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Effects of Temperature and Storage Conditions on the Electrophoretic, Toxic and Enzymatic Stability of Venom Components

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ABSTRACT. Rattlesnake venoms are complex biological products containing potentially autolytic components, and they provide a useful tool for the study of long-term maintenance of enzymes in a competent state, both *in vivo* and *in vitro*. To evaluate the stability of venom components, 15 aliquots of freshly extracted venom (from *Crotalus molossus molossus*) were subjected to 15 different temperature and storage conditions for 1 week and then lyophilized; conditions varied from storage at −80°C (optimal preservation of activities) to dilution (1:24) and storage at 37°C (maximal degradation potential). Effects of different storage conditions were evaluated using SDS-PAGE, metalloprotease zymogram gels, a cricket LD_{50} assay and enzyme assays (metalloprotease, serine proteases, phosphodiesterase, l-amino acid oxidase and phospholipase A_2). Venom samples were remarkably refractive to widely varying conditions; enzyme activities of some samples were variable, particularly l-amino acid oxidase, and one sample treatment showed higher toxicity, but electrophoretic results indicated very little effect on venom proteins. This study suggests that most venom activities should remain stable even if stored or collected under potentially adverse conditions, and freezing samples is not necessarily advantageous. Proteins in the crude venom are not as labile as has been previously thought, and endogenous mechanisms present in the venoms likely inhibit autolysis during long-term storage that occurs *in vivo* in the gland. comp biochem physiol 119B;1:119–127, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. Autolysis, l-amino acid oxidase, metalloprotease, phospholipase A_2, phosphodiesterase, rattlesnake, serine protease, snake venom

INTRODUCTION

Animal venoms are an important source of enzymes including proteases, phospholipase A_2 (PLA_2), phosphodiesterase and other activities (12,29,36). In addition, the study of venoms, specifically snake venoms, is an important area of biomedical research because of the abundant neurotoxic, hemorrhagic and tissue-damaging activities they possess

oms and other animal products are most often frozen immediately and lyophilized to preserve maximal activities [i.e., (17)], but the necessity for these treatments has not been demonstrated unequivocally. Therefore, investigation of the effects of storage conditions on venom components may have important implications for improving techniques used to collect and preserve venoms. These considerations are of

TABLE 1.

Electrophoresis

Reconstituted venoms were analyzed by SDS-PAGE for treatment-induced changes in electrophoretic mobilities of components. Tris–glycine 14% acrylamide gels were run (without 2-mercaptoethanol or boiling) as recommended by the manufacturer [essentially the method of (11)]. Thirty-five micrograms of venom was loaded in each lane.

Venom samples were analyzed for metalloprotease activities using 10% acrylamide ''Zymogram'' gels obtained from Novex, Inc. These gels are copolymerized with gelatin substrate. Electrophoresis and development of gels followed a published method (8). Each lane contained 0.5 μg crude venom. All gels were imaged using a charge coupled device (CCD) video camera with a yellow filter.

Toxicity Assays

Domestic crickets (*A. domesticus*) were used to evaluate potential changes in toxicity induced by storage conditions. Previous experiments (24) demonstrated that more consistent data were obtained using a 48-hr period for LD_{50} determinations with snake venoms (rather than 24 hr), and this interval was used for all experiments.

Crickets were obtained from Fluker Farms; all were three- a total volume of 1.0 ml. After centrifugation, absorbance of the supernatant was read at 285 nm; activity was expressed as ΔA_{285nm}/min/mg protein.

Serine Protease Assays

Substrates for thrombin-like (BzPheValArg pNA) and kallikrein-like (BzProPheArg pNA) activities were used to assay samples for two serine proteases common in crotalid venoms (14,23). Activity, based on a *p*-nitroaniline standard curve, was expressed as nmol product formed/min/mg protein. Activities toward five additional pNA-derived peptides (BzArg pNA, GluPheArg pNA, N-CbzGlyProCit pNA, N-methoxysuccinylAlaAlaProMet pNA, and GlyArg pNA) were also assayed.

L-*Amino Acid Oxidase Assay*

l-AAO activity was assayed as described previously (17). The activity, based on a kynurenic acid standard curve, was expressed as nmol product formed/min/mg protein.

Phospholipase A_2 Assay

BL Acid 26795(by)-26than method 10(8).)-25[(Th.)]TJ874.805 -06975

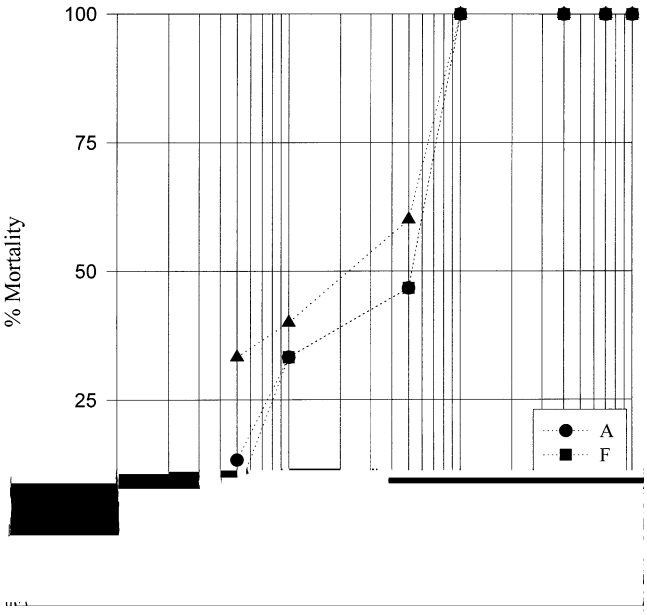

FIG. 3. Lethality of venom samples stored under several conditions: semilog plot. Toxicity of three venom samples (A, F and L) was evaluated in crickets as described in Materials and Methods. Samples A (Lyophilized immediately) and F (diluted 1:24 and freeze/thawed) had identical values (0.54 μg/g); sample L (diluted 1:24 and stored at 37°C) had a greater apparent toxicity (0.23 μg/g).

5C) were somewhat more variable than the thrombin-like activity; however, most sample activities, in particular those stored at above-freezing temperatures, were uniform. Sample E was slightly lower than the average and again, the treatment that was much higher than average was sample L. All venom samples showed extremely low or no activity toward five other pNA substrates (BzArg pNA, GluPheArg pNA, N-CBzGlyProCit pNA, *N*-methoxysuccinylAlaAlaProMet pNA and GlyArg pNA).

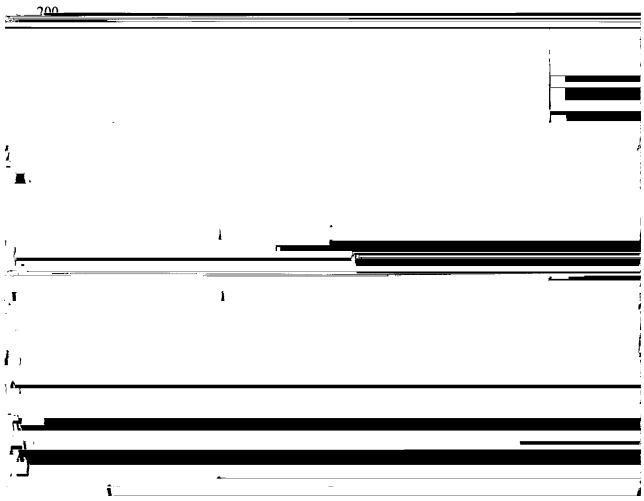

FIG. 4. Effect of storage conditions on enzyme activities. The average value of each enzyme assay346(aszyme0vothesi7k1[(treatmeser)-35watus)]TJ/F3 1 TT9.948 0 0 9.965 311.4317.6351 Tmemor

The activities of the l-AAO (Fig. 5D) were most variable. The lowest activity level was found in sample I (diluted 1:24 and stored at room temperature); samples A (frozen at −20°C and lyophilized immediately) and L showed the highest activities. A 3-fold range of variation (13–38 nmol/min/mg) was seen in activities of this enzyme. PLA_2 activities (Fig. 5E) were fairly uniform. The lowest activities, only slightly lower than the average, were found with samples C (diluted 1:24 and stored at −20°C), E and I. Samples J (stored at room temperature and air dried) and L had the highest activities. Phosphodiesterase activity of all eight samples that were frozen showed higher activities than those not frozen, except samples J and L (Fig. 5F). The lowest activity levels were seen in samples E and I, and sample L once again showed much higher activity than all other treatments.

DISCUSSION

Results of this study are consistent with several earlier studies (1,31). Previous investigators (1) found that the effects of preparatory procedures had little effect on the stability of *C. m. molossus* venom protein banding patterns after isoelectric focusing. An early study (31) found that LD_{50} values were largely unaffected by crude or lyophilized venom storage conditions. However, lethality of venom results from a synergistic interplay of venom components, including enzymes, peptides and specific toxins, and it is possible that isoelectric focusing patterns could remain stable even if components were denatured by storage conditions. The present study examined protein banding patterns (electrophoretic profiles), toxicity and specific enzyme activities of venom components with presumed differential stabilities. Venom from *C. m. molossus* was used in this study because metalloprotease activity is extremely high and autolytic degradation loss of activity should be quite pronounced for this venom. Further, as demonstrated by comparative enzyme assays and zymogram gel assays (Mackessy, unpublished data), *C. m. molossus* venom has many of the same enzymes present in the venoms of other large species of *Crotalus* (such as *C. atrox*, *C. mitchelli*, *C. scutulatus*), and results should therefore be generalizable to other viperid species.

Although it was initially hypothesized that the integrity and stability of venom components would be adversely affected by dilution, freeze-thawing cycles and higher storage temperatures, the results from electrophoretic assays dem-

believe that the results obtained with *C. m. molossus* venom can be generalized to all front-fanged snake venoms; nevertheless, venoms from other species will need to be examined to confirm or refute this hypothesis.

Results of this study have shown that electrophoretic, toxic and representative enzymatic activities of blacktail rattlesnake venom, a complex biological secretion containing at least 24 distinct protein components, are largely unaffected by storage conditions varying by as much as 117°C. The fact that no loss of activities was observed in the normal range of ambient temperatures experienced by the snake in the field (approximately 0–37°C) demonstrates that endogenous stabilizers or inhibitors must be present in the gland and the venom stored in the lumen. Further, as demonstrated electrophoretically for all sample treatments except sample L, protein components did not undergo detectable autolytic degradation, despite the presence of at least four discrete size classes of metalloproteases with endoprotease (casein, gelatin) activity. Snake venoms

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