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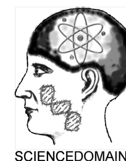

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Characterization of Dipeptidyl Peptidase IV Enzyme Activity in Serum of the Komodo Dragon (*Varanus komodoensis*)

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Authors' contributions

This work was carried out in collaboration between all authors. Author MM provided the conceptual basis for the study, funding, experimental design and writing of the manuscript. Authors DH and RF conducted the experiments, analyzed and presented the results. Authors BM and JB collected blood from animals, coordinated the study and helped to write the manuscript. All authors read and approved the final manuscript. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Aims: Soluble serum dipeptidyl peptidase IV (DPPIV) is a protease that cleaves dipeptides from proteins that have alanine or proline next to the N-terminal amino acid. This enzyme demonstrates substantial immune function by regulating T-lymphocyte activity, T-cell chemotaxis, growth, and proliferation during an inflammatory response. The goal of this study was to characterize DPPIV activity in the serum of the Komodo dragon (*Varanus komodoensis*).
Study Design: Serum from captive Komodo dragons were pooled and the DPPIV activities were

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determined at a range of serum dilutions, temperatures, and time points. The effects of Diprotin A, a specific DPPIV enzyme inhibitor, on the generation of fluorescent product were also determined. All samples were analyzed in quadruplicate such that meaningful statistical evaluations could be conducted.
Place and Duration of Study: Serum was collected from eight Komodo dragons at the San Antonio Zoo (n=5) and Houston Zoo (n=3) in June of 2012. The samples were analyzed for DPPIV enzyme activity in the Department of Chemistry at McNeese State University in Lake Charles, Louisiana, USA
Methodology: We used Ala-Pro-AFC, a dipeptide conjugated to a fluorescent probe *via* an amide linkage, to measure the activity of DPPIV in the serum of Komodo dragons (*Varanus komodoensis*). The fluorescent intensity of the product formed was measured at excitation and emission wavelengths of 395 and 530 nm, respectively, in a fluorimeter.
Results: Incubation of different volumes of serum from the Komodo dragon with Ala-Pro-AFC resulted in a volume-dependent increase in fluorescent intensity, which was decreased in a concentration-dependent manner by diprotin A, a specific inhibitor of DPPIV activity. Kinetic analysis showed that the DPPIV enzyme activity was detectable after five minutes, and that was nearly linear for three hours. A thermal profile showed that Komodo dragon DPPIV exhibited dramatically reduced activities at low temperatures (5-10°C), but activity increased linearly with temperature and was maximal at the highest temperature tested (40°C).
Conclusion: These results from this study indicate that Komodo dragons exhibit considerably high serum DPPIV activities, which are likely to contribute to T-cell activation and function, and act as a bridge between innate and adaptive immunity in these ancient vertebrates.

Keywords: DPPIV; Innate immunity; reptile; varanid.

1. INTRODUCTION

Dipeptidyl peptidase IV (DPPIV) is a multifunctional protein whose function depends upon the cell type, intracellular or extracellular location, and the specific physiological and cellular conditions under which it is expressed [1]. It exhibits proteolytic activity towards proteins that contain either alanine or proline next to the N-terminal position [2]. It is expressed as a membrane protein with an extracellular domain, or a soluble serum protein with similar catalytic activity [3]. The natural substrates for DPPIV are many, and include proteins such as glucose-like peptide 1 [4], natriuretic peptide [5], and the RANTES (Regulated on Normal T-cell Expressed and Secreted) protein [6], which are involved in a broad spectrum of biological activities such as glucose metabolism, diuresis, apoptosis, cell adhesion, and immune modulation [7]. Because of its wide array of clinically-relevant functions, DPPIV has been the target of many inhibitors that are used as drugs [8]. It is also used as a diagnostic/prognostic factor for various tumors, inflammatory disorders, and viral infections [8].

The immunological activity of DPPIV stems from its proteolytic activities, and also from its interaction with chemokines [9]. It is an important factor for T-cell growth and proliferation during the inflammatory response, and is a modulator of T-cell chemotaxis [2]. The enzyme activity of DPPIV has also been associated with immune competency *in vivo* [10]. In addition, decreased DPPIV activity has been identified in patients with immunodeficiency diseases, such as HIV-1 [11], and also has been shown to be increased in some autoimmune diseases [12].

Despite the endangered status of the Komodo dragon, little is known about the biochemistry and physiology of these Varanid lizards. In order to manage populations of a species properly, it is necessary to understand their general health, disease, and immunology [13-14]. Very few studies have been conducted to investigate the immune system of the Komodo dragon. In 2011, Merchant et al. showed that the serum of Komodo dragons exhibited potent antibacterial activities [15]. Another study from this group demonstrated that these animals have a very effective serum complement innate immunity [16]. This study was undertaken to identify and characterize soluble DPPIV activity in the serum of these ancient vertebrates.

2. MATERIALS AND METHODS

2.1 Biochemicals

Ala–Pro–AFC and AFC (7-amino-4-trifluoromethyl coumarin) was purchased from Anaspec (San Jose, CA, USA). Diprotin A (Leu–

Pro–Leu) was purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.2 Treatment of Animals

Blood samples were collected from Komodo dragons at the Houston and San Antonio zoos. 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 of three adults (20-81.5kg) 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 the average DPPIV activities for this species could be generated [16].

2.3 DPPIV Assay

For the experiment in which the dependence of DPP4 activity on serum concentration was investigated, different volumes of Komodo dragon serum were diluted to 750µL with assay buffer (100mMTris–HCl, pH 7.4), and then incubated for 60 min with 10µL of 1.0mg/mL Ala–Pro–AFC. Stop buffer (750µL, 100mMTris–HCl, pH 7.4, 15mM EDTA) was added, the samples were transferred to cuvettes and the fluorescent intensities were measured at an excitation λ of 395 nm (slit = 2nm) and an emission λ of 530 nm (slit=2nm) in a Horiba JobinYvonFluoromax™-4 fluorimeter. The presence of DPPIV activity resulted in cleavage of AFC from the dipeptide, which produced large increases in fluorescent intensities under these spectrofluorimetric conditions.

The effects of diprotin A on the hydrolysis of Ala–Pro–AFC were determined. Different amounts of diprotin A were added to Komodo dragon serum preparations, followed by the addition of Ala–Pro–AFC substrate to a final concentration of 40 µM. Samples were incubated at ambient temperature for 1 h, and the fluorescent intensity was measured as described above.

For the kinetic experiment, 2.0mL of Komodo dragon serum were added to 27.7mL of assay buffer. Upon the addition of 400µL of 1.0mg/mL Ala–Pro–AFC, samples were removed to 750mL of stop buffer at different time points, and the samples were transferred to cuvettes and the fluorescent intensities were measured as described above.

To examine the effects of temperature on enzyme activity, 10µL of 1.0mg/mL Ala–Pro–AFC was diluted to 750mL with assay buffer, incubated at different temperatures (5-40℃, at 5℃ intervals) for 15 min, and then the reaction was started by the addition of 10mL of Komodo dragon serum. After 60min, 750mL of stop buffer was added, the samples were transferred to cuvettes, and the fluorescent intensities were measured as described above.

2.4 Statistics and Controls

The DPPIV activities are expressed as nmol of product formed and represent the mean ± standard deviations of four independent determinations for each data point. The fluorescent intensities derived from each assay were compared to a linear standard curve generated with AFC, and the mass of product formed was calculated using the linear regression analysis of the data. The statistical comparisons between groups were conducted using analyses of variance (P<0.5 as significant) and Duncan's post-hoc comparisons.

3. RESULTS AND DISCUSSION

Incubation of different volumes of serum from Komodo dragons with the Ala–Pro–AFC substrate resulted in a near linear increase in DPPIV activity from, 1-100µL (Fig. 1). Substantial production of fluorescent product (5.8±0.8) was observed with only oneµL of serum. Increased volumes of 10, 20, 50, and 100µL resulted in 26.7±1.1, 47.7±1.9, 100.2±5.0, and 160.5±3.6 nmol of product formed, respectively.

The amount of product formed, using different volumes of Komodo dragon serum, is higher that observed for crocodilian species when comparable volumes were used. By way of comparison, the maximum activity observed in this study was 160.5±3.6 nmol of product formed (100µL serum), while 200µL of serum from the American alligator (*Alligator mississippiensis*) produced a maximum of 90nmol [17]. Likewise, serum from the American crocodile (*Crocodylus acutus*) produced only 8 nmol [18], and sera from the broad-snouted caiman (*Caiman latirostris*) and the yacare caiman (*Caiman yacare*) produced approximately 120 and 100 nmol of product, respectively [19]. It is unclear whether the increased activity correlates to a more effective innate immunity *in vitro*. However, other

studies have shown that the serum of the Komodo dragon has extremely effective antibacterial properties [15], and an extremely potent serum complement activity [16]. Therefore, although we present no evidence in this study for increased immune activity due to a relatively high serum DPPIV activity in the Komodo dragon, it would not be surprising if this activity correlated to an effective immune system.

Diprotin A is a tripeptide (Leu-Ala-Leu) that has been shown to be a specific competitive inhibitor of DPPIV activity [20]. Coincubation of Komodo dragon serum with 3.5µM Ala–Pro–AFC substrate and diprotin A resulted in a concentration-dependent reduction in DPPIV activity (Fig. 2). Coincubation with 0.1, 0.5, and 1.0mMdiprotin A and 3.5 µM Ala–Pro–AFC 17.8%, 71.0%, and 95.2% inhibition of DPPIV activities.

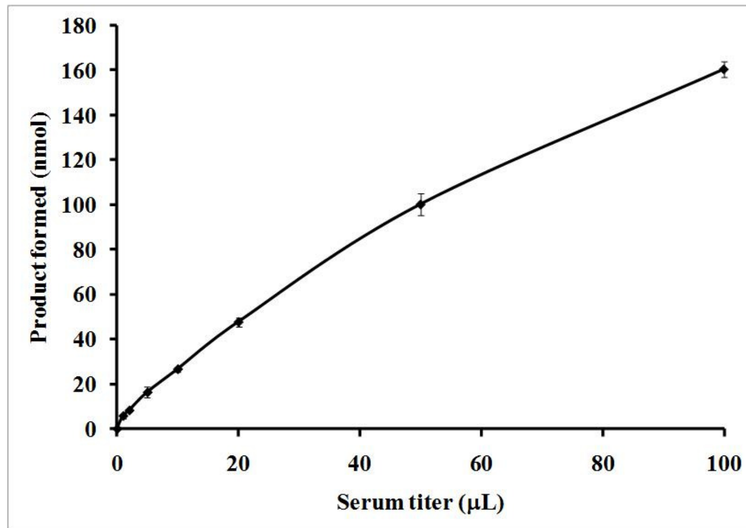

Fig. 1. Volume-dependent DPPIV activity in Komodo dragon serum

Different volumes of Komodo dragon serum were diluted with isotonic saline, and incubated with Ala-Pro-AFC for 15 min, and the DPPIV activity was measured as described in the Materials and Methods. The data are expressed as nmol of product formed, and represent the means ± standard deviations for four independent determinations

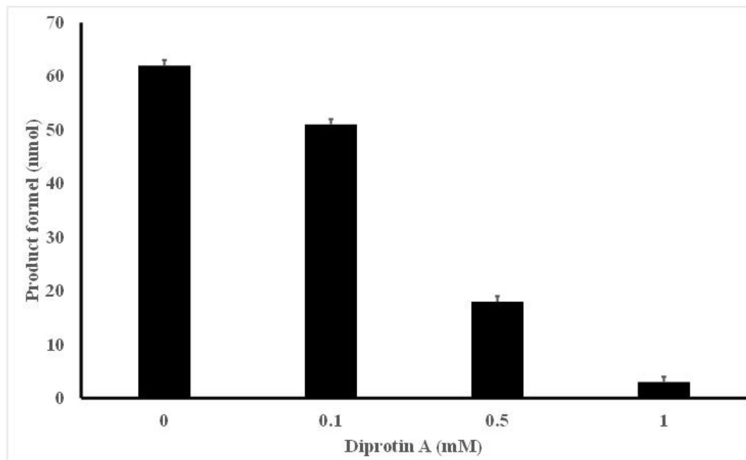

Fig. 2. Inhibition of DPPIV activity in Komodo dragon serum by diprotin A

Komodo dragon serum was incubated with Ala-Pro-AFC and different concentrations of diprotin A at ambient temperature, and the DPPIV activity was measured as described in the Materials and Methods. The data are expressed as nmol of product formed, and represent the means ± standard deviations for four independent determinations

The sera from vertebrates contain many proteases that capable of catalyzing the cleavage of peptide bonds, either in a nonspecific, or sequence-specific manner. It is a common practice in the biochemistry laboratory to use protease inhibitors, specifically designed to inhibit a specific protease (or class of proteases), to determine if a measured enzyme activity is due to the presence of a specific protease. Inclusion of 0.1, 0.5, and 1.0 mMdiprotin A, a specific DPPIV inhibitor, resulted in 17.8, 71.0, and 95.2% inhibition of DPPIV activities, respectively. This concentration-dependent inhibition of DPPIV activity is expected of diprotin A. These results are an indication that the cleavage of AFC from the Ala-Pro dipeptide is due to the presence of DPPIV enzyme activity.

Fig. 3 illustrates the DPPIV-dependent accumulation of product at different times after incubation with serum from Komodo dragons. Incubation of substrate with 67µL of serum (per 1000 µL reaction volume) for only 5 min resulted in the formation of 13.8±0.6 nmol of fluorescent product. Further incubation at 30, 60, 120, 1nd 180 min resulted in 46.8±0.6, 78.9±1.6, 129.6±3.0, and 170.1±2.4 nmol of product, respectively. The product concentration increased throughout the entire 180 min time period.

Kinetic analysis of the rate of product AFC formation by Komodo dragon peripheral blood DPPIV showed a near-linear increase in the rate of accumulation of approximately 1.12nmol/min from 5-180 min. The rate of product formation observed using a constant volume of Komodo dragon serum, was higher than that observed for similar volumes of serum from a variety of crocodilian species [17-19,21]. The increase in DPPIV activity shows that the Komodo dragon has a high capacity for N-terminal proteolysis of specific sequences, and a potentially high capability for facilitation of immune competence [2,7].

Incubation of serum from Komodo dragons with Ala-Pro-AFC at different temperatures resulted in increased activities at higher temperatures (Fig. 4). Enzyme activity increased in a linear fashion (R^2=0.962) as the assay temperature increased. The increase amounted to an increase of approximately 7.5nmol of product formed per one degree C. At low temperatures of 5℃ and 10°C, production of 80.1±0.3 and 97.4±1.6 nmol of product was observed, while the higher assay temperatures (35℃ and 40℃) resulted in 285.7±5.5 and 346.3 ± 2.7 nmol, respectively.

To a large extent, biochemical and physiological processes in ectotherms are dependent on external temperatures. The thermal profile of Komodo dragon DPPIV shows increased activities at elevated temperatures (Fig. 4).

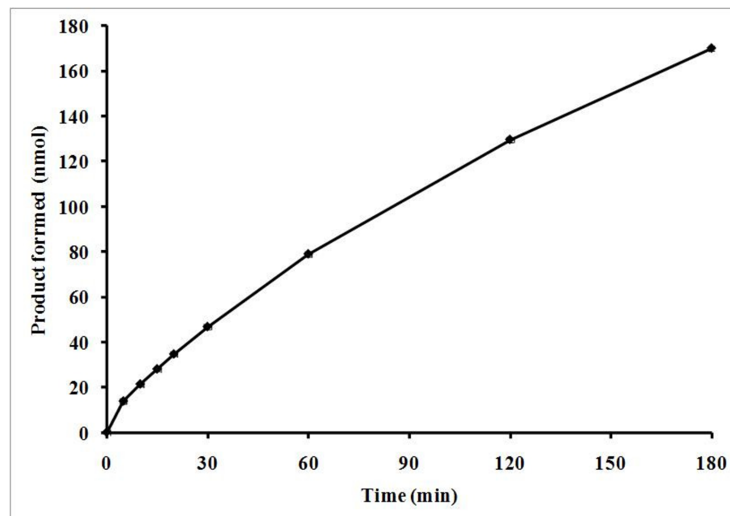

Fig. 3. Kinetic analysis of DPPIV activity in the serum of the Komodo dragon

Komodo dragon serum was incubated with Ala-Pro-AFC for different amounts of time, and the DPPIV activity was measured as described in the Materials and Methods. The data are expressed as nmol of product formed, and represent the means ± standard deviations for four independent determinations

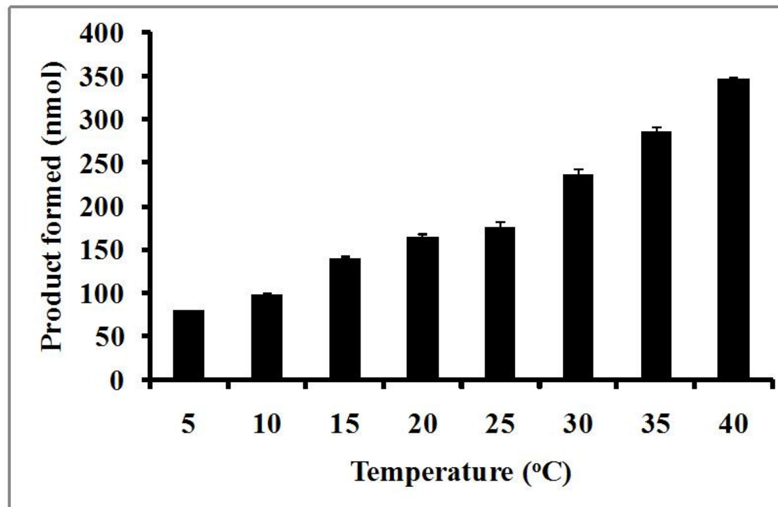

Fig. 4. Effects of temperature on DPPIV activity in Komodo dragon serum

Samples of Komodo dragon serum were incubated with Ala-Pro-AFC for 15 min at different temperatures, and the DPPIV activity was measured as described in the Materials and Methods. The data are expressed as nmol of product formed, and represent the means ± standard deviations for four independent determinations

Consequently, since these animals live in regions with tropical climates (approximate latitude of 8.4 - 8.8°S), there may be an advantage for higher immune-related activities at higher temperatures. The results displayed in Fig. 4 show that the DPPIV activity *in vitro* increases in a linear fashion ($y=37.009x+24.263$, $R^2=0.962$) with temperature. This model estimates an approximate 7.5nmol of product formed for every 1°C increase in temperature. Komodo dragons have been shown to thermoregulate to 37-38°C [22], and therefore the higher activity near this temperature range could potentially result in increased DPPIV-mediated immune activity. In addition, many poikilotherms have been shown to exhibit behavioral fever during infection [23-24].Thus, elevated body temperatures may be advantageous for activation and maintenance of potent and sustained immune responses. Therefore, the high activities of Komodo dragon DPPIV could serve as a survival advantage during infection, allowing higher activation of T-cell function, and thus humoral immunity.

4. CONCLUSION

To date, the majority of work with DPPIV in reptiles has been focused on snake venoms [25]. However, a few studies exist in which the soluble serum DPPIV have been characterized in crocodilians [17-19,21]. Although these studies did not link DPPIV activity to immune competency, it was assumed that these activities were immunologically important, due to the presence in the peripheral circulation.

This is the first study to characterize DPPIV in any Varanid lizard. Although the results to not show a definitive link between DPPIV activity and immune function, it is likely that this enzyme plays an important role in the regulation of both innate and adaptive immune processes. It is critically important to understand the disease and immunity of wildlife populations in order to understand susceptibilities to pathogenic colonization [13-14]. The results from this study will add to the growing body of knowledge concerning the immunological capabilities of these endangered reptiles.

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ETHICAL APPROVAL

The collection of blood from these animals was conducted in accordance with the Animal Care and Use institutional policies of the Houston Zoo, the San Antonio Zoo, and McNeese State University. All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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