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Comparison of plasma dipeptidyl peptidase IV activity in two caiman species: *Caiman latirostris* and *Caiman yacare* (Crocodylia, Alligatoridae)

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Abstract

Dipeptidyl peptidase IV (DPPIV) is a well-characterized protease with broad substrate specificity, functionally-related to the activity of many bioactive peptides. It plays an important role as physiological regulator of a number of peptides that serve as biochemical messengers within the immune system. Plasma DPPIV activity was characterized with respect to temperature, kinetics and concentration dependence in two species of caiman, the broad-snouted caiman (*Caiman latirostris*) and the black yacare (*Caiman yacare*). DPPIV activity showed a significant positive correlation from titrations carried out in the presence of different plasma concentrations. DPPIV activity was lower in *C. yacare* than in *C. latirostris* at all temperatures tested. *C. yacare* DPPIV activity showed a significant increase only at higher temperatures whilst *C. latirostris* plasma demonstrated a strong positive correlation starting at the lowest temperature, probably due to an adaptation for the tolerance of lower temperatures. Exposure of *C. latirostris* and *C. yacare* plasma at different time points showed that plasma DPPIV activities were time-dependent, and that the titer-dependent curves were different for the two species. These results revealed that plasma DPPIV activities were different between these two crocodilian species, which could contribute to the differences in susceptibility to infection between them.

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Introduction

Membrane peptidases are a group of ectoenzymes with a broad range of functions. Their importance in protein metabolism has been well-documented, especially in peptide degradation and amino acid scavenging (Scharpé and De Meester, 2001). Dipeptidyl peptidase IV (CD26/DPPIV) can be considered a multifunctional protein because it exerts different functions depending on cell type and intracellular (mainly in the membrane of cells) or extracellular (enzymatically active plasma isoform) locations (Boonacker and Van Noorden, 2003). DPPIV was first discovered in rat liver and kidney (Hopsu-Havu and Glenner, 1966) and subsequently found in human salivary gland (Oya et al., 1972), pig small intestine (Svensson et al., 1978), human serum (Hino et al., 1975) and human peripheral blood cells (Lojda, 1977).

The *in vivo* expression of DPPIV in epithelial, endothelial and lymphoid cells is compatible with its role as a physiological regulator of a number of peptides that serve as biochemical reporters between, and within, the immune and neuroendocrine systems (Ludwig et al., 2002). DPPIV, which is identical to the lymphocyte surface glycoprotein CD26, is unique among these peptidases due to its ability to liberate Xaa-Pro and, less efficiently, Xaa-Ala dipeptides from the N-terminus of regulatory peptides (Boonacker et al., 2002). It has an important role in immune function as a co-stimulatory molecule in T cell activation and as a regulator of the functional effect of selected biological factors. Its well-characterized protease enzymatic functionality is related to the activity of many bioactive peptides, with broad substrate specificity for which at least 62 natural substrates have been identified (Chen, 2006). These substrates include neuropeptides, circulating peptide hormones such as peptide YY, growth hormone-releasing factor, glucagon-like peptides (GLP)-1 and -2, gastric inhibitory polypeptide, paracrine chemokines (regulate normal T cell activation, expression, and segregation), stromal cell-derived factor, as well as eotaxin and macrophage-derived chemokine (Mentlein, 1999; Gorrell, 2005), among others. Based on its multiple functions, DPPIV has been proposed as a diagnostic or prognostic marker for various tumors, hematological malignancies, viral infections, as well as immunological, inflammatory, and psychoneuroendocrine disorders (Lambeir et al., 2003).

Few studies have been focused on reptilian DPPIV and most of them are limited to snakes. DPPIV substrate specificity, susceptibility to inhibitors, and optimum pH of the partially purified enzyme was investigated in venoms glands (Ogawaa et al., 2006), seasonal variation of peptidase activity was studied in the reproductive tract (Marinho et al., 2009), and the taxonomic distribution of DPPIV activity was examined in venoms of 59 ophidian taxa (Aird, 2008). Recently, a work in crocodilians detected and characterized DPPIV activities in the plasma and whole blood of the American alligator (*Alligator mississippiensis*; Merchant et al., 2009).

Figure 1. Adult female of *Caiman latirostris*.

Figure 2. Geographic distribution map of *C. latirostris* and *C. yacare* in Argentina.

The goal of this study was to identify, characterize, and compare DPPIV activities between *Caiman latirostris* (fig. 1) and *Caiman yacare*, the only two crocodilian species living in Argentina (fig. 2).

Materials and methods

Chemical and biochemicals

Pro-Ala-AFC was purchased from Anaspec (San Jose, CA). Diprotin A (Leu-Pro-Leu) was purchased from Sigma Aldrich (St. Louis, MO).

Animals

All animals had been treated following the Reference Ethical Framework for Biomedics Researches: ethical principles for research with laboratory, farm and wild animals (National Scientific and Technical Research Council, 2005), using non-invasive techniques of blood collection and minimizing stress and suffering by suitable management methods.

C. latirostris (n = 13, 7 females and 6 males; from 1.51 to 2.31 m) and *C. yacare* (n = 14, 7 males and 7 females 1.44 to 2.11 m) wild adults from Santa Fe and Corrientes provinces (Argentina), respectively, were captured during the month of March. Animals were bled from the spinal vein (Tourn et al., 1994; Zippel et al., 2003) using 3.81 cm 18-gauge needle and a 20-ml syringe. Blood was then transferred to heparinized tubes and plasma was removed (5 ml per animal, approximately) and pooled after centrifugation at 2500 rpm for 15 min.

DPPIV assays

To characterize *C. latirostris* and *C. yacare* DPPIV activity, we used the assay adapted from American alligator plasma and whole blood by Merchant et al. (2009).

This assay is based on the effects of DPPIV on the hydrolysis of Ala-Pro-AFC. Assay buffer to initiate the reaction (100 mM Tris HCl, ph 7.4) and stop buffer to finished it (100 mM Tris HCl, ph 7.4, 15 mm EDTA), were used. DPPIV requires divalent cations (Ca^{2+} and Mg^{2+}) to exert its functions. Based on its activity as chelant of divalent cations, stop buffer includes EDTA as a common protease inhibitor (Ghersi et al., 2006).

To evaluate the effects of temperature on *C. latirostris* and *C. yacare* DPPIV plasma activity, mixture incubation was carried out at different temperatures (5°C to 40°C, at 5°C intervals). Aliquots of caiman plasma (50 μl) and assay buffer (700 μl) were incubated for 15 min at the corresponding temperatures prior to the addition of the substrate. Then, 10 μl of 2.5 mM Ala-Pro-AFC were added and incubated for 60 min at the same thermal environments. After incubation, 700 μl of stop buffer were added.

To study the concentration-dependence of DPPIV plasma activity, different volumes of caiman plasma (0, 1, 2, 5, 10, 20, 50, 100, or 200 μl) were added to assay buffer with 10 μl of 2.5 mM Ala-Pro-AFC. The reactions were balanced to a final volume of 750 μl using assay buffer, and stop buffer was added after incubation time.

Kinetic parameters of DPPIV plasma activity were determined at different time intervals. Two ml of caiman plasma, 27.7 ml of assay buffer and 400 μl of 2.5 mM

Ala-Pro-AFC were mixed. At different time intervals (0, 5, 10, 15, 20, 30, 60 and 120 min), 760 μl of this mixture were removed and added to 700 μl of stop buffer.

All assays were carried out at ambient temperature (25°C, approximately) to be able to compare with results reported by previous studies (except during differential temperature assays), and for 60 min (except for kinetics assays). The samples were transferred to cuvettes and the fluorescent intensities were measured at an excitation λ of 395 nm (slit = 2 nm) and an emission λ of 530 nm (slit = 2 nm). The presence of DPPIV activity resulted in cleavage of AFC from the dipeptide, producing large increases in fluorescent intensities that were measured spectrofluorimetrically.

The effects of Diprotin A on the hydrolysis of Ala-Pro-AFC were also determined. Different amounts of Diprotin A were added to plasma. Samples were incubated at ambient temperature for 1 h, and the fluorescent intensity was measured as described above.

Statistics analysis

Statistical analysis was performed using Stata 8.0 software (Statacorp, USA). All assays were carried out by quadruplicate and the DPPIV activities are expressed as nmol of product created (means ± standard errors). Linear regressions were used to analyze the effect of temperature, kinetics, and concentration on DPPIV activity for each species. A p value $\leqslant 0.05$ was considered statistically significant.

Results

The effects of temperature on caiman plasma DPPIV activity are shown on fig. 3. DPPIV activity was lower in *C. yacare* than in *C. latirostris* at all temperature tested. At the lowest temperature (5°C) both species presented similar plasma DPPIV activities ($p = 0.618$), but the patterns of increase varied greatly thereafter between the two species, with *C. latirostris* activity significantly higher at each temperature between 10 and 40°C. Between 5 and 35°C, DPPIV activity for *C. yacare* increased steadily at a rate of 2.63 nmol/°C ($r^2 = 0.99$, $p = 0.001$), and increased at a higher rate after 35°C to a maximum activity of 177.98 nmol at 40°C. It is not known whether activity would have continued to increase after 40°C. In contrast, for *C. latirostris* there was a marked increase in activity between 5 and 10°C, followed by a steady increase at a rate of 3.64 nmol/°C between 10 and 25°C ($r^2 = 0.95$, $p = 0.026$), and stable activity between 30 and 40°C (mean = 301.41 nmol) ($p = 0.011$).

Examination of DPPIV activity over time indicated that both species had biphasic patterns of activity, but they varied significantly with respect to rates of increase over different periods of time (fig. 4). For *C. latirostris*, plasma DPPIV activity increased rapidly between 0 and 10 minutes (25.22 nmol/min; $r^2 = 0.99$, $p = 0.07$), before leveling off at a much reduced rate (99% reduction) between 15 and 120 minutes (0.25 nmol/min; $r^2 = 0.93$, $p = 0.008$). For *C. yacare*, the initial rate of increase in activity occurred between 0 and 30 minutes (1.94 nmol/min; $r^2 = 0.99$,

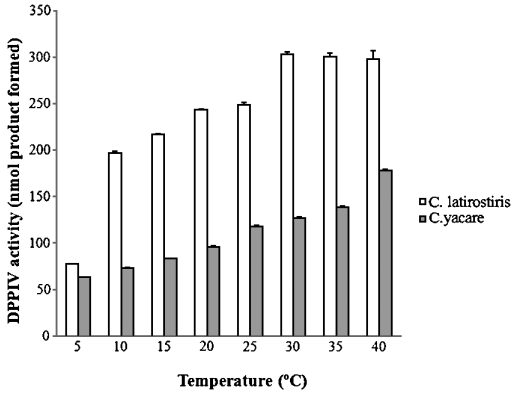

Figure 3. Abilities of DPPIV to cleave Ala-Pro-AFC were tested at different temperatures. Fluorescence caused by AFC released was recorded as DPPIV activity. Results are displayed as means ± standard error for four independent determinations for each temperature tested.

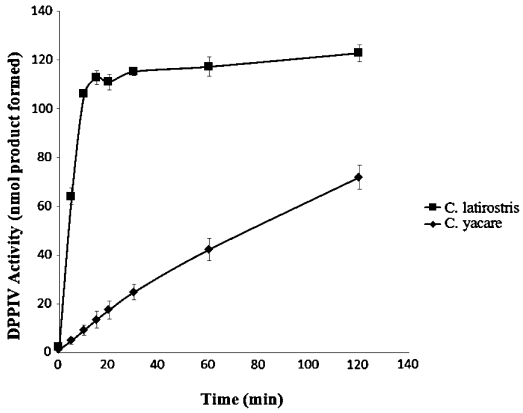

Figure 4. Soluble plasma caiman DPPIV activity at different times at ambient temperature (25°C). Fluorescence of the cumulative AFC proteolytic products were detected and expressed as DPPIV activity. Results are expressed as means ± standard deviation for four independent determinations.

$p = 0.0001$), and then decreased by around 35% between 30 and 120 minutes (1.26 nmol/min; $r^2 = 0.99$, $p = 0.026$). With neither species has DDPIV appeared to stabilise.

DPPIV activity of *C. latirostris* and *C. yacare* plasma exhibited titer dependence (fig. 5). Both species demonstrated similar positive relationship between activity and plasma titer increase ($p < 0.001$), showing no differences in the shape of the curves until 100 μl. DPPIV activity for *C. latirostris* plateaued after 100 μl, whereas for *C. yacare* it continued to increase, albeit at a much reduced rate relative to titers less than 100 μl.

The effects of a specific DPPIV inhibitor, Diprotin A, on the hydrolysis of AFC by *C. yacare* and *C. latirostris* serum are on fig. 6. Inclusion of 0.15 mM Diprotin A inhibited *C. yacare* and *C. latirostris* DPPIV activity by 38% and 49% respectively.

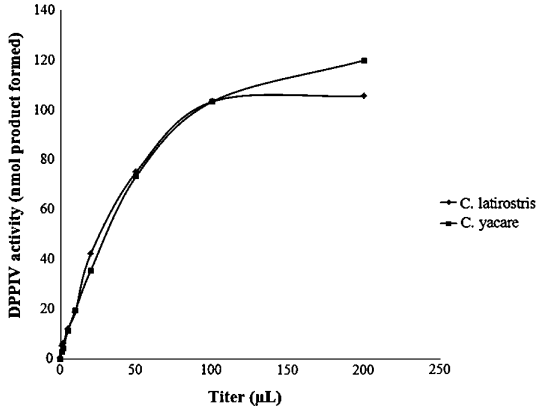

Figure 5. Titration of DPPIV enzyme assayed at different plasma concentrations. Caiman plasma was diluted and flurorescence was recorded as DPPIV activity after 60 minutes (25°C) of incubation. Results are expressed as means ± standard deviation for four independent determinations.

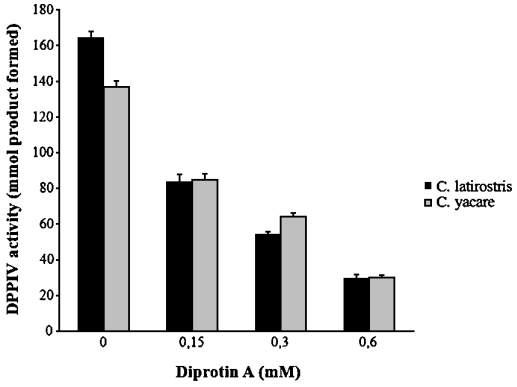

Figure 6. Concentration-dependent inhibition of caiman DPP4 activity by Diprotin A. Caiman plasma was incubated in the absence, and in the presence of different concentrations of Diprotin A for 1 h at ambient temperature. The fluorescence of the AFC proteolytic product was measured fluorimetrically. Results are expressed as the means ± standard deviation for four independent determinations.

Concentrations of 0.3 and 0.6 mM Diprotin A caused reductions of 53% and 78%, and 67% and 82% in *C. yacare* and *C. latirostris* activity respectively.

Discussion

In this report, we identified and described the activities of the soluble form of DPPIV in *C. latirostris* and *C. yacare* plasma. Recently, DPPIV has been reported in serum and whole blood of American alligator (Merchant et al., 2009) where it was confirmed that the proteolytic activity observed was due to DPPIV because they based its identification using a specific DPPIV inhibitor, Diprotin A (Ile-Pro-Ile) (Rahfeld et al., 1991). The concentration-dependent reduction of these serum ac-

tivities by a specific DPPIV inhibitor, Diprotin A (fig. 6), strongly suggests that the hydrolysis of AFC is due to the presence of DPPIV. Our results confirm the presence of DPPIV in plasma of the two species of caiman studied.

DPPIV activity showed a significant positive correlation based on the data obtained from titrations of different plasma concentrations. Peptidase activity showed concentration-dependent activity for both *C. latirostris* and *C. yacare* plasma. These activities were much higher compared with murine *in vitro* activity (Kubota et al., 1992), and pig (Faidley et al., 2006) and human serum (Lefebvre et al., 2002; McKillop et al., 2008). Similar results were found in American alligator (Merchant et al., 2009) but the values detected in whole blood were higher than in plasma at all titrations, which was attributed to the presence of DPPIV on the surface of T-cells (Mentlein et al., 1984). In general, the shape of the curves (concentration-, temperature-, and kinetics-dependence) and rates of product formation obtained in our study were similar with that in the American alligator (Merchant et al., 2009).

Ectothermic vertebrates are suitable models for studies regarding the influence of temperature on a variety of physiological functions (Pxytycz and Zkowicz, 1994). Previous studies indicated that environmental temperature plays a fundamental role in the physiology of ectothermic vertebrates, including antibody formation and immune response (Klesius, 1990), and specifically in crocodilians, affecting sex determination and clutch size (Simoncini et al., 2008), as well as components of innate immune system (Merchant et al., 2005a; Siroski et al., 2010). Crocodilians prefer to maintain body temperatures within a narrow range of 28-33°C by using thermal gradients in their natural environment: sunshine and shade, warm surface and cold deep waters (Huchzermeyer, 2002). Temperature influenced DPPIV activities in caiman plasma, and this influence seems to be more pronounced in *C. latirostris* than in *C. yacare*. At low temperatures, both species showed very low activities and coincide with the fact that ectothermic vertebrates' metabolisms slow down to a minimum (Johnson et al., 2008). Maximum DPPIV activities were detected at those temperatures in which crocodilian metabolic processes, including immune responses, are optimal, as demonstrated by Glassman and Bennett (1978). Merchant et al. (2009) showed that the response of American alligator to infection is temperature-dependent, with maximal activity around 30°C.

The differences found between both species studied here could be attributed to optimal temperature range where these species live. *C. latirostris* has a broader geographic distribution in South America than *C. yacare*, both occur in the north of Argentina, but the range of *C. latirostris* reaches farther south than *C. yacare*, being the most austral crocodilian in the American continent. In this part of the country, there are important differences in temperature with changes in latitude. At the limit of *C. yacare* population range (30°S latitude) (Verdade, 1998), the mean temperature during colder months (June to August) is around 17°C. In the case of *C. latirostris*, the limit of distribution appears at 32°S latitude (Verdade, 1998), where the mean temperature during same months is approximately 10°C (Servicio Meteorológico Nacional Website: www.smn.gov.ar). The southern distribution of

C. latirostris in Argentina indicates that it has a greater climatic tolerance (fig. 2) (Siroski, 2004). Our results showed that *C. latirostris* stabilized DPPIV activity between 30 and 40°C whereas it would stabilize at higher temperatures for *C. yacare*. Hence, we could postulate that the difference found in DPPIV activity in relation to temperature is due to an adaptation of *C. latirostris* to exert its metabolic functions at lower temperatures than *C. yacare*. Previous studies had shown similar behavior in relation to temperature on the complement system between *C. latirostris* and *C. yacare* (Siroski et al., 2010). DPPIV activities may be enhanced at lower temperatures as an immunological adaptation.

C. latirostris and *C. yacare* DPPIV plasma activities increase with time. The kinetics of caiman plasma DPPIV activity was similar to that observed *in vivo* in mice (Kubota et al., 1992), and the values detected in this study were similar to those found in American alligator (Merchant et al., 2009). According to Merchant et al. (2009), the increment in caiman DPPIV activity with time could be due to the enzymatic activity requiring a certain time to achieve its catalytic efficiency by the same level of protein. We observed that caiman plasma DPPIV has a fast reaction and could be considered as a property of caiman serum; the same results were found in the serum complement system of *C. latirostris* (Siroski et al., 2010).

There is much evidence for a role of DPPIV in the regulation of the immune response which focuses on the putative role of the catalytic domain (Kahne et al., 1999; Ludwig et al., 2002) among other functions. The results from this study demonstrate and characterize plasma DPPIV activity in two species of caiman, and show that they are similar to that of American alligator (Merchant et al., 2009), probably due to their common phylogenetic origin. Based on the DPPIV immunological functions previously noted, this could be related to the efficiency of the crocodilian immune system (Merchant et al., 2003, 2004, 2005b; Siroski et al., 2009), showing another advantage of this successful group of ancient vertebrates. Differences in susceptibilities to factors that affect their survival (such as stress agents and pathogenic microorganisms) between the two species studied have been observed in different Argentinean caiman farms. Mortality reports from caiman farms indicated that under the same raising conditions (temperature, diet, stress factors, feeding and cleaning regimes, etc.) *C. yacare* has higher mortality rates than *C. latirostris* (A. Larriera, personal communication). This suggests that *C. latirostris* may be more "resistant" to infection than *C. yacare*. The variations found in plasma DPPIV activities between these two crocodilians could be one of the reasons for different susceptibility to infection observed in these species.

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