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KALLIKREIN-LIKE AND THROMBIN-LIKE PROTEASES FROM THE VENOM OF JUVENILE NORTHERN PACIFIC RATTLESNAKES *(CROTALUS VIRIDIS OREGANUS)*

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ABSTRACT

Venoms of juvenile and adult northern Pacific rattlesnakes (*Crotalus viridis oreganus*) contain numerous proteolytic enzymes. A thrombin-like enzyme (34 kD) and a kallikrein-like enzyme (31 kD) were purified from juvenile rattlesnake venom and shown to have high activity toward BzPheValArg-pNA and BzProPheArg-pNA, respectively. Neither were inhibited by EDTA and both showed ~34-fold greater activity toward the preferred substrate than toward 10 other pNA derivatives. The thrombin-like enzyme was inhibited directly by heparin, and the kallikrein-like enzyme was partially inhibited by aprotinin and completely inhibited by the active site affinity label PPACK. The thrombin-like enzyme produced a non-hydrolyzed clot when incubated with fibrinogen, and SDS-PAGE analysis of a digestion time revealed no secondary degradation of the three subunits. The thrombin-like and kallikrein-like proteases likely contribute to defibrination syndrome and hypotension often accompanying rattlesnake bites, and unlike crotalase, the activities in *Crotalus viridis oreganus* venom reside on separate molecules which can be separated from one another.

INTRODUCTION

Rattlesnake venoms are complex biological products which induce numerous profound physiological changes when injected into an animal (Hawgood, 1982). Crotalid venoms in general are a rich source of proteolytic enzymes with varying specificities (Tu, 1982). Some appear to be non-specific metalloproteases (e.g., Bjarnason and Fox, 1983; Kurecki and Kress, 1985; Mackessy, 1985, 1993), hydrolyzing many substrates, while others are much more specific, hydrolyzing, for example, arginine esters and amides (Schwartz and Bieber, 1985; Samel et al., 1987) but not general protease substrates such as casein. The latter enzymes are of particular interest due to their effects on various components of the hemostatic system (Markland, 1983; Kornalik, 1986). Proteases with thrombin-like activity (Markland and Damus, 1971; Selistre and Giglio, 1987) and kallikrein-like activities (Markland et al., 1982; Schwarz and Bieber, 1985; Ohtani et al., 1988) appear to be common features of crotalid venoms and are of clinical importance. Thrombin-like proteases from snake venoms may be useful in human medicine by controlling some thrombotic disorders, because an *in vivo* action is defibrination (Markland and

Pirkle, 1977). One of these, batroxobin (from *Bothrops* venoms) has been cloned and sequenced (Itoh et al., 1987, 1988).

One of the actions of kallikrein, a serum serine protease, is the liberation of a nonapeptide, bradykinin is a potent vasodilator and increases vascular permeability (Roche e Silva et al., 1949; Komori and Sugihara, 1988), and the venom enzyme liberating this peptide may be clinically useful in treating certain types of hypertension. Venoms from crotalid and viperid snakes usually contain both of these activities, and a related toxin from beaded lizard (*Heloderma horridum*) venom, a serine protease, has recently been sequenced (Utaisincharoen et al., 1993).

Venoms from juvenile and adult *Crotalus viridis oreganus* have been shown to contain several proteases capable of hydrolyzing fibrinogen, and some of these promote clot formation (Mackessy, 1993). In the present study, kallikrein-like and thrombin-like proteases were isolated from juvenile *C. v. oreganus* venom, and unlike crotalase from *C. adamanteus* venom, the two activities resided in two separate enzymes. These enzymes contribute to specific facets of envenomation, and the biological role of the serine proteolytic enzymes to prey capture and handling are discussed.

MATERIALS AND METHODS

Venoms and Reagents

Adult (<350 mm total length) snakes. Venoms were extracted manually by gently massaging the glands, quick-frozen with dry ice/methanol, lyophilized, and stored at -20°C with desiccant until used.

Peptide paranitroaniline (pNA) derivatives were purchased from U. S. Biochemical Corp. and Sigma Chemical Co. Electrophoresis reagents were obtained from BioRad Laboratories. Casein yellow was purchased from CalBioChem. DEAE-Sephacel, Sephadex G-75 (fine) and Sephadex G-200 (fine) were obtained from Pharmacia LKB. Porcine heparin was from Bel-Mar Labs, Inc. Molecular weight standards, aprotinin (A4529), human fibrinogen (type I) and all other biochemicals (analytical grade) were purchased from Sigma Chemical Co.

Enzyme Assays

Phosphodiesterase activity was assayed by the method of Bjork (1963) using 25 μl column effluent. L-amino acid oxidase activity was assayed by the method of Weissbach et al. (1960) using 25 μl column effluent. Caseinolytic activity was assayed as described (Mackessy, 1985) using 10 μl column effluent (ion exchange fractions - adult venom) or 25 μl column effluent (ion exchange fractions - juvenile venom; gel filtration fractions).

Amidolytic activity toward the paranitroaniline-derived peptides was assayed using a method similar to that of Stocker et al. (1986). The standard reaction mixture for column effluent assays included 20 μl effluent, 25 μg substrate and 655 μl 0.1 M HEPES buffer pH 7.8 @ 37°C. Substrates were dissolved initially in DMSO and brought to 0.5 μg/μl with HEPES buffer (total DMSO = 1%). Incubation was at 37°C (after vortexing) for 6 min and was terminated by the addition of 75 μl 50% acetic acid. Absorbance was read at 405 nm and readings were stable for at least 1 hr.

For substrate preference assays utilizing pNA derivatives, 10 μg protease in 475 μl HEPES buffer was added to 50 μl buffer containing $CaCl_2$ and $MgCl_2$ (final concentration of each: 0.5 mM). Tubes were incubated at 37°C for 3 min and 150 μl substrate (0.5 μg/μl 0.1 M HEPES pH 7.8 containing 1% DMSO) was added, tubes vortexed and incubated at 37°C for 3 min. 75 μl 50% acetic acid terminated the reaction. Paranitroaniline derivates used, together with the enzyme for which they were developed as a substrate, are listed in Table 1.

Inhibition assays contained the same volumes but metal ions were omitted; inhibitors at the appropriate concentrations and 10 μg enzyme (total volume: 525 μl) were allowed to stand at room temperature (21°C) for 30 min before incubating and assaying as above.

For pH profiles, 10 μg enzyme in 0.9% NaCl (50 μl) was added to 475 μl of various Good buffers and incubated at 37°C for 3 min. Substrate (150 μl in 1% DMSO) was then added, hydrolysis proceeded for 3 min and was measured as in substrate preference assays. Substrates (0.5 μg/μl) were α-N-Bz-L-Pro-L-Phe-L-Arg-pNA (ProPheArgpNA) for juvenile kallikrein-like protease and α-N-Bz-L-Phe-L-Val-L-Arg-pNA (PheValArgpNA) for thrombin-like protease. Buffers (0.1 M) used were: sodium acetate, pH 5.0; MES, pH 5.5 and 6.0; PIPES, pH 6.5 and 7.0; HEPES, pH 7.4, 7.6, 7.8 and 8.0; TAPS, pH 8.5; and CHES, pH 9.0, 9.5 and 10.0.

For kinetic measurements, 10 μg enzyme in 100 μl HEPES buffer pH 7.8 was added to varying amounts of buffer containing metal ions (as above) within a 1.0 cm cuvette at 21°C. Measurements

Table 1. Paranitroaniline derivatives used in substrate specificity studies.

NITROANILIDE DERIVATIVE	POTENTIAL SUBSTRATE FOR:
α-N-Bz-L-Pro-L-Phe-L-Arg-pNA	Kallikrein
α-N-Bz-L-Phe-L-Val-L-Arg-pNA	Thrombin
CBZ-L-Arg-pNA	Arginine Esterase
N-α-Bz-DL-Arg-pNA	Trypsin
Succ-L-Ala-L-Ala-L-Ala-pNA	Elastase
N-MeO-Succ-L-Ala-L-Ala-L-Pro-L-Val-pNA	Leukocyte Elastase
CBZ-Gly-Gly-L-Leu-pNA	Subtilisin
Gly-L-Phe-pNA	Cathepsin C
Gly-L-Pro-pNA	Dipeptidyl Peptidase
γ-L-Glu-pNA	γ-Glutamyl-Transpeptidase

were made for the kallikrein-like protease using ProPheArgpNA and for the thrombin-like protease using PheValArg-pNA. An appropriate amount of substrate was added to give a total volume of 675 μl. Reactions were followed at 405 nm for 3 min, and the linear portion of curves (between 30-45 sec) were used to estimate rate of product formation (v, in mM/min/mg enzyme). The extinction coefficient used for paranitroaniline at 405 nm was 10,300 (Stocker et al., 1986), and the double reciprocal method of Lineweaver and Burke (1934) was used to plot data and estimate the Michaelis constant, Km, a measure of enzyme affinity for the substrate, and the maximum rate constant, Vm ($=k_{cat}/E_e$).

Isolation of Venom Enzymes

Ion Exchange Chromatography

31 mg of juvenile venom (first venom samples taken from each of nine littermates) were dissolved in 3.0 ml 10 mM tris-HCl buffer pH 8.2 @ 5°C and applied to a 1.5 cm x 30 cm column of DEAE Sephacel. The column was washed with starting buffer at a flow rate of 12 ml/hr, 4 fractions/hr, established with a peristaltic pump. A 0-0.5 M NaCl linear gradient was started (as described above) at fraction 104. Caseinolytic, L-amino acid oxidase and phosphodiesterase activities were located as described below. Fractions 127-134 contained thrombin-like activity and were combined, dialyzed, and lyophilized. Fractions 145-160 contained kallikrein-like activity and were combinedd, dialyzed, and lyophilized.

A. Isolation of thrombin-like protease

The protease peak containing thrombin-like activity (fractions 127-134) was redissolved in 1.5 ml 50 mM tris-HCl pH 8.0 and applied to a 1.5 cm x 75 cm Sephadex G-75 gel filtration column. Flow rate was 4.8 ml/hr with four fractions collected/hr. Fractions 50-60 containing only thrombin-like activity were combined, dialyzed and lyophilized, and used for further studies.

B. Isolation of kallikrein-like protease

The protease peak containing kallikrein-like activity (fractions 145-160) was redissolved in 2.5 ml 50 mM tris-HCl pH 8.0 and applied to the Sephadex G-75 gel filtration column at a flow rate of 5.2 ml/hr with four fractions collected/hr. Fractions 48-59 containing only kallikrein-like activity were combined, dialyzed and lyophilized, and used for further studies.

Electrophoresis

SDS-polyacrylamide gel electrophoresis of β-mercaptoethanol treated and untreated venom proteins was performed in 10% total acrylamide gels (0.75 mm thick) as described by Hames and Rickwood (1985). Stacking voltage was 50 V for ~1 hr followed by separation at 300 V for ~3 hr.

Fibrinogenolytic activity of the thrombin/kallikrein-like preparation was examined using the method of Ouyang and Huang (1979) with human fibrinogen. A 9% total acrylamide gel was used to follow digestion of fibrinogen and electrophoresis was performed using 3 μl/well.

RESULTS

Fractionation of 31 mg juvenile venom resolved ~9 peaks with absorbance

at 280 nm; caseinolytic activity was evident in 4 of these (Fig. 1A). L-amino acid oxidase and phosphodiesterase activities were located but were not further characterized. A thrombin-like protease coeluted with a caseinolytic protease at ~0.15 M NaCl, and a kallikrein-like protease coeluted with another caseinolytic protease at ~0.21 M NaCl.

Gel filtration of the thrombin-like peak (fractions 127-134) completely separated caseinolytic and thrombin-like activities (Fig. 1B), and the two proteases were dialyzed and lyophilized. The thrombin-like protease (Mr ~33.5 kD) showed high activity toward the thrombin substrate ProPheArgpNA and very low activity toward ProPheArgpNA (kallikrein substrate; see Fig. 2) and BAPNA (a trypsin substrate; Table 2); none of the other pNA substrates tested (Table 1) were hydrolyzed. A pH profile for the thrombin-like enzyme is shown in Fig. 3; maximum activity was observed at pH 8.0. Kinetic analyses of enzyme activity toward BzPheValArgpNA gave a Km value of 222 μM and a Vm value of 1,143 μmol/min/mg enzyme (Fig. 4). This protease was directly inhibited by porcine heparin (Fig. 5) but not EDTA.

Gel filtration of the lyophilized kallikrein-like peak (fractions 145-160) separated caseinolytic and kallikrein-like activities (Fig. 1C), and the two proteases were dialyzed and lyophilized (kallikrein-like: fractions 48-59; caseinolytic: 34-44). Relative molecular weight (determined electrophoretically) was 31,000 for the kallikrein-like protease. The kallikrein-like protease had a somewhat broader pH profile than the thrombin-like protease, and it showed a pH optimum of ~8.5 (Fig. 6).

Activity toward ProPheArgpNA was used to determine kinetic constants for the kallikrein-like enzyme (Fig. 7). The Michaelis constant, Km, determined by extrapolation, was 13.0 μM, and the maximum rate constant, Vm, was 1,130 μmol/min/mg enzyme. The active site inhibitor PPACK (Fig. 8) showed high affinity for this enzyme, and approximately 100 nM PPACK resulted in complete inhibition of the kallikrein-like protease (~0.5 nM) within one minute (Fig. 9).

DISCUSSION

Venoms from juvenile and adult *Crotalus viridis oreganus* have been shown to contain different proteases which can be distinguished on the basis of gel filtration and action on bovine fibrinogen (Mackessy, 1993). These analyses were extended in the present study to determine more specifically the types of peptide bonds preferentially hydrolyzed as a means of inferring the probable activity of these proteases.

Venoms from *Crotalus viridis oreganus* contained several proteases with a high degree of substrate specificity, as judged by their actions on paranitroaniline-peptide derivatives. The thrombin-like protease showed a narrow pH preference (using PheValArg-pNA) and, like most of the *C. v. oreganus* proteases, it is active at neutral to somewhat alkaline pH (optimum pH = 8.0). The molecular weight estimate for the enzyme was 33,500, similar to those of other thrombin-like enzymes such as ancrod from *Calloselasma rhodostoma* venom (35 kD; Esnouf and Tunnah, 1967), gyroxin from *C. durissus terrificus* venom (33-35 kD; Alexander et al., 1988) and crotalase (33 kD; Markland and Damus, 1971). The *C. v. oreganus* protease caused clot formation with human fibrinogen which was not dissolved, and no significant degradation products were noted on SDS-

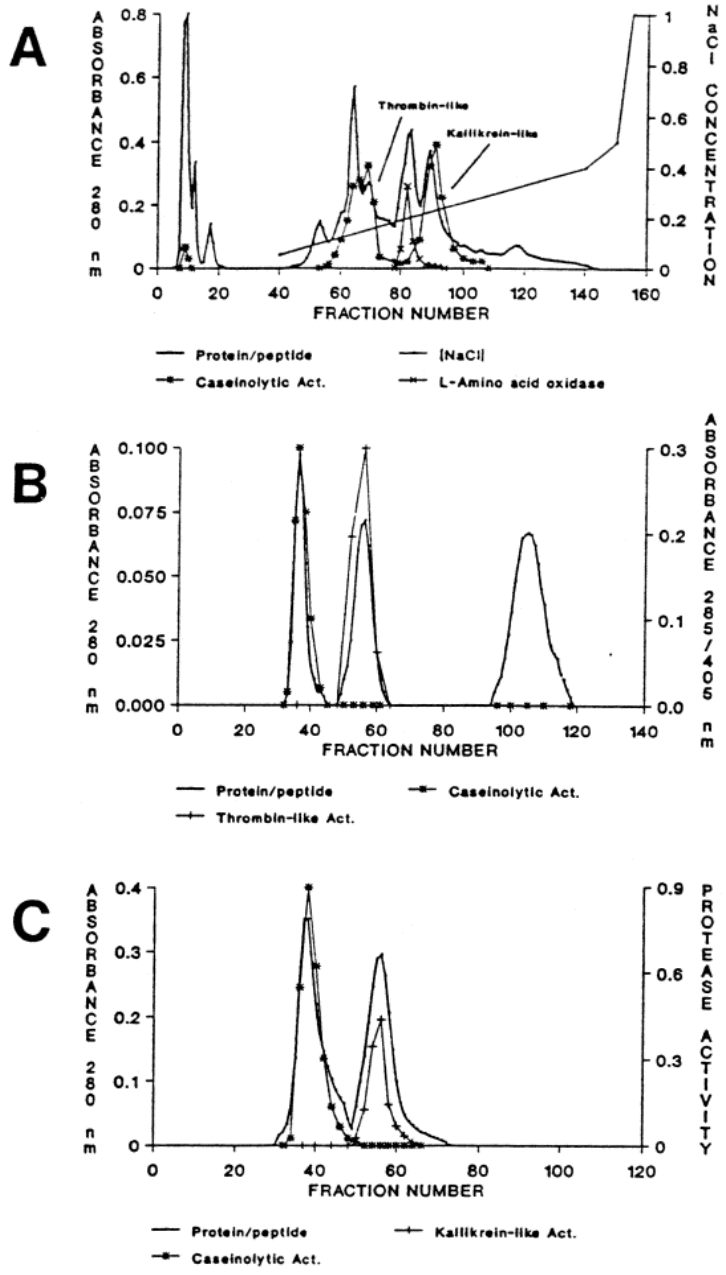


Figure 1. A. Elution profile of 31 mg of juvenile venom from *Crotalus viridis oreganus* fractionated on a 1.5 cm x 30 cm DEAE Sephacel ion exchange column. B. Elution profile of combined fractions from the thrombin-like protease peak chromatographed on a 1.5 cm x 75 cm Sephadex G-75 gel filtration column. Thrombin-like activity was completely separated from caseinolytic activity. C. Elution profile of combined fractions from the kallikrein-like protease peak chromatographed on a 1.5 cm x 75 cm Sephadex G-75 gel filtration column. Kallikrein-like activity was also separated from caseinolytic activity.

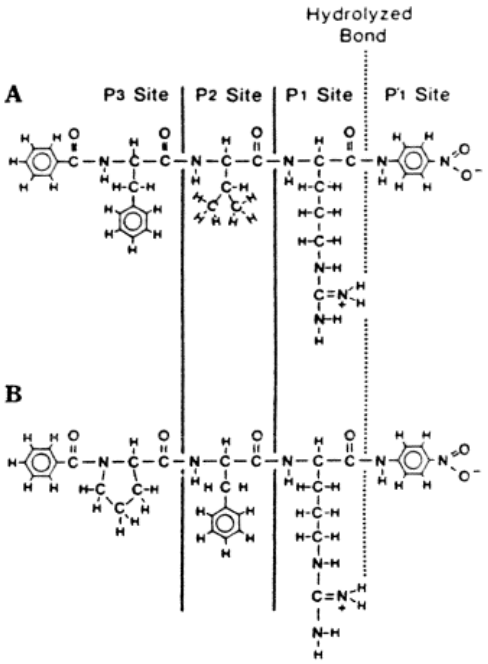


Figure 2. Two-dimensional structures of the thrombin and kallikrein paranitroaniline-derived substrates. Potential recognition sites for the protease molecules are indicated as P sites. Hydrolysis of the amide bond released the chromophore, allowing the reaction to be followed spectrophotometrically.

Table 2. Relative substrate preference of several venom proteases. No other paranitroaniline substrates were hydrolyzed by these enzymes. Relative preferences are given as percentages of the highest activity shown.

	PROTEASE	
pNA SUBSTRATE	Kallikrein-like	Thrombin-like
PheValArg	3.4	100.0
ProPheArg	100.0	3.0
GlyGlyLeu	0	0
SuccAlaAlaProVal	4.6	0
BAPNA	0	2.0

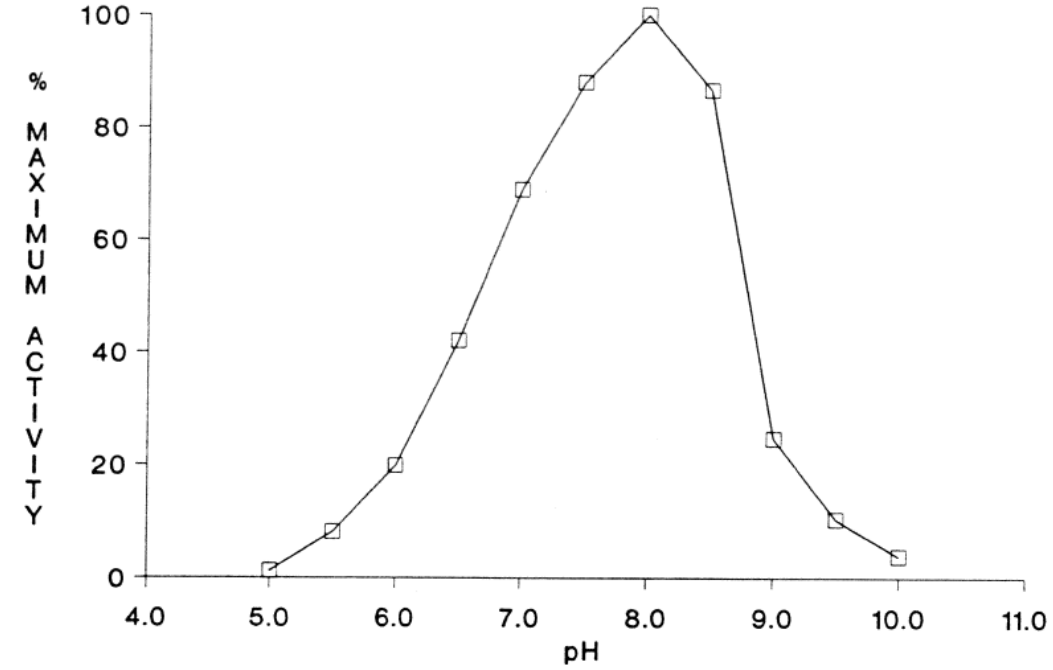


Figure 3. pH profile for the hydrolysis of α-N-Bz-L-Phe-L-Val-L-Arg-pNA by the adult venom thrombin-like protease. % maximal activity is based on highest activity observed (at pH 8.0).

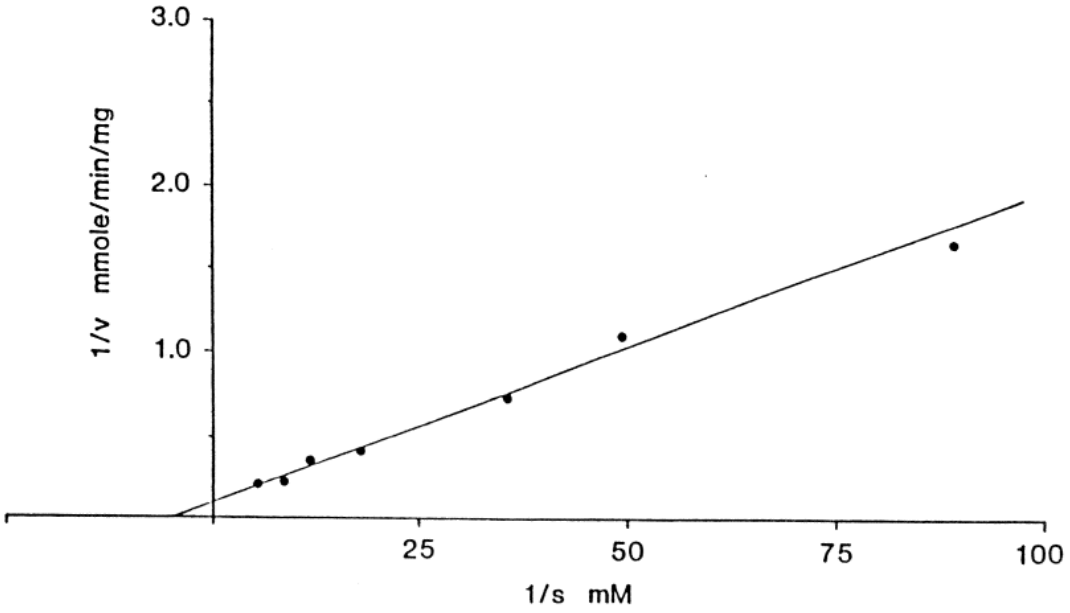


Figure 4. Double reciprocal plot of rate vs. substrate concentration for the hydrolysis of -N-Bz-L-Phe-L-Val-L-Arg-pNA by juvenile thrombin-like protease. Estimated kinetic constants were: Km = 222 μM; Vm = 1,143 μmol/min/mg.

PAGE. The venom protease (amidase activity) was also directly inhibited by heparin, in contrast to thrombin, which is believed to be primarily inhibited by heparin via its acceleration of the action of antithrombin III in serum (Olson et al., 1986). However, other workers have shown that bovine thrombin is directly inhibited by equimolar or greater concentrations of heparin, producing 70% inhibition (Smith, 1977). 100 units of porcine heparin resulted in 70% inhibition of the *C. v. oreganus* enzyme, further indicating its similarity to thrombin. This protease showed high substrate specificity for the thrombin substrate PheValArg-pNA (~33-fold greater than for ProPheArg-pNA) and no activity toward CBZ-L-Arg-pNA, indicating that an aliphatic amino acid side chain may be preferred in the P2 position but that the aromatic ring of phenylalanine may impede the fit of the substrate to enzyme (see Fig. 2).

The kallikrein-like enzymes from juvenile venoms had a relative molecular weight of 31,000. Mammalian kallikrein (Pisano and Austin, 1974) and kallikrein-like enzymes from other snake venoms (Komori and Sugihara, 1988; Ohtani et al., 1988) showed *in vivo* and *in vitro* hydrolytic activity toward the serum protein high molecular weight (HMW) kininogen, liberating the nonapeptide bradykinin. Bradykinin is a potent vasodilator (Roche e Silva et al., 1949) which causes rapid hypotension via its action on smooth muscle of blood vessels. The carboxy-terminus of bradykinin is -ProPheArg, hence the use of the chromogenic substrate α-N-Bz-ProPheArg-pNA to assay venom components. The action of the kallikrein-like protease from *Crotalus viridis oreganus* venom toward HMW kininogen was not assayed, but based on amidolytic activity assays, bradykinin-releasing activity is likely.

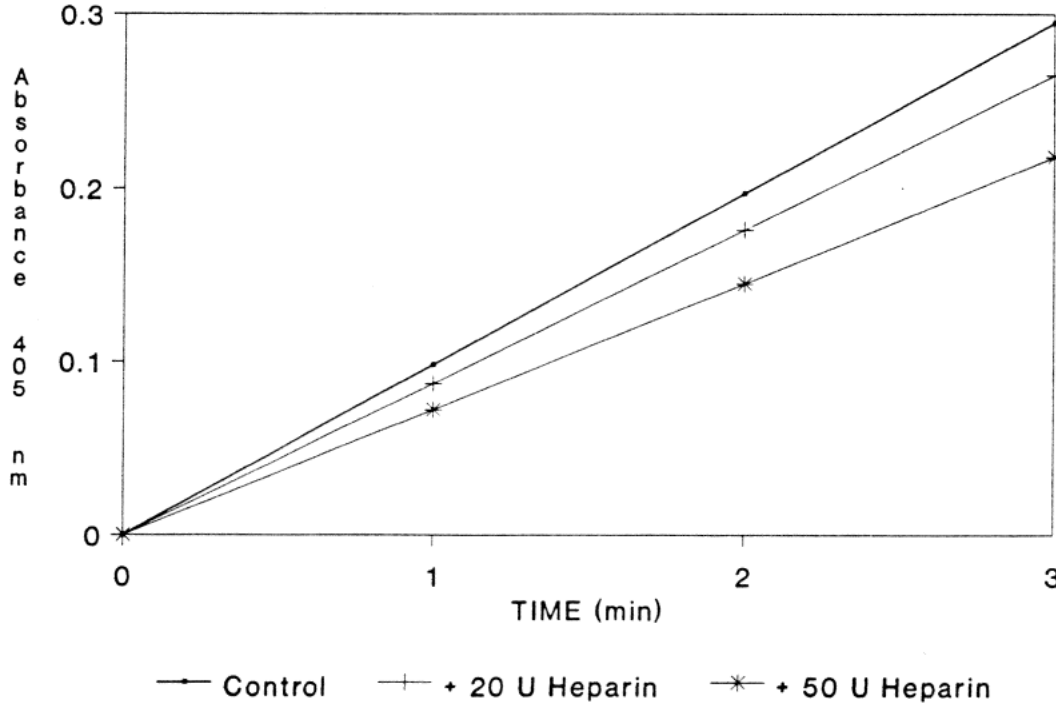


Figure 5. Inhibition of venom thrombin-like protease by heparin.

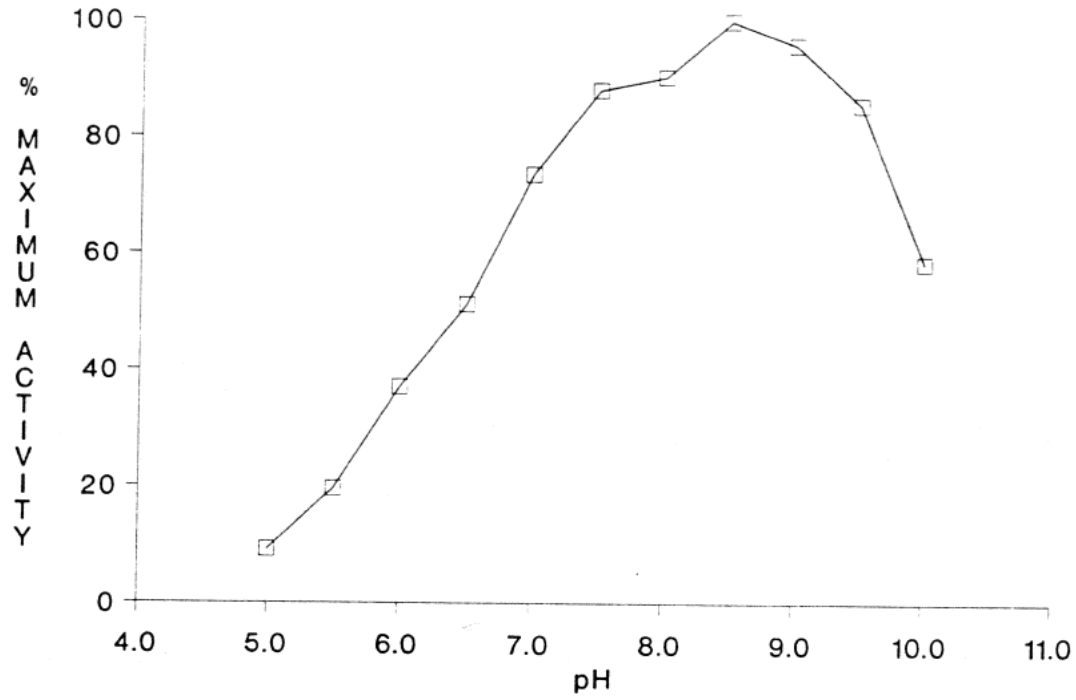


Figure 6. pH profile for the hydrolysis of α-N-Bz-L-Pro-L-Phe-L-Arg-pNA by juvenile kallikrein-like protease. % maximal activity is based on highest activity observed (at pH 8.5).

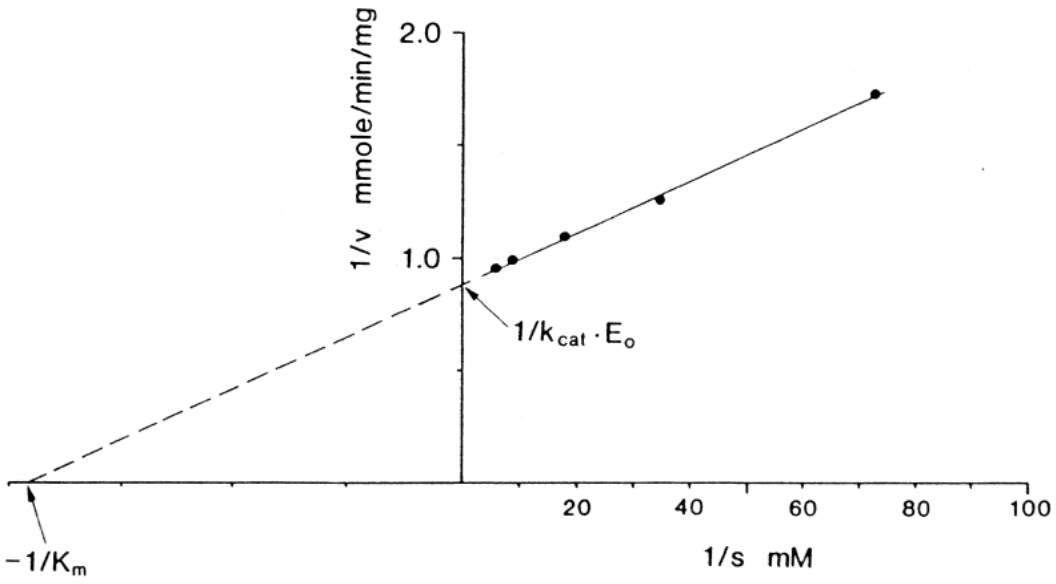
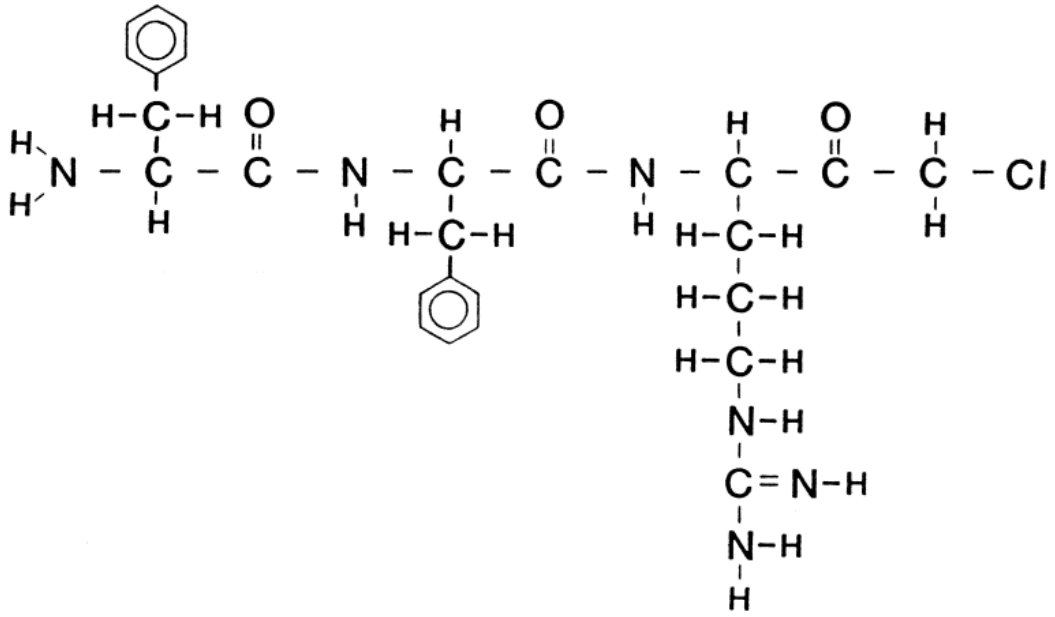


Figure 7. Double reciprocal plot of rate vs. substrate concentration for the hydrolysis of -N-Bz-L-Pro-L-Phe-L-Arg-pNA by juvenile kallikrein-like protease. Estimated kinetic constants were: Km = 13.0 μM; Vm = 1,130 μmol/min/mg.

The kallikrein-like proteases showed a high degree of specificity for the kallikrein substrate ProPheArg-pNA, with little activity toward the somewhat similar thrombin substrate PheValArg-pNA. Proteases typically have a specificity pocket (see e.g., Zubay, 1983) which "fits" the side chain of the amino acid immediately toward the amino-terminal side from the peptide bond to be hydrolyzed (the P1 site). Other aspects of the substrate affect whether or not it is recognized by an enzyme. Amino acids on the amino-terminal side (designated P2, P3, etc.) as well as on the carboxy-terminal side (designated P'1, P'2, etc.) of the bond to be hydrolyzed may affect how well the substrate fits the enzyme. Since all pNA derivatives used have the same group at the P'1 site (paranitroaniline), and those hydrolyzed to a significant extent had arginine at the P1 site, amino acid identity at the P2 and P3 sites were at least partly responsible for determining specificity of action of the venom proteases (kallikrein-like and thrombin-like). Kallikrein and thrombin pNA substrates and P-sites are shown in Fig. 2; CBZ-L-Arg was not hydrolyzed by either enzyme, indicating the importance of the P2 and P3 amino acids for substrate binding. The thrombin substrate was hydrolyzed at ~30-fold lower rate, indicating a "poorer fit" of this substrate for the kallikrein-like protease. It is assumed that some substrates not tested might be hydrolyzed by this protease, par-



D-Phe-L-Phe-L-Arg-CMK: Inhibitor of Kallikrein

Figure 8. Two-dimensional structure of PPACK, an active site affinity label inhibitor of kallikrein.

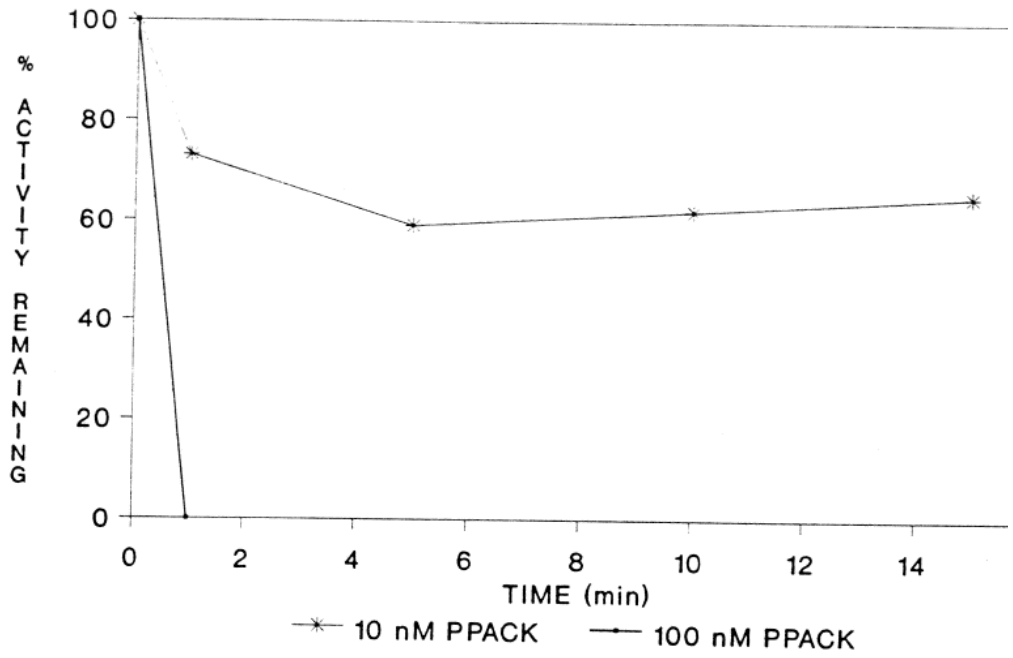


Figure 9. Inhibition of venom kallikrein-like activity by PPACK. 100 nM PPACK resulted in the complete inhibition of ~0.5 nM enzyme within one minute.

ticularly ArgMetLys-pNA (a homolog of the sequence cleaved from the amino-terminal end of bradykinin), and kallikrein-like enzymes from other snake venoms have been shown to hydrolyze several amide bonds in addition to ProPheArg-pNA (e.g., Ohtani et al., 1988).

The kallikrein-like protease from juvenile *C. v. oreganus* differed from crotalase, an enzyme from the venom of *C. adamanteus* (Markland and Damus, 1971), in several respects; the *C. v. oreganus* protease lacked thrombin-like activity, while crotalase had both thrombin-like and kallikrein-like activities (Markland et al., 1982). Crotalase had a molecular weight of 33 kD, intermediate between the *C. v. oreganus* proteases (31 and 34 kD), and crotalase was shown to be a homogeneous preparation. While *C. v. oreganus* and *C. adamanteus* are not thought to be closely related rattlesnakes (Klauber, 1956; Brattstrom, 1964), it was nonetheless somewhat surprising to find these activities combined in a single enzyme in one rattlesnake venom and in two separate enzymes in another rattlesnake venom.

The kallikrein-like protease from *C. v. oreganus* venom was partially inhibited by relatively high levels of aprotinin, a kallikrein, plasmin, and trypsin inhibitor (Trautschold et al., 1967), further suggesting a similarity to kallikrein. It was not inhibited by EDTA or 1,10-phenanthroline (thus probably not a metalloprotease) and based on inhibition by the active site affinity label PPACK, it is likely a serine protease (as are several other kallikrein-like enzymes from snake venoms; see Fox and Bjarnason, 1983; Schwarz and Bieber, 1985; Komori and Sugihara, 1988). Km and Vm values for the juvenile *C. v. oreganus* protease were 13.0 μM and 1,130 μmol/min/mg, respectively. These values are similar to those obtained for the hydrolysis of arginine esters by two

kallikrein-like enzymes isolated from *C. scutulatus* venom (Schwarz and Bieber, 1985) and by a kallikrein-like enzyme isolated from *Bitis gabonica* venom (Viljoen et al., 1979).

Biological Roles of Specific Venom Components

Rattlesnake venoms have two major biological functions related to feeding: rapid immobilization of prey with minimal struggle and digestion of prey (Klauber, 1956; Russell, 1980; Kardong, 1986; Mackessy, 1988). In many snakes, particularly the sea snakes (Hydrophiidae) and elapids, prey immobilization is of primary importance and is accomplished by the injection of venom containing large amounts of potent post-synaptic neurotoxins (Lee, 1972, 1979; Tu, 1988). Proteases and other enzymes are unusual in these venoms (Lee, 1979; Tu, 1988). In rattlesnake venoms, the reverse is true. Hydrolytic enzymes predominate (Iwanaga and Suzuki, 1979), and specific toxins, though present in some venoms, are few in number (Hendon and Bieber, 1982). Crotalids, as adults, typically feed on large prey (Greene, 1983), and proteolytic activity of the venom (as discussed below) helps to offset digestive difficulties caused by large prey (Thomas and Pough, 1979; Mackessy, 1988). Various investigators have questioned, perhaps rhetorically, whether enzymes were involved in the action of snake venoms or whether the effects of envenomation were due to specific toxins. Though many snake toxins have discrete pharmacological actions (Lee, 1972; Hendon and Bieber, 1982), hydrolytic enzymes are the major constituents of crotalid and viperid venoms (based on quantities present). Venom enzymes are unquestionably involved in both localized and systemic effects of envenomation. Of necessity, the various components of a venom must be purified to determine the particular activities attributable to that component, but this approach abolishes the potential synergistic effects of venom constituents. In order to understand the biological role of venoms as biological products which have evolved primarily as trophic adaptations, one must attempt to reassemble the summed effects of isolated venom components. Rather than being treated as individual independent compounds, venom components should be analyzed for their potentially interdependent effects. Zeller (1977) has made the point that by studying venom components from the perspective of venom-prey interactions (that is, approach-ing venom biochemistry with the view that venoms have evolved to serve specific biological roles), one could derive much biologically relevant information on venoms. This point will be developed below.

Thrombin-like and Kallikrein-like Proteases

Earlier attempts to classify rattlesnake venoms as procoagulant or anticoagulant were stymied by conflicting results, most of which may not be attributed to the fact that venoms may contain one or the other or both activities (Kornalik, 1986; Pirkle and Markland, 1988). Although the presence of procoagulant and anticoagulant proteases in a single venom has led to contradictory interpretations (as described by Kornalike, 1986), the presence of both in a single venom, such as the venom of adult *C. v. oreganus*, may be quite important to the biological role of venoms. The thrombin-like protease and crotalid protease both initiated clot formation, which *in vivo* could result in thrombosis and circulatory dysfunction. Added to this is the action of the kallikrein-like protease, causing rapid hypotension, and the venom could rapidly

cause circulatory shock. In humans, anaphylactic shock resulting from rattlesnake bites can quickly be fatal (Russell, 1980). In prey animals, with a much smaller body mass than humans, this reaction is likely accentuated by venom enzymes interfering with hemostasis.

Enzymes as Toxins

Venom proteases may act as toxins initially and as digestive enzymes later in the course of envenomation. In the blood, the thrombin-like and kallikrein-like enzymes have relatively specific actions, namely the hydrolysis of fibrinogen to produce fibrin clots and the release of bradykinin from kininogen. Other venom enzymes may also act initially as toxins. For example, phospholipase A2 hydrolysis of phosphatidylcholine releases arachidonic acid, a fatty acid with numerous direct activities and a precursor to the hormone-like prostaglandins and thromboxanes.

The immediate action of enzymes as toxins is via the release of potent metabolites such as bradykinin and arachidonic acid from larger precursors and structural molecules rather than by a direct blocking action (such as several snake venom neurotoxins). The toxic action of these metabolites likely facilitates rapid prey immobilization and quiescence. Complementary to this toxic effect is the later digestive action of many venom enzymes. For example, though the major metalloprotease of *C. v. oreganus* venom initially promotes clot formation, a later phase of its activity on fibrin(ogen) is clot dissolution (Mackessy, 1993). *In vivo*, a long-term effect of venom thrombin-like proteases is defibrination (Markland and Pirkle, 1977; Russell, 1980). Metallo-proteases and the thrombin-like protease may thus act in tandem to clear the serum of much of fibrinogen and fibrin (at least locally), impeding defense against hemorrhagic proteases. Most rattlesnake venoms produce severe local hemorrhage (Ownby, 1982), and the synergistic actions of the thrombin-like and metallo-proteases may be responsible for much of this tissue damage. It is of interest to note that the major metalloprotease is absent from juvenile snake venoms (Mackessy, 1993), and bites by smaller snakes rarely result in local tissue necrosis (unpub. obs.; Russell, 1980).

The *C. v. oreganus* thrombin-like and kallikrein-like proteases are common to both adult and juvenile venoms, and appear to exhibit a rather high degree of substrate specificity *in vitro*, though they may produce several *in vivo* effects. Human thrombin and proteolytically-generated fragments have been shown to have numerous actions both *in vivo* and in cell cultures (Bar-Shavit et al., 1986; Alexander et al., 1988), and the venom thrombin-like proteases may also have effects other than the hydrolysis of fibrinogen. The relative abundance of these two proteases does not appear to change ontogenetically, suggesting that their role in prey capture is probably similar for both juvenile and adult snakes.

The enzymes described above can have profound effects on living tissues and can explain certain clinical features noted in cases of human and lab animal envenomation. However, the venom of *Crotalus viridis oreganus* (and other rattlesnakes) is a complex mixture, primarily proteins, with diverse biological activities. While there are many studies of single venom components, some in great detail, there are few studies attempting to characterize the many different components of a single venom and to link specific activities to particular biological roles the whole venom provides for the snake. This study represents a

IN VIVO CLEAVAGE SITES OF THROMBIN AND KALLIKREIN

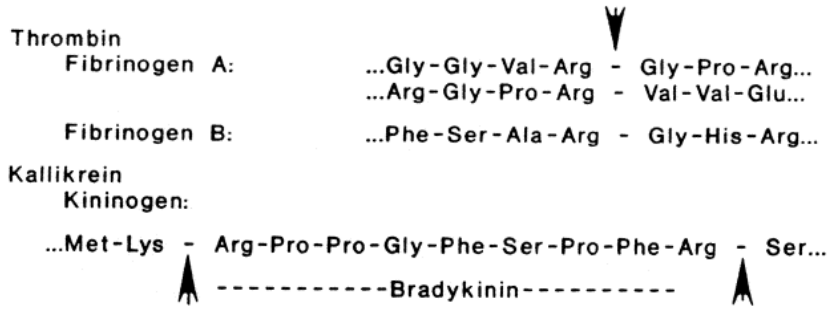


Figure 10. Several of the *in vivo* cleavage sites of thrombin and kallikrein. Paranitroaniline-derived substrates are based on these recognition sequences.

beginning toward the elucidation of these roles.

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