 alone (Table I).

The sum of the compositions of peptides 1A and 1B (Figure 3 and Table II) corresponds closely to the composition of peptide 1, the amino-terminal fragment resulting from cleavage of the B chain at the Ala_{14}–Leu_{15} bond. Individually, the compositions of peptides 1A and 1B correspond very well with the compositions expected for the sequence segments Phe_1–His_{10} and Leu_{11}–Ala_{14} (inclusive). It is reasonable, therefore, to conclude that peptides 1A and 1B result from cleavage of peptide 1 at the His_{10}–Leu_{11} bond.

Conspicuously absent from the chromatogram in Figure 3 is a spot corresponding to the dipeptide L-Leu-L-Tyr. This dipeptide would be produced if the primary proteolytic fragment designated as "peptide 2" (the result of cleavage of the Ala_{14}–Leu_{15} bond) were to undergo subsequent cleavage of the Tyr_{16}–Leu_{17} bond. The dipeptide L-Leu-L-Tyr could also arise from cleavage of peptide 3 at the Ala_{14}–Leu_{15} bond. When L-Leu-L-Tyr was chromatographed along with the

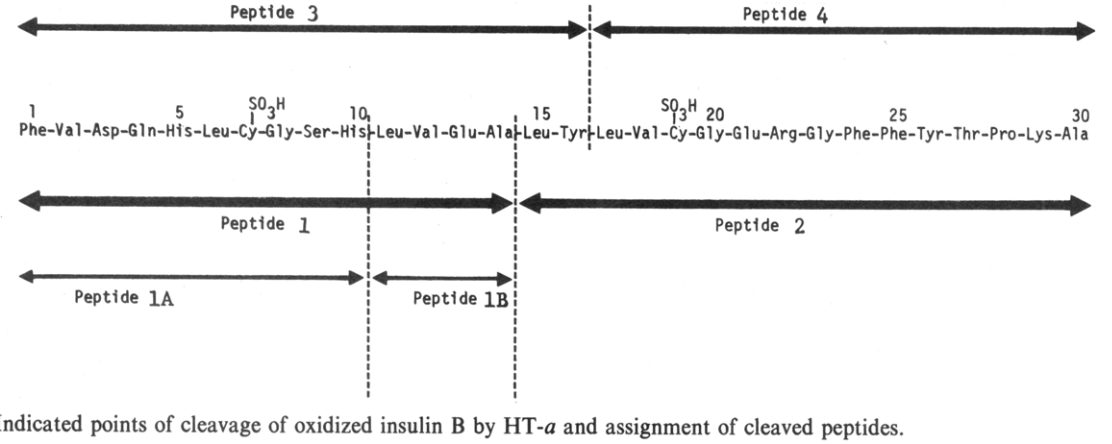

FIGURE 4: Indicated points of cleavage of oxidized insulin B by HT-*a* and assignment of cleaved peptides.

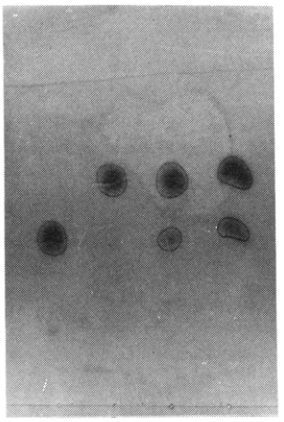

FIGURE 5: Cleavage of Phe–Met bond of pentapeptide Tyr-Gly-Gly-Phe-Met. (A) Methionine; (B) a pentapeptide only; (C, D) a pentapeptide incubated with hemorrhagic toxin *a*. Note methionine spot in lanes C and D.

cleavage products resulting from a 415-min incubation, the dipeptide migrated significantly faster than peptide 1B, the most mobile of the proteolytic cleavage fragments (R_f 0.57 for the dipeptide and R_f 0.48 for peptide 1B). There was no separate spot in Figure 3 which could represent L-Leu-L-Tyr, nor did the band from which peptide 1b was eluted show any significant tyrosine content (Table II), which might have indicated contamination by the dipeptide in question.

Other Peptides Hydrolyzed. Several leucine- and methionine-containing substrates were also investigated to further clarify substrate specificity. All leucine-containing peptides investigated were hydrolyzed at the aminoacyl–leucine bond. In Tyr-Gly-Gly-Phe-Leu, the phenylalanylleucine bond was cleaved. Similarly the alanylleucine bond of Phe-Ala-Leu was hydrolyzed. The dipeptide Ala-Leu was also hydrolyzed with hemorrhagic toxin *a*. Since insulin B chain does not contain a methionine residue, the methionine-containing peptide Tyr-Gly-Gly-Phe-Met was investigated, and it was found that the aminoacyl–methionine bond was cleaved, although a dipeptide Gly-Met was not hydrolyzed. An example of hydrolysis on Tyr-Gly-Gly-Phe-Met is shown in Figure 5. Upon 16 h of incubation, the dipeptide Tyr-Leu was hydrolyzed, although no hydrolysis had been observed after a 4-h incubation.

Trypsin and Chymotrypsin Substrates. None of these substrates, TAME, and ATEE were hydrolyzed with hemorrhagic toxin *a*, suggesting that the toxin does not have trypsin-like or chymotrypsin-like activity.

Small Substrates Not Hydrolyzed. Furylacryloylglycyl-L-leucinamide (FAGLA) (Feder, 1968) is commonly used to measure neutral protease activity (Wilkes & Prescott, 1976). Although the Gly–Leu bond of FAGLA is somewhat similar to the Ala_{14}–Leu_{15} bond of insulin B chain, hemorrhagic toxin *a* shows no activity against FAGLA.

It has been noted in at least one instance (Spiekermann et al., 1973) that a venom protease may act against small substrates by cleaving bonds quite different from those attacked in larger substrates. With this in mind, we tested hemorrhagic toxin *a* against a variety of small substrates having bonds that involve amino acid residues of types different from those involved in the susceptible bonds of the insulin B chain. The hemorrhagic toxin failed to cleave either of the dipeptides Cbz-Gly-L-Glu and Cbz-L-Glu-Gly. Also, no activity was detected against either the chromogenic trypsin substrates benzoyl-L-arginine-*p*-nitroanilide and L-lysine-*p*-nitroanilide or the chromogenic elastase substrate $(L\text{-Ala})_3$-*p*-nitroanilide.

Table III: Comparison with Representative Proteases of Non-Snake Venom Origin[a]

enzyme	1 Phe	5 Val-Asp-Gln-His	SO_3^- Leu-Cy	10 Gly-Ser-His-Leu-Val-Glu-Ala	15 Leu-Tyr-Leu-Val	SO_3^- Cy	20 Gly-Gly-Glu-Arg-Gly-Phe-Phe	25 Tyr-Thr-Pro-Lys	30 Ala
hemorrhagic toxin *a*[a]				↑	↑	↑	↑		
thermolysin[b]			↑	↑	↑		↑	↑	
trypsin[c]								↑	
α-chymotrypsin[c]					↑	↑			
subtilisin Carlsberg[d]			↑	↑	↑	↑		↑	
elastase[e]		↑		↑	↑	↑	↑		
papain[f]	↑		↑	↑	↑	↑		↑	↑

[a] This study. [b] Matsubara et al. (1966). [c] Sanger & Tuppy (1951). [d] Johansen et al. (1968). [e] Narayanan & Anwar (1969). [f] Johansen & Ottesen (1968).

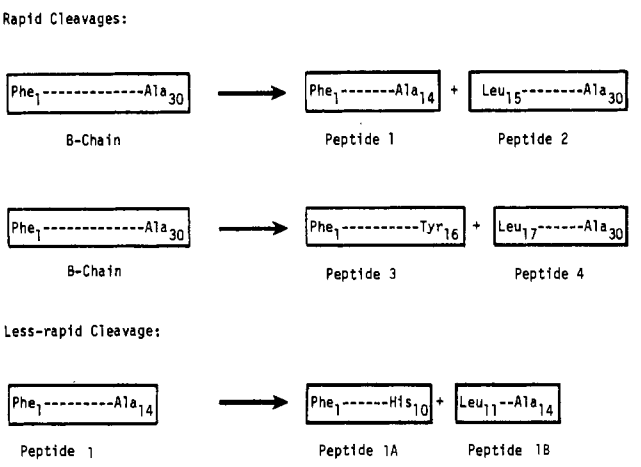

FIGURE 6: Cleavage scheme with comprehensive listing of cleavage fragments, showing mutual exclusivity, of Ala_{14}–Leu_{15} and Tyr_{16}–Leu_{17} cleavages.

Unlike Tyr-Gly-Gly-Phe-Met, the dipeptide Gly-Met was not hydrolyzed. In addition, no hydrolytic activity was observed in Gly-Phe-Phe and Gly-Gly-Ala.

Discussion

From the order of appearance of the proteolytic fragments (monitored by analytical thin-layer chromatography, Figure 3), it appears that the Ala_{14}–Leu_{15} and Tyr_{16}–Leu_{17} bonds are the primary sites of cleavage of the oxidized insulin B chain by hemorrhagic toxin *a*. A third bond, His_{10}–Leu_{11}, is cleaved more slowly than the first two but is still cleaved fairly rapidly.

The absence of the dipeptide L-Leu-L-Tyr from the thin-layer chromatograms of incubation mixtures indicates that the Ala_{14}–Leu_{15} and Tyr_{16}–Leu_{17} cleavages are mutually exclusive. Cleavage of either of the two highly susceptible bonds results in the other bond being located only two residues from the end of one of the resulting fragments. Hemorrhagic toxin *a* apparently will not cleave an otherwise susceptible bond located this close to either the amino or carboxyl terminus. According to this idea, the major cleavages of insulin B by hemorrhagic toxin *a* are comprehensively described by the scheme of Figure 6. The observation that only one bond per B chain molecule appears to be cleaved rapidly (Figure 2) is consistent with the idea that the Ala_{14}–Leu_{15} and Tyr_{16}–Leu_{17} cleavages are mutually exclusive.

Table III compares the proteolytic specificity of hemorrhagic toxin *a* with representative members of several major classes of non-snake venom proteases. It is evident that hemorrhagic toxin *a* is quite different in its specificity from the well-known proteases trypsin, chymotrypsin, subtilisin, elastase, and papain. Hemorrhagic toxin *a* shows more similarity to the microbial neutral protease, thermolysin, but even here there are notable differences. Additional evidence is that the specific substrates for trypsin (TAME) and chymotrypsin (ATEE) are not hydrolyzed with hemorrhagic toxin *a*. Most striking is the much greater preference hemorrhagic toxin *a* appears to show for bonds involving the amino groups of leucine. This specificity is also substantiated by the cleavage of the aminoacyl–leucine bond for Ala-Leu, Phe-Ala-Leu, and Tyr-Gly-Gly-Phe-Leu. Whereas thermolysin has been shown to hydrolyze bonds involving the amino groups of phenylalanine almost as rapidly as those involving the amino group of leucine (Morihara et al., 1968; Morihara, 1974), hemorrhagic toxin *a* fails to show any activity against bonds involving the amino groups of phenylalanine (Gly_{23}–Phe_{24} and Phe_{24}–Phe_{25}) in the insulin B chain. After a 420-min incubation, at which time the Ala_{14}–Leu_{15} and Tyr_{16}–Leu_{17} bonds have been almost quantitatively hydrolyzed (allowing for mutual exclusivity), there is no evidence of significant cleavage of the Gly_{23}–Phe_{24} and Phe_{24}–Phe_{25} bonds. To the contrary, peptides 2 and 4, which contain these bonds as internal bonds, are quite resistant to further attack of any kind, as shown by their persistence as prominent spots (Figure 3) even after 20 h of incubation. This unusually tight specificity for bonds involving the amino group of leucine suggests that this proteolytic toxin may, in addition to its biological significance as a potent hemorrhagin, be of considerable interest for use in protein sequencing studies.

In view of the proteolytic activity of hemorrhagic toxin *a*, combined with its clearly demonstrable biological effect, it seems that rather selective proteolyses of the type shown by hemorrhagic toxin *a* may be of far-reaching significance in the understanding of venom action.

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