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Presence of chitinase enzymes in crocodilians

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Abstract. Chitin is an abundant bio-polymer present as a structural component of many organisms such as arthropods, nematodes, mollusks, insects, and fungi, among others. Chitinolytic enzymes are synthesized for organisms to defend themselves against chitin-containing pathogens. Chitotriosidase (CHT) is a chitinase enzyme and one of the main proteins secreted by activated macrophages. It plays an important role in mechanisms of immunity by hydrolyzing chitin, thus protecting against chitin-containing pathogens. In this study, CHT was detected in *Caiman latirostris* plasma, and characterized under laboratory controlled conditions of temperature, reaction time, plasma concentration, pH and salinity. The results complement other immunological studies performed in caimans and demonstrate that they possess an efficient and well-developed immune system that resists the attack of some pathogens. Based on the current knowledge of the properties and homologies of CHT, it would be highly valuable to evaluate its possible therapeutic application in the veterinary clinical setting.

Keywords. Chitinases, crocodilians, innate immunity, immunology.

INTRODUCTION

Chitin, a repeating β (1→4)-linked homopolymer of N-acetyl-β-D-glucosamine (GlcNAc), is the second most abundant biopolymer on earth next to cellulose (Daimon et al., 2003). It can be found as a structural component in coatings of many species, including the integument of arthropods (Neville et al., 1976), the microfilarial sheath of parasitic nematodes (Fuhrman and Piessens, 1985; Araujo et al., 1993), mollusks (Badariotti et al., 2007), the gut linings of insects (Shahabuddin and Kaslow, 1994), the cell walls of fungi (Kuranda and Robbins, 1991) and some algae (Badariotti et al., 2007). Production of chitin depends on chitin synthase, while degradation or remodeling of chitin-containing structures requires chitinases. These enzymes are essential for maintaining normal life cycle functions such as morphogenesis of arthropods (Merzendorfer and Zimoch, 2003) or cell division and sporulation of yeast and other fungi (Kuranda and Robbins, 1991). Chitinolytic enzymes have also been found in organisms that do not contain chitin polymers themselves, such as viruses, bacteria, plants and animals (Badariottia et al., 2007). Indeed, chitinases also have an important role in parasitic invasion of chitinous hosts. Several studies have demonstrated that chitinases are also produced constitutively, or inducibly, as pathogen resistant proteins; thus vertebrates synthesize chitinases to defend themselves against chitin-containing pathogens such as protozoa, fungi, insects and nematodes (Renkema et al., 1995; Gooday, 1999).

Mammals express two active chitinases, chitotriosidase (CHT, EC Number: 3.2.1.14) and acidic mammalian

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chitinase (AMCase). Only AMCase has been detected in the gastro-intestinal tract, probably due to its acidic pH (Bussink et al., 2008). Chitotriosidase enzyme glycosyl hydrolase, is one of the main proteins secreted by activated macrophages and has been characterized at the protein and gene level (Hollack et al., 1994). The enzyme is synthesized in massive amounts and is predominantly secreted as a 50 kDa protein by specifically-activated macrophages. About one third of the enzymes synthesized are proteolytically processed to a 39 kDa form that accumulates in lysosomes. In the blood stream, the secretory 50-kDa CHT occurs predominantly, whereas in tissues, the 39-kDa form is most abundant (Renkema et al., 1997). The 39 kDa enzyme is C-terminally truncated and lacks a chitin-binding domain.

Chitotriosidase is located in specific granules of polymorphonuclear cells and secreted following stimulation with granulocyte macrophage colony-stimulating factor (GM-CSF). In addition, GM-CSF induces expression of CHT in macrophages, not expressed in monocytes that constitutively secrete the enzyme and partially accumulate it in their lysosomes in a number of pathological settings period. It is also produced in vitro after prolonged cultivation (Renkema et al., 1998). Chitotriosidase activity had been known to cleave both colloidal chitin and the cell wall chitin of *Candida albicans*, and inhibits *Cryptococcus neoformans,* and *Mucor rouxii* proliferation, suggesting a role in defense against chitinous human pathogens (Boot et al., 2001; van Eijk et al., 2005). Elevated chitinase activity has also been detected in the blood and tissues of guinea pigs with systemic *Aspergillus fumigatus* infection (Overdijk et al., 1996; 1999).

Plasma chitotriosidase activity is increased in several lysosomal (Brinkman et al., 2005; Ries et al., 2006; Vedder et al., 2006) and nonlysosomal diseases (Michelakakis et al., 2004). Serum and plasma CHT is used as diagnostic marker of Gaucher disease. Elevated CHT reflects a gradual intralysosomal accumulation in the liver, spleen, or bone marrow, which is indicative of Gaucher cell (lipid-loaded macrophages). Macrophages overloaded with the enzyme accumulated in lysosomal material (lipids) were shown to secrete CHT. In contrast, low levels of CHT have been associated with susceptibility to infection by chitin containing parasites (Bussink et al., 2006). In fact, many studies have been performed to detect CHT activity towards chitin-containing pathogens both *in vitro* and *in vivo* (van Eijk et al., 2005).

Crocodiles exhibit a versatile and efficient nonspecific immune system adapted to the environments in which they commonly live. Generally, these animals live in environments (both natural and captive) that contain high concentrations of pathogenic microorganisms. For different reasons, serious injuries occur and sometimes animals lose entire limbs as a consequence of social disputes (Webb and Manolis, 1983). However, crocodilians normally tolerate these injuries and generally do not show signs of illness (Siroski et al., 2009).

Crocodilians have been shown to have immune components with an apparently higher activity than others animals, including humans (Siroski, 2011), which make them interesting models for the elucidation of those mechanisms. These immunological effector mechanisms necessary for the efficient control of infectious agents are dependent on the distinct defense strategies. Some components involved in these routes of the defense system have been identified and characterized. These include serum complement cascades (Merchant et al., 2003; Siroski et al., 2009), dipeptidilpeptidase (Merchant et al., 2010; Siroski et al., 2011), and phospholiphases (Merchant et al., 2009a; 2011; Siroski et al., 2013). All these features may converge in the antimicrobial properties detected in different crocodilian tissues (Shaharabany et al., 1999; Merchant et al., 2003; Siroski et al., 2009).

To our knowledge, CHT activity in the plasma of crocodilians has not been studied to date. Due to the important role of CHT enzymes in the generation of the innate immune system, we aimed to analyze the presence of this enzyme in *Caiman latirostris* plasma under different biochemical conditions (plasma concentration, time, temperature, pH, and salinity-dependence) to characterize its activity.

MATERIAL AND METHODS

For this study, wild *Caiman latirostris* (N=13; 7 females and 6 males) were captured from different pristine natural areas in the province of Santa Fe, Argentina. Due to the influence of temperature on the physiology of these animals, all samples were collected during the summer.

Animals were bled and measured (range 1.51 to 2.31 m of total length), and then returned to their environment within an hour of capture. The blood samples were collected immediately after capture to avoid stress in the animals. Blood samples were obtained from spinal vein (Zippel et al., 2003) using heparin as an anticoagulant. Whole blood was centrifuged immediately at 1000 *g* for 20 min, at room temperature (approximately 24°C). The plasma was frozen at -20°C and CHT enzyme assays were conducted within seven days of the collection of samples.

Plasma CHT enzyme activity was determined as described in Hollack et al. (1994) using the artificial substrate 4-methylumbelliferyl-β-D-N,N′,N′′-triacetylchitotrioside (4 MU-chitotrioside; Sigma Chemical Co., St. Louis, MO). The enzyme assay mixture contained 15 µL of plasma and 200 µL (0.022 mM) of the substrate dissolved in citrate-phosphate buffer, pH 5.2, in a total volume of 215 µL. The reaction was stopped with 2 mL of glycine-sodium hydroxide (stop buffer),

pH 10.6. This mixture was incubated under different conditions depending on the type of assay, and the fluorescence of each sample was determined with a spectrofluorimeter (excitation and emission wavelength of 365 and 450 nm, respectively). The CHT activity was determined by plotting a standard curve of the 4-methylumbelliferone product against the relative fluorescence units.

Caiman CHT activity temperature dependence. To evaluate the effect of temperature on enzyme activity, CHT assays were performed at different temperatures (from 5°C to 40°C, with intervals of 5°C). Prior to the onset of the reaction, aliquots of plasma and substrate dissolved in buffer were incubated separately for 15 min to reach the corresponding temperature of the study. Components of the reaction were mixed and incubated for 30 min at the same temperatures.

Caiman CHT activity plasma concentration dependence. To determine the effect of plasma concentration on CHT activity, different amounts of caiman plasma (0, 1, 2, 5, 10, 20, 50 and 100 µl) were used. The reaction was stopped after 30 min of incubation.

Caiman CHT activity time dependence. To evaluate the kinetics of the CHT activity, the substrate was incubated with the caiman plasma for varying amounts of time (0, 5, 10, 15, 20, 30, 60, and 90 min). Aliquots of plasma and substrate dissolved in citrate-phosphate buffer were mixed by quadruplicate. The reaction was stopped with 2 mL of glycine-sodium hydroxide buffer after the different time points.

Caiman CHT activity pH dependence. The effects of the pH on the CHT activity were evaluated using several buffers (McIlvain buffer containing dihydrogen phosphate in different concentrations to regulate the pH) varying from pH 4 to 10. After 30 min incubations, stop buffer was used to terminate the reaction.

Caiman CHT activity salinity dependence. To determine the effects of salinity on CHT activity, different volumes of 1 M NaCl solution (from 0 to 100 µl) were added to the enzyme assay mix (plasma and substrate) and the total volume was balanced with distilled water and incubated for 30 min.

Statistical analyses. All assays were performed in quadruplicate and the results are expressed as µM ± standard error (SE). Statistical analyses were performed using the software SPSS 14.0 for Windows (SPSS for Windows, 2005). Nonlinear regression analyses were used to describe the effects of each variable on CHT activity, and a p value ≤ 0.05 was considered statistically significant.

RESULTS

The CHT activity of *C. latirostris* was dependent on the incubation temperature during the reaction, showing a positive relationship up to 35°C (R=0.8907) (Fig. 1). Chitotriosidase activity was compromised at low temperatures (5, 10 and 15°C; p <0.05), and at 40°C.

In the assays performed to determine the influence of plasma concentration on CHT of *C. latirostris*, the activity exhibited an increase when volumes of plasma increased

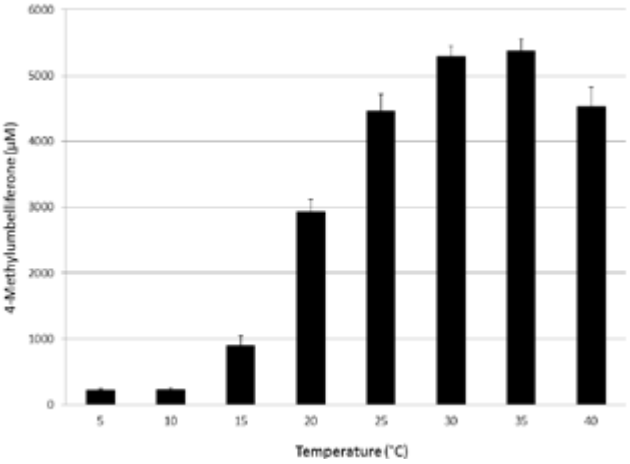

Fig. 1. Chitotriosidase activities in caiman plasma increased with incubation temperature (R=0.8907). Samples were analyzed in quadruplicates, and are presented as means ± standard error.

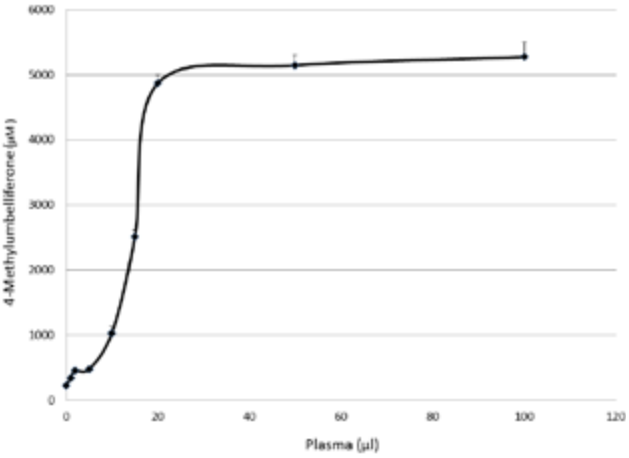

Fig. 2. The increment of plasma concentration was positively correlated with the product formed as consequence of chitotriosidase activity (R= 0.8956; p <0.05). Samples were treated as independent in quadruplicates, and presented as means ± standard error.

(R^2= 0.8956, Fig. 2) showing a volume-dependent activity. Low volumes of plasma produced a significant increase in CHT activity. The 50% of the maximum activity was reached with approximately 15 µL of caiman plasma and the higher CHT activity was obtained with the addition of 30 µL of plasma. Time of incubation was found to have a positive relationship with the substrate (R^2=0.6425). Caiman plasma CHT exhibited high activity immediately after the addition of substrate, gradually increased with time. Activity was elevated from 5 to 40 min of incubation with fluorescent substrate, the moment at which a plateau was reached by the curve until the last point of time studied

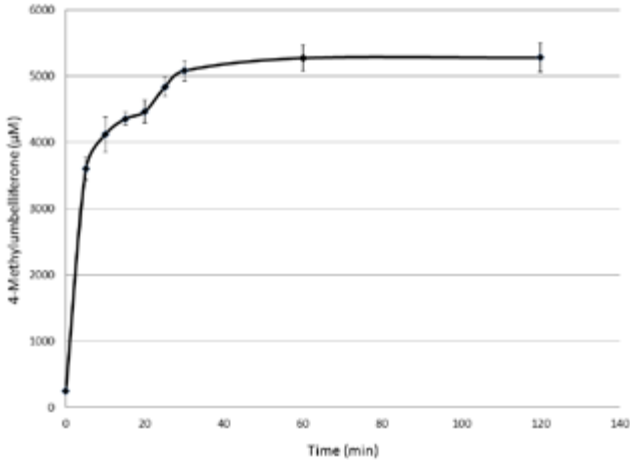

Fig. 3. Chitotriosidase activity in *Caiman latirostris* plasma reached a very high level over a short period. Similar behavior was detected in others enzymes activities studied involved in the caiman immune system.

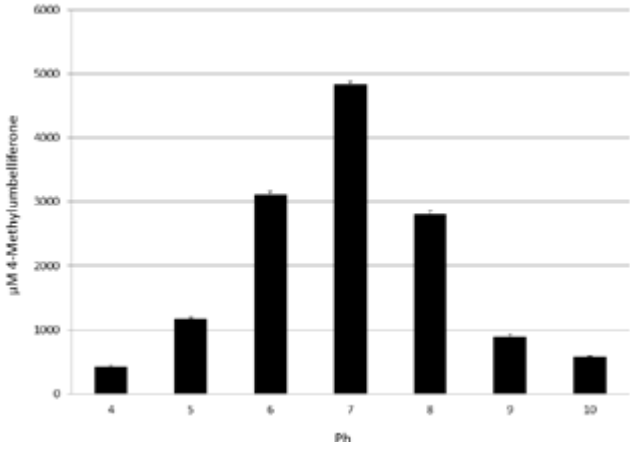

Fig. 4. The pH functioning for chitotriosidase activity in *Caiman latirostris* plasma showed the highest enzyme activity around 7 and a wide tolerance to work under and above this.

(Fig. 3). Figure 4 displays the assay performed to evaluate the optimum functioning pH for CHT activity in *C. latirostris* plasma (R^2=0.7497). Incubation of caiman plasma at different pH showed that the optimum pH for enzyme activity is 7. Activity decreased to 50% of the maximum at pH 6 and 8, and practically no activity was detected at both pH extremes tested. The CHT activity of *C. latirostris* plasma was assayed under different salinity conditions and increased with salinity (R^2=0.5697) at low concentrations (Fig. 5), reaching the highest value with the addition of different volumes of 1 M NaCl. The addition of 80 µL (or more) of saline solution resulted in a reduction of CHT activity at levels even lower than base line.

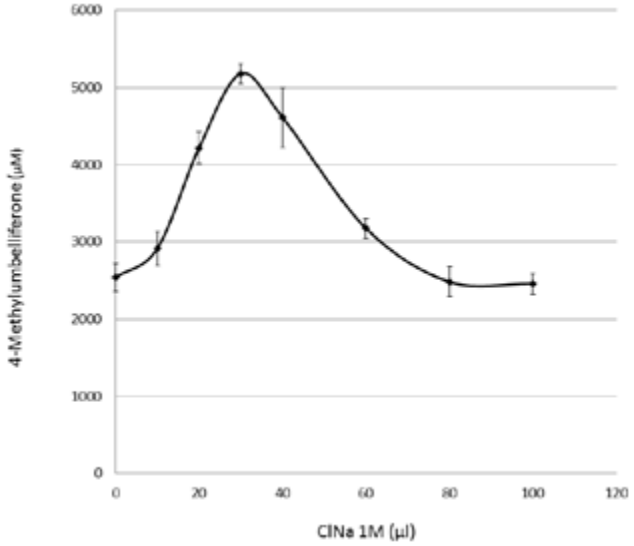

Fig. 5. The effects of the several salinities conditions reached with differents quantities of NaCl on the chitotriosidase activity in samples of *Caiman latirostris* plasma in quatriplicate and presented as means ± standard error.

DISCUSSION

Chitinases are ubiquitous enzymes that hydrolyze chitin, and have been identified in various organisms; they are involved in several biological processes including defense against chitin containing pathogens, such as fungi (Sahai and Manocha, 1993). Presently, these enzymes are receiving considerable attention due to their importance in very diverse processes such as the life cycles of chitin-containing organisms, food processing, defense against pathogens, and innate immunity. Particularly, some of these processes have been proposed to be very effective in crocodilians (Siroski et al., 2011, 2013). Based on the results obtained from the reaction between caiman plasma and 4 MU-(chitotrioside), the specific substrate of CHT allowed us to identify the first known crocodilian chitinase, *C. latirostris* CHT. This CHT is structurally homologous to chitinases from various species (Barone et al., 2007). They are secreted by activated macrophages and probably play a role in the defending against chitin-containing pathogens, in tissue remodeling and cell migration. In this study, we report similar values in animals apparently healthy or without any signs of illnesses.

There are few studies of chitinase activity in other reptiles. Several genera of Old World lizards including, *Anguis, Uromastyx, Chamaeleo* and *Lacerta* (Jeuniaux, 1961, 1962, 1963); and two New World lizards, *Anolis carolinensis* (Jeuniaux, 1962, 1963) and *Sceloporus undulatus garmani* (Marsh et al., 2001) have been reported

to secrete chitinase. These studies have shown that some vertebrates produce chitinase in the whole digestive tube in order to metabolize chitin as a food source (Karasov, 1989). Thus, the production of chitinase would allow them to utilize the chemical potential energy in their food more efficiently.

Polymorphonuclear leukocytes, but not lymphocytes and monocytes, are a major source of CHT in blood. Chitotriosidase hydrolyzes chitin substrates similarly to chitinases that are found in a variety of species (Boot et al., 1998), but this study is the first report of the presence and characterization of CHT in crocodilian plasma, or for that matter in any reptile species. In our study, we learned that CHT exhibits optimal activity in a narrow range of biochemical conditions. We chose several factors that are known to affect the rate at which the enzymes work, including temperature, salinity, pH, plasma concentration, and kinetic of incubation. Because any of these parameters might affect the rate of CHT enzyme reaction, each must be carefully controlled if we attempt to study the effects of changes in the enzyme itself. In all assays carried out the CHT enzymes of *C. latirostris* demonstrated high levels of activity.

Ectothermic vertebrates are considered appropriate models to assess the influence of temperature on a variety of physiological functions (Pxytycz and Zkowicz, 1994). Plasma CHT activity demonstrated a positive relationship with temperature (Fig. 1). When the incubation temperature is 15°C, CHT thermal activity was found to be very low, but increased at higher temperatures. Crocodilian physiology is considered practically "null" at temperatures below 15°C, but *C. latirostris* plasma CHT activity detected at these temperatures could be attributed to the greater climatic tolerance of *C. latirostris* compared with other related species (Siroski, 2004). Maximum CHT activity was detected between 30 and 35°C. This is reasonable because crocodilians have been demonstrated to maintain body temperature within a range of 28-33°C by using natural thermal gradients, and this range is close to the preferred temperature of *C. latirostris* for carrying out normal physiological processes (Bassetti, 2002). To our knowledge, there are no published studies concerning the temperature dependence of CHT activity in other species, and we have found that caiman CHT requires a specific environment and conditions within which to function. This is to be expected in ectotherms in which biochemistry and physiology are highly dependent on temperature (Siroski et al., 2012; Siroski et al., 2013).

The effect of increased volumes of caiman plasma with constant amounts of substrate solution was associated with increased CHT activity up to a peak at which activity remains high, independent of the plasma volume added. With this extreme sensitivity reproducible results with minimal variance can be obtained with small volumes of plasma, which can be extremely useful for studies in which it is not possible to obtain high volumes of blood. Similarly low volumes of plasma were found to be sufficient to produce measurable activities with high precision in this and related crocodilian species in the determination of crocodilian plasma phospholipase, PLA_2 (Merchant et al., 2010; Siroski et al., 2013) and dipeptidilpeptidase, DPPIV enzymes (Merchant et al., 2009b; Nevalainen et al., 2009; Siroski et al., 2012), as well as with respect to serum complement activities (Merchant et al., 2005; Merchant and Britton, 2006; Siroski et al., 2010).

Our findings demonstrated that caiman CHT exhibited time-dependent activity. After only five minutes of incubation of substrate with caiman plasma, CHT activity rapidly increased. High concentrations of the reaction products were detectable at 2 min, and steadily increased up to a maximum at 30 min, at which time, the CHT activity showed a plateau until the last time point evaluated. These results support those observed in similar studies performed in DPPIV and PLA_2 in the same species (Siroski et al., 2011). Observations such as these highlight immunological properties of caiman serum to to respond to challenges from microbial infection.

The pH of a solution is important for the structure and function of enzymes. Outside the range of the optimum pH of an enzyme, its structure can be compromised, and thus the activity can decrease dramatically. High temperatures and changes in pH can denature the enzyme, or result in conformation changes. The pH-activity relationship observed in this species differs from that in other species and other types of chitinases. At intermediate pH values (6-8), plasma CHT activities were elevated. It is expected that the pH optimum for each chitinase isoform is close to the pH of the environment in which it is active *in vivo*, and may differ depending on tissue origin. Chitinolytic activity was present in extracts of stomach, small intestine and pancreas, with the stomach and pancreas having the highest specific activities (Jeuniaux et al., 1982). For reptiles, optimum pH for intestinal and pancreatic chitinase is 4.5, with no activity at pH 1.0, whereas stomach chitinase has an optimum at pH 3.0, with strong activity at pH 1.0 (Micha et al., 1973; Jeuniaux et al., 1982). In this context, it seems desirable to investigate why the CHT activity in our samples occurred over such a broad pH range, but this feature could be another adaptive advantage that is part of an efficient defense mechanism.

It is expected that CHT isoforms are close to the osmolality of environmental conditions and may be seasonally-related. Under these conditions, maximum activi-

ties correspond to each species and may differ depending on tissue origin, or in response to tissue-specific metabolic demands. In this study, the effect of increasing concentrations of salinity affected CHT caiman plasma activity. Such ionic strength dependence is characteristic of a number of enzymes whose activities are inhibited or diminished at low or high salt concentrations; one would expect that changes in salt concentration cause conformational changes in these enzymes. To our knowledge, there are no studies concerning the salinity tolerance of proteins in crocodilians. However, those few crocodilian species that inhabit water of high or low salinity, or estuarine waters with fluctuating salinities probably exhibit enzymes that can act across a broader range of salinities than those of caimans. Such species have the ability to actively increase their body fluids in low or high salinity environments. Hyperregulation is accompanied by permanent exchange of ions from the internal and external milieu, and thus, mechanisms to counteract dilution or concentrated of body fluids had to be developed. Changes in salinity can alter the functions of enzymes and hormones with serious consequences, resulting in a variety of metabolic changes identical to those caused by water stress, as detected by Lance et al. (2010), and are probably mediated by hormonal signals (Elsey, 2005). Out of this tolerance range, one would expect CHT activity to fall under the severity of the imposed salinity stress.

We found the highest enzyme activity values reasonably close to the physiological pH and salinity conditions of caiman plasma. Despite this, caiman plasma CHT activities also were detected under extreme pH conditions, but at lower activities. The fact that this enzyme exhibits activity outside of normal physiological conditions (at which the enzymes of only a few species would be active) could possibly be another reason for the effectiveness of the caiman's immune system. Additional evidence for a role of CHT during immunological responses is the observation that the enzyme is rapidly and acutely up-regulated with pro-lactin, IFN-γ, tumour necrosis factor α, and LPS, but not with IL-10 (Malaguarnera et al., 2004; Di Rosa et al., 2005). Involvement of CHT in caiman immune defenses was not investigated but it would be very interesting to measure its plasma and tissue expression level after both bacterial and LPS challenges.

The high activity of CHT detected in caiman plasma suggests that future investigations may reveal interesting information regarding the properties and homologies of this enzyme in crocodilians. Based on the importance and function of CHT enzyme, it could be used to study molecular immune mechanisms in a phylogenetic context or could have a possible clinical application as a biomarker of individual health status.

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