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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 (20,21,23,25). An enigmatic quality of animal venoms is that although potent biological activities are secreted and stored in the lumen of the gland [e.g., (16)] only millimeters from the animal's brain, autolytic and autopharmacological reactions apparently do not occur. Endogenous protective components active against some venom enzymes have been demonstrated (2,3,32,38), and other protective mechanisms undoubtedly exist as well.

Once expressed from the animal, many secretory products are unstable and are subject to (auto)lytic degradation. Venoms 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 particular importance when ideal conditions cannot be met, such as during lengthy isolation procedures or when venom is collected in the field. In addition, knowledge of the effects of storage conditions on enzymatic and biological activities of venoms and other natural products may reveal novel mechanisms by which these compounds are maintained in a competent but inactive state *in vivo*.

Previous studies have shown that many biological and enzymatic activities of such venoms are stable over many years (1,30,31,33). However, although some studies have indicated that prompt lyophilization prevents the degradation of protein components and does not cause a decrease in biological activities (30,31) or produce alterations in electrophoretic mobilities of protein components (5,7), other reports have suggested that such treatments can adversely affect electrophoretic mobilities (39) or enzymatic/

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TABLE 1. Storage conditions of aliquots of *Crotalus molossus molossus* venom

Samples	Storage Temperature	Conditions
A	None	Immediately frozen and lyophilized.
B	−20°C	Frozen for 1 week.
C	−20°C	Diluted (1:24) and frozen for 1 week.
D	4°C	Stored for 1 week.
E	4°C	Diluted (1:24) and stored for 1 week.
F	−20°C	Diluted (1:24) and frozen and thawed daily for 1 week.
G	−20°C	Frozen and thawed daily for 1 week.
H	~20°C (room temperature)	Stored for 1 week.
I	~20°C (room temperature)	Diluted (1:24) and stored for 1 week.
J	~20°C (room temperature)	Continuously exposed to air and stored for 1 week.
K	37°C	Stored in a water bath for 1 week.
L	37°C	Diluted (1:24) and stored in a water bath for 1 week.
M	−80°C	Frozen for 1 week.
N	−80°C	Frozen and thawed daily for 1 week.
O	−80°C	Diluted (1:24) and frozen and thawed daily for 1 week.

biological activities (37). From these conflicting reports, it became clear that a more systematic and extensive approach to the stability of venom components was needed. Specifically, very few studies have utilized electrophoresis, toxicity assays and enzymatic activity assays to evaluate the effects of storage conditions on venom stability [but see (22)].

We hypothesized that extended exposure to temperatures above freezing, particularly after dilution, would result in the degradation of enzymatic activities and change electrophoretic mobilities of some venom components. Because lyophilized venom is often reconstituted, frozen and then subjected to freeze/thaw cycles as it is used, we also hypothesized that this process of freezing and thawing could damage venom components, resulting in enzymatic degradation and thus reducing the overall activities of the venom. In the present study, the effects of varying storage conditions on protein electrophoretic mobility, zymogram metalloprotease activity, lethal toxicity and enzymatic activities of venom were investigated. We used venom from the black-tailed rattlesnake (*Crotalus molossus molossus*) because this species produces a venom containing several potent metalloproteases (26,27) and overall caseinolytic protease activity is much higher than that observed in venoms from several other rattlesnake species (17). Changes in venom properties resulting from specific storage conditions should therefore be more pronounced in this species' venom. Activities assayed included those considered thermally more stable (PLA_2 and serine proteases) and those that are thermally labile (L-amino acid oxidase [L-AAO]).

MATERIALS AND METHODS

Materials

Casein yellow (Lot 610029) was obtained from CalBioChem, Inc., La Jolla, CA, USA. Protein concentration reagents and γ-globulin were purchased from BioRad, Inc., Hercules, CA, USA. All other reagents (analytical grade or better) were obtained from Sigma Chemical Co., St. Louis, MO, USA. Tris-glycine 14% acrylamide gels, Zymogram gels and Mark 12 molecular weight standards were obtained from Novel Experimental (Novex), Inc., San Diego, CA, USA. Crickets (*Acheta domesticus*) were purchased from Fluker Farms, Inc., Baton Rouge, LA, USA.

Venom Extraction and Treatments

Venom was extracted from a healthy adult male black-tailed rattlesnake (*C. m. molossus*) collected in Cochise County, Arizona. The crude venom was centrifuged for 5 min at approximately 4000 rpm to pellet cellular debris. It was then aliquoted into 15 portions (labeled A–O) of 25 μl each. Each of these aliquots was then subjected to a different storage condition (Table 1). To evaluate the stability of the whole venom, extremes in storage conditions were used (−80°C to +37°C). After 1 week, all samples, except sample A (which was frozen and lyophilized immediately after centrifugation), were lyophilized. The venoms were then dissolved in 50 μl Millipore-filtered H_2O, and aliquots were diluted 1:9 with Millipore-filtered H_2O. This final dilution was used in all experiments.

Protein Concentration Assay

Protein concentration was determined by a slight modification of the Bio-Rad method, using bovine gamma globulin as a standard (0, 5, 10, 15, 20, 30 and 50 μg). To each new test tube, the following was added: Millipore-filtered H_2O (795 μl), reconstituted crude venom (5 μl), and the Bio-Rad dye reagent (200 μl). The absorbance of both the samples and standards were read at 595 nm after 5 min and within 20 min. Protein concentrations (determined in duplicate three times) were used to calculate gel loads, LD_{50} dosages and enzyme specific activities.

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-quarter-inch sub-adults of approximately 0.5 g weight. For all experiments, the averaged weight of 15 representative crickets was used to calculate doses. Venom (treatments A, F, and L) was diluted to the appropriate dose/g body weight in cricket Ringer solution (150 mM NaCl, 7 mM KCl, 8 mM $CaCl_2$, 4 mM $MgCl_2$) (40). Initial experiments showed that handling of crickets and injection with cricket Ringer solution had no effect on 48-hr LD_{50} (i.e., control injections produced no mortality).

Intraperitoneal injections of 10 μl were administered to 15 crickets at each dose level; seven dose levels were evaluated (0.05–10.0 μg/g body weight). Crickets were gently restrained while the needle of a 25-μl Hamilton syringe was inserted 4–5 mm at an angle of approximately 30 degrees. All injections were made behind the center of the fifth abdominal segment. All crickets were housed with food and water *ad libitum* at 26°C and checked at 24 and 48 hr post-injection. Percent mortality at 48 hr as a function of dose (semi-log plot; Sigma Graphics) was used to determine LD_{50} values. The cricket model, although obviously differing physiologically from mammalian models, provides repeatable data on acute toxicity (LD_{50}). Additionally, initial testing with invertebrates can help decrease the number of vertebrates used in subsequent assays, a goal consistent with concerns about vertebrate use in toxicity tests.

Caseinolytic Protease Assay

Caseinolytic protease activity was assayed by a method described previously (15) using 5 μl reconstituted venom in 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

PLA_2 activity was assayed by an aqueous method (10). The absorbance of the samples was read at 425 nm after 5 min and within 1 hr. Activity, based on a nitrobenzoic acid standard curve, was expressed as nmol product formed/min/mg protein.

Phosphodiesterase Assay

Phosphodiesterase activity was assayed as described previously (18). Activity was expressed as ΔA_{400nm}/min/mg protein.

RESULTS

Protein electrophoretic mobilities, toxicity and enzymatic activities of black-tail rattlesnake venom were largely unaffected by a wide variety of treatment and storage conditions. The various treatments showed only a few conditions that caused significant changes in activity of the venom components.

All samples had the same major banding patterns on 14% acrylamide gels (SDS-PAGE). At least 24 protein bands, ranging in size from approximately 6–250 kD, were visualized in all sample treatments (Fig. 1A and B). Nearly all of these protein bands had the same intensity, with the exception of sample L. In this sample treatment (diluted 1:24 and stored for 1 week at 37°C), several higher molecular weight bands were missing or less intense. This included a minor band at approximately 78 kD and two major bands at approximately 63 and 58 kD. A minor lower molecular

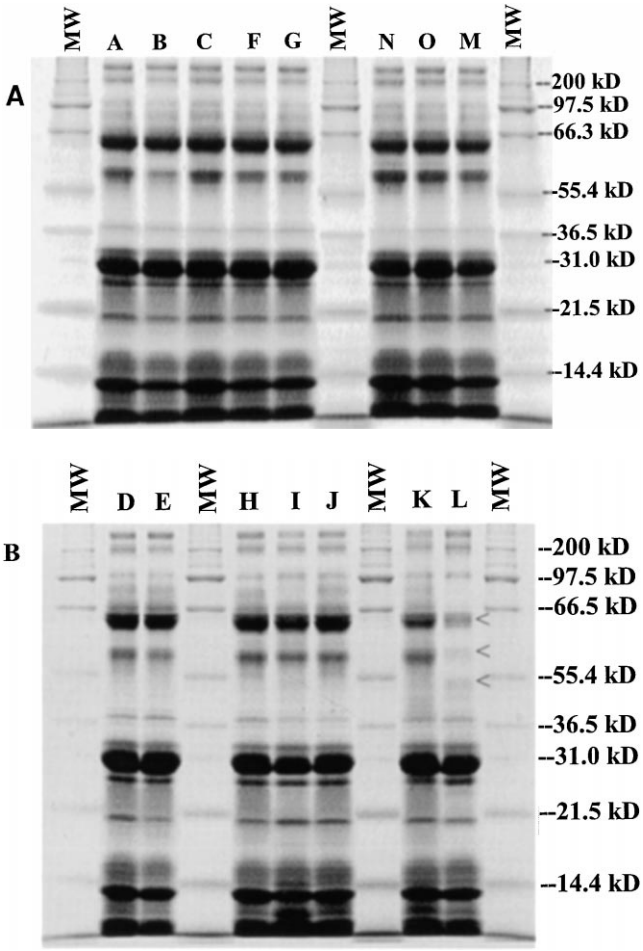

FIG. 1. (A and B). Electrophoretic mobility of *C. m. molossus* venom components after storage under various conditions (see Table 1). Sample concentrations were 35 μg/lane. Note similarities of banding patterns in all lanes except lane L. Sample L shows decreased intensity of major bands at 63 and 58 kD, and an additional band at 53 kD (arrowheads).

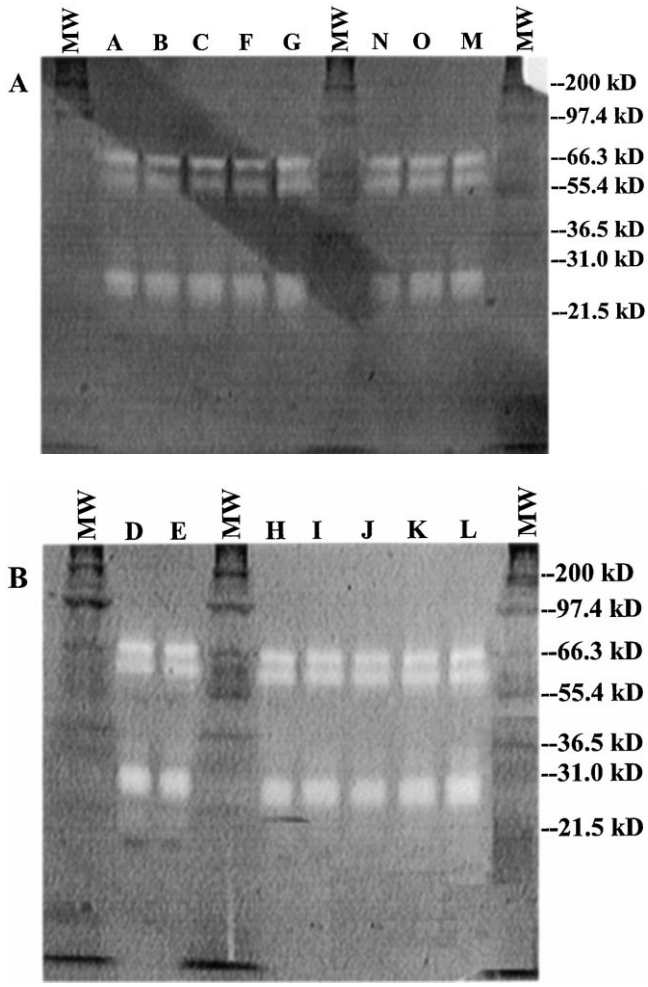

FIG. 2. (A and B). Electrophoretic zymogram of venom stored under various conditions. Sample concentrations were 0.2 μg/lane. Metalloproteases appear as white bands at approximately 61, 56, 27 and 24 kD. Note the constancy of protease banding patterns regardless of storage treatment. The diagonal dark bar in A is a photographic artifact.

weight band (approximately 53 kD) was present in sample L, but absent from other samples, indicating a potential product of autolysis.

A high degree of similarity was also seen in the 10% acrylamide Zymogram gels, which detect metalloprotease activity (Fig. 2A and B). Four distinct regions of protease activity were observed for all samples at approximately 61, 56, 27 and 24 kD. No differences in band position or intensity were noted between samples regardless of the treatment.

Toxicities of samples A (immediately frozen and lyophilized), F (diluted 1:24 and frozen/thawed daily) and L (diluted 1:24 and stored at 37°C) are shown in Fig. 3. Samples A and F showed identical toxicity profiles and a 48-hr LD_{50} in crickets of 0.54 μg/g. Sample L showed an apparent 2-fold greater toxicity, and the 48-hr LD_{50} was 0.23 μg/g. All samples produced 100% mortality at 48 hr at doses of 1.0 μg/g or greater.

Enzyme activities were somewhat variable but largely unaffected by extremes in storage conditions. Figure 4 summarizes effects of storage treatment on six enzyme activities. In virtually all cases, sample L (diluted 1:24 and stored at 37°C) showed higher activity than all other treatments, and in most cases, sample E (diluted 1:24 and stored at 4°C) showed the lowest activity.

Figure 5 illustrates the effects of each differential storage condition on enzyme activities. Caseinolytic protease activity of sample A (frozen at −20°C and lyophilized immediately) showed the lowest level (Fig. 5A); two treatments, sample J (stored at room temperature and air dried) and sample L, had significantly higher activity.

Thrombin-like venom protease activities (Fig. 5B) were largely uniform but lower in the sample treatment E (diluted 1:24 and stored at 4°C). The treatment that showed significantly higher activity was sample L (diluted 1:24 and stored at 37°C). The kallikrein-like protease activities (Fig.

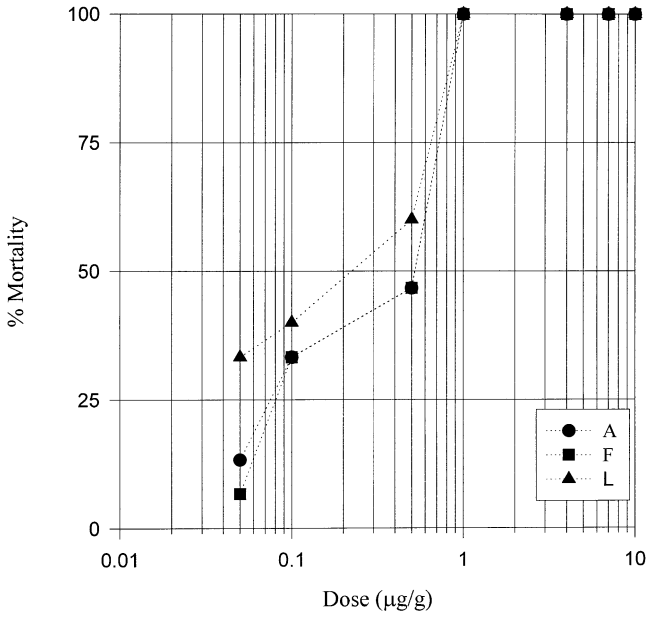

FIG. 3. Lethality of venom samples stored under several conditions: semilog plot. Toxicity of three venom samples (A, F and L) was evaluated in crickets as described in Materials and Methods. Samples A (Lyophilized immediately) and F (diluted 1:24 and freeze/thawed) had identical values (0.54 μg/g);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

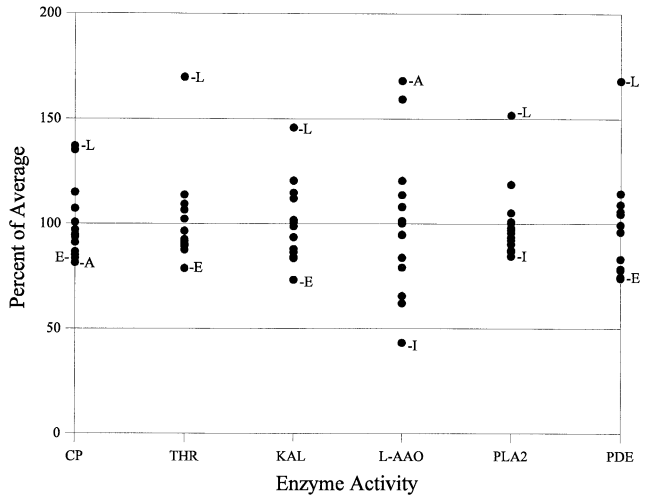

FIG. 4. Effect of storage conditions on enzyme activities. The average value of each enzyme assay for all treatments was set at 100%; each point represents [treatment value ÷ average value] × 100%. CP, caseinolytic protease; THR, thrombin-like protease; KAL, kallikrein-like protease; L-AAO, L-amino acid oxidase; PLA2, phospholipase A_2; PDE, phosphodiesterase.

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).

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 demonstrated that the venom protein components were not altered electrophoretically when stored and lyophilized, consistent with previous reports (1,5,7). Additionally, the major banding patterns observed after the different treatments in this study were all similar with those observed in

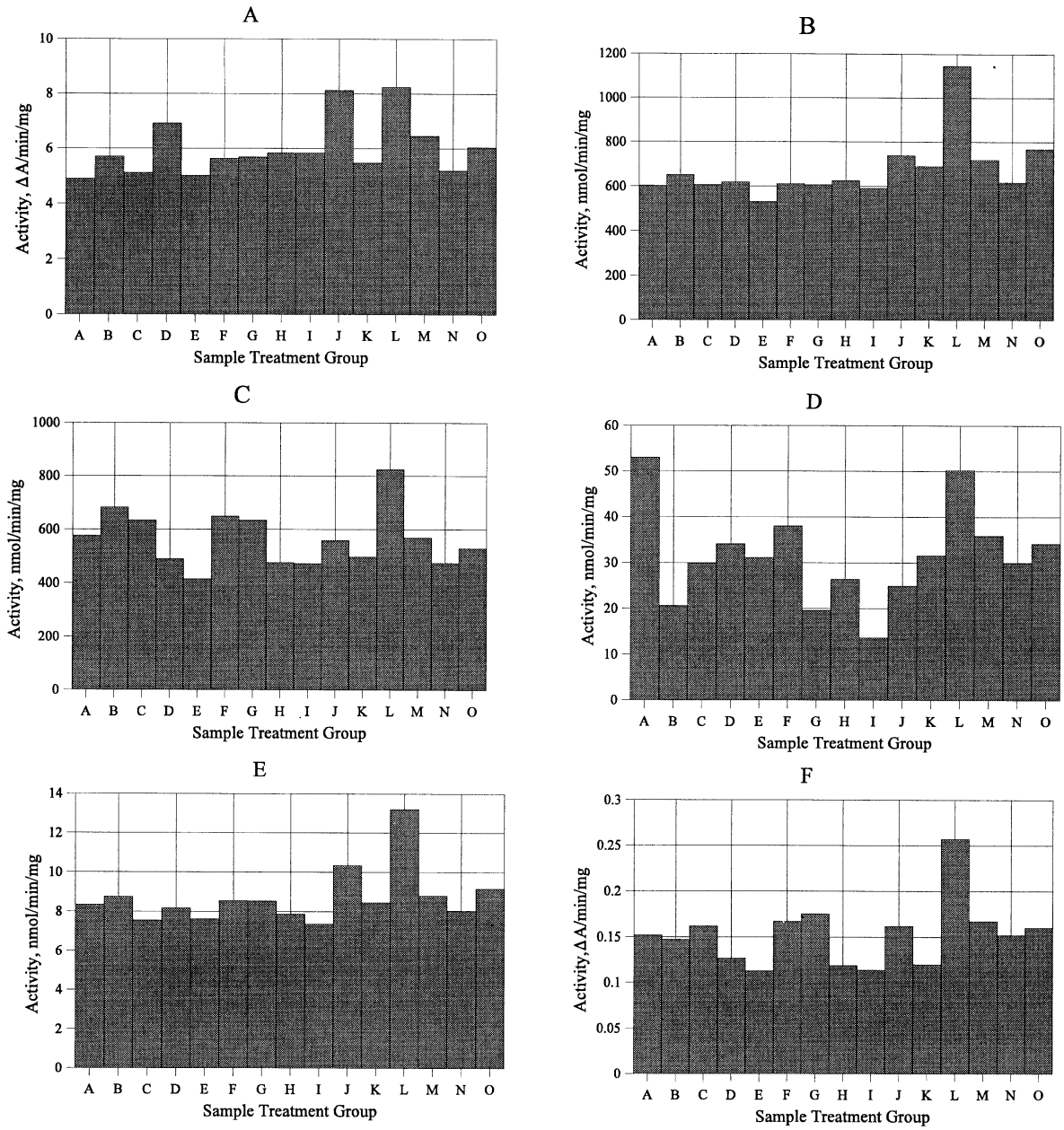

FIG. 5. Histogram distribution of specific activity levels of six enzyme activities for venom samples stored under 15 different conditions (see Table 1). A, Caseinolytic protease; B, thrombin-like protease; C, kallikrein-like protease; D, L-amino acid oxidase; E, phospholipase A_2; F, phosphodiesterase.

other electrophoretic studies (4,34) of *C. m. molossus* venom.

The pattern of major and minor bands for *C. m. molossus* venom were very similar for all storage conditions; only sample L (diluted 1:24 and stored at 37°C) had some minor differences. In this sample, two major bands (approximately 63 and 58 kD) were less intense than in other samples and one minor band (approximately 78 kD) was not observed in sample L but was present in all other samples. A second minor band (approximately 53 kD) was observed in sample L but was not seen in any other sample. The lower intensity of the two major bands and the absence of the minor band may be attributed to autolytic degradation promoted by dilution and high temperature conditions. The additional minor band could likely have resulted from degradation of higher molecular weight components.

Electrophoretic consistency was also observed for the zymogram gels, which provide a first approximation of the number of molecular weight classes of metalloproteases in venoms. Regardless of treatment, all lanes showed the same

pattern of four protease bands, an observation that is particularly noteworthy because purified metalloprotease from *Crotalus viridis oreganus* venom, which is homologous with the 61-kD band, underwent autolytic degradation when the purified protease was stored in solution (13). Apparently, components present in the intact crude venom prevented autolysis of metalloproteases, even after storage at 37°C.

The cricket LD_{50} assay was developed as an alternative to the mouse model, in part in response to growing criticism of vertebrate toxicity studies. Although mammalian and insect physiology differ significantly, the cricket model allowed comparison of organism-level toxicity of complex materials such as venoms (24). All three *C. m. molossus* samples tested showed comparable toxicity; however, sample L (diluted and stored at 37°C) showed half the apparent LD_{50} value. It should be noted that using identical routes of administration, different investigators have found mouse LD_{50} values for the same venom that have differed by as much as a factor of 4 (6). The cricket assays demonstrated that differential storage of venom did not result in a loss of biological activity (toxicity) and the cricket 48-hr LD_{50} model is a useful alternative for initial toxicity screening.

The cricket 48-hr LD_{50} model has several advantages over vertebrate model systems. It retains a desirable aspect of the toxicity assays (complex systems interactions, whole organism instead of cellular toxicity or *in vitro* diagnostics), but it avoids the use of vertebrates. Many researchers have been attempting to minimize the use of vertebrates in acute studies, and the cricket model provides a means for initial toxicity screenings that will decrease the number of vertebrates needed for secondary tests. Because crickets have an adult body weight of ~0.5 g (vs a 20 to 25-g mouse), very small amounts of valuable samples can be assayed at numerous dosages using a statistically significant number of animals. In addition, because crickets are commercially available, are small and are easily housed and maintained, overall costs for LD_{50} assays are significantly reduced. It should be reiterated that this model was designed to augment existing models and to decrease the numbers of vertebrates required for secondary testing; it is *not* suggested to replace mouse models.

Although venom samples appeared electrophoretically stable under nearly all temperature and storage conditions, venom enzyme activity was somewhat variable. Several general trends are apparent from the enzyme assays. Unlike many macromolecules in solution, venom samples were remarkably stable to freeze/thaw cycles, and most activities assayed were not negatively affected by such cycling. One notable exception was L-AAO, the enzyme that showed the greatest overall variation with storage condition; previous studies have demonstrated the thermal lability of L-AAO (35). For this activity only, −20°C/+20°C freeze/thaw cycles of undiluted venom significantly decreased apparent enzyme activity; all other enzymes assayed were unaffected by freeze/thaw cycles. In contrast, the sample that was diluted and stored at 4°C for 1 week (sample E) showed the lowest levels of activity for all enzymes *except* L-AAO. Because lyophilized venoms are typically rehydrated at concentrations well below original protein concentrations (~80–120 mg/ml), storage of rehydrated samples frozen between assays should preserve most activities and is recommended. Appropriate storage of rehydrated samples is particularly important for comparative studies.

Caseinolytic protease activity generally was higher after storage at ≥4°C, whereas preservation of kallikrein-like activity was promoted by freezing storage. Under short-term intense heating (100°C for 5 min), a recent study (22) found *C. m. molossus* venom showed an increase in caseinolytic protease activity in comparison with those samples that were not heated. Under long-term and less intense heating (37°C for 7 days, samples K and L), sample L (diluted 1:24) showed a large increase in activity (approximately 35% above average); activity of sample K (undiluted) did not vary significantly from the mean value. An unexpected and difficult to explain observation is the increased toxicity and enzyme activities of sample L. For all activities assayed, dilution and warm storage significantly increased apparent activities and toxic potency to the highest levels noted. Only slight changes in sample L were observed after SDS-PAGE, but it may be possible that a concentration and/or temperature-dependent inhibitor was degraded during this treatment; conversely, an activator may have been stimulated. Peptide and protein inhibitors of several venom components have been isolated from snake venoms [e.g., (2,9,28)], so it is conceivable that degradation of a functionally similar component could have produced the increased activity levels observed. In the absence of an endogenous peptide inhibitor, proteases from *Bothrops asper* venom degraded several venom myotoxins (2). This phenomenon of heat activation of venom enzymes is being investigated further using venoms from different species of rattlesnakes.

One potential limitation of this study is that venom from only one species of rattlesnake was tested. It is conceivable that venoms from different species and/or different families of snakes could show differential sensitivities to the storage conditions reported above. However, this is unlikely for several reasons. First, many, perhaps most, species of rattlesnakes produce venoms containing numerous metalloproteases (most likely to destabilize other venom components), and many of these proteases are homologous between species (Mackessy, unpublished data). Second, among PLA_2s from venoms, for which many sequences of enzymes from three families of snakes are available (29), a high degree of homology is observed, indicating that venoms from very different snakes share some basic similarities; this suggests that storage mechanisms may also show similarities. Finally, endogenous enzyme inhibitors have been isolated from crotalid, elapid and viperid snakes (2,3,9,28), further indicating a common response to potential storage problems. We

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 in the crude expressed state appear quite stable regardless of whether or not freezing occurs promptly. It is probable that for most species, collection of venom samples in the field followed by cool storage (i.e., non-extreme temperatures) will produce samples of quality comparable with those obtained in the laboratory. We have used this procedure to obtain venom samples of an endangered species occurring in a remote location (19) where removal of animals to a laboratory setting was not possible, and activities of samples are comparable with those of samples obtained from two captive specimens. For isolation of components with the highest specific activities, it may be necessary to proceed from venom extraction to the isolation procedure directly, because lyophilization has been suggested to decrease some activities (37); however, this will rarely be possible. To preserve maximum activity for venom samples that cannot be processed immediately, it is recommended that whenever possible, expressed venoms should be frozen and lyophilized promptly.

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References

1. Egen, N.B.; Russell, F.E. Effects of preparatory procedures on the venom from a rattlesnake (*Crotalus molossus molossus*), as determined by isoelectric focusing. Toxicon 22:654–56;1984.
2. Francis, B.; Kaiser, I.I. Inhibition of metalloproteinases in *Bothrops asper* venom by endogenous peptides. Toxicon 31: 889–899;1993.
3. Francis, B.; Seebart, C.; Kaiser, I.I. Citrate is an endogenous inhibitor of snake venom enzymes by metal-ion chelation. Toxicon 30:1239–1246;1992.
4. Foote, R.; MacMahon, J.A. Electrophoretic studies of rattlesnake (*Crotalus* and *Sistrurus*) venom: Taxonomic implications. Comp. Biochem. Physiol. 57B:235–41;1977.
5. Gené, J.A.; Lomonte, B.; Gutiérrez, J.M.; Cerdas, L. Changes in the electrophoretic pattern of the venom of the bushmaster (*Lachesis muta stenophrys*) stored under various conditions. Rev. Biol. Trop. 33:63–65;1985.
6. Glenn, J.; Straight, R. The rattlesnakes and their venom yield and lethal toxicity. In: Tu, A.T. (ed). Rattlesnake Venoms. Their Actions and Treatments. New York: Marcel Dekker; 1982:3–119.
7. Gregory-Dwyer, V.M.; Egen, N.B.; Bianchi Bosisio, A.; Righetti, P.G.; Russell, F.E. An isoelectric focusing study of seasonal variation in rattlesnake venom proteins. Toxicon 24: 995–1000;1986.
8. Heussen, C.; Dowdle, E.B. Electrophoretic analysis of plasminogen activators in polyacrylamide gels containing sodium dodecylsulfate and copolymerized substrate. Anal. Biochem. 102:196–202;1980.
9. Hokama, Y.; Iwanaga, S.; Tatsuki, T.; Suzuki, T. Snake venom proteinase inhibitors. III. Isolation of five polypeptide inhibitors from the venoms of *Hemachatus haemachatus* (Ringhal's cobra) and *Naja nivea* (Cape cobra) and the complete amino acid sequences of two of them. J. Biochem. 79:559–578;1976.
10. Holzer, M.; Mackessy, S.P. An aqueous endpoint assay of snake venom phospholipase A_2. Toxicon 34:1149–1155;1996.
11. Laemmli, U.K. Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. Nature 227:680–85;1970.
12. Lee, C.Y. (ed). Snake Venoms. Handbook of Experimental Pharmacology, Vol. 52. Berlin: Springer-Verlag; 1979.
13. Mackessy, S.P. Isolation and characterization of the major metalloprotease of northern Pacific rattlesnake (*Crotalus viridis oreganus*) venom. Toxicon 34:1277–1285;1996.
14. Mackessy, S.P. Kallikrein-like and thrombin-like proteases from the venom of juvenile northern Pacific rattlesnakes (*Crotalus viridis oreganus*). J. Nat. Toxins 2:223–39;1993.
15. Mackessy, S.P. Fibrinogenolytic proteases from the venoms of juvenile and adult northern Pacific rattlesnakes (*Crotalus viridis oreganus*). Comp. Biochem. Physiol. 106B:181–89;1993.
16. Mackessy, S.P. Morphology and ultrastructure of the venom glands of the northern Pacific rattlesnake *Crotalus viridis oreganus*. J. Morphol. 208:109–128;1991.
17. Mackessy, S.P. Venom ontogeny in the Pacific rattlesnakes *Crotalus viridis helleri* and *C. v. oreganus*. Copeia 1988:92–101; 1988.
18. Mackessy, S.P. Fractionation of red diamond rattlesnake (*Crotalus ruber ruber*) venom: Protease, phosphodiesterase, L-amino acid oxidase activities and effects of metal ions and inhibitors on protease activity. Toxicon 23:337–340;1985.
19. Mackessy, S.P.; Holycross, A. Analysis of U.S. montane rattlesnake venoms with an emphasis on *Crotalus willardi obscurus* venom. Abstracts, Society for the Study of Amphibians and Reptiles 38th annual meeting, 1995.
20. Mackessy, S.P.; Tu, A.T. Biology of the sea snakes and biochemistry of their venoms. In: Tu, A.T. (ed). Toxin-related Diseases: Poisons Originating from Plants, Animals, and Spoilage. New Delhi: Oxford and IBH Publishing Co.; 1993: 305–351.
21. Mebs, D. Venom components with other important biological activities. In: Shier, W.T.; Mebs, D. (eds). Handbook of Toxinology. New York: Marcel Dekker, Inc.; 1990:761–76.
22. Ownby, C.L.; Colberg, T.R.; Li, Q. Presence of heat stable hemorrhagic toxins in snake venoms. Toxicon 32:945–54; 1994.
23. Pirkle, H.; Markland, F.S., Jr. Hemostasis and Animal Venoms. New York: Marcel Dekker, Inc., 1988.
24. Quinn, B.; Mackessy, S.P. Determination of snake venom

LD_{50} values using a cricket model system. J. Colo.-Wyo. Acad. Sci 34:28–29;1994.
25. Ramírez, G.A.; Fletcher Jr, P.L.; Possani, L.D. Characterization of the venom from *Crotalus molossus nigrescens* Gloyd (black tail rattlesnake): Isolation of two proteases. Toxicon 28:285–297;1990.
26. Rael, E.D.; Rivas, J.Z.; Chen, T.; Maddux, N.; Huizar, E.; Lieb, C.S. Differences in fibrinolysis and complement inactivation by venom from different northern blacktailed rattlesnakes (*Crotalus molossus molossus*). Toxicon 35:505–513;1997.
27. Rael, E.D.; Martinez, M.; Molina, O. Isolation of a fibrinolytic protease, M4, from venom of *Crotalus molossus molossus* (northern blacktail rattlesnake). Haemostasis 22:41–49;1992.
28. Ritonja, A.; Meloun, B.; Gubenšek, F. The primary structure of *Vipera ammodytes* venom chymotrypsin inhibitor. Biochim. Biophys. Acta 746:138–145;1983.
29. Rosenberg, P. Phospholipases. In: Shier, W.T.; Mebs, D. (eds). Handbook of Toxinology. New York: Marcel Dekker, Inc.; 1990.
30. Russell, F.E.; Emory, J.A.; Long, T.E. Some properties of rattlesnake venom following 26 years storage. Proc. Soc. Exp. Biol. Med. Hyg. 103:737;1960.
31. Russell, F.E.; Eventov, R. Lethality of crude and lyophilized *Crotalus* venom. Toxicon 2:81–82;1964.
32. Straight. R.; Glenn, J.L.; Snyder, C.C. Antivenom activity of rattlesnake blood plasma. Nature 261:259–260;1976.
33. Sugihara, H.; Nikai, T.; Moriura, M.; Kaimya, K.; Tanaka, T. Enzymochemical studies on snake venoms 1. Changes in biologic and enzymatic activities of snake venoms on long standing at room temperature. Jpn. J. Bact. 27:47;1972.
34. Tan, N.H.; Ponnudurai, G. A comparative study on the electrophoretic patterns of snake venoms. Comp. Biochem. Physiol. 102B:103–109;1992.
35. Tan, N.H.; Ponnudurai, G. A comparative study of the biological activities of rattlesnake (genera *Crotalus* and *Sistrurus*) venoms. Comp. Biochem. Physiol. 98C:455–61;1991.
36. Tu, A.T. Chemistry of rattlesnake venoms. In: Tu, A.T. (ed). Rattlesnake Venoms: Their Actions and Treatment. New York: Marcel Dekker, Inc.; 1982:247–314.
37. Villegas, L.; Aguirre, E.; Zavaleta, A.E. Effects of lyophilization on four biological activities of *Bothrops atrox* venom (Serpentes: Viperidae). Rev. Biol. Trop. 41:851–53;1993.
38. Weissenberg, S.; Ovadia, M.; Fleminger, G.; Kochva, E. Antihemorrhagic factors from the blood serum of the western diamondback rattlesnake *Crotalus atrox*. Toxicon 29:807–818; 1991.
39. Willemse, G.T.; Hattingh, J. Effect of drying and storage on electrophoretic properties of venom from puff adders (*Bitis arietans*) and cape cobras (*Naja nivea*). Herpetologica 36:170–74;1980.
40. Woodring, J.P. Circulatory systems. In: Blum, M.S. (ed). Fundamentals of Insect Physiology. New York: Wiley-Interscience; 1985:5–57.