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Bioweapons synthesis and storage: The venom gland of front-fanged snakes ☆

Stephen P. Mackessy*, Louise M. Baxter

School of Biological Sciences, University of Northern Colorado, 501 20th St., CB 92, Greeley, CO 80639-0017, USA

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Abstract

A paradoxical task of the venom gland of snakes is the synthesis and storage of an instantly available suite of toxins to immobilize prey and the protection of the snake against its own venom components. Furthermore, autolysis of the venom constituents due to the action of venom metalloproteases is an additional problem, particularly among viperid venoms, which are typically rich in lytic enzymatic proteins. To address questions concerning these problems, the structure of the venom gland was investigated using light microscopy, SEM and TEM. The composition of the venom originating from the intact venom apparatus or from the main venom gland alone was analyzed by electrophoresis, and the pH of freshly expressed venom as well as pH optima of several representative enzymes was evaluated. Results from several species of rattlesnakes demonstrated that the venom gland is structurally complex, particularly in its small rostral portion called the accessory gland, which may be a site of activation of venom components. Secreted venom is stable in extremes of temperature and dilution, and several proximate mechanisms, including pH and endogenous inhibitors, exist which inhibit enzymatic activity of the venom during storage within the venom gland but allow for spontaneous activation upon injection into prey. Whereas acid secretion by the parietal cells activates digestive enzymes in the stomach, within the venom gland acidification inhibits venom enzymes. We propose that the mitochondria-rich cells of the main venom gland, which are morphologically and histochemically very similar to the parietal cells of the mammalian gastric pit, play a central role in the stabilization of the venom by secreting acidic compounds into the venom and maintaining the stored venom at pH 5.4. Hence, our results indicate yet another trophic link between the processes of venom production and of digestion, and demonstrate that the venom glands of snakes may represent an excellent model for the study of protein stability and maintenance of toxic proteins.

Keywords: Accessory gland; Autolysis; Enzymes; Gland; Mitochondria-rich cell; Parietal cell; pH; Rattlesnake; Stabilization; Toxin; Venom

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*Corresponding author. Tel.: +1 979 351 2429; fax: +1 970 351 2335.
E-mail address: stephen.mackessy@unco.edu (S.P. Mackessy).

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1. Introduction

The biochemical ecology of venomous snakes involves a fascinating blend of protein chemistry, structural biochemistry, evolution and ecology. Venomous snakes utilize numerous protein and peptide enzymes and toxins to dispatch prey and deter predators, yet the compounds themselves are often highly toxic, inherently unstable, or both. Venomous snakes are thus confronted with a conundrum: How does one produce, store and deliver these substances, without inflicting toxic effects on oneself and without losing the biological potency due to autolysis and other degradative processes?

The venom glands of snakes, particularly those of rattlesnakes (Viperidae), represent an excellent model system for the study of the synthesis, secretion and long-term storage of toxic proteins. Following the depletion of stored venom by manual extraction, secretory epithelial cells lining the tubules of the venom gland initiate rapid protein synthesis (Carneiro et al. 1991; Kochva et al. 1980; Mackessy 1991; Warshawsky et al. 1973), apparently in response to stimulation by the autonomic nervous system (Kerchove et al. 2004; Yamanouye et al. 1997). Within 4–8 days, proliferation of the rough endoplasmic reticulum and mRNA levels have reached a maximum (Carneiro et al. 1991; Kochva et al. 1980; Mackessy 1991; Rotenberg et al. 1971; Yamanouye et al. 1997), and subsequent merocrine exocytosis results in the replenishment of venom in the epithelial ductules and large basal lumen. Completion of the synthesis and secretion stage occurs approximately 16 days after depletion of the gland, and during this period, cells cycle from cuboidal to columnar and back to cuboidal morphology (Kochva 1987; Kochva et al. 1975; Mackessy 1991). The venom is then stored in the basal lumen and ductules of the venom gland for varying periods of time and is available when needed. No evidence of venom protein turnover in the glandular lumen or ductules has been presented, but when secretory cells assume a cuboidal morphology, the rough endoplasmic reticulum resumes a minimal state, suggesting inactivity (Kochva 1987; Kochva et al. 1975; Mackessy 1991). Snakes in captivity, which have not had venom extracted in several years and which are maintained on dead prey, show a large amount of cellular debris in the venom, in contrast to snakes whose venom is extracted regularly (pers. obs.). The presence of cellular debris demonstrates that at least large particles are not reabsorbed within the gland, further demonstrating the static nature of the secreted and stored venom. Because snakes in general are adapted to withstand long fasting periods, the venom may be stored for months and in captivity even for several years and will remain active.

Redundant protective mechanisms exist to protect a snake from its own toxins and to maintain the potency of the stored venom, and the expressed venom remains remarkably stable under a wide variety of conditions (Munekiyo and Mackessy 1998). Numerous inhibitors of enzymes, which may be responsible for this stability, have been described, including peptides from venoms of several viperids (Francis et al. 1992; Huang et al. 1998, 2002; Munekiyo and Mackessy 2005; Robeva et al. 1991), zymogen activation of a metalloprotease via a "cysteine switch" mechanism (Grams et al. 1993), and enzyme inhibition by citrate (Fenton et al. 1995; Francis et al. 1992; Odell et al. 1998). Mechanisms that protect a snake from its own venom include the presence of antibodies in the blood to venom proteins (Straight et al. 1976) and structural modifications of specific receptors, such as the acetylcholine receptor of skeletal muscle, which inhibit toxin binding (Servent et al. 1998; Takacs et al. 2001). General protective mechanisms against circulating "rogue" enzymes, such as those produced by invasive microorganisms or resulting from activation of hemostatic mechanisms, are typified by the actions of α_2-macroglobulins against proteases, and these may also protect the snake. However, the mechanisms that allow the long-term storage of venom within the venom gland have not been fully addressed yet, nor is it fully understood how venom that has been released from the secretory cells may be stored in the glandular lumen and ductules for many months and yet remains active upon demand.

To address these questions, we analyzed both the structure of venom glands and the biochemistry of venoms of the Prairie Rattlesnake [*Crotalus viridis viridis* (Rafinesque, 1818)], the Northern Pacific Rattlesnake [*C. oreganus oreganus* (Holbrook, 1840)] and the Western Diamondback Rattlesnake [*C. atrox* (Baird & Girard, 1853)] as models of the viperid design of the venom gland. There are many variations in venom gland design and venom composition among viperids and all other venomous snakes, but the observations presented here may be common among venomous caenophidians generally, a conclusion that is supported by comparative observations of the histology of venom glands (e.g., Kochva and Gans 1966) and on the chemistry of snake venoms (Freitas et al. 1992; Munekiyo and Mackessy 2005; Odell et al. 1998).

2. Materials and methods

2.1. Collection of snakes and venoms

Rattlesnakes were collected in several locations in the western US with permission of appropriate local

agencies. The taxonomy of the Western Rattlesnake (*viridis/oreganus*) complex follows that of Ashton and de Queiroz (2001); *C. atrox* is monotypic. All snakes were housed in the UNC Animal Facility in accordance with UNC-IACUC protocol no. 9204.1 and ASIH/SSAR guidelines. Venoms were manually extracted from captive animals using standard methods (Mackessy 1988), and lyophilized venom was stored frozen and desiccated until used. Venoms from a subset of *C. atrox* specimens from southcentral Texas were collected during surgery to remove the venom glands. Venom was collected at the fang tip on one side of the snake; this venom passes through the entire venom apparatus (designated as "whole gland" venom) and is identical to venom collected from snakes as described above. On the contralateral side, venom was collected from the main gland only (designated as "main only" venom) after isolation via occlusion of the primary duct, thereby bypassing the primary duct, the accessory gland, the secondary duct and the fang. Both of these venom preparations from Texas *C. atrox* were supplied by Bruce Young. The pH of freshly expressed whole venom was measured using a microelectrode and a Beckman pH meter.

2.2. Preparation of venom glands

Venom glands were removed from snakes that had been lightly anesthetized with Halothane or Fluothane and subsequently sacrificed by decapitation. One group of snakes had not been extracted of venom for at least two months and provided "unextracted venom glands"; these venom glands are not actively synthesizing venom proteins and their epithelial cells are cuboidal. The other group of snakes were extracted of venom as above, sacrificed 4 days after venom extraction, and produced "extracted venom glands". These glands had been stimulated to synthesize venom proteins by venom extraction, and their epithelial cells are columnar. The venom glands and tissues were prepared for light microscopy or electron microscopy as previously described (Mackessy 1991). Briefly, samples for electron microscopy were cut into 1 mm blocks while immersed in 100 mM sodium cacodylate buffer (pH 7.2) containing 5% (v/v) glutaraldehyde. Fixation proceeded for three hours, followed by three 10 min washes with sodium cacodylate buffer (100 mM, pH 7.2) containing 10% sucrose. Samples were post-fixed in 1% osmium tetroxide in 100 mM sodium cacodylate buffer (pH 7.2). Specimens for transmission electron microscopy were dehydrated in an ethanol series followed by propylene oxide and then embedded in Epon 812 resin. Thin sections prepared with a diamond knife on a Reichert OM-U2 microtome, lifted onto Formvar-coated grids, stained with uranyl acetate and lead citrate and viewed with a Hitachi 300 (75 kV) transmission electron microscope.

Tissues for histochemistry and light microscopy were preserved in 10% formalin buffered with 100 mM HEPES (pH 7.4), embedded in paraffin and sectioned to 5 μm. Because initial observations suggested structural similarities between mitochondria-rich cells of the venom gland and parietal cells of the stomach, rattlesnake and mouse stomach tissues were also prepared for comparative purposes and as a positive control for histochemical staining. Slides were deparaffinized and hydrated (xylene followed by an ethanol series) using standard protocol (e.g., Humason 1972). Aqueous periodic acid-Schiff's reagent (PAS) staining was immediately followed by hematoxylin and then Orange G counterstain (Wheater et al. 1979); this method results in preferential light orange staining of acid-secreting cells such as parietal cells. Tissue samples were also stained with nitro-BT (2,2′-di-*p*-nitrophenyl-5,5′-diphenyl-3,3′-(3,3′-dimethoxy-4,4′-biphenylene; Nachlas et al. 1957), a method that has correlated positive staining of parietal cells with the production of H^+ in both frog and rat stomachs (Villarreal and Burgos 1955). The sections were dehydrated in an ethanol series followed by xylene, mounted in Per-Mount and photographed using a Nikon 35 mm camera mounted on a Nikon light microscope. Slide film and electron micrograph negatives were then digitized (Epson scanner) and labeled using Adobe Photoshop software.

2.3. Electrophoresis

Analysis of venoms was performed using 17 lane Invitrogen NuPage gels (12% acrylamide; SDS–PAGE) as described previously (Huang and Mackessy 2004). Venoms were dissolved in Invitrogen sample buffer containing 15 mM dithiothreitol at a final concentration of 2.0 mg/mL. Varying amounts of venom (20–50 μg protein/lane) from adult *C. atrox* were loaded to visualize potential differences between intact whole venom and main gland only venom (described above). Eight microliters/lane of Mark 12 molecular weight standards (Invitrogen, Inc.) was also loaded onto gels as a protein mass reference. Electrophoresis at 200 V in MES/SDS buffer (50 mM MES, 50 mM Tris–HCl, 1.0 mM EDTA, 0.1% SDS, pH 7.3) proceeded for approximately 40 min until the tracking dye reached the bottom of the gel. The gel was then removed and stained overnight in 0.1% Coomassie Brilliant Blue R-250 in 30% methanol and 10% glacial acetic acid. The gel was then destained in 30% methanol and 10% glacial acetic acid and photographed using an Alpha Imager.

2.4. Enzyme pH optimum assays

Purified enzymes from whole venoms of several species were assayed for optimal activity pH. These included thrombin-like and kallikrein-like serine proteases (Mackessy 1993) and Cvo PrV metalloprotease (Mackessy 1996) from *C. o. oreganus* venom, Cmp venom phosphodiesterase from Southwestern Speckled Rattlesnake [*C. mitchelli pyrrhus* (Cope, 1866] venom (Perron et al. 1993), Cc L-amino acid oxidase from Colorado Desert Sidewinder Rattlesnake [*C. cerastes laterorepens* (Klauber, 1944)] venom (Mackessy, unpub. data) and Cmp venom phospholipase A_2 from *C. m. pyrrhus* venom (Mackessy, unpub. data). All enzymes were purified using size exclusion chromatography on a 98×2.8 cm column of BioGel P-100 resin in 10 mM HEPES buffer containing 100 mM NaCl at pH 6.8. Enzyme activities were located using assays specific for each activity (Mackessy et al. 2003). Peaks containing enzymes of interest were then dialyzed to remove salts, lyophilized and reconstituted in buffer. Samples were then subjected to ion exchange chromatography using either DEAE-Sephadex or CM-Sephadex (Amersham-Pharmacia). Enzymes obtained by this two-step procedure were typically 90% pure or better, as evaluated by SDS–PAGE. Enzymes were assayed for activity from pH 5 to 10 (0.5 pH unit intervals) using 0.1 M "Good" buffers as described previously (Huang and Mackessy 2004). Activity values were normalized to the highest activity level observed for each enzyme ($= 100\%$ activity). Results from venom enzyme purification studies for two additional species, *C. mitchelli pyrrhus* and *C. cerastes laterorepens*, are included here because they provide data on pH optima for several additional venom enzymes.

3. Results

The venom gland apparatus of rattlesnakes contains four discrete regions: the main gland, the primary duct, the accessory gland and the secondary duct (Fig. 1). Storage of venom occurs in the large basal lumen, the smaller ductules of the main gland and the primary duct (Fig. 2); venom appears to be absent from the accessory gland when the venom gland apparatus is at rest. Just proximal to the accessory gland, the primary duct narrows, forming a glandular isthmus, which restricts the passage of venom to the distal components of the venom gland apparatus (Fig. 3). It is unclear whether this glandular isthmus contains myoepithelial cells, which could constrict or close the primary duct and prevent venom flow forward. However, during manual extraction of venom from snakes, considerable back-pressure is frequently encountered (pers. obs.), perhaps as a consequence of the glandular isthmus, and this structural feature could limit passive anterior flow of venom.

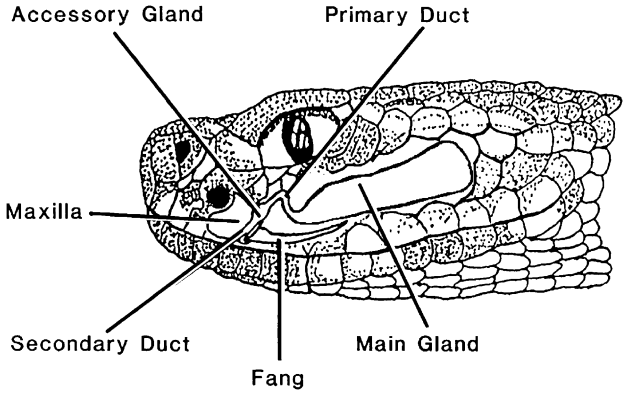

Fig. 1. Diagram indicating location of various components of the venom gland apparatus of the Northern Pacific Rattlesnake *Crotalus oreganus oreganus* after removal of the skin (redrawn from Mackessy 1991).

The accessory gland contains numerous ductules that are oriented radially at approximately 45° to the central duct and run rostrocaudally from the center (Fig. 3). As noted previously (Mackessy 1991), there are seven cell types in the accessory gland, and the proximal part of the accessory gland contains a large number of serous secretory cells and mitochondria-rich cells; some of the latter are also ciliated. At its distal end, the accessory gland primarily contains large mucus-secreting cells (Fig. 4). This arrangement of a serous portion followed by a portion dominated by mucus-secreting cells is similar to the arrangement of cells in the gastric glands of the mammalian stomach (e.g., Csendes et al. 1992; Zalewsky and Moody 1979; Fig. 5A) and in the stomach of the Prairie Rattlesnake (Fig. 5B). In the mammalian gastric glands, parietal, chief and other serous cells are located deep in the pit, while mucus-secreting cells dominate the epithelium of the distal portion of the pit tubules and the gastric surface generally. The same spatial arrangement is seen in rattlesnake gastric glands, but here mixed function chief/parietal cells perform the roles of the individual cells in the mammalian gastric gland. Secreted mucus protects the gastric epithelium in both taxa against the effects of hydrochloric acid and activated gastric enzymes. The sequentially homologous arrangement of the cell types in the rattlesnake accessory gland suggests that venom components from the main gland could become activated by proximally located cell secretions of the accessory gland, requiring mucus secretion to protect the distal parts of the venom gland apparatus from the hydrolytic effects of venom.

However, electrophoretic analysis of venom that was collected from either the intact venom gland apparatus or from the main gland only shows no difference in

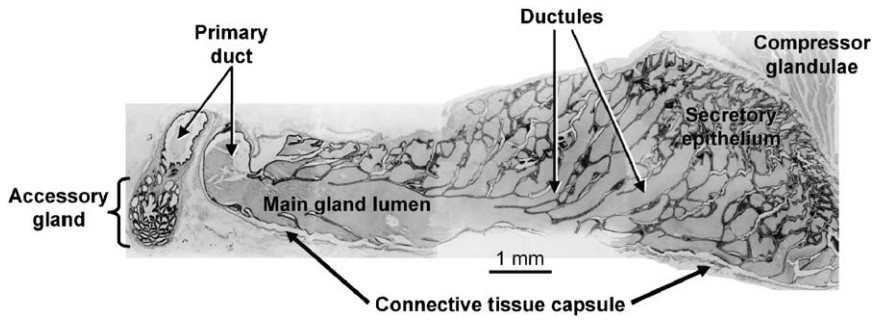

Fig. 2. Longitudinal histological section through the venom gland apparatus of *Crotalus oreganus oreganus*. The secretory epithelium of the accessory gland is quite small relative to that of the main gland. Venom produced by the secretory epithelium is stored within the ductules of the main gland, the main gland lumen and the primary duct. Stained with hematoxylin/eosin.

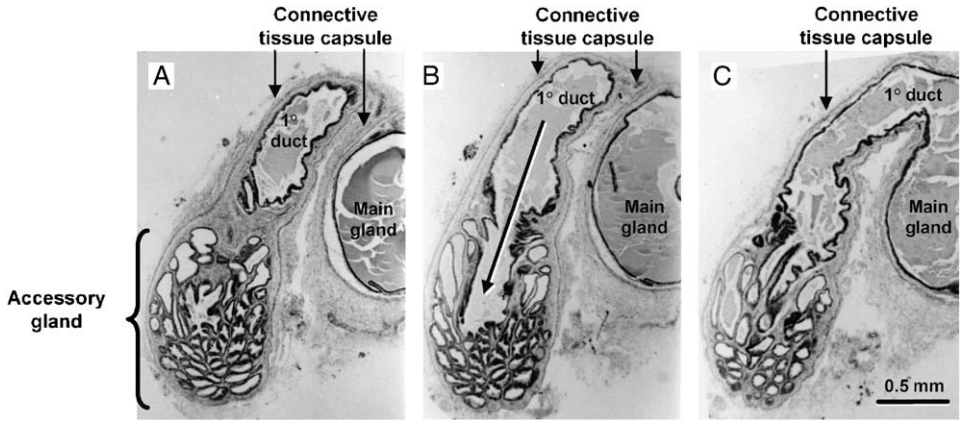

Fig. 3. Three longitudinal histological sections through the accessory gland of *Crotalus oreganus oreganus*. (A) Lingualmost section showing dense tubules of the accessory gland. (B) Medial section showing the continuity between the primary duct and the central lumen of the accessory gland (arrow), and the constriction at the junction of the primary duct and the accessory gland. (C) Lateralmost section showing the continuity between the main gland, primary duct and accessory gland. Stained with hematoxylin/eosin.

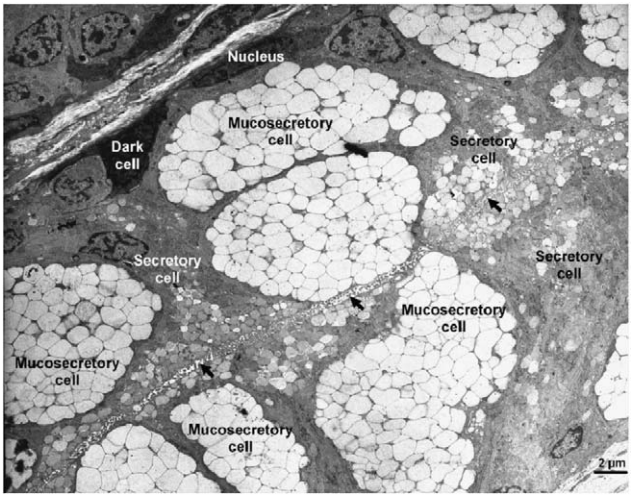

Fig. 4. Transmission electron micrograph (TEM) of mucus-secreting cells in the distal part of the accessory gland of *Crotalus oreganus oreganus*. These cells are filled with large electron-lucid granules (mucus), while adjacent serous secretory cells have smaller electron-dense granules. Dark cells of unknown function lie basally. Arrows point to the lumen of a narrow ductule.

protein profile between the two sample types (Fig. 6). Preliminary reversed phase high-pressure liquid chromatography (RP-HPLC) analysis also indicates that these secretions have identical composition (data not shown). Although the accessory gland is certainly contributing to the entire venom bolus, its protein/peptide contributions have not been identified.

The majority of the cells in the venom gland of *C. o. oreganus*, as in the venom glands of all venomous snakes, are serous secretory epithelial cells (approximately 79%; Mackessy 1991). Another cell type, the mitochondria-rich cell, represents about 2% of the epithelial cell population. Like the serous secretory cells, the mitochondria-rich cells change in height during the venom synthesis cycle (Figs. 7A and B); however, these cells do not contain large secretory granules, in contrast to the secretory cells. Like the parietal cells of the mammalian stomach (Black et al. 1982; Berglindh et al. 1980; Forte et al. 1977), mitochondria-rich cells of the venom gland are pyramidal with a basal nucleus, contain numerous mitochondria, apical vesicles and elongate microvilli, and are recessed into shallow pits (Fig. 7C; see also Fig. 11A). In addition,

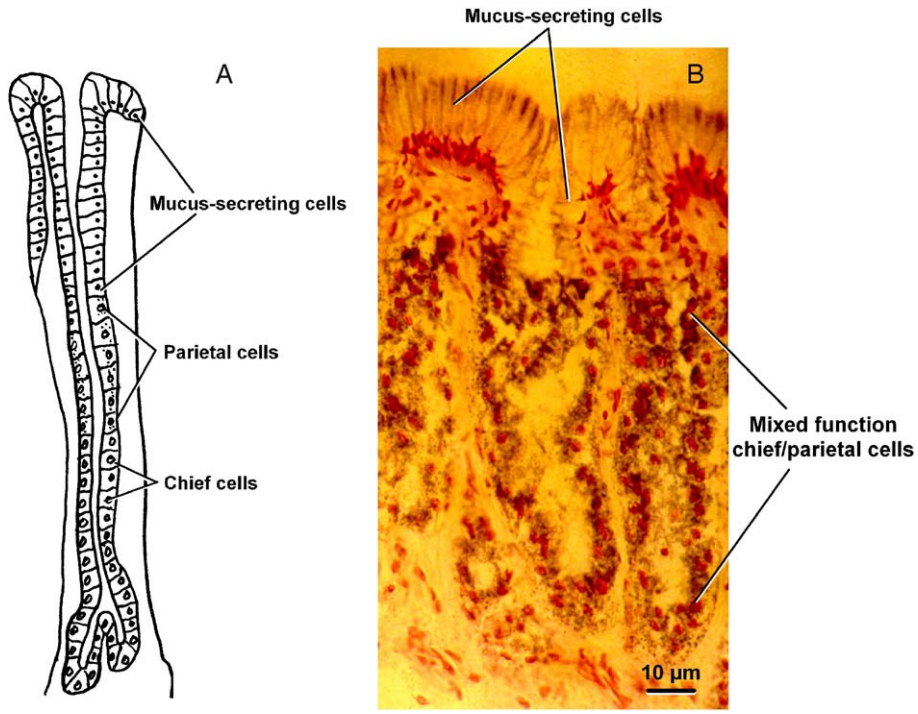

Fig. 5. Comparison of the gastric glands in a mammal and a rattlesnake. (A) Diagram of a gastric gland of the mammalian stomach. Note that the arrangement of cells is similar to that in the rattlesnake stomach, but that the parietal and chief cells are individual entities. (B) Histological section through a gastric gland of the stomach of a Prairie Rattlesnake (*Crotalus viridis viridis*) as stained with nitro BT. Mucus cells predominate along the luminal surface of the stomach and along the distal portion of the gastric gland, while mixed function chief/parietal cells, which stain dark and granular with nitro BT, extend to the base of the gastric gland. In both the mammal and rattlesnake, mucus cell secretions protect gastric tissues from the effects of HCl and activated enzymes released by parietal and chief cells (in mammals) or mixed function cells (in rattlesnakes).

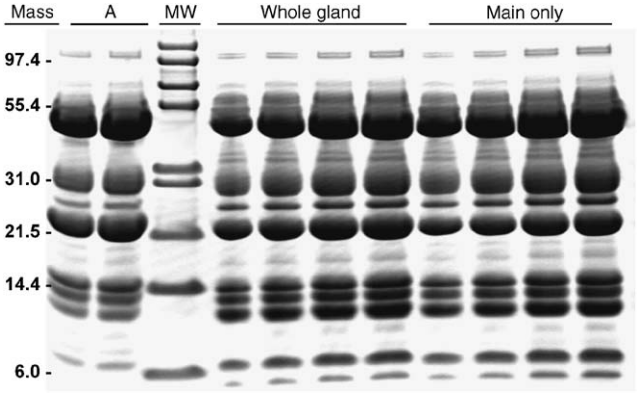

Fig. 6. Electrophoretic analysis of the venom of *Crotalus atrox*, which was collected from the fang tip (''Whole gland'' venom) or directly from the main gland only after primary duct occlusion (''Main only'' venom). The samples are from the same individual snake. The banding patterns are identical in both venom types as well as in the unrelated sample A. Mass, mass of standards in kilodaltons; A, pooled venom from five *C. atrox*, 30 and 40 µg per lane; MW, molecular mass standards. Protein amounts per electrophoretic lane are 20, 30, 40 and 50 µg (left to right) for ''whole gland'' and ''main only'' venoms.

mitochondria-rich cells in the extracted (stimulated) venom gland, when secretory cells are beginning to secrete venom proteins, have numerous canaliculae which extend to the base of the cell (Figs. 8A and B) and which appear to be absent in the mitochondria-rich cells of the unextracted gland (Fig. 7A). These differential features are also similar to those observed in the mammalian parietal cell during active secretion of HCl versus a nonsecretory state. Because these features are all highly reminiscent of the morphology of the parietal cell of the mammalian stomach (Fig. 9), we hypothesized that the mitochondria-rich cells of the main gland epithelium have a similar role to that of the parietal cells of the mammalian stomach in producing an acidic secretion. Therefore, we used specific histochemical stains for revealing acidophilic cells. As a positive control, stomach tissue from rattlesnakes, which contain mixed function chief/parietal cells (Luppa 1977; Fig. 10A) and from mouse stomach tissue (not shown), which contains parietal cells, were stained with Orange G. Both tissue samples demonstrated that the staining reactions were specific for acid-secreting cells, and mitochondria-rich cells from the main venom gland

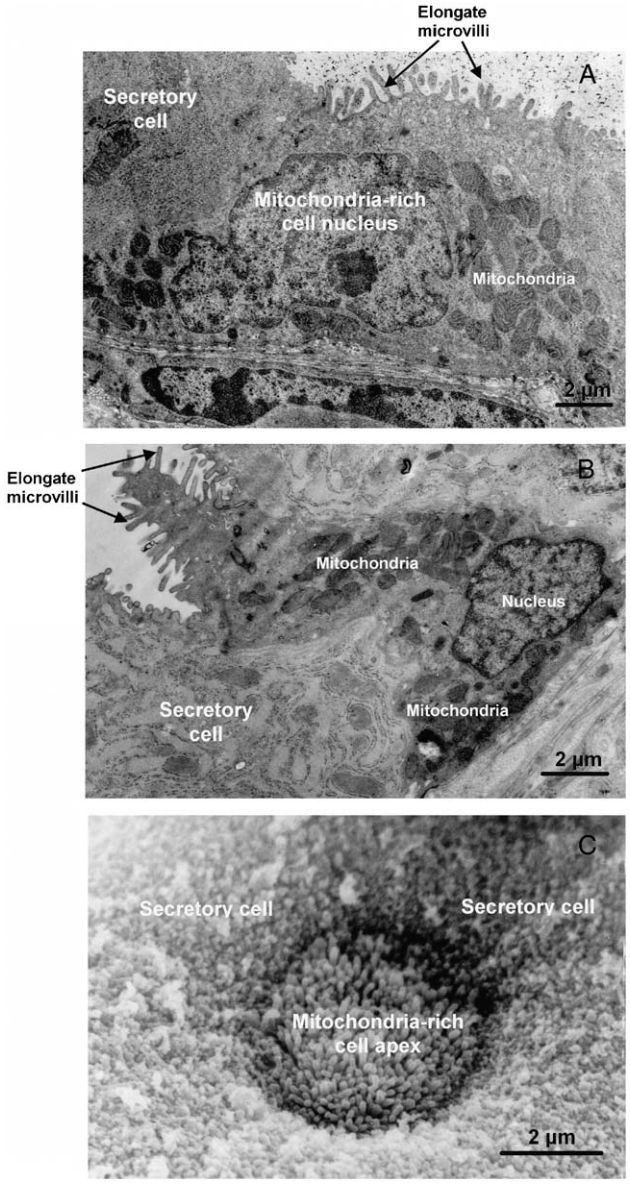

Fig. 7. Electron micrographs of the main venom gland of *Crotalus oreganus oreganus*. (A) TEM of mitochondria-rich cell from an unextracted gland. The short cell (approx. 9.5 µm) is roughly pyramidal, and the elongate microvilli are apparent. (B) TEM of mitochondria-rich cell from an extracted gland. The cell (approx. 14 µm) has increased in height approximately 50%, cycling with the serous secretory cells. (C) Scanning electron micrograph of mitochondria-rich cell, extracted gland. Note that the cell apex (with elongate microvilli) is recessed into a pore-like depression relative to the surrounding serous secretory cells.

also stain differentially (Fig. 10B). The recessed position and pyramidal morphology of the mitochondria-rich cells was apparent following staining with hematoxylin (Fig. 11A), and histochemical staining with either Orange G (Fig. 11B) or Nitro BT (Fig. 11C) revealed that the mitochondria-rich cells of the main venom glands of rattlesnakes, like the parietal cells of the mammalian gastric pit, are strongly acidophilic and stain differently from the surrounding secretory cells. Parallel results were obtained for both mouse (data not shown) and rattlesnake stomach tissues (Fig. 5B, 10A) that were stained with Nitro BT or Orange G. The pH of freshly extracted venom from several rattlesnake species is approximately 5.4 (Table 1), and this slightly acidic nature is functionally important. At this pH, well below the slightly alkaline pH (7.4) of recipient prey tissues (cf. Guyton and Hall 2000), purified enzymes from rattlesnake venoms, including the major metalloprotease from *C. o. oreganus* venom, several serine proteases (thrombin-like and kallikrein-like), a venom phosphodiesterase, an L-amino acid oxidase and a phospholipase A_2, show no or very low enzymatic activity (Fig. 12).

4. Discussion

Although considerable variation exists in the configuration and architecture of the venom gland apparatus of front-fanged snakes, the basic morphology of the venom gland appears to be conserved across taxa (e.g., Gopalakrishnakone and Kochva 1990a; Kochva and Gans 1970; Lake et al. 1983; Mackessy 1991), and the general regionalization of the venom gland as exemplified by *C. o. oreganus* is typical of most species. However, several notable exceptions occur among elapids in the genus *Maticora* (Gopalakrishnakone 1986; Gopalakrishnakone and Kochva 1990b), in the viperid genus *Causus* (Shayer-Wollberg and Kochva 1967), and in the atractaspidid genus *Atractaspis* (Kochva 1959; Kochva et al. 1967), particularly with respect to the length of the venom gland. The general configuration, however, with a caudal main gland, a primary duct (typically absent in elapids), a rostral accessory gland, and a secondary duct leading to an enlarged maxillary fang, is typical of front-fanged snakes.

A conserved morphology and a distal accessory gland suggests an important functional role for the accessory gland, particularly in light of the striking regional differentiation and the high number of cell types present within the accessory gland (Mackessy 1991). The presence of an epithelium rich in mucus-secreting cells in the rostral portion, directly following the serous caudal half, strongly implies that lytic venom components passing through the accessory gland during venom injection have been activated by secretions from the caudal serous portion (see also Hattingh et al. 1984). The spatial arrangement of the components of the

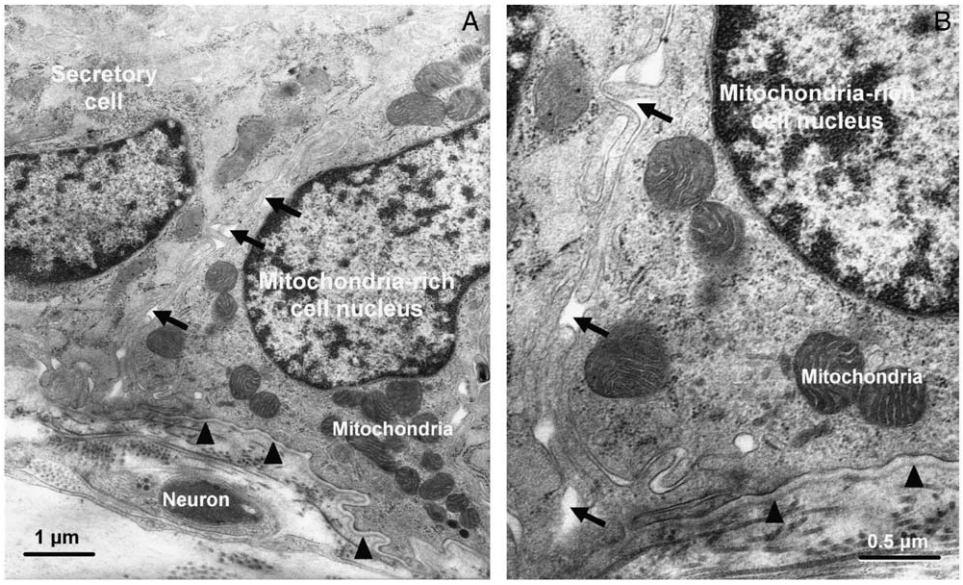

Fig. 8. Transmission electron micrographs of the basal region of a mitochondria-rich cell of an extracted main venom gland of *Crotalus oreganus oreganus*. Note the presence of canaliculae (arrows), similar to those seen in a mammalian stomach parietal cell. In A, a neuron lies in close proximity to the base of the mitochondria-rich cell. In B, the elongate microvilli are folded over within the canalicular space (arrows), and microvilli originating from both sides of the space are apparent. Arrowheads indicate the basal lamina.

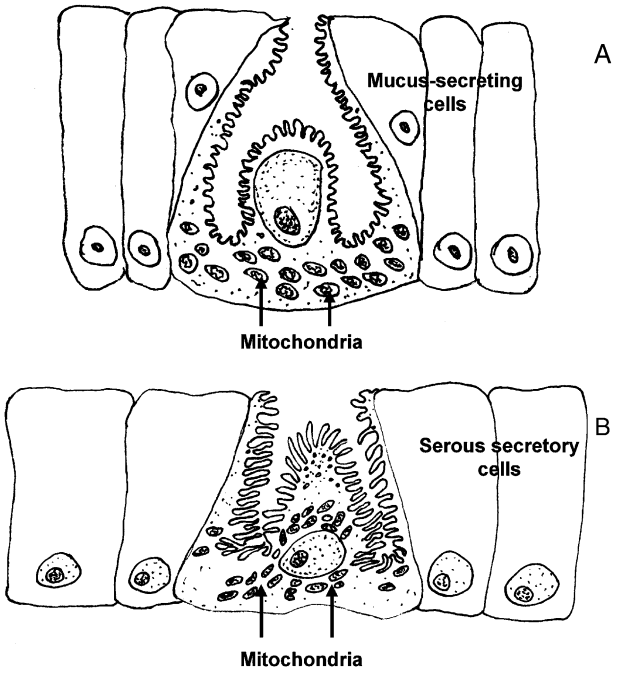

Fig. 9. Comparison of the morphology of an active parietal cell (flanked by mucus-secreting cells) of the mammalian gastric gland (A) and a mitochondria-rich cell (flanked by serous secretory cells) of the extracted rattlesnake main venom gland (B). Note that both cells are characterized by a large nucleus, a large number of mitochondria, elongate microvilli and a prominent canalicular space.

venom gland in rattlesnakes is quite similar to the condition in the stomach (i.e., gastric gland) of both the mouse and the rattlesnake, in which the mucus-secreting cells are abundant near the opening of the gastric glands and in the mucosa of the stomach, providing protection against HCl and digestive enzymes (Csendes et al. 1992). The morphology of the accessory gland in rattlesnakes also suggests that substances are contributed to the venom bolus as it passes through the proximal region of this gland, perhaps activating venom components. However, based on the data from electrophoresis and the preliminary HPLC analyses, no peptide or protein components appear to be added to the venom. Further, whole venom expressed at the fang tip has an acidic pH (Table 1), indicating that neutralization of pH does not occur via the accessory gland. A role in venom activation by the accessory gland is not supported by these data but it also cannot be ruled out, as small amounts of peptides or other organic components may be added by the accessory gland and may not be detected by the methods currently employed. It is also unlikely that the small accessory gland simply provides mucus to lubricate prey and assist in swallowing it (Gans and Kochva 1965; Gopalakrishnakone 1986), because little venom is typically expressed onto the surface of prey during a bite (Hayes et al. 1992) and because many other oral glands that produce copious quantities of saliva are present (Kochva 1978; Kardong 2002).

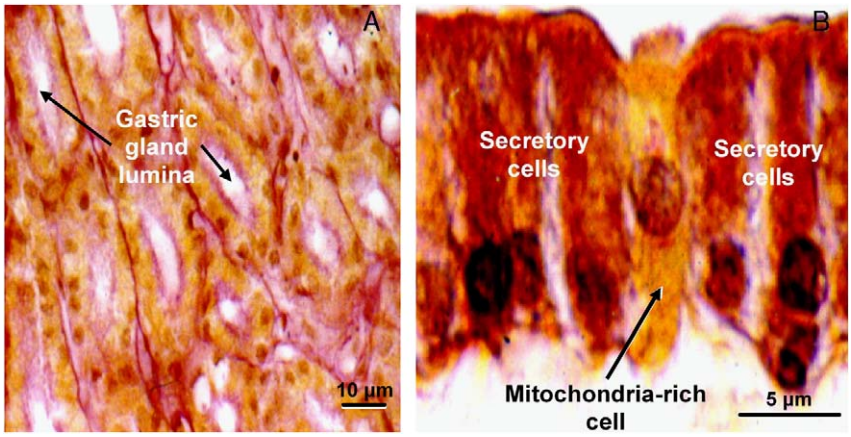

Fig. 10. Light micrographs of histochemical demonstration of acid-secreting cells in *C. viridis viridis* stomach tissues (A) and *C. viridis viridis* main venom gland (B). Tissues were stained with Orange G, and acid-secreting cells stain light orange. (A) Cross-section of rattlesnake stomach through numerous gastric glands showing mixed function chief/parietal cells lining the lumina. (B) Sagittal section of main venom gland epithelium showing differential staining of a mitochondria-rich cell; the recessed apex is apparent.

The cell morphology of the main gland also shows similarities to that of the mammalian stomach, particularly with respect to the mitochondria-rich cells of the venom gland and the parietal cells of the mammalian gastric glands. Because of these similarities, we hypothesized by analogy that the mitochondria-rich cells of venom glands might participate in the acidification of contents of the venom gland lumen, like the parietal cells for the stomach contents. Histochemical staining of the main venom gland confirmed that the mitochondria-rich cells share acidic properties with the parietal cells of the stomach, and they stain very differently from the much more numerous serous secretory cells of the rest of the gland epithelium. This evidence strongly suggests that the mitochondria-rich cells secrete acidic materials into the glandular lumen, and this proposal is supported by our measurements of pH of freshly expressed venom (5.4). Additionally, all purified venom enzymes assayed have pH optima of approximately 7.5–9.5 and are inactive (or nearly so) at the pH of stored venom. As noted previously, mitochondria-rich cells comprise only about 2% of the secretory epithelium cell population (Mackessy 1991); this lower density of acid-producing cells relative to that of the mammalian stomach is also reflected by the higher pH of the luminal contents of the venom gland as well as by mammalian stomach acid concentrations that are 3–4 orders of magnitude *higher* than those of the venom gland (e.g., Guyton and Hall 2000).

Therefore, we propose that the role of the mitochondria-rich cells in the venom glands is to acidify the venom gland luminal contents, and that the morphological and functional similarities between the parietal cell of the mammalian stomach and the mitochondria-rich cell of the snake venom gland illustrate yet another link between venom production and food digestion. The acidification of venom likely occurs in concert with the release of newly synthesized and exocytosed venom, because mitochondria-rich cells cycle in height with secretory epithelial cells and the canaliculae of mitochondria-rich cells are apparent only during venom protein synthesis, which follows extraction of venom. After synthesis in the rough endoplasmic reticulum of the secretory cells, venom proteins are contained within large intracellular granules which appear to rupture into the main lumen upon release from the cell (Mackessy 1991); rate of acid secretion by mitochondria-rich cells is thus predicted to peak at this time. Mitochondria-rich cells may also have a limited role in water resorption (e.g., Warshawsky et al. 1973), but we believe that the primary role is to acidify stored venom and inhibit venom enzymes. It remains unclear whether free acid (such as HCl) is released from mitochondria-rich cells, and the mechanism of transport to the lumen is not known, but acidification may involve release of citrate via specific transporters. Citrate ($pKa_3 = 5.19$), which could buffer secreted venom at the acidic pH observed (pH 5.4), is an abundant component of many snake venoms (Francis et al. 1992; Odell et al. 1998) and can inhibit many enzymes via metal ion chelation (Francis and Kaiser 1993). However, at the basic pH levels of optimum enzyme activity, citrate, even at very high concentrations, does not show significant inhibition of the major metalloprotease (Cvo Protease V) of *C. o. oreganus* venom (Mackessy 1996). Storage of venom enzymes at an acidic pH is necessary for inhibition of enzyme activities, and upon injection into prey tissues,

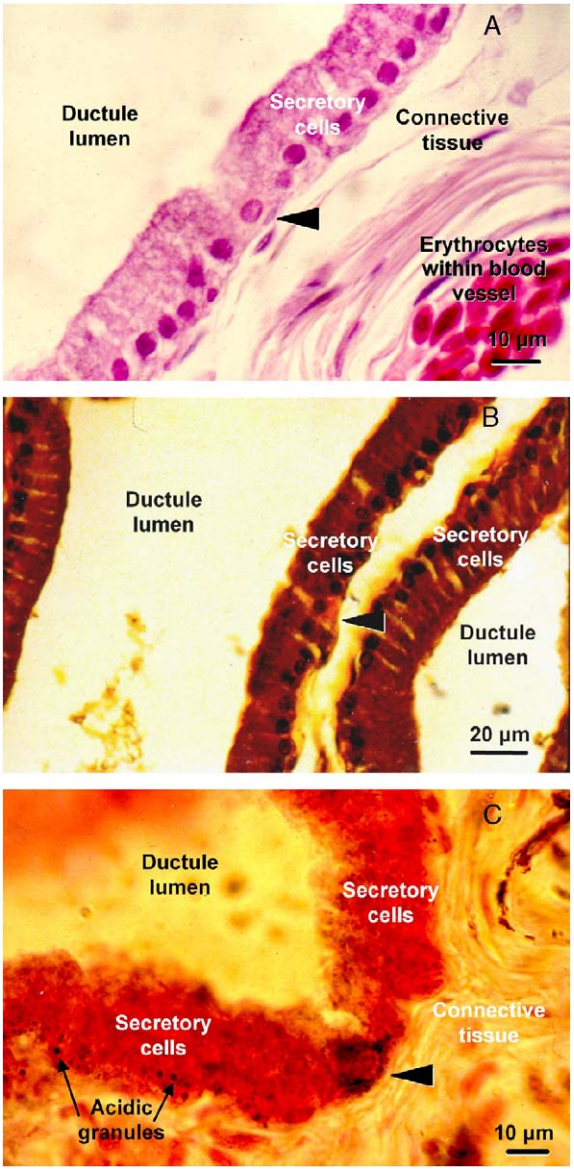

Fig. 11. Light micrographs of the extracted secretory epithelium of the main venom gland of *C. viridis viridis*; mitochondria-rich cells typically show triangular/pyramidal morphology. A mitochondria-rich cell is indicated in each panel by an arrowhead; note the differential staining of mitochondria-rich cell in B and C, indicating a high probability of acid-secretory function. Stains: (A) hematoxylin/eosin; (B) PAS/Orange G/hematoxylin and (C) nitro BT.

Table 1. pH of freshly extracted rattlesnake venom samples

Species	Total length (mm)	pH
Crotalus molossus molossus	930	5.43
C. mitchelli pyrrhus	780	5.52
C. mitchelli pyrrhus	850	5.52
C. ruber ruber	750	5.25
C. ruber ruber	770	5.47
C. ruber ruber	1000	5.61
C. ruber ruber	1200	5.70
C. ruber ruber	1400	5.50
C. oreganus helleri	1100	5.57
C. o. oreganus	310	5.52
C. o. oreganus	730	5.79
C. o. oreganus	760	5.43
C. o. oreganus	775	5.56
C. o. oreganus	860	5.75
C. o. oreganus	890	5.29
C. o. oreganus	890	5.58
C. o. oreganus	1030	5.30
C. viridis viridis	760	5.42
Mean ± SD		5.42 ± 0.15

Note that all samples are mildly acidic and that the variation in pH between species and between size classes is quite low.

spontaneous activation occurs due to the pH of recipient tissues (~7.2–7.4).

Acidification of stored venom by the mitochondria-rich cells is a primary mechanism that allows storage of potentially dangerous and unstable venom components in an inactive state that is readily and instantaneously reversed upon injection, permitting long-term storage and on-demand deployment of a potent biological weapon. However, inhibition by low pH alone is apparently insufficient to protect venom components from autolysis, and additional protective mechanisms exist. Several tripeptide inhibitors of venom metalloproteases are present in many rattlesnake venoms (Munekiyo and Mackessy 2005), and during storage in the venom gland these peptides stabilize and inhibit the abundant venom proteins that might otherwise catalyze autolytic degradation of venom components. Organic acid inhibitors, such as citrate, also appear to have a role in stabilizing venom (Fenton et al. 1995; Francis et al. 1992; Odell et al. 1998), and zymogen activation of at least one venom metalloprotease is required for activity (Grams et al. 1993). These additional redundant mechanisms allow safe venom storage, and upon injection, the venom bolus encounters an environment where pH favors high enzyme activity, peptide inhibitors dissociate and citrate inhibitor is diluted. The snake is further protected from potential effects of venom by modified receptor binding sites (Servent et al. 1998; Takacs et al. 2001) and circulating antibodies to venom components (Straight et al. 1976). Because several of these mechanisms also promote stabilization of protein components, the venom gland of front-fanged snakes could serve as a model for stabilization of protein products with pharmacological applications (e.g., Dong et al. 1995; Ó Fágáin 1995; van den Burg and Eijsink 2002). Future work is being directed toward the identification of transport mechanisms and of the acidifying components expressed by the mitochondria-rich cells of the venom gland.

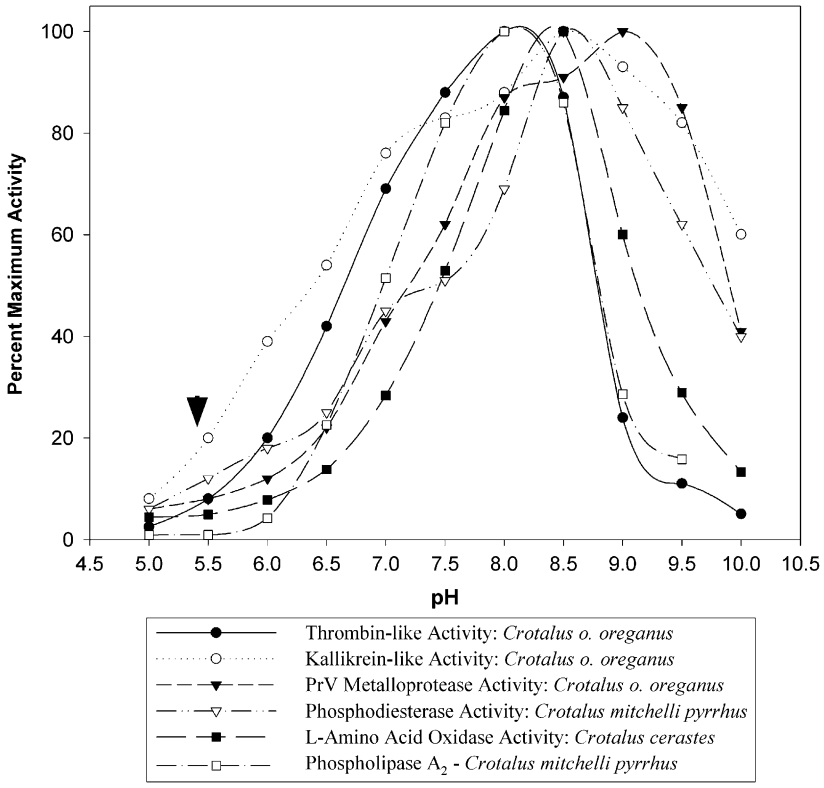

Fig. 12. Effect of pH on activity level for six different purified enzymes from rattlesnake venoms. Percent maximum activity is based on the highest activity level obtained for each enzyme. pH optima vary somewhat between enzymes, but note that at the pH of expressed venom (5.4; arrow), activity of all enzymes is very low. Data for thrombin-like and kallikrein-like proteases is from Mackessy (1993), data for Cvo PrV metalloprotease is from Mackessy (1996), and data for Cmp venom phosphodiesterase is from Perron et al. (1993).

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