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Characterization of plasma secretory phospholipase A_2 activity in the prairie rattlesnake (*Crotalus viridis*)

Sarah Baker[1,2*] and Mark Merchant[3]

Abstract

Background: Plasma phospholipase A_2 (PLA_2) enzyme activity is a key component of innate immunity in most vertebrates. We evaluated circulating secreted PLA_2 activity of prairie rattlesnakes (*Crotalus viridis*) by incubation of plasma with bacteria labeled with fluorescent membrane lipids.

Results: Incubation of bacteria with increasing volumes of plasma resulted in volume-dependent lysis of fatty acids from bacterial membranes. The activity was rapid, with substantial activity recorded after only 5 min of incubation with labeled bacteria, and a linear response for 20 min. In addition, the lysis activity was temperature-dependent, increasing activities from 5 to 20 °C, peak activities at 25–30 °C, and then decreasing activities from 35 to 40 °C. Furthermore, the activity was inhibited in a concentration-dependent manner by *p*-bromophenacyl bromide, a specific inhibitor of PLA_2 activity, which indicated that the observed activities were due to the presence of PLA_2 in the plasma of *C. viridis*.

Conclusions: This study represents the first description of secretory PLA_2 activity in the plasma of a snake. Our study shows that in addition to being an important component of snake venom, PLA_2 enzymes play an important role in the snake's immune response.

Keywords: Antimicrobial, Crotalid, Innate immunity, Reptilian, Vipers

Background

Phospholipase A_2 (PLA_2) is a member of a superfamily of enzymes, the phospholipases, that function to remodel membranes (Rocha et al., 2014) and liberate fatty acids from the *sn*-2 position of membrane lipids to generate a broad spectrum of paracrine hormones (Murakami & Kudo, 2002; Dennis, 2000). In general, members of the PLA_2 family of enzymes can be divided into two main groups: intracellular and extracellular (secreted) PLA_2 forms. Intracellular PLA_2 serves the important function of membrane remodeling (Brown et al., 2003) and the generation of arachidonic acid (Balsinde et al., 2002, Fonteh et al., 1994) for the biosynthesis of a wide array of eicosanoids that serve a broad spectrum of biological functions (Dennis, 2000). Extracellular PLA_2 enzymes also play diverse biological roles, and some bind to extracellular receptors that mediate biological responses (Murakami et al., 2014).

More recently, attention has been focused on soluble, circulating secretory PLA_2 that has been deemed important in innate immunity (Nevalainen et al., 2008). This group of enzymes, called secretory PLA_2 ($sPLA_2$), contains 11 isoforms in mammals (Murakami et al. 2016) and was first described by Vadas et al. (1993). Secretory PLA_2 has a substantial role in innate immune activities, including antibacterial defense and inflammation (Murakami et al. 2016). This enzyme has been shown to be protective against *Staphylococcus aureus* (Laine et al., 1999; Dominiecki & Weiss, 1999),

* Correspondence: sbaker@azgfd.gov
[1]Illinois Natural History Survey, Prairie Research Institute, Champaign, IL, USA
[2]Arizona Department of Game and Fish, Phoenix, AZ, USA
Full list of author information is available at the end of the article

experimental anthrax (Piris-Jimenez et al., 2005), and *Helicobacter pylori* (Huhtinen et al., 2006).

The antibacterial activity of PLA_2 has been attributed to the cationic properties of this enzyme, which allows it to selectively utilize prokaryote membranes as substrates (Buckland & Wilton, 2000). The presence of this enzyme in human tears (Qu & Lehrer, 1998), and inflammatory fluids (Dennis, 1994), and its expression in macrophages (Murakami et al., 1997) and intestinal Paneth cells (Harwig et al., 1995) are consistent with its role as a modulator of immune activity.

Secretory PLA_2 has been identified as an immune component in a variety of ectothermic vertebrates. Members of the families Alligatoridae (Merchant et al., 2009; Siroski et al., 2013) and Crocodylidae (Merchant et al., 2011; Merchant et al., 2017) express high levels of circulating secretory PLA_2 enzymes. In addition, Komodo dragons (*Varanus komodoensis*, Merchant et al., 2018) and several species of turtles (Merchant, unpublished results) also exhibit relatively high circulating levels of this enzyme. This study was conducted to identify and characterize PLA_2 activity in the plasma of the prairie rattlesnake (*Crotalus viridis*).

Methods

Chemicals and biochemicals

4,4-Difluoro-5,7-dimethyl4-bora-3a,4a-diaza-s-indacene-3-hexadecanoic acid (BODIPY FL C16) was purchased from Invitrogen (Carlsbad, CA, USA). Ethylene glycol tetraacetate (EGTA), *p*-bromophenacyl bromide (BPB), $CaCl_2$, nutrient broth, sodium hydroxide, and tris-HCl were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Treatment of animals

Plasma samples were obtained from 21 (9F:12M) adult long-term captive *C. viridis*. Rattlesnakes were housed in plastic enclosures (Neodesha Plastics, Inc.) on newspaper substrate. Temperature was maintained between 18 and 24 °C and photoperiod on a 12L:12D cycle. Water was provided ad libitum, and snakes were fed pre-killed mice biweekly. Blood samples were collected from the caudal vein and did not exceed 0.08% of total snake body mass per sampling event. Whole blood was centrifuged to separate plasma from red blood cells, and the collected plasma was pooled and stored at − 20 °C until use.

Labeling of bacteria

One-milliliter cultures of *E. coli* bacteria were grown overnight at 37 °C in nutrient broth. These cultures were used to inoculate 1-L cultures. These cultures were incubated for 24 h in the presence of 1 mg of BODIPY FL C16 (dissolved in 1 mL of dimethyl sulfoxide). The bacteria were centrifuged at 8000×*g* for 15 min, the cultures were decanted, and the bacteria were resuspended in 30 mL of sterile isotonic saline. The bacteria were again centrifuged 8000×*g* for 15 min to remove unincorporated BODIPY, and the bacterial pellet was resuspended in 30 mL of sterile isotonic saline, divided into 2-mL aliquots, and frozen at − 20 °C until ready for use.

PLA_2 assay

The method used for the determination of PLA_2 in the plasma enzyme activity of *C. viridis* was described by Merchant et al. (2009). Rattlesnake plasma was incubated with 250 μL of assay buffer (1 mM Ca^{2+} in 100 mM tris-HCl, pH 7.4) and 100 μL of BODIPY-labeled bacteria. The balance of the 750-μL reaction consisted of isotonic saline. For the determination of the effects of plasma volume on PLA_2 activity, different volumes of *C. viridis* plasma (1–100 μL) were incubated with 50 μL of BODIPY-labeled *E. coli* bacteria in assay buffer for 30 min ambient temperature. The reactions were terminated by the addition of 750 μL of stop buffer (100 μM tris-HCl, pH 7.4 with 15 mM EDTA) and were then centrifuged at 16,000×*g* to pellet the labeled bacteria, and approximately 1500 μL of each reaction was removed to a 1-mL plastic cuvette. The fluorescent intensity of each reaction was measured at an excitation λ of 500 nm and an emission λ of 510 nm (excitation and emission slit widths = 1 nm) in a Horiba Jobin Yvon Fluoromax-4 fluorimeter. The same procedure was followed to determine the effects of time, temperature, and inhibitors on PLA_2 activities in plasma from *C. viridis*.

Statistics and controls

The fluorescent intensity of each sample was compared to a standard curve of pure product to determine the nanomoles of product formed. The fluorescent intensities of each sample were corrected for background fluorescence by subtraction of a reagent blank in the absence of plasma. Each data point represents the means ± standard deviation for four independent determinations.

Results

The incubation of plasma derived from *C. viridis* with bacteria labeled with BODIPY resulted in a volume-dependent increase in fluorescent product (Fig. 1). The relation between soluble fluorescence and plasma volume was biphasic, with a slow increase at low volumes (0–10 μL) and a near linear increase from 20 to 100 μL. At these higher volumes, an average increase of 1.35 nmol product formed/μL of plasma was observed (Fig. 1).

The accumulation of fluorescent product was relatively rapid, with substantial amounts of product (5.8 ± 5.6 nmol) detected after incubation of bacteria with *C. viridis* plasma for only 5 min (Fig. 2). The accumulation of product appeared to be asymptotic, while the initial

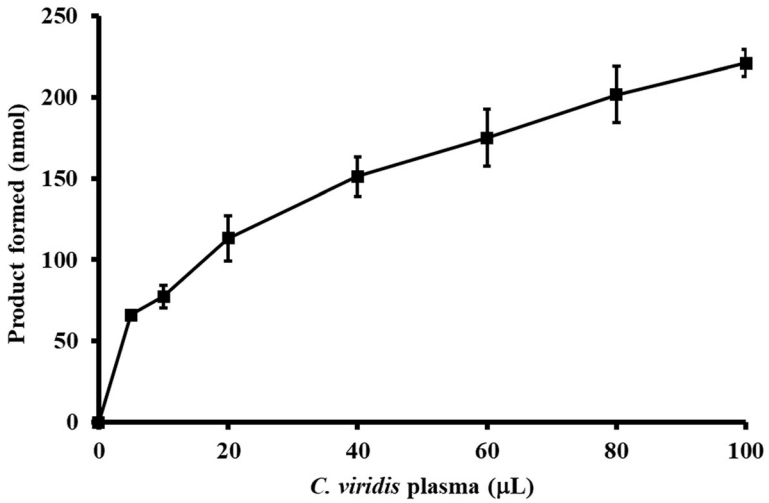

Fig. 1 Volume-dependent lysis of labeled membrane fatty acids from bacteria by plasma derived from *Crotalus viridis*. The results represent the means ± sd of four independent trials

formation of product was near linear (from 0 to 20 min), and product was generated at a rate of 2.5 nmol/min during this time frame. However, between 20 and 30 min, the rate of product formation fell to 0.9 nmol/min and dropped further to 0.5 nmol/min between 30 and 60 min.

The thermal profile for PLA_2 activity was shown to be highly temperature-dependent (Fig. 3). From 5 to 10 °C and from 10 to 15 °C, the plasma exhibited a stepwise increase of 38 and 55 nmol of product formed, respectively. However, from 15 to 30 °C, a relatively small increase of 36 nmol was observed for the 15° increase in temperature. At 35 °C, the activity of 129 nmol was 22.8% lower than that measured at 30 °C (167 nmol), which fell further to 113 nmol at 40 °C.

The plasma-dependent generation of fluorescent product was inhibited in a concentration-dependent manner by BPB (Fig. 4). Incubation of *C. viridis* plasma with fluorescently labeled bacteria led to the generation of 71.5 ± 2.7 nmol of product. The inclusion of only 1 mm BPB inhibited the activity by 17.5 ± 4.6%. The inhibition was further increased to 35.7 ± 3.1 and 70.3 ± 7.8 when the concentration of BPB was increased to 2 and 5 mM, respectively. Furthermore, the addition of 50% unlabeled *E. coli* bacteria caused a 43.7 ± 7.3% reduction of activity relative to cultures of only labeled bacteria.

Discussion

Multicellular organisms must continuously defend against invasion and colonization by potentially infectious microbes. Innate immunity has been identified as a

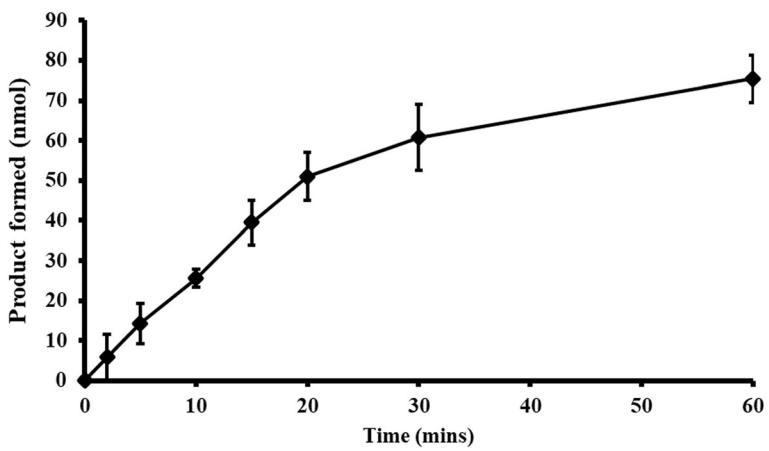

Fig. 2 Time-dependent lysis of fatty acids from the membrane of fluorescently labeled *E. coli* incubated with plasma derived from *Crotalus viridis*. The results represent the means ± sd of four independent trials

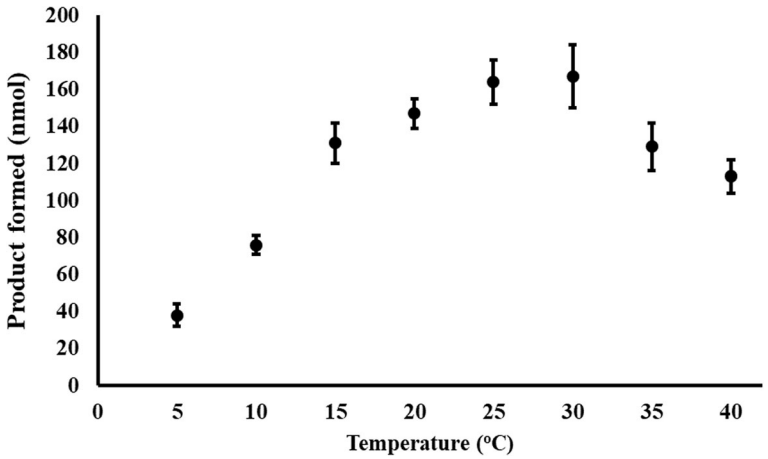

Fig. 3 Thermal profile of the lysis of fluorescently labeled fatty acids from the membranes of *E. coli*. The results represent the means ± sd of four independent trials

primary mechanism of defense in ectotherms (Romo et al., 2016). However, our knowledge of the function of reptile immune systems lags behind other vertebrates (Warr et al., 2003). Recent studies have described the broad-acting antibacterial properties of plasma from *C. viridis* (Baker & Merchant, 2018a) and have assigned much of those properties to an active serum complement system of proteins (Baker & Merchant, 2018b).

With the exception of crocodylians (Merchant et al., 2009), PLA_2 activity has not been well described in reptiles. The exception is the role of PLA_2 as a component of snake venoms. When injected, PLA_2 enzymes can contribute to envenomation morbidity via hemorrhage and edema, with the severity being highly species specific. However, PLA_2 enzymes also play an important role in immunity, with some venom PLA_2 showing bactericidal ability equivalent to that of commercially produced drugs (Samy et al., 2007). Fewer studies have examined the role of non-venom secretory PLA_2 in the snake's own immune system.

Activity of PLA_2 increases in a concentration-dependent manner but does not appear to reach an asymptote. Overall activity is less than that observed in American alligators, and higher concentrations of *C. viridis* plasma are needed to approach maximum activity (Merchant et al., 2009). We found PLA_2 activity is rapid,

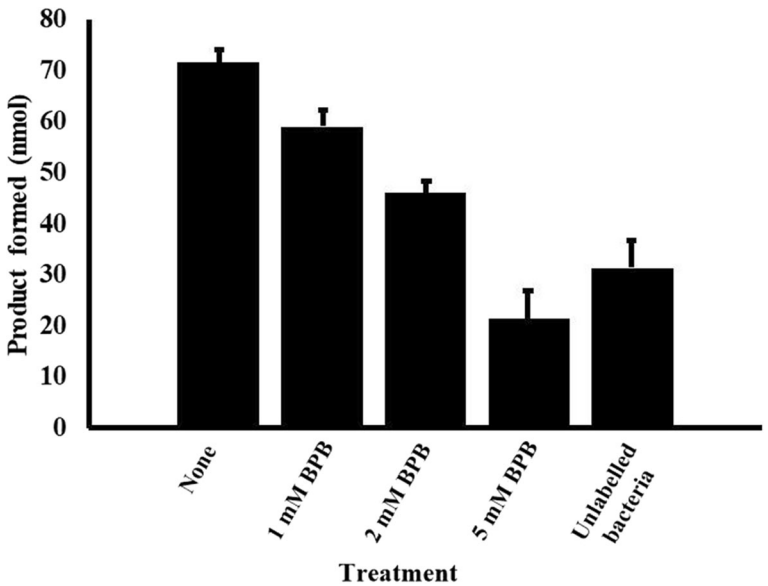

Fig. 4 Inhibition of the accumulation of fluorescent of product generated by plasma from *Crotalus viridis*. The addition of different amounts of BPB, a specific inhibitor of PLA_2, resulted in a concentration-dependent inhibition of the accumulation of fluorescent fatty acid product. The results represent the means ± sd of four independent trials

rising sharply from 5–20 min post exposure. Quick response to pathogens is an important component of the innate immune response. American alligators are frequently injured in combat with conspecifics and are found in swamps and marshes of the southeastern USA where temperatures remain warm year-round and potentially pathogenic organisms are common. In contrast, *C. viridis* inhabit dry regions of the western USA and must hibernate through the winter months throughout most of their range (Ernst & Ernst, 2003), reducing both the number of pathogenic organisms they are likely to encounter and the season when infection is likely.

We found PLA_2 activity was maximal between 25 and 30 °C, which approximates the preferred body temperature of *C. viridis* (28–32 °C; Gannon & Secoy, 1985). Previous studies have found that the antibacterial ability of plasma and serum complement activity also peak at approximately 30 °C (Baker & Merchant, 2018 a, b). At higher temperatures, PLA_2 activity is reduced. This is not the case in the American alligator, or in two species of caiman native to South America which show an increase in activity at temperatures above 30 °C (Merchant et al., 2009; Siroski et al., 2013). This may be partially due to higher preferred body temperatures in crocodylians; however, neither American alligators nor spectacled caiman has been found to voluntarily tolerate temperatures above 38 °C (Diefenbach, 1975; Johnson et al., 1978).

Conclusions

Our results indicate that secretory PLA_2 is an important component of the innate immune response in rattlesnakes. While the activity of this enzyme is well known as a component of venom, this is the first study to describe PLA_2 activity as it relates to the snake's own immune system.

Abbreviations

PLA_2: Phospholipase A_2; *C. viridis*: *Crotalus viridis*, prairie rattlesnake; BODIPY FL C16: 4,4-Difluoro-5,7-dimethyl4-bora-3a,4a-diaza-s-indacene-3-hexadecanoic acid; EGTA: Ethylene glycol tetraacetate; BPB: *p*-Bromophenacyl bromide

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Authors' contributions

SJB collected the plasma samples, conducted the lab work, and contributed to the writing of the manuscript. MEM provided funding, conducted the lab work, and contributed to the writing of the manuscript. The authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Work was conducted under approved University of Illinois Institutional Animal Care and Use Committee Protocol #17013.

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

Author details

[1]Illinois Natural History Survey, Prairie Research Institute, Champaign, IL, USA. [2]Arizona Department of Game and Fish, Phoenix, AZ, USA. [3]Department of Chemistry and Physics, McNeese State University, Lake Charles, LA, USA.

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