The symptoms which immediately follow envenomation by many crotalid snakes include hypotension, hypovolemia, hemoconcentration, and shock. We have isolated and characterized two proteases (E1 and E11) from the venom of Crotalus atrox which may be involved in the onset of these symptoms. E1 and E11 have molecular weights of 27,500 and 29,200 and isoelectric points of 4.7 and 4.3, respectively. Specific esterolytic activities of E1 and E11 on N\(^{-}\)p-tosyl-L-arginine methyl ester are 51.5 \(\mu\)mol min\(^{-\text{mg}^{-1}}\) and 48.1 \(\mu\)mol min\(^{-\text{mg}^{-1}}\), respectively. Both enzymes are rather specific in their substrate requirements in that neither was demonstrated to have any proteolytic activity against either of the oxidized chains of insulin, or glucagon. Neither enzyme was shown to have plasmin or fibrinolytic activity. Both enzymes are able to cleave a kininogen analog to release bradykinin. This proteolytic activity is inhibited by aprotinin and phenylmethanesulfonyl fluoride but not by ethylenediaminetetraacetate. The enzymes are active upon the kallikrein substrates S2666 and S2302. The \(K_m\) values of the enzymes with these substrates are similar to those reported for kallikrein. Structural similarity between the two enzymes was demonstrated by ultraviolet and circular dichroic spectroscopy, and amino acid analysis. Tryptic peptide mapping of the two native enzymes also suggested a large degree of structural similarity. Furthermore, sequence studies on the NH\(^{-}\)terminal regions of the enzymes indicate that they share a significant degree of sequence homology with porcine kallikrein and crotalase, a kallikrein-like enzyme from Crotalus adamanteus. The main physical difference between the two kallikreins reported here appears to be due to the carbohydrate moieties on the enzymes. At present the in vitro role of venom kallikreins in envenomation pathology is uncertain; however, it is possible that they play an important part in giving rise to the initial symptoms of hypotension and shock.

The Western diamondback rattlesnake (Crotalus atrox) is indigenous to a large area spanning the Southwestern United States. Upon envenomation, there are marked effects on the victim's cardiovascular system, respiratory system, somatic nerve system, and skeletal muscle (1). Since death is a relatively uncommon consequence of envenomation, much emphasis has been placed on research involving the dramatic local effects caused by envenomations such as hemorrhage, myonecrosis, inflammation, edema, and pain (2-7). Another area of focus by investigators has been on the systemic effects of crotalid envenomation. These effects play an important role in giving rise to hemorrhage, hypotension, coagulation, hemolysis, and hemoconcentration. All of these factors serve to produce the overall symptom of crotalid poisoning sometimes called rattlesnake venom shock (8). It is the rapid onset of venom shock which probably plays a major role in prey immobilization and possibly death.

In the past, there have been reports of the hypotensive nature of certain crotalid venoms (9-11) and also of the isolation of kallikrein-like enzymes from viper and crotalid venoms (12-14). It has been proposed that these venom kallikreins along with other hypotensive factors in the venom serve in the production of venom shock (8). In this report, we discuss the isolation from the venom of C. atrox of two proteases with similar structural and functional properties as certain snake and mammalian kallikreins.

**EXPERIMENTAL PROCEDURES AND RESULTS**

Amino Acid Composition and NH\(^{-}\)-terminal Sequence Analysis—Table I contains the amino acid composition of E1 and E11. As can be seen, the compositions of each protein are nearly identical, suggesting homology between the two proteins. The total number of residues/molecule for E1 and E11 based upon the estimated protein fraction of the molecular weights are 216 and 219 residues, respectively.

Fig. 1 shows the NH\(^{-}\)-terminal sequences of E1 and E11 compared to another snake venom protease, crotalase (from Crotalus adamanteus venom), and porcine \(\alpha\) chain kallikrein. In each case, the HPLC analysis of the first Edman degradation cycle on E1 and E11 showed the presence of only one PTH derivative (see Table VI in Miniprint). The NH\(^{-}\)-terminal sequences of E1 and E11 are identical to each other up to residue number 21. A notable degree of sequence homology is also observed between E1, E11, crotalase, and kallikrein (15, 16).

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Kallikrein-like Enzymes from Crotalus atrox Venom

The overall mechanism of the toxic action of rattlesnake poisoning is somewhat unclear due to the complex nature of crotalid venoms. In general, rattlesnake venoms are not strongly neurotoxic with the exceptions being venoms from Crotalus durissus terrificus and Crotalus atrox. This is in sharp contrast to the venoms from Hydrophiidae and Elapidae snakes which are extremely neurotoxic. The primary, initial, overt action of crotalid envenomation seems to be onset of local tissue damage at the site of envenomation. To date, significant progress has been made in the understanding of the mechanism and factors involved in local tissue damage. The initial transient hypotension that is common in rat-}

**DISCUSSION**

The overall mechanism of the toxic action of rattlesnake poisoning is somewhat unclear due to the complex nature of crotalid venoms. In general, rattlesnake venoms are not strongly neurotoxic with the exceptions being venoms from *Crotalus durissus terrificus* and *Crotalus atrox*. This is in sharp contrast to the venoms from Hydrophiidae and Elapidae snakes which are extremely neurotoxic. The primary, initial, overt action of crotalid envenomation seems to be onset of venom shock symptoms such as hypotension and hypovolemic shock, followed by rather considerable local tissue damage at the site of envenomation. To date, significant progress has been made in the understanding of the mechanism and factors involved in local tissue damage. The initial transient hypotension that is common in rattlesnake envenomation is probably due, for the most part, to two types of factors present in the venom; one of the factors is the presence in some crotalid venoms of angiotensin-converting enzyme inhibitors. These interesting peptides act by inhibiting the conversion of angiotensin I to angiotensin II by the converting enzyme and thereby additionally serve to potentiate the pharmacological actions of bradykinin (31).

The other group of important factors involved in hypotension is the kallikrein-like enzymes. Bradykinin has been demonstrated to be released by the proteolytic action of venom kallikreins on bradykininogen in plasma, intestine, uterus, and smooth muscle (31, 32). Additionally, bradykininogen levels have been shown to be decreased following rattlesnake envenomation (9). Prior to this investigation, there have been reports of at least two well characterized snake venom proteases which were identified as having kallikrein-like enzymatic activity. One of these proteases was isolated from the venom of *Vipera ammodytes ammodytes* (12). This kallikrein was shown to be a glycoprotein of molecular weight 34,300. The protease had an isoelectric point of 7.2 and was six times as active as trypsin in releasing a kinin from plasma kininogen. As to whether the kallikrein released was lysyl-bradykinin or bradykinin was not discussed. Another kallikrein-like enzyme called crotalase, which was originally identified as a thrombin-like enzyme, has been isolated from the venom of *C. adamanteus* (13). Some of the distinguishing properties of this enzyme are molecular weight of 32,700, glycoprotein, serine esterase, and inhibition by specific chloromethylketone kallikrein inhibitors (13, 33). This enzyme was shown to make up approximately 0.2% of the crude venom (13).

The two kallikreins (E1 and EII), as we now term the enzymes, isolated from *C. atrox* venom show some similarity to the enzymes mentioned above, particularly crotalase. E1 and EII are also very similar with regard to each other. The amino acid compositions and NH$_2$-terminal amino acid sequences of both enzymes are similar. The conformations of E1 and EII as examined by UV and CD spectroscopy also appear similar to a degree. However, the spectroscopic studies did demonstrate some differences in the fine structure of the two enzymes' conformations. These similarities of native conformations were further demonstrated by tryptic digestion studies on the two native enzymes. The HPLC elution profiles of the digestions were overall notably alike although at least one major difference was observed. As to whether this difference is a result of a slight conformation difference due to different primary structures or different carbohydrate moieties between the two enzymes is at present unclear. However, this study has shown that there are both qualitative and quantitative differences in the carbohydrates present in these enzymes. The effects these may have had on the mapping and spectroscopic studies is uncertain.

The two enzymes share like specificity for releasing the same peptides in identical order from the KS-1, KS-2, and KS-3 substrates. However, when their activities were examined with the chromogenic kallikrein substrates S2266 and S2302, some kinetic differences became evident. The relative reaction rates (normalized against trypsin) of E1 on both substrates were nearly identical whereas EII demonstrated a higher rate with S2266. E1 had nearly identical $K_m$ values with both substrates; however, EII had a larger $K_m$ with the glandular kallikrein substrate S2302 compared to the plasma kallikrein substrate S2266.
to 0.13% and 0.26% for EI and EII, respectively. Finally, EI, EII, crotalase, and porcine kallikrein do possess homologous amino acid sequences in the NH₂-terminal region, and, as more sequence data are reported on both C. atrox kallikreins and crotalase, it is likely further sequence homology will be uncovered.

One very interesting difference between EI and EII and crotalase is the strong thrombin-like activity demonstrated by crotalase whereas no significant clotting ability was observed with either EI or EII. In light of the many similar biochemical properties of EI, EII, and crotalase, far from insignificant is the observed amino acid sequence homology. The comparison of the complete primary structures of EI, EII, and crotalase may well lead to very interesting findings from the proteins’ structures which shed light on the dual enzymatic activities of crotalase and the mechanism of action of EI and EII. Additionally, it is noteworthy that these two crotalid snakes are closely related taxonomically, and there-to uncovered.

One very interesting difference between EI and EII and crotalase is the strong thrombin-like activity demonstrated by crotalase whereas no significant clotting ability was observed with either EI or EII. In light of the many similar biochemical properties of EI, EII, and crotalase, far from insignificant is the observed amino acid sequence homology. The comparison of the complete primary structures of EI, EII, and crotalase may well lead to very interesting findings from the proteins’ structures which shed light on the dual enzymatic activities of crotalase and the mechanism of action of EI and EII. Additionally, it is noteworthy that these two crotalid snakes are closely related taxonomically, and therefore it is not surprising that the two snakes share similar toxins in their venoms. Consequently, in the future, it will also be interesting to examine what similarities and differences the other toxins in the respective venoms possess and their effects in the overall pathological nature of the venoms.

Finally, the isolation of kallikrein enzymes from crotalid venoms further emphasizes that one of the important aspects of crotalid poisoning is the hypotensive venom shock which occurs almost immediately following envenomation. It is this aspect of envenomation which probably plays an important, immediate role in debilitating the victim, which is then followed by the local effects at the site of envenomation. Further investigation of these and other kallikrein-like enzymes in crotalid venoms may lead to a more complete understanding of the nature of crotalid envenomation.

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**Method**

The Tris PAGE stacking gel buffer. The pooled fractions of the samples acetate buffer pH 5.6. 0.5% NaCl, 0.1% CdCl₂ for 40h at 37°C. Control digests in 500 μl of 5.7N HCl containing methanol acetic acid solution. All polyacrylamide gels were run with analytical grade.

**Results**

The activity of the two proteases was inhibited by 5% TAME 8 µM. E₂ was assayed spectrophotometrically. The spectrophotometric reading was measured at 25°C with a Cary model 11 UV/vis spectrophotometer.

**Discussion**

The proteases were digested in 1 ml of 10M TAME, pH 8.5, 2M CaCl₂ at a concentration of 1mg/μl. Trypsin (VSLK) was added to the digestion mixture to give a ratio w/w of 1:1. The digestion mixture was then dialyzed at 37°C. Aliquots of 100 μl were taken at timed intervals and then assayed with a polyclonal anti-tryptic antibody to a p/a of 2.8. The aliquots were then inserted into a Novex Mini-PROTEAN apparatus. The gels were developed with a w/v buffer system A, 0.1% TFA in 80% TFA in acetonitrile. A linear gradient in buffer B was produced 14%ac/m with a Beckman 115i liquid chromatograph.

**UDP-GlcNAc Specificity**

The proteases were each digested in 10M TAME buffer, pH 8.5, 2M CaCl₂ at a concentration of 1mg/μl. The protease dialysates were then assayed for their ability to produce substrate peptide at a concentration of 1mg/μl. All aliquots were incubated at 37°C with 0.1% Trypsin. The second digest was then analyzed by HPLC for the presence of digestion products.

**Protein and Carbohydrate Analysis**

The two proteases were each digested in 10M TAME buffer, pH 8.5, with 0.1% Trypsin. The digests were then assayed for their ability to produce substrate peptide by HPLC for the presence of digestion products.

**Sequence Analysis of E₁ and E₂**

The amino terminal primary structure analyses were performed on E₁ and E₂ with a Beckman 125. The two proteases were digested in 10M TAME buffer, pH 8.5, with 0.1% Trypsin. The digests were then analyzed by HPLC for the presence of digestion products.

**Molecular Weights**

The molecular weights of the two proteases were estimated by SDS-PAGE using a 12% gel. The proteases were digested in 10M TAME buffer, pH 8.5, with 0.1% Trypsin. The digests were then analyzed by HPLC for the presence of digestion products.

**Isolation**

The total amount of the two proteases was determined by polyacrylamide gel electrophoresis with and without SDS, until and also by spectrophotometry. The Tris PAGE stacking gel was 2.5% cross-linked with a p/a of 7.75. The resolving gel was 7.5% cross-linked with a p/a of 8.5. The Tris-acetate buffer was carried out with increasing concentrations of the proteases in order to detect the presence of any faint bands. Electrophoresis was performed according to the method of Walsh et al. (21). The proteases were digested in 10M TAME buffer, pH 8.5, with 0.1% Trypsin. The digests were then analyzed by HPLC for the presence of digestion products.

**UDP-GlcNAc Specificity**

The proteases were each digested in 10M TAME buffer, pH 8.5, 2M CaCl₂ at a concentration of 1mg/μl. The protease dialysates were then assayed for their ability to produce substrate peptide at a concentration of 1mg/μl. All aliquots were incubated at 37°C with 0.1% Trypsin. The second digest was then analyzed by HPLC for the presence of digestion products.
Kallikrein-like Enzymes from Crotalus atrox Venom

Fig. 2. Fractionation of Peak A-2 on Sephadex G-75-42. Column (1.5 x 95cm) was equilibrated and developed with 50mM Tris buffer, pH 8.5 containing 0.1M NaCl and 2M CaCl2. Fraction size was 6.0ml/tube and the flow rate was 30m1/h. E-2 = 2 x 2 TAME activity (units/ml). Chromatograph represents 1 of 3 runs of A-2 pool.

Fig. 3. Fractionation of Peak A-2 on DE-32. Column (1.5 x 20cm) was equilibrated with 10mM Tris buffer, pH 8.5 containing 450mM NaCl and 2M CaCl2. Column was developed with a linear gradient of the equilibration buffer and 1M acetic acid (300ml in each reservoir). Gradient was started at fraction 10. Fraction size was 6.0ml/tube and the flow rate was 30ml/h. Vertical dashed lines indicate fractions pooled. (See Table II for peak activities).

Fig. 4. Fractionation of Peak C-1 on DE-32. Column (1.5 x 40cm) was equilibrated with 10mM Tris buffer, pH 8.5 containing 2M CaCl2 and 450mM NaCl. Column was developed with a linear salt gradient of 0.5M to 0.1M NaCl in equilibration buffer. Fraction size was 6.0ml/tube and flow rate was 4ml/h. Dashed horizontal line depicts salt gradient; vertical dashed line depicts fractions pooled. Indicates TAME activity of pooled peak fractions.

Fig. 5. Rechromatography of peak D-2 on DE-32. Conditions same as Fig. 4.

Fig. 6. Rechromatography of peak D-3 on DE-32. Conditions same as Fig. 4.

Table II

| Fraction | Total Protein (mg) | Total Activity (Units) | Specific Activity (Units/mg) | Recovery of Activity (% of Initial) | Fold Purification Based on Activity Against TAME |
|----------|--------------------|------------------------|-----------------------------|---------------------------------|---------------------------------------------|
| Crude venom | 20,000             | 24,800                 | 1.2                         | 100.0                           | 1.0                                         |
| A-1       | 6,200              | 23,035                 | 3.8                         | 91.3                            | 3.0                                         |
| B-2       | 1,260              | 17,130                 | 13.6                        | 69.1                            | 11.0                                        |
| C-1       | 319                | 7,146                  | 22.4                        | 28.8                            | 18.1                                        |
| D-2       | 68.4               | 3,755                  | 47.9                        | 13.2                            | 38.6                                        |
| D-3       | 45.0               | 2,106                  | 46.8                        | 8.5                             | 37.7                                        |
| EI        | 51.3               | 2,821                  | 51.1                        | 10.6                            | 41.2                                        |
| EII       | 26.1               | 1,255                  | 48.1                        | 5.1                             | 38.8                                        |

Fig. 7. SDS-polyacrylamide electrophoresis of EI and EII. Gel conditions in text. CV is crude venom (50.g); EI and EII (10.g).
Kallikrein-like Enzymes from Crotalus atrox Venom

Fig. 8. SDS-polyacrylamide electrophoresis of EI and EII following neuraminidase digestion. Gel condions in text. Lane S: molecular weight standards from top to bottom, bovine albumin, egg albumin, tryptophan, B-lactoglobulin, and lysozyme. Lane A: EI + incubation mixture of EII and neuraminidase. Lane B: EII + incubation mixture of EI and neuraminidase. Lane C: incubation mixture of EI and neuraminidase. Lane D: incubation mixture of EI and neuraminidase. Lane E: EII. Lane F: EII. All samples contained 30μg of the proteins. The high molecular weight band seen in lanes A, B, C, and D is neuraminidase.

Table III. Carbohydrate Analysis of EII and EIIa

| Carbohydrate | EI (mol carbohydrate/mo1 enzyme) | EII (mol carbohydrate/mol enzyme) |
|--------------|----------------------------------|-----------------------------------|
| Fucose       | 1.8                              | 5.8                               |
| Mannose      | 4.9                              | 1.9                               |
| Galactose    | 3.2                              | 11.7                              |
| N-ac-Glucosamine | 6.3                       | 1.6                               |
| Sialic Acid  | 0.1                              | 0.5                               |

a Determined according to the method of Tomans et al. (1974).

Fig. 9. HPLC peptide map of EI and EII. Tandem digestion of EI and EII by trypsin (conditions in text) were analyzed by HPLC on a a Bondapak C18 column (0.39 x 30cm). Buffer system used was: A. 0.1% TFA in H2O B. 0.14% TFA in CH3CN. A linear gradient in B was applied from 0 to 100% over 60 min at a flow rate of 1ml/min.

Pepptide Mapping of EI and EII - Figure 9 shows the HPLC elution profiles of the tandem digests of EI and EII by trypsin. The elution profiles following 8h digestion times for both EI and EII appear somewhat similar with the most readily discernable difference observed in the heights of peaks a and b for EII and EI respectively. This difference remains in the 24h diges-
tions. Additionally, there appears to be more fine structure in the EI 24h profile in the 10 to 20 minute elution time range than in the EII 24h pro-

Kinetic Analysis of EI and EII - The km and relative reaction rates of the enzymes with substrates S2266 and S302 are shown in Table IV. The km values for EI with S2266 and S302 were approximately the same whereas EII showed a difference with km values of 2.8 x 10^-7 and 9.9 x 10^-7 mol/L with S2266 and S302 respectively. Similar results were seen in the relative reaction rates of EI and EII. EII showed similar relative reaction rates normalized against the trypsin rate with S2266 and S302 whereas the reaction rates of EII with the two substrates were different. Both enzymes were demonstrated to have reaction rates with the two chromogenic substrates notably greater than those of either trypsin or plasmin.

Table IV

| Enzyme | Substrates | S2266 | S302 |
|--------|------------|-------|------|
|EI      | B-Val-Leu-Arg-pNA | 4.2 ± 1.3 | 4.2 ± 1.2 |
|EI      | B-Arg-Pro-Arg-pNA | 2.6 ± 1.2 | 6.9 ± 0.3 |
|EI      | S2266       | 54.1 ± 0.5 | 59.0 ± 0.4 |
|EI      | S302        | 29.7 ± 0.3 | 13.6 ± 0.3 |
|EI      | Plasmin     | 1.4 ± 0.08 | 1.3 ± 0.08 |
|EI      | Trypsin     | 1.0     | 1.0   |

a All experiments performed in triplicate.
b km values are mol/L x 10^-7.
c Reaction rates normalized against trypsin values. [S] = 0.2mM.

Hydrolytic Activity of EI and EII on Proteins and Peptide Substrates - Neither EI or EII noticeably cleaved either the A or B oxidized chains of insulin or glycogen within incubation times of up to 4h. This is indicative of the strict substrate requirements of these enzymes.

Both EI and EII showed the same sites of cleavage on the kallikrein substrates KS1, KS2, and KS3. In the case of KS3, the only peptide bond to be cleaved was demonstrated by analysis of the digestion products was the Arg1-Arg4 peptide bond (Fig. 10). Cleavage was complete (est. 98%) within 60 min. KS2 was completely cleaved (est. 95%) at one specific site within 120 min. The KS-2 cleavage site was the Gly1-Arg4 peptide bond (Fig. 10).

Sites of Action of EI and EII on Kallikrein Model Substrates

KS1: Arg-Val-Asn-Lys-Leu-Arg-Pro-Arg-Lys-Pro-Arg-His-Pro-Phe-Pro-Arg-Lys-Arg-pNA
KS2: Arg-Val-Asn-Lys-Leu-Arg-Pro-Arg-Lys-Pro-Arg-His-Pro-Phe-Pro-Arg-Lys-Arg-pNA
KS3: Arg-Val-Asn-Lys-Leu-Arg-Pro-Arg-Lys-Pro-Arg-His-Pro-Phe-Pro-Arg-Lys-Arg-pNA

Fig. 10. Sites of bond cleavage of a kallikrein model substrates by EI and EII. Arrow denote peptide bonds cleaved. In KS1 the Arg5-Val14 bond was cleaved prior to the Lys12-Arg15 bond.
The kallikrein analog KS-1 was cleaved at two sites by E1 and EII. The following is the description of E1 digestion of KS-1. At 9h incubation time the major peak at approximately 45 min was identified by amino acid analysis as the intact KS-3 peptide (Figure 11 and Table VI). At 30 min the intact KS-1 is no longer present; however, four new peaks appeared. From amino acid analysis of the four digestion peptides, identifications were made (Table VI). Peak 1 was the KS-1 terminal peptide Ac-Arg-Lys-Met-Lys-Arg-Pro-Gly-Der-Pro-The-Arg- resulting from the bond cleavage between -Arg-Lys- Met-Lys-Arg-Pro-Gly-Der-Pro-The-Arg-. Peak 2 was identified as the carboxyl-terminal fragment from the first cleavage site -Arg-Lys-Met-Lys-Arg-Pro-Gly-Der-Pro-The-Arg-. In the 40 and 120 min digests one can see that the peak 1 peptide is being digested to yield two new fragments located at peaks 3 and 4. Amino acid analysis identified peak 4 as the C-terminal peptide -Arg-Pro-Gly-Der-Pro-The-Arg- from the peak 1 peptide resulting from the bond cleavage at Lys-Arg-. Peak 3 was identified as the major terminal fragment of the peak 1 peptide. No further digestion was detected after 120 min. Both enzymes appeared to cleave the two peptide bonds of KS-3 in the same order and with nearly identical rates.

![Fig. 11. HPLC of timed digestions of kallikrein analog KS-1 by EII. Peptides were analyzed by HPLC under conditions identical to those described in Fig. 9. Description of peaks given in text.](image)

| Table VI: Amino Acid Composition of KS-1 Digestion Products by E1 |
|----------------|------------------|----------------|------------------|------------------|------------------|
| Peptides       | 1    | 2    | 3    | 4    | KS-1 |
| Residues       |      |      |      |      |      |
| Arg             | 1.4(2)| 1.4(2)| 0.6(1)| 0.7(1)| 3.4(4) |
| Thr             | 2.6(3)| 2.6(3)| 1.8(1)| 1.8(1)| 3.1(1) |
| Ser             | 1.2(1)| 1.2(1)| 1.1(1)| 1.1(1)| 2.2(1) |
| Tyr             | 1.7(2)| 1.7(2)| 1.7(2)| 1.7(2)| 3.4(4) |
| Cys             | 0.8(1)| 0.8(1)| 0.8(1)| 0.8(1)| 1.6(2) |
| Met             | 1.1(1)| 1.1(1)| 1.1(1)| 1.1(1)| 2.2(2) |
| Leu             | 2.9(1)| 2.9(1)| 2.9(1)| 2.9(1)| 5.7(2) |
| Ile             | 1.9(1)| 1.9(1)| 1.9(1)| 1.9(1)| 3.8(2) |
| Val             | 1.0(1)| 1.0(1)| 1.0(1)| 1.0(1)| 2.0(2) |
| Ala             | 0.9(1)| 0.9(1)| 0.9(1)| 0.9(1)| 1.8(2) |
| Thr             | 1.9(1)| 1.9(1)| 1.9(1)| 1.9(1)| 3.8(2) |
| Total Residues  | 13   | 13   | 13   | 13   | 26 |

*Subsequently time of 28-38 minutes. All entries are expected theoretical values. Where no values are shown, the amino acid was not detected or present at an amount too low to quantitate.

Aprotinin and PMSF were both able to inhibit all cleavage of KS-1 by either E1 or EII. EDTA did not affect the digestion of KS-1 by E1 or EII. Neither E1 nor EII demonstrated any thrombin-like activity when compared with trypsin, plasmin, or the crude venom in the fibrinolysis assay. Also both enzymes were unable to produce typical fibrin clots in the thrombin assay. However, after extended periods of time (48h) very small, typical clots were sometimes observed. The mechanism of production of the typical clots is unknown, therefore the thrombin-like activity of E1 and EII was considered to be negligible.

Spectroscopic Analysis of E1 and EII - The UV spectra of both E1 and EII appear to be identical in peak positions, intensities, and fine structure (Fig. 11, presented on CD).

The CD spectra for E1 and EII do appear to have some minor differences. In the peptide region (Figure 11), the E1 spectrum has minima at 211 and 220nm with corresponding mean residue weight ellipticities of -690 and -617 respectively. In the case of EII a minimum is observed at 212nm and a broad minimum at 226nm with mean residue weight ellipticities of -695 and -617 respectively. Both spectra appear to represent proteins with predominantly helical structure (29, 30). However, as apparent from their CD spectra, the conformations of both proteins do differ to some small extent.

![Fig. 12. Circular dichroism spectra of E1 and EII in the peptide region.](image)
Kallikrein-like Enzymes from Crotalus atrox Venom

![Circular dichroism spectra of E1 and E11 in the aromatic region. Buffer and temperatures same as in Fig. 11. Molar ellipticities \([\theta]_M\) are based upon molecular weights of 27,500 and 29,200 for E1 and E11 respectively.](image)

Table VI: Edman Degradation of Amino Terminal Region of E1 and E11

| Cycle | E1 | E11 | E1 | E11 | Yield (mol) |
|-------|----|-----|----|-----|-------------|
| 1     | His | His | 7.5| 6.8 |             |
| 2     | Val | Val | 7.1| 6.7 |             |
| 3     | Gly | Gly | 7.0| 6.2 |             |
| 4     | Gln | Gln | 5.9| 5.6 |             |
| 5     | Asp | Asp | 5.3| 5.6 |             |
| 6     | Gln | Gln | 5.1| 5.4 |             |
| 7     | CH-OH | CH-OH | 5.0| 5.2 |             |
| 8     | Asp | Asp | 5.0| 5.1 |             |
| 9     | Ile | Ile | 5.4| 5.4 |             |
| 10    | Asp | Asp | 5.3| 5.1 |             |
| 11    | Gln | Gln | 5.4| 5.0 |             |
| 12    | His | His | 5.7| 5.6 |             |
| 13    | Gln | Gln | 5.4| 5.0 |             |
| 14    | Ser | Ser | 4.0| 4.0 |             |
| 15    | Ser | Ser | 4.0| 4.0 |             |
| 16    | Val | Val | 4.7| 4.5 |             |
| 17    | Ala | Ala | 4.1| 4.0 |             |
| 18    | Ile | Ile | 4.1| 4.1 |             |
| 19    | Phe | Phe | 2.8| 2.5 |             |
| 20    | Val | Val | 2.8| 2.5 |             |
| 21    | Thr | Thr | 2.2| 2.2 |             |
| 22    | Thr | Thr | 2.2| 2.2 |             |
| 23    | Gln | Gln | 2.1| 2.1 |             |
| 24    | Phe | Phe | 2.1| 2.1 |             |
| 25    | Phe | Phe | 2.1| 2.1 |             |

*Initial yields of E1 and E11 were 73.2% and 67.8% respectively. The average repetitive yields of E1 and E11 were 95.7% and 97.3% respectively.*
Kallikrein-like enzymes from Crotalus atrox venom.
J B Bjarnason, A Barish, G S Direnzo, R Campbell and J W Fox

J. Biol. Chem. 1983, 258:12566-12573.

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