ISSN 2347-6893
Volume 11 Number1
Journal of Advances in Biology

Characterization Of Phospholipase A_2 Enzyme Activity In Serum Of The Komodo Dragon (Varanus Komodoensis)

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ABSTRACT

Soluble phospholipase A_2 ($sPLA_2$) is an enzyme found in the peripheral circulation of vertebrates which has significant immunological activity. This enzyme exerts immune activity by the hydrolysis of fatty acids from the sn-2 position of membrane glycerophospholipids of microbes, thus compromising membrane integrity and casing eventual lysis. We utilized membrane fatty acids labeled with a fluorescent probe (BODIPY) at the sn-2 position fatty acid to label the membranes. Incubation of different volumes of serum from Komodo dragons with BODIPY-labeled bacteria resulted in liberation of labeled fatty acid in a serum volume-dependent manner. This cleavage of fatty acid occurred rapidly, with a biphasic production of fluorescent product. An immediate accumulation of product was noted, which increased steadily for a 30-minute period, followed by a slower hydrolysis between 30 and 180 min. The activity was temperature-dependent, with low activities observed at 5^{o}C and a linear increase up to 40^{o}C. The liberation for fatty acid was inhibited by *p*-Bromo phenacyl bromide, a specific phospholipase A_2 (PLA_2) inhibitor, in a concentration-dependent manner, indicating that the activity was due to the presence of $sPLA_2$.

Indexing terms/Keywords

innate immunity, reptile, varanid.

Academic Discipline And Sub-Disciplines

Biology; Biochemistry; Conservation; Ecology

SUBJECT CLASSIFICATION

Class Q: Science; subclass QL: Zoology, Herpetology

TYPE (METHOD/APPROACH)

Experimental. Laboratory Research

INTRODUCTION

Phospholipase A_2 (PLA_2) is an enzyme that cleaves fatty acids from the sn-2 position of membrane structural lipids. This enzyme serves a variety of important biological and cellular functions including remodeling of membranes [1]. Among its most important functions is the cleavage of intracellular arachidonic acid from membranes, which then serves as a precursor for eicosanoid synthesis [2-3]. However, another form of PLA_2 ($sPLA_2$), first described by Vadas et al. [4], which circulates as a free enzyme in the serum. This enzyme is found in the peripheral circulation and cleaves Sn-2 fatty acids from the membranes of microbes, thus compromising membrane integrity and function. As a result, $sPLA_2$ exhibits important immune activity [5-7] and is thought to be a critical component of innate immunity.

The Komodo dragon (*Varanus komodoensis*) is a critically-endangered species that is native to only three islands on the Indonesian archipelago. The largest lizard in the world, Komodo dragons are fierce predators that have been known to feed on prey more than ten times their own size [8-9]. These animals have saliva which contains large loads of pathogenic bacteria. They deliver a lethal bite, and follow their prey for days, or even weeks, until it succumbs to systemic infection. For this reason, they are able to feed on prey items much larger than themselves [8-9]. In addition, it has been suggested that these reptiles deliver toxins in their bite, based on skull morphology [10]. Although these toxins have not been isolated or described to date, they are reported to induce systemic shock and anticoagulant effect. Komodo dragons are territorial, and often engage in territorial battles with members of their own species. In addition, these animals often inflict wounds on each other during feeding frenzies in which multiple dragons feed on large prey items. However, despite the fact that their saliva contains large loads of infectious bacteria, Komodo dragons do not seem to suffer the same fate as their prey. This would seem to indicate that these animals have evolved an effective immune system to combat these potentially lethal wounds.

Little is known concerning the immune system of Varanids. Several recent studies have characterized antibacterial activity [11], serum complement [12], and dipeptidyl peptidase [13] activities of serum from the Komodo dragon. In addition, these animals have been reported to express antimicrobial peptides [14]. Because Komodo dragons are endangered, it would be useful to further understand their mechanisms of immunity to disease and infection. This study was conducted to characterize the expression of circulating PLA_2 enzyme activity in the serum of the Komodo dragon.

MATERIALS AND METHODS

Chemicals and Biochemicals

4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-hexadecanoic acid (BODIPY™ FL C_{16}) was purchased from Invitrogen (Carlsbad, CA, USA). Ethylene glycol tetraacetate (EGTA), *p*-bromophenacyl bromide (BPAB), $CaCl_2$, nutrient broth, sodium hydroxide, and tris HCl were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Treatment of Animals

Blood samples were collected from Komodo dragons at the Houston and San Antonio zoos. Blood was drawn from the caudal vein, transferred to Vaccutainer™ tubes, and allowed to clot for at least five h before the serum was collected be centrifugation. The amount of blood collected from each individual depended on the size of the animal, and was at the discretion of the attending veterinarian at each institution. Blood was collected from the tail caudal veins three adults (20 - 81.5 kg) and five juveniles (1.5 - 6.2 kg), transferred to Vaccutainer™ tubes, and allowed to clot for at least five hr before serum was collected by centrifugation. The serum was pooled so that average antibacterial values for this species could be generated. The collection of blood from these animals was conducted in accordance with the Animal Care and Use institutional policies of the Houston and San Antonio Zoos.

Preparation of PLA_2 substrate

Escherichia coli (ATCC 11105 strain) was used to inoculate 1 L of nutrient broth. Subsequently, 1 mg of BODIPY® FL C16 (4,4-difluoro-5, 7-dimethyl-4-bora-3a, 4a-diaza-s-indacene-3-hexadecanoic acid; Invitrogen, Carlsbad, CA, USA) dissolved in 1 ml of DMSO (dimethyl sulfoxide) was added to this culture and incubated at 37 °C for 18 h. The culture was then centrifuged at 8000 *g* for 15 min. The supernatant was discarded and the bacterial pellet resuspended in a solution of 0.9% NaCl (w/v). This process was carried out twice. Finally, the pellet was resuspended in 30 ml assay buffer (1 mM Ca^{2+} in 100 mM tris-HCl, pH 7.4).

PLA_2 assays

This assay was developed to measure the secretory PLA_2 enzyme activity in serum. We used fluorescent BODIPY® to label a fatty acid that binds occupies the *sn*-2 position of phospholipids. Bacterial cultures add these macromolecules as component of the outer membrane during growth and proliferation. When bacteria are exposed to the activity of the PLA_2 enzyme, the labeled *sn*-2 fatty acid is hydrolyzed from the membrane and released from the into the assay buffer. After a brief centrifugation, the pellet formed contains bacteria with the labeled fatty acid in the membrane, while the supernatant has free labeled fatty acid that can be measured by spectrofluorometry. PLA_2 activity was measured in a spectrofluorometer at an excitation λ of 500 nm and an emission λ of 510 nm, and excitation and emission slit widths of 1 nm.

To evaluate the kinetic parameters of the PLA_2 enzyme activity in Komodo dragon serum, 3.5 ml of serum, 1.75 mL of the solution containing the fluorescent marked bacteria and 33.25 ml of assay buffer were mixed. At different time intervals (0, 5, 10, 15, 20, 30, 60, 90 min), 1.1 mL of the mixture was removed and added to 900 μl of stop buffer (100 mM tris-HCl, 50 mM EDTA, pH 8.0), centrifuged, and the supernatant was measured spectrofluorometric ally as previously described.

To determine the effect of serum titer on PLA_2 activity, different amounts of serum (0, 1, 2, 5, 10, 20, 50, 100, 200 and 500 μl) derived from Komodo dragons were added and then balanced to a final volume of 750 μL with assay buffer. Then, 100 μL of the solution containing labeled *E. coli* were added and incubated for 30 min. After incubation time, the reaction was terminated with the addition of 750 μL stop buffer, then centrifuged, and the fluorescence intensity of supernatant determined spectrofluorometric ally.

Statistics and controls

The concentrations of each sample were compared to that of a standard curve developed for pure product, and the nmol of product formed for each assay was calculated. The fluorescent intensities for each sample were corrected for background by the subtraction of florescence measured in the absence of alligator serum. Each data point represents the mean ± standard deviation of four independent determinations

RESULTS AND DISCUSSION

Eukaryotic organisms must constantly protect against colonization by potentially pathogenic microbes. Host defense can be accomplished by two different, but highly interrelated immunological systems of protection, innate and adaptive immunity. While adaptive immunity affords high specificity, it typically requires previous exposure and can take several days to develop following initial infection. In contrast, the innate immune system, while less specific in activity, is based on microbial molecular pattern recognition [15-16], is available immediately upon infection, and acts as a first line of defense against infection. Secretory PLA_2 act as a significant component of innate immunity, hydrolyzing fatty acids from glycerophospholipids of microbial membranes, thereby compromising membrane integrity and function.

Phospholipase A_2 has been found in a few reptiles, but has been primarily characterized in snake venoms [reviewed in 17]. More recently, circulating PLA_2 has been identified in a variety of crocodilian species. Nevalainen et al. [18] detected PLA_2 in the serum of both saltwater (*Crocodylus porosus*) and Siamese crocodiles (*C. siamensis*) using radiolabeled fatty

acids. More recent studies in our laboratory have identified circulating $sPLA_2$ in the American alligator (*Alligator mississippiensis*) [19], broad snouted-caiman (*Caiman latirostris*) and yacare caiman (*Caiman yacare*) [20]. In addition, $sPLA_2$ activity has been identified three species of African crocodiles, the Nile crocodile (*C. niloticus*), dwarf crocodile, dwarf crocodile (*Osteolaemus tetraspis*), and the slender-snout crocodile (*Mecistops cataphractus*) [21].

The data displayed in Figure 1 illustrate the titer-dependent PLA_2 activity in Komodo dragon serum. Inclusion of only one µL of serum resulted in the formation of 9.7 nmol of product after 30 min of incubation with substrate. The activity increased dramatically when 2-50 µL of serum were used, and then increased less dramatically from 100-500 µL. The curve appeared to be asymptotic in nature, approaching a maximum product formation of approximately 85-90 nmol. This strong activity is not surprising considering the potent and broad-acting antibacterial activity of the Komodo dragon serum [11], and also the effective serum complement activity [12] previously described for these animals. These animals are known to have a broad spectrum of both gram positive and negative bacterial species in their saliva [22]. Therefore, the strong PLA_2 activity shown in Figure 1 might help defend against infection of injuries due to interspecies fighting during territorial disputes, feeding frenzies, and during mating conflicts.

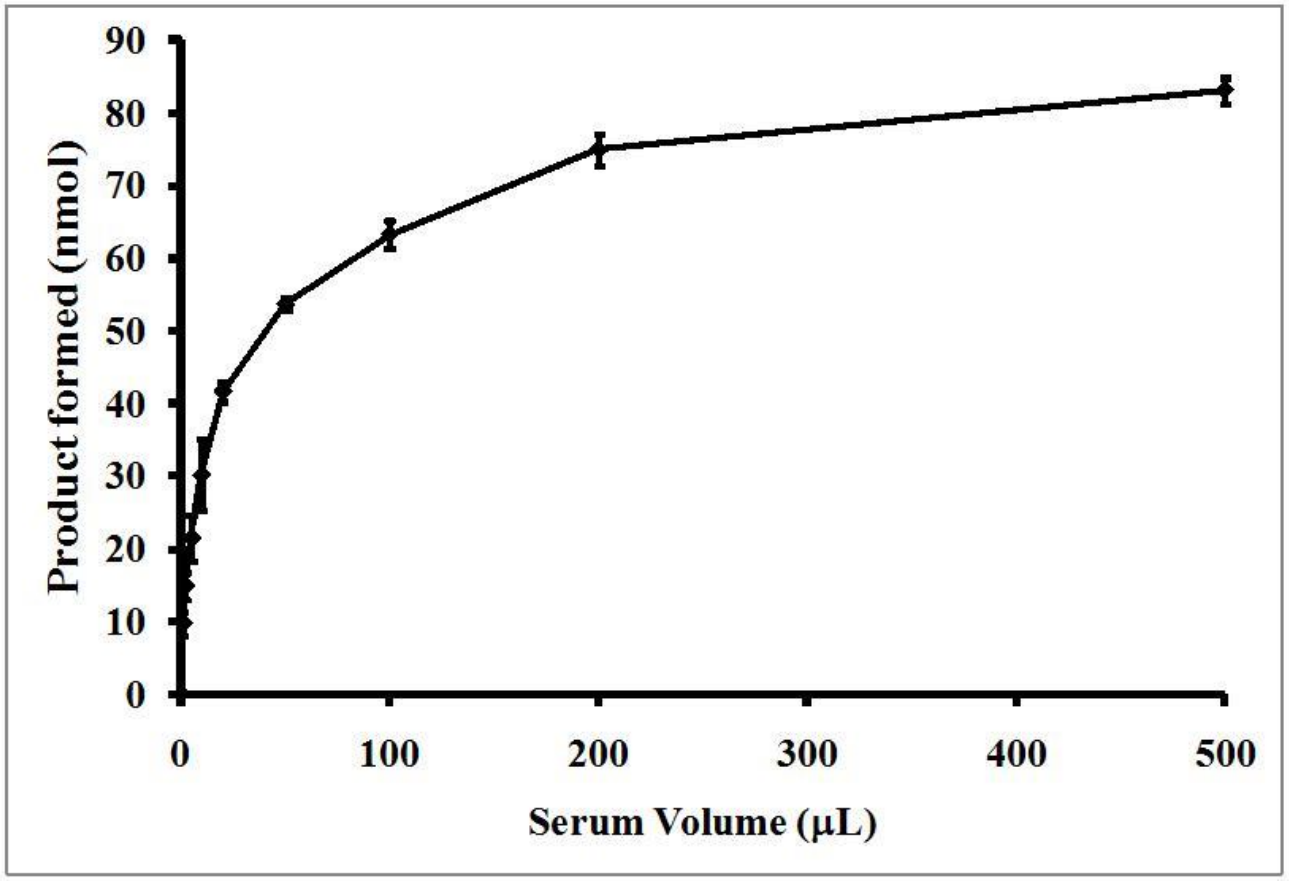

Figure 1. Serum volume-dependent PLA_2 activity in serum from the Komodo dragon

Different volumes (1-500 µL) were incubated with 100 µL of BODIPY-labeled E. coli bacteria in a 750 mL reaction. After one hour, the samples were centrifuges and the fluorescent activity of the resulting supernatant was determined as described in the Materials and Methods section. The results are presented as the means standard deviations of four independent determinations and are expressed as nmol of product formed/30 min reaction incubation.

The data in Figure 2 demonstrate the kinetic curve of Komodo dragon serum PLA_2 activity. The response was biphasic and showed the high rate of conversion of substrate to product. The accumulation of enzymatic product occurred very rapidly, with 11.2 nmol of product formed only five minutes after the addition of substrate. The response was linear for 30 minutes, and then a strong change in slope occurred, and the response exhibited another linear portion from 30-180 min. The initial rate of product formation during the first thirty minutes of incubation, was approximately 1.3 nmol/min. Since a ten-fold dilution of plasma was used for this assay, we can assume a 10-fold increase of product formation to 13 nmol/min in undiluted plasma. This is a very high rate of product formation for PLA_2. These data indicate that serum from Komodo dragons might potentially have a high capacity to kill bacteria due to the PLA_2 activity. This rapid activity would be useful to suppress microbial growth and proliferation within a short time after an aseptic injury or infection occurs.

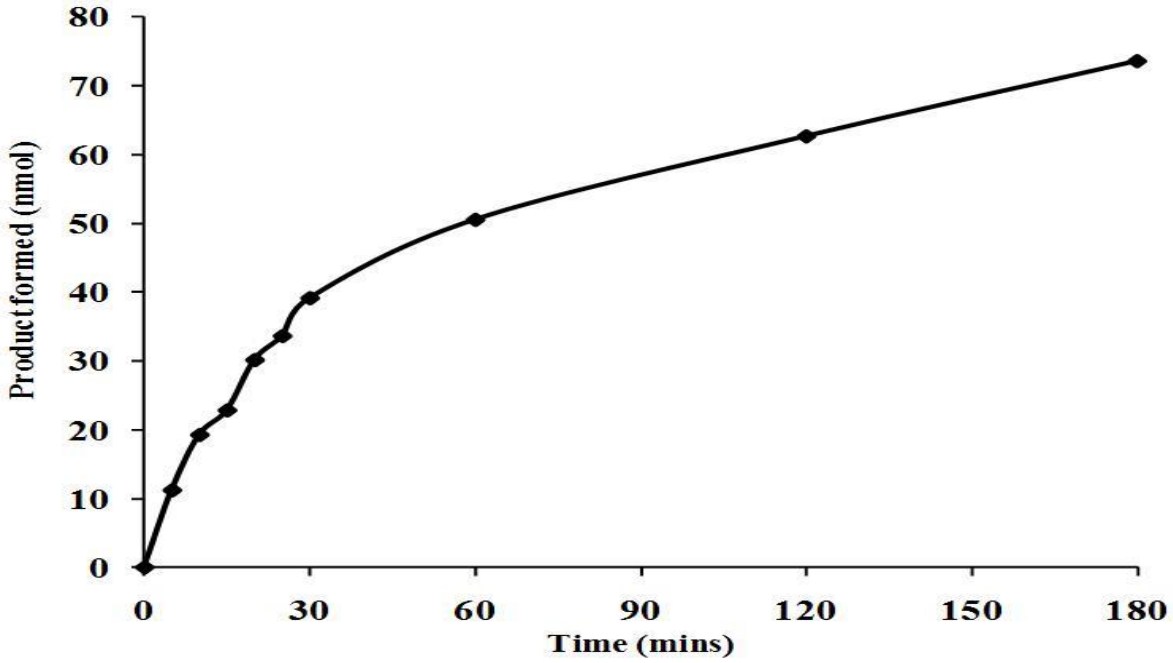

Figure 2. Time-dependent PLA_2 activity in serum from Komodo dragons

Aliquots (1.1 mL) of a 38.5 mL reaction (3.5 mL serum, 1.75 mL of BODIPY-labeled E. coli bacteria, and 33.25 mL assay buffer) were stopped at different time intervals and the fluorescent product was measured as described in the Methodology section. The results are presented as the means standard deviations of four independent determinations and are expressed as nmol of product formed/30 min reaction incubation.

Figure 3 shows the temperature-dependent PLA_2 activity of Komodo dragon serum. At lower temperature ranges, the Komodo dragon serum exhibited relatively low activities. However, the PLA_2 activity increased in a near-linear fashion from 10 to 40°C ($y = 8.5579x + 46.071$, $R^2 = 0.9451$). The production of product at 10°C (61.0 nmol) was only half of that produced at 40°C (122.7 nmol). The slope of the linear increase suggests an approximate increase of 2.1 nmol of product per 1°C of temperature increase. Harlow et al. [23] showed that the preferred body temperature of Komodo dragons is 34.0-35.6°C, and thus these animals have a relatively high PLA_2 activity (111.1 – 114.3 nmol/30 min) in this temperature range. The PLA_2 thermal profile presented in Figure 3 exhibits different characteristics than the temperature dependent serum complement activity reported by Merchant et al. [12], but was almost identical to the dipeptidyl peptidase IV activity [13]. These data would seem to indicate that an innate immune response in Komodo dragons would be optimal at 35-40°C. It would be reasonable to expect that these animals would need optimal immune activity during warmer weather when they are more active, and might be more likely to engage in territorial disputes.

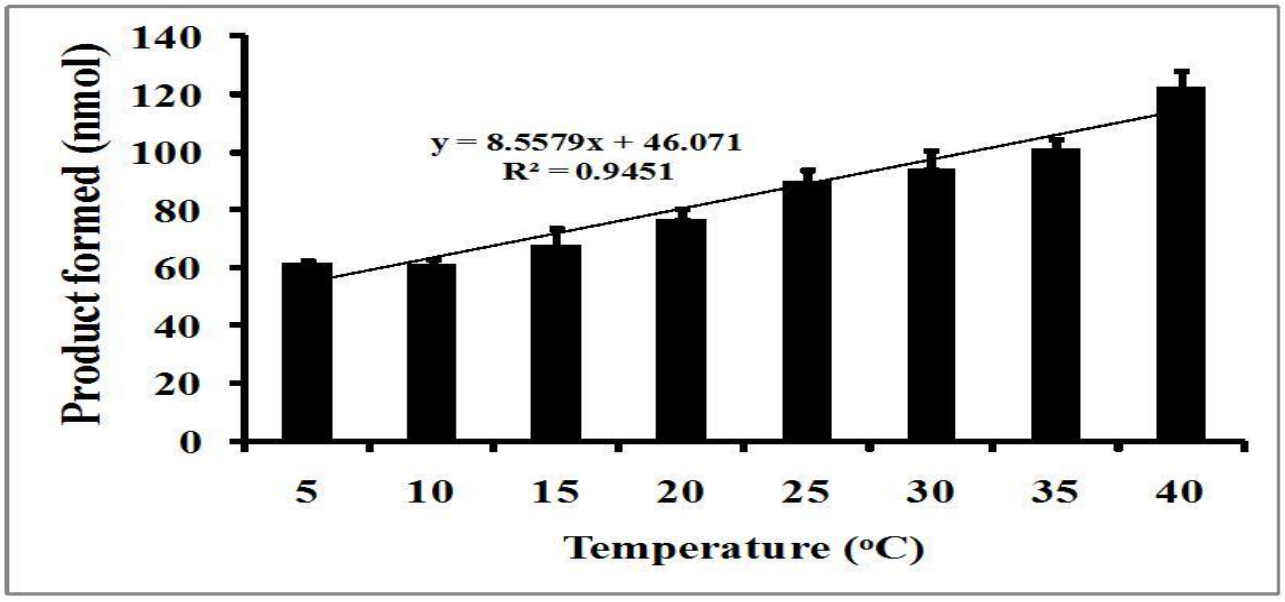

Figure 3. Temperature-dependent PLA_2 activity in Komodo dragon serum

Serum from pooled serum from Komodo dragons were incubated at different temperatures with BODIPY-labeled E. coli bacteria in a 750 µL reaction. After one hour, the samples were centrifuges and the fluorescent activity of the resulting supernatant was determined as described in the Materials and Methods section. The results are presented as the means standard deviations of four independent determinations and are expressed as nmol of product formed/30 min reaction incubation.

Longo et al. [24] showed that BPAB is a specific inhibitor of both secretory and intracellular PLA_2. This compound acts as a suicide substrate for PLA_2, and alkylates a specific histidine residue in the active site [25]. Figure 4 shows the concentration-dependent effects of BPAB on fluorescent product formation by serum from Komodo dragons. Treatment with 50 µ M BPAB produced no discernable change in activity, but inclusion of 100 µ M BPAB resulted in a 30.4% reduction in accumulation of fluorescent product, compared to the plasma-mediated reaction in the absence of BPAB. Further treatment with 500 and 1000 µ M BPAB produced stepwise decreases of 76.5 and 85.3% in activity ($p<0.01$). The inverse relation of BPAB concentration with fluorescent product formation indicates that the enzymatic activity is due to the presence of $sPLA_2$ in the serum of the Komodo dragon.

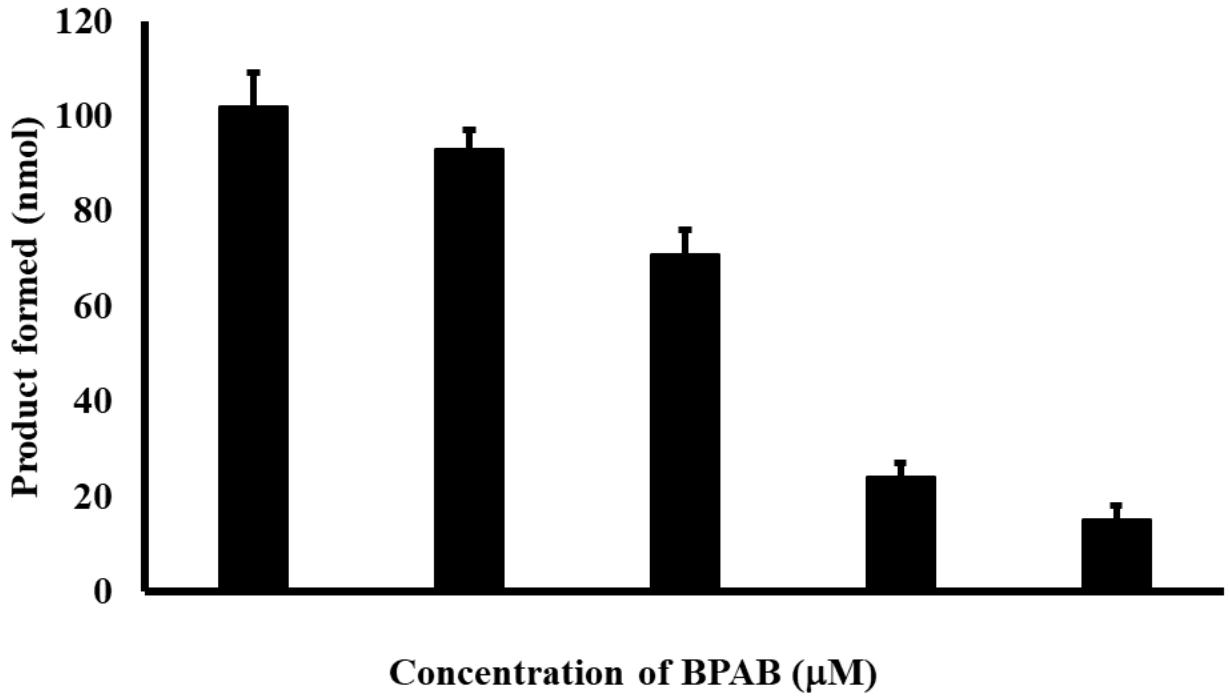

Figure 4. Inhibition of PLA_2 activity in Komodo dragon serum by BPAB

Pooled samples of serum from Komodo dragons was incubated with BODIPY-labeled E. coli bacteria in the absence, and presence of different concentrations of BPAB. After one hour, the samples were centrifuges and the fluorescent activity of the resulting supernatant was determined as described in the Materials and Methods section. The results are presented as the means standard deviations of four independent determinations and are expressed as nmol of product formed/30 min reaction incubation.

The data presented in this study provides strong evidence for immunological activity in the Komodo dragon via PLA_2 activity. The activity is dependent on plasma volume, occurs rapidly, and temperature-dependent as well. Although this study did not include *in vitro* studies, it is reasonable to expect that this circulating PLA_2 activity constitutes an important part of the immune system of the Komodo dragon.

ACKNOWLEDGEMENTS

The authors wish to acknowledge the help of San Antonio Zoo employees: J. Stephen McCuskey (Director), Alan Kardone (Curator of Herpetology and Aquarium), and Rob Coke (Senior Veterinarian), and Houston Zoo employees Rick Barongi (Director), Stan Mays (Curator of Reptiles), and Maryanne Tocidlowski (Veterinarian), for permission to work with zoo animals and collection of samples. In addition, we also thank technicians Courtney Threadgill and Candice Robinson (San Antonio Zoo) for help in restraining animals during blood collection. This research was funded by a McNeese State University Alumni Association Faculty Development Award. In addition, support was provided by a NSF Research Commercialization/Educational Enhancement Plan grant administered by the Louisiana Board of Regents.

REFERENCES

1. Brown, W. J., Chambers, K., and Doody, A. 2003. Phospholipase A2 (PLA2) enzymes in membrane trafficking: mediators of membrane shape and function. Traffic 4, 214-221.
2. Fonteh, A., N., Bass, D. A., Marshal, L. A., Seeds, M., Samet, J. M., and Chilton, F. H. 1994. Evidence that secretory phospholipase A2 plays a role in arachidonic acid release and eicosanoid biosynthesis by mast cells. J. Immunol. 152, 5438-5446.
3. Balsinde, J., Winstead, M. V., and Dennis, E. A. 2002. Phospholipase A_2 regulation of arachidonic acid mobilization. FEBS Lett. 531, 2-6.
4. Vadas, P., Browning, J., Edelson, J. and Pruzanski, W. 1993. Extracellular phospholipase A2 expression and inflammation: the relationship with associated disease states. J. Lipid Mediat. Cell Signal. 8, 1–30.
5. Dominiecki, M. E., and Weiss, J. 1999. Antibacterial action of extracellular mammalian group IIA phospholipase A2 against grossly clumped Staphylococcus aureus. Infection and Immunity, 67(2), 2299–2305.
6. Beers, S. A., Buckland, A. G., Koduri, R. S., Cho, W., Gelb, M. H. and Wilton, D. C. 2002. The antibacterial properties of secreted phospholipases A2," J. Biol. Chem. 277, 1788–1793.
7. Huhtinen, H. T., Gronroos, J. O., and Gronroos J. M. 2003. Antibacterial effects of human group IIA and group XIIA phospholipase A2 against Helicobacter pylori in vitro. Acta Pathol. Microbiol. Scand. 114, 127– 130.
8. Jessop, T. S., Madsen, T., Sumner, J., Rudiharto, H., Phillips, J. A., and Ciofi, C. 2006. Maximum body size among insular Komodo dragon populations covaries with large prey density. Oikos 112, 422-429. doi:10.1111/j.0030-1299.2006.14371
9. Bull, J. J., Jessop, T. S., and Whiteley, M. 2010. Deathly drool: evolutionary and ecological basis of septic bacteria in Komodo dragon mouths. PLoS ONE 5(6): e11097. doi:10.1371/journal.pone.0011097.
10. Fry B. G., Wroe S., Teeuwisse, W., van Osch, M. J. P., Moreno, K., Ingle, J., McHenry, C., Ferrara T., Clausen P., Scheib H., Winter K. L., Greisman L., Roelants K., van der Weerd L., Clemente C. J., Giannakis E., Hodgson W. C., Luz S., Martelli P., Krishnasamy K., Kochva E., Kwok H. F., Scanlon D., Karas J., Citron D. M., Goldstein E. J. C., McNaughtan, J. E., Normalna, J. A. 2009. A central role for venom in predation by *Varanus komodoensis* (Komodo Dragon) and the extinct giant *Varanus* (*Megalania*) *priscus.* Proc. Natl. Acad.Sci. USA, 106, 8969-8974.
11. Merchant, M., Henry, D., Falconi, R., Muscher, B., and Bryja, J. 2013. Antibacterial activity of serum from the Komodo dragon (*Varanus komodoensis*). Microbiol. Res. 4, 16-20.
12. Merchant, M., Falconi, R., Muscher, B., and Bryja, J. 2013. Characterization of serum complement activity in serum of the Komodo dragon (*Varanus komodoensis*). Adv. Biol. Chem. 2, 353-359.
13. Merchant, M., Henry, D., Falconi, R., Musher, B., and Bryja, J. 2015. Characterization of dipeptidyl peptidase enzyme activity in serum of the Komodo dragon (*Varanus komodoensis*). *Int. J. Biochem. Res. Rev*. 5(2), 145-152.
14. Bishop, B., Juba, M., Russo, P., Barksdale, S., Scott, S., Settlage, R., Michalak, P., Gupta, K., Vliet, K., Schnur, J., and Van Hoek, M. 2017. Discovery of Novel Antimicrobial Peptides from Varanus komodoensis (Komodo Dragon) by Large-Scale Analyses and De-Novo-Assisted Sequencing Using Electron-Transfer Dissociation Mass Spectrometry. J. Proteom. Res. 16, 1470-1482.
15. Ozinsky, A., Underhill, D. M., Fontenot, J. D., Hajjar, A. M., Smith, K. D., Wilson, C. B., Schroeder, L., and Adrem, A. 2000. The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between Toll-like receptors. Proc. Natl. Acad.Sci. USA 97, 13766–13771.
16. Kawai, T., and Akira, S. 2010. The role of pattern-recognition receptors in innate immunity: update on toll-like receptors. Nature Immunol. 11, 373-384.
17. Fraenkel-Conrat, H. 2008. Snake Venom Neurotoxins Related to Phospholipase A2. J. Toxicol. 1, 205-221.
18. Nevalainen, T. J. Kanchanapangka, S., Youngprapakorn, P., Webb, G. J. W., Manolis, S. C. and Scott, K. F., Phospholipase A2 activity of crocodile serum. Reptilia-Amphibia 30, 119–125.
19. Merchant, M., Heard, R., and Monroe, C. 2009. Characterization of phospholipase A_2 activity in serum of the American alligator (*Alligator mississippiensis*). J. Exp. Zool. A 311, 662-666.
20. Siroski, P., Merchant, M., Poletta, G., Larriera, A., and Ortega, H. 2013. Detection and characterization of phospholipase A_2 (PLA_2) in *Caiman latirostris* and *Caiman yacare* plasma. Zool. Sci. 30, 35-41.
21. Merchant, M., Royer, A., Broussard, Q., Gilbert, S., and Shirley, M.H. 2011. Characterization of serum phospholipase A2 activity in three diverse species of West African crocodilians. Biochem Res. Int. 1-7.

22. Montgomery, J. M., Gillespie, D., Sastrawan, P., Freeking, T. M., and Stewart, G. L. 2002. Aerobic salivary bacteria in wild and captive Komodo dragons. J. Wildl. Dis. 38, 545-551.

23. Harlow, H., Purwandana, D., Jessop, T., and Phillips. J. 2010. Size-Related Differences in the Thermoregulatory Habits of Free-Ranging Komodo Dragons. Int. J. Zool. **http://dx.doi.org/10.1155/2010/921371**.

24. Longo, W. E., Grossman, E; M;, Erickson, B., Panesar, N., Mazuki, A. E., Kaminski, D. L. 1999. The effect of phospholipase A2 inhibitors on proliferation and apoptosis of murine intestinal cells. J. Surg. Res. 84, 51–56.

25. Volwerk, J.J., Pieterson, W.A., de Haas, G.H., Histidine at the active site of phospholipase. Biochem. 13, 1446-1454.