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# Characterization of Chitotriosidase Enzyme Activity in the Serum of the American Alligator (*Alligator mississippiensis*)

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## Abstract

Chitotriosidase (ChT) is an endoglucosaminidase enzyme that cleaves chitinous substrates and has been strongly associated with innate immune activity and the ability to identify non-selftissues. This enzyme activity was detected and characterized the serum from the American alligator (*Alligator mississippiensis*) using a fluorometric probe. Alligator serum exhibited volume-dependent activity, with activity (2.1 ± 0.3 μmol/min) observed at dilutions as low as a 1:150, and maximum activity (5.2 ± 0.6 μmol/min) measured at a dilution of 1:30. Alligator serum ChT showed linear activity for approximately 20 min, at which time activity decreased exponentially, presumably due to the depletion of substrate. In addition, the ChT activity in alligator serum was temperature-dependent with low activity at 5˚C, a sharp increase from 10˚C - 30˚C, and maximal activity from 30˚C - 40˚C. The activity was inhibited in the presence of water-soluble chitin, but not mannan, indicating the specificity of the enzyme. The presence of ChT in alligator serum is likely to be partially responsible for the potent innate immune system of these crocodylians, and particularly antifungal activities.

## Keywords

Antifungal, Crocodilian, Crocodylian, Innate Immunity

## 1. Introduction

Chitin is a major component of the cell walls of fungi, and also in some bacteria, and the microfilarial sheath of parasitic nematodes [1] [2]. Chitinase enzymes are used by these organisms to remodel their outer walls. In addition, some organisms that do not have chitin polymers express chitinase enzymes to degrade

chitin for carbon and energy [3], while others use it as a form of microbial and/or parasitic defense [4] [5] [6].

Chitotriosidase (ChT) is an endoglucosaminidase that cleaves, and exhibits transglycosylation activity towards chitin, a polymer of N-acetyl-D-glucosamine present in the coatings of many pathogens [7]. It belongs to the family of 18 glycosyl hydrolases. Chitinases, in general, have been found in a variety of organisms and are believed to play an important role in innate immunity, especially against viral, bacterial, protozoan, fungal and nematode parasites [7] [8]. This enzyme has also been adapted by some organisms as a means of defense against pathogenic fungi and other chitinous pathogens [9] [10].

There are six recognized classes of chitinases, and ChT belongs to class III [11]. Chitotriosidase exists in two forms, a 50-kDa precursor protein and a 39-kDa active enzyme that is produced through proteolytic processing. The 50-kDa protein consists of a C-terminal chitin-binding domain (hinge region) and a 39 kDa N-terminal domain that has chitinase activity. Both forms are present in human serum [7] [8]. The 39-kDa isoform is able to cleave chitotriose and also hydrolyzes colloidal chitin to yield chitobiose. The C-terminal domain also plays a role in processing colloidal chitin [8].

Chitotriosidase appears to be expressed in all mammalian species [12]. Studies conducted with goats and mice indicate a direct relationship between the lack of occurrence of pathogens and the presence of ChT activity in the serum. Studies among human populations also indicate a relationship between the exposure to pathogens and production of ChT [13]. Studies of Gaucher disease led to the isolation and identification of ChT [14]. Among human populations, a 24-base pair duplication in exon-10 of ChT activates a 3' splice and results in an enzymatically-inactive protein deficient in 29 amino acids [15]. The clinical result is an accumulation of glucosylceramide in the lysosomes of macrophages (mΦ) which, in turn, accumulates in various tissues of the patient (Gaucher Disease) [14] [15]. Further investigations lead to the identification of AMCase (Acidic Mammalian Chitinase), which is present in two isoforms [16]. ACMase has an optimal pH of 2.3 and is capable of cleaving artificial and natural chitin-like substances [12] [14]. In populations lacking ChT, AMCase may offer significant resistance to chitin-based parasites as evidenced in natives of Papua New Guinea and India [17]. Among populations inhabiting environments with endemic parasitic diseases, the occurrence of non-functional ChT genes is much lower. Deficiencies in ChT production are common among human populations in France, Sicily and Asia, but are rare African populations [17].

In human studies, polymorphonuclear neutrophils (PMNs), not lymphocytes nor monocytes, were found to be the major source of ChT in blood. The enzyme is selectively expressed and released upon specific stimuli by human PMNs as well as mΦ—a macrophage produced by the differentiation of monocytes. Granulocyte macrophage colony-stimulating factors (GM-CSFs) induce the release of ChT from human PMNs, indicating the enzyme is not present in lysosome-like (blue staining) granules, but in specific granules [7]. When exposed to

the proper stimuli, ChT is released. Serial plasma samples reveal plasma ChT and lactoferrin occur in parallel, but independent of neutrophil cell increase which occurs much later [7].

Due to its role in mammalian immunology, this study was conducted to characterize ChT in the American alligator (*Alligator mississippiensis*), an organism that lives in pathogen-laden environments. Only recently has attention been focused crocodylian immune systems (reviewed in [18]). The focus of this study was on the detection and characterization of ChT enzyme activity in the serum of the American alligator (*Alligator mississippiensis*).

## 2. Materials and Methods

Chemical and Biochemicals: 4-methylumbelliferyl-$\beta$-D-N, N', N''-triacetylchitotrioside (4 MU-chitotrioside), 4-methylumbelliferone (4-MUB), and glycine were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO).

Collection of Samples: Alligators (n = 12, 190 - 243 cm) were captured from a boat at night, using a spotlight and cable snares. Whole blood (10 - 20 mL, depending on the size of the alligator) was collected from the spinalvein using 18 g a needles and a 20 mL syringes as described by Zippel *et al.* [19]. The animals were released immediately after the collection of samples. All of the activities concerning the treatment of alligators were approved by the McNeese State University Animal Care and Use Committee.

Enzyme assays were conducted in quadruplicate. Alligator serum was diluted to six different titers (0.5% to 12.5%, v/v) using isotonic saline. To each 100 µL of diluted serum sample, 100 µL of 25 µM 4-MU-chitotrioside, dissolved in citrate-phosphate buffer (pH 5.2), was added to initialize the reaction, and allowed to proceed for 15 min at ambient room temperature. At the end of the 15 min incubation, one mL of stop solution (3M glycine-NaOH buffer, pH 10.6) was added and fluorescent activity was recorded.

An enzyme kinetics experiment was conducted by adding 4 mL of 25 µM 4-MUB substrate, 200 µL serum, 1.8 mL isotonic saline. At various times after the addition of substrate (0, 1, 2, 5, 10, 15, 20, 30, 60 min), 150 µL were withdrawn and placed in cuvettes containing one mL of stop solution. Time zero samples were withdrawn as soon as the substrate was added. Product formation was recorded in Horiba Jovin Yvon Fluoromax4fluorimeterat an excitation $\lambda$ of 365 nm (slit width = 2 nm), and an emission $\lambda$ of 450 nm (slit width = 2 nm).

Thermodynamic stability of alligator ChT activity was evaluated at a broad range of temperatures (5˚C - 40˚C). Alligator serum (5 µL) diluted with 195 mL of isotonic saline was incubated with 25 µM substrate, and the reaction was allowed to proceed for 15 min. The reaction was halted with the addition of one mL of stop solution, and the fluorescent activity was measured as described above.

Statistics and Controls: The fluorescence resulting from each reaction was compared to a standard curve developed from the pure product (4-MUB), and

the nmol of product formed for each assay were calculated. The fluorescent intensities for each sample were corrected for background by the subtraction of florescence measured in alligator serum devoid of substrate. Each data point represents a mean ± standard deviation of four independent determinations. The results obtained from each experiment were subjected to analysis of variance using Schee's post hoc comparisons [20].

## 3. Results

Upon exposure of different volumes of serum derived from the American alligator (*A. mississippiensis*) alligator serum to the fluorogenic substrate 4 MU-chitotrioside, a positive relation of serum volume with ChT activity was observed. Enzyme activity effectively catalyzed the target substrate at 1.0 µL of serum, producing 31.5 ± 5.22 µmoles of product. An increase to only 5 µL of serum resulted in maximal activity (78.3 ± 8.43 µmoles) and remained constant ($p > 0.05$) at higher volumes (10 - 50 µL).The increase at low volumes (≤5 µL) was nearly linear ($y = 14.5x + 7.5$, $R^2 = 0.976$), with 50% of maximal activity calculated to occur at 3.2 µL of alligator serum.

The kinetic character of alligator serum ChT activity is shown in Figure 2. Alligator serum showed substantial ChT activity within one min of substrate addition (17.0 ± 0.3, $p < 0.05$) and maximal activity (301 ± 14.8 µmol) within 30 min. The activity was observed to be relatively linear for the first 20 min, with a slope of approximately 13 µmoles of product generated per minute.

Figure 3 displays the ChT activity of alligator at a broad spectrum of temperatures (5˚C - 40˚C). The generation of product occurred slowly at low temperatures, as 43.0 ± 4.9 and 65.2 ± 5.3 µmol of product were generated at 5˚C and 10˚C, respectively, but increased greatly from 10˚C - 30˚C. The maximal ChT remained constant from 30˚C - 40˚C ($p > 0.05$).

The addition of various amounts of water-soluble chitin to serum from alligators decreased the ChT activity in a concentration-dependent manner (Table 1). The inclusion of a 5-, 20- and 100-fold molar excess of chitin, relative to the

Table 1. Effects of water-soluble chitin and mannose on ChT activity in alligator serum. The fluorogenic substrate (4-MU-chitotrioside) was incubated alone, or with an excess of water-soluble chitin or mannose. The results are presented as a mean ± standard deviation for four independent determinations and are expressed in µmol product formed. * = statistically lower ($p < 0.01$).

| Incubation | ChT Activity | % Inhibition |
|---|---|---|
| 1 mM 4-MU-chitotrioside | 21.6 ± 5.3 | - |
| 1 mM 4-MU-chitotrioside + 5 mM chitin | 17.9 ± 2.3 | 17 |
| 1 mM 4-MU-chitotrioside + 20 mM chitin | 7.1± 1.3* | 67* |
| 1 mM 4-MU-chitotrioside + 50 mM chitin | 1.7 ± 2.1* | 92* |
| 1 mM 4-MU-chitotrioside + 20 mM mannose | 21.0 ± 3.5 | 3 |
| 1 mM 4-MU-chitotrioside + 50 mM mannose | 20.5 ± 1.9 | 5 |

fluorogenic chitinous substrate, resulted in a 17%, 67%, and 92% decrease in ChT-mediated cleavage of the chitinous substrate, respectively. Furthermore, the addition of a 20- or 100-fold molar excess of mannan resulted in no change ($p > 0.05$) in ChT activity.

## 4. Discussion

Ectothermic vertebrates rely heavily on innate immunity as the major mechanism of host defense [21]. Innate immunity is relatively rapid and nonspecific in nature, relying on molecular pattern recognition to discriminate self from non-self tissues [22] [23]. Although American alligators have less-developed adaptive immune systems relative to endothermic vertebrates [24], they exhibit well-developed innate immune mechanisms (reviewed in [25]). It is thought that the biological cost of development and maintenance of adaptive immunity is a limiting factor for ectotherms [26]. Because ectotherms cannot regulate body temperature by internal mechanisms, they undergo seasonal shift in their biochemistry and physiology which is associated with environmental conditions, and constrains available mechanisms of immunity [27].

The addition of increasing volumes of alligator serum to enzyme assays resulted in more enzyme activity (**Figure 1**). The amount of product formed was parallel to that observed with serum from *Caiman latirostris* [28]. The shapes of the curves were virtually identical when serum from these two Alligatorids was used in ChT assays. Human serum ChT levels in normal patients have been reported to be similar [29]. The increase of 14.5 μmoles of product formation per one μL of serum is extremely high and was similar to that produced by serum from the broad-snouted caiman [28], when standardized for substrate concentration. It is likely that the enzyme activity observed is due to the presence of

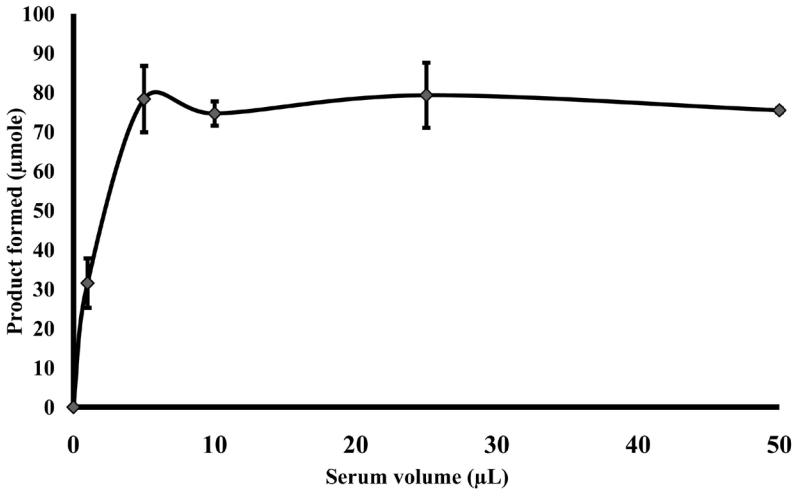


**Figure 1.** Relation of the volume of alligator serum with ChT activity. Difference volumes of alligator serum were incubated with the fluorogenic substrate, and the fluorescence product was measured at an excitation $\lambda$ of 365 nm (slit width = 2 nm), and an emission $\lambda$ of 450 nm (slit width = 2 nm). The results are presented as a mean ± standard deviation for four independent determinations and are expressed in μmol product formed.

ChT due to the fact that the activity could be inhibited by unlabeled chitin, but not by another polysaccharide such as mannan (**Table 1**).

The kinetic character of alligator ChT (**Figure 2**) was also very similar to that measured in the broad-snouted caiman [28]. Small volumes of serum (2 - 5 μL) resulted in rapid accumulation of product. The rapid rise in accumulated fluorescent product is an indication of the high capacity for cleavage of chtinous substrates. These results seem to indicate that this enzyme activity in serum derived from alligators has the ability to rapidly compromise the structural integrity of microbes with chitinous outer cell walls.

The thermal profile of alligator ChT activity reflects the preferred body temperatures of these animals. Alligators thermoregulate to an internal body temperature of 31˚C - 32˚C [30], where many physiological parameters such as cardiac function [31] and general metabolism [32], are optimized. In addition, many immunological parameters, such as serum complement [33], phospholipase $A_2$ [34], and dipeptidyl peptidase IV [35] activities have been shown to exhibit comparable thermal characteristics. At temperatures higher than the preferred body temperature the activity ChT remains high (**Figure 3**). Alligators are known to exhibit a febrile response to infection, which is not controlled by internal biochemical mechanisms, but rather by basking behavior [36]. During this response, infected alligators achieve higher daily internal body temperature spikes relative to untreated animals or those injected with saline controls. It is notable that, at lower temperatures, the activity of alligator ChT in the plasma is depressed; however, at temperatures above 30˚C, the activity remains high (**Figure 3**). The potential function of this elevated internal body temperature during

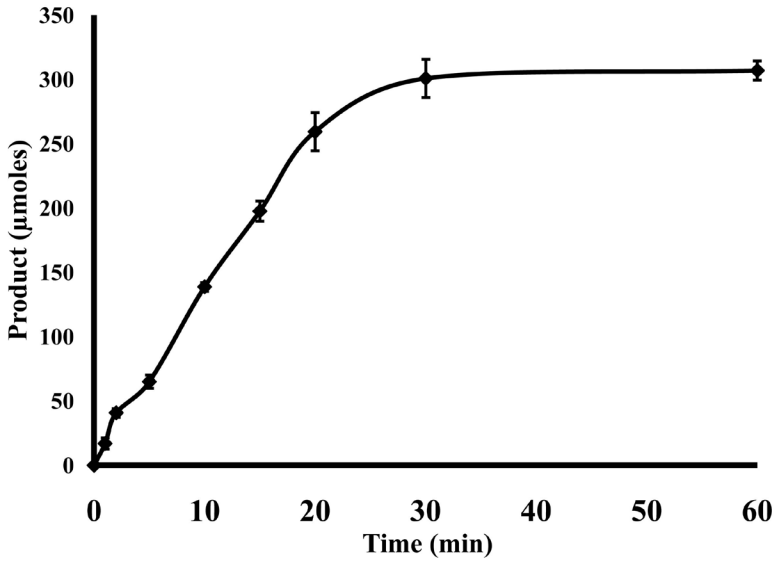


**Figure 2.** Kinetic profile of ChT activity at 25˚C. Alligator serum was incubated for different amounts of time with a fluorogenic substrate, and the fluorescence product was measured at an excitation $\lambda$ of 365 nm (slit width = 2 nm), and an emission $\lambda$ of 450 nm (slit width = 2 nm). The results are presented as a mean ± standard deviation for four independent determinations and are expressed in μmol product formed.

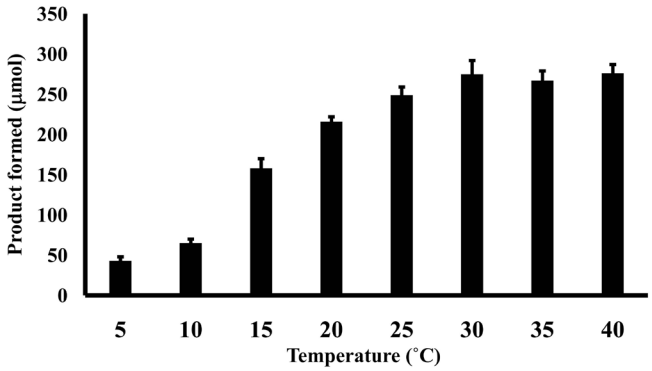


**Figure 3.** Thermal profile of alligator serum ChT activity. Alligator serum was incubated in the presence of fluorogenic substrate for one hour at different temperatures. The fluorescence product was measured at an excitation $\lambda$ of 365 nm (slit width = 2 nm), and an emission $\lambda$ of 450 nm (slit width = 2 nm). The results are presented as a mean ± standard deviation for four independent determinations and are expressed in µmol product formed.

infection might be to increase the temperature above the optimal growing range for pathogenic microbes while still maintaining a potent immune defense.

Alligators, like most crocodylians, spend appreciable amounts of time shuttling from terrestrial to aquatic environments in an attempt to regulate body temperature. This behavior results in these animals having moist skin much of the time, which would seem to be a suitable substrate for fungal colonization and growth. Because many fungi have chitinous outer membranes [37], ChT activity is thought to have evolved as an innate immune defense against infection by potentially pathogenic fungi [38] [39]. The high levels of circulating ChT activities in crocodylian blood may serve to protect against fungal infections.

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## Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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