Characterization of Serum Complement Activity in Three Species of Crocodilians from Southeast Mexico

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Abstract

Aims: To characterize the serum complement innate immune system in three species of crocodilians native to southeast Mexico.

Methodology: Plasma collected from three wild crocodilian species native to southeast Mexico were exposed to sheep red blood cells (SRBCs) to measure hemolysis, which is used as an indication of serum complement immune activity.

Results: Incubation of different volumes of plasma from *Crocodylus acutus*, *Crocodylus moreletii*, and *Caiman crocodilus* resulted in a volume-dependent increase in SRBC hemolysis. However, while maximum hemolysis for *C. acutus* and *C. moreletii* were both approximately five-fold higher than that of *Ca. crocodilus*. A kinetic study revealed that the hemolysis was rapid, with near-maximum activity recorded at 30 min for *C. acutus* and *C. moreletii*. However, *Ca. crocodilus* activity exhibited a significant increase ($P < .5$) only between one and two hours. A thermal analysis showed that the SRBC hemolysis was maximal at temperatures to which these species thermoregulate. The thermal profiles were similar for all three species, although the activity was lower for *Ca. crocodilus* ($P < .01$). The SRBC hemolysis was strongly inhibited by mild heat treatment ($56^{o}C$, 30 min) and also by EDTA, indicating that the hemolytic activity was probably due to the presence of crocodilian serum complement activity. The EDTA-inhibited activity was restored by the addition, of a 20 mM excess of Ca^{2+} or Mg^{2+}, but not Fe^{2+} or Cu^{2+}, thus exhibiting the specific need for Ca^{2+} or Mg^{2+}.

Conclusions: The serum complement activities of *C. acutus* and *C. moreletii* are much higher ($P < .01$) than *Ca. crocodilus*, which may be related to the maximum sizes and increased aggressive natures of *C. acutus* and *C. moreletii.*

Key Words: American crocodile, spectacled caiman, Morelet's crocodile, serum complement, innate immunity, crocodilian, sheep red blood cells, *Crocodylus acutus*, *Crocodylus moreletii*, *Caiman crocodilus*

1. **Introduction**

The American crocodile (*Crocodylus acutus*) primarily inhabits coastal mangrove swamps, the lower brackish sections of rivers, and coastal lagoons in eastern Mexico [1]. The Morelet's crocodile (*Crocodylus moreletii*) is found in freshwater lakes, marshes and rivers, but is occasionally found in brackish areas [2]. The ranges and habitats of these two species overlap, and interbreeding in Mexico is known to occur [3-4]. The spectacled caiman (*Caiman crocodilus*) is an extremely adaptable species that lives in a wide variety of habitats and a broad range of salinities, and can be found in practically all wetlands and riverine systems throughout its range. In Mexico, this species is restricted to the extreme southeastern portion of the country.

Although *C. acutus* is listed as CITES appendix I in Mexico, and *C. moreletii* was only recently down-listed to appendix II, little is known about the biochemistry, physiology, and immunology of these apex predators. It is important to understand health and disease of animals in order to effectively manage populations [5-7]. A better understanding of the immune systems of these animals will lead to a better apprehension of their susceptibilities and resistances to diseases. Although many species of crocodilians are critically endangered, there is little information concerning their immunology and susceptibility/resistance to disease states. This could be due to the fact that crocodilians in captivity are chronically stressed, and stress is immunosuppressive in vertebrates. Therefore, captive crocodilians are poor models for immunological studies. In addition, it is often difficult to obtain fresh tissue samples from wild crocodilians and transport them to the laboratory due to the remote habitats in which they live, the preservation of samples in adverse field conditions in which many of these animals must be captured, and the difficulties in obtaining permits due to the laws governing the collection and transfer of crocodilian tissues. Although there are many deterrents for the study of crocodilian immunity, there have been recent efforts to study their immune systems, and in particular, the crocodilian serum complement [reviewed in 8]. This study was conducted to characterize and compare serum complement innate immune activity in three different crocodilian species native to southeast Mexico.

2. Materials and Methods

2.1. Reagents – Sheep red blood cells (10% v/v, washed and packed) were purchased from Rockland Immunochemicals (Gilbertsville, PA, USA). Ethylene diamine tetraacetate (EDTA), and all other salts, was purchased from Sigma-Aldrich Chemicals (St. Louis, MO, USA).

2.2. Treatment of animals – crocodilians were captured at night, from a boat with the aid of a spotlight and a stainless steel cable-locking snare. *Crocodylus acutus* specimens (n=18, 0.85 - 2.62 m) were captured from Boca del Cielo on the Pacific coast. *Crocodylus moreletii* (n=12, 0.67 - 2.26 m) were captured from Laguna de las Illusiones in Villahermosa, and from the Rio Hondo near Cacao. *Caiman crocodilus* (n=11, 0.62 - 1.24 m) samples were collected from Puerto Arista. After collection of blood samples, the animals were released at the capture site. All of the methods for the capture, handling, and collection of tissues from animals was approved by the McNeese State University Animal Care and Use Committee.

2.3. Collection of blood – Blood was collected from crocodilians via the spinal vein [9-10] using a 3.8 cm 18 ga. needle. Approximately 5-7 mL of blood were collected from animals with a total length of 0.6-1.2 m, while 20 mL were collected from animals greater than 1.2 m. Equal volumes of the plasma from all animals was pooled so that average values for each species could be obtained. All of the activities involving handling of the animals used in this study were approved by McNeese State University Animal Care and Use Committee.

2.4. SRBC hemolysis assay – crocodilian plasma was incubated with an equal volume of 2% SRBCs for 30 min. The samples were centrifuged for 5 min at 3000x*g*, and 200 μL of supernatant was transferred

to a well of a 96-well microtiter plate. The absorbance values were measured with a BioRad Benchmark Plus™ microtiter plate reader at 540 nm.

For experiments in which the effects of different volumes of plasma were determined, 0-200 μL of plasma were added to 200 μL of 2% SRBCs, and the volume was kept constant by the addition of 0.7% saline (final concentration of SRBCs was 1%). The samples were allowed to incubate at ambient temperature for 30 min, centrifuged, 200 μL of supernatant was removed to a microtiter plate, and the OD_{540} was measured as described above.

Time-dependence of SRBC hemolysis was determined by an incubation of 1.0 mL of 50% plasma (diluted with isotonic saline) with 2.0 mL of 2% SRBCs. Aliquots were removed, at different times (0, 2, 5, 10, 15, 30, 60, and 120 min), to 0.05 volumes of 1 M EDTA (pH 8.0). The samples were immediately centrifuged (5000x*g* for 3 min), and 200 μL of supernatant were removed to microtiter plates for determination of OD_{540}.

To determine the effects of temperature on the hemolysis of SRBCs by crocodilian plasma, 150 μL aliquots of crocodilian plasma and 2% SRBCs were incubated at different temperatures (5-40°C) for 15 min, and then mixed to start the reaction. After 30 min, the reactions were stopped by the addition of 3 mL of 1 M EDTA (pH 8.0), and then centrifuged immediately at 5000x*g* for 3 min at ambient temperature. The supernatants (200 μL) were removed to wells of a microtiter plate, and the OD_{540} was determined as described above.

The effects of mild heat treatment on SRBC hemolysis was determined by incubation of the crocodilian plasma at 56°C for 30 min prior to coincubation with 2% SRBC (ambient temp, 30 min). In addition, the contribution of divalent metal ions was probed by inclusion of 50 mM EDTA to an incubation of 2% SRBCs and 50% crocodilian plasma. The effects of Mg^{2+}, Ca^{2+}, Fe^{2+}, and Cu^{2+} on hemolysis by inclusion a 20 mM excess of the chloride salts of these metals with 2% SRBCs and 50% crocodilian plasma. The samples were centrifuged, and the OD_{540} of 150 μL were determined as described above.

2.5. Statistics and controls – For each assay, the results were compared to a positive hemolysis control in which a sample of 1% SRBCs were exposed to 0.05% Triton-X detergent, and vortexed vigorously for one min. The OD_{540} for each sample was compared to that of the positive control, and the results are expressed as % maximum hemolysis. Each sample was analyzed in quadruplicate, and the results presented represent the means ± standard deviations. The samples were subjected to analysis of variants, using Duncan's post-hoc comparisons [11], with $P = 0.05$ chosen as the level of significance.

3. Results

The results displayed in Figure 1 show the volume-dependent complement activity for plasma derived from *C. acutus*, *C moreletii*, and *Ca. crocodilus*. Hemolysis of SRBCs was measured in 20 μL of plasma of *C. acutus* (25.4 ± 1.2%) and *C. moreletii* (20.4 ± 0.1%). In contrast, significant activity was not observed in *Ca. crocodilus* plasma until 40 μL (7.5 ± 1.9%). Hemolytic activities peaked at approximately 70 μL for *C. acutus* (103.1 ± 1.3%), *C moreletii* (103.6 ± 2.0%), and *Ca. crocodilus* (22.8 ± 0.4%). The SRBC hemolysis was clearly lower in Ca. crocodilus ($P > .01$), relative to the other two species, at all volumes greater than

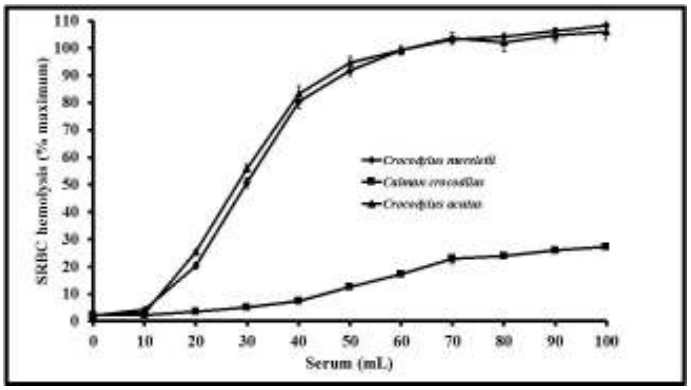

Figure 1. Volume-dependent hemolysis of SRBCs by plasma from *C. acutus*, *C. moreletii*, and *Ca. crocodilus*. Different volumes of crocodilian plasma was incubated with 1% (v/v) SRBCs for 30 min at ambient temp, and then the hemolysis was measured spectrophotometrically at 540 nm as described in Material and Methods. The data represent the means ± sd for four independent determinations.

Figure 2 exhibits the kinetic analysis of serum complement activities for all three crocodilian species studied. The hemolytic activity increased rapidly for both *C. acutus* and *C. moreletii*, and peaked between 30 and 60 min. Hemolytic activity for *Ca. crocodilus* exhibited no significant increase from time zero for up to 30 min ($P > .05$), and showed only a small increase to 21.1% at 60 min. The maximum activity observed for *Ca. crocodilus* (36.6 ± 0.8%) was approximately three-fold lower ($P < .01$) than for the other two species (98.8 - 99.2 %), but still increased only slightly between 60 and 120 min.

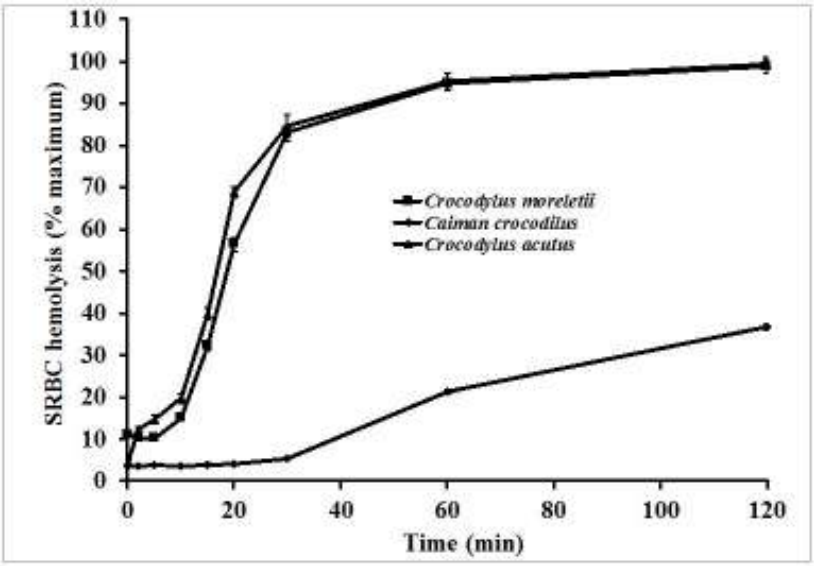

Figure 2. Kinetic analysis of the hemolysis of SRBCs by plasma from *C. acutus*, *C. moreletii*, and *Ca. crocodilus*. Crocodilian plasma was incubated with 1% (v/v) SRBCs for different amounts of time at ambient temp, and then the hemolysis was measured spectrophotometrically at 540 nm as described in Material and Methods. The data represent the means ± sd for four independent determinations.

The results shown in Figure 3 demonstrate the thermal profile of the serum complement activity of all three crocodilian species. The plasma from all three species exhibited relatively low activities at temperatures below 20°C. The hemolysis activities increased in a linear fashion from 5-30°C for plasma from both *C. acutus* and *C. moreletii*. The hemolytic activity peaked at 30°C for *C. acutus* (89 ± 1.5%),

and then decreased ($P < .05$) to 81 ± 1.3 and 74 ± 1.1% at 35 and 40°C, respectively. Likewise, the activities for *C. moreletii* also peaked at 30°C (88 ± 1.4), and then decreased at 35°C (73 ± 2.3) and 40°C (71 ± 1.6). Although the activities were lower for plasma from *Ca. crocodilus* at all temperatures ($P < 0.01$), the same pattern of activities were observed, as the activity peaked at 30°C (40 ± 0.4%) and decreased at 35°C (35 ± 0.7%) and 40°C (33 ± 1.2%).

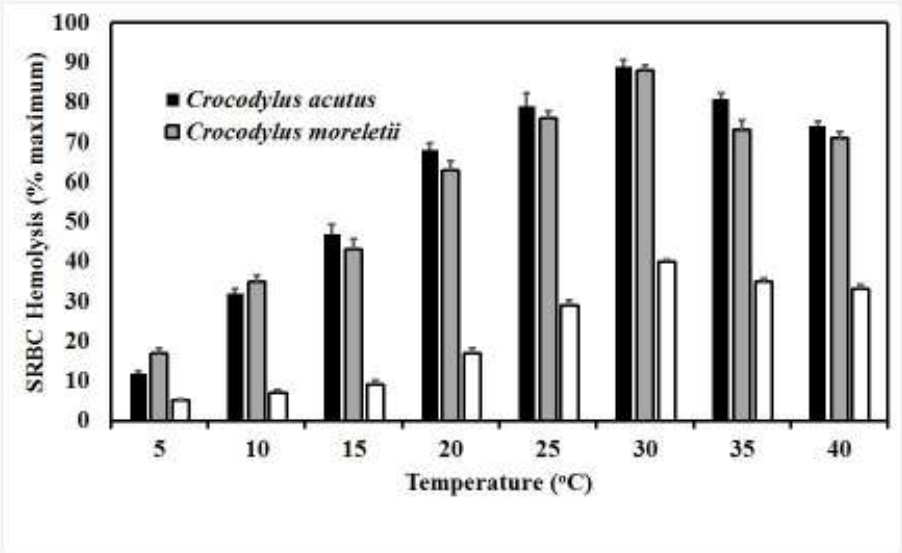

Figure 3. Temperature-dependent hemolysis of SRBCs by plasma from *C. acutus*, *C. moreletii*, and *Ca. crocodilus*. Crocodilian plasma was incubated with 1% (v/v) SRBCs at different at different temps for 30 min, and then the hemolysis was measured spectrophotometrically at 540 nm as described in Material and Methods. The data represent the means ± sd for four independent determinations.

The results of a mechanistic study on the effects of mild heat treatment and divalent metal ions on the hemolytic activity of crocodile plasma toward SRBCS are shown in Figure 4. Incubation of plasma at 56°C for 30 min, prior to incubation with SRBCs, reduced ($P < .01$) the hemolytic activity to 5.6-9.6% of the untreated plasma. Treatment of plasma with 30 mM EDTA produced an 86.2, 91.7, or 93.7% reduction ($P < .01$) in SRBC hemolysis by *C. acutus*, *C. moreletii*, or *Ca. crocodilus* plasma, respectively. However, the inhibitory effects of EDTA were almost completely overcome ($P < .01$) by the addition of 50 mM Mg^{2+} (89.9-103.6%) or Ca^{2+} (96.8-108.6%). The addition of Fe^{2+} or Cu^{2+} exhibited no effect ($P > .05$) on the inhibition of SRBC hemolysis by crocodilian plasma.

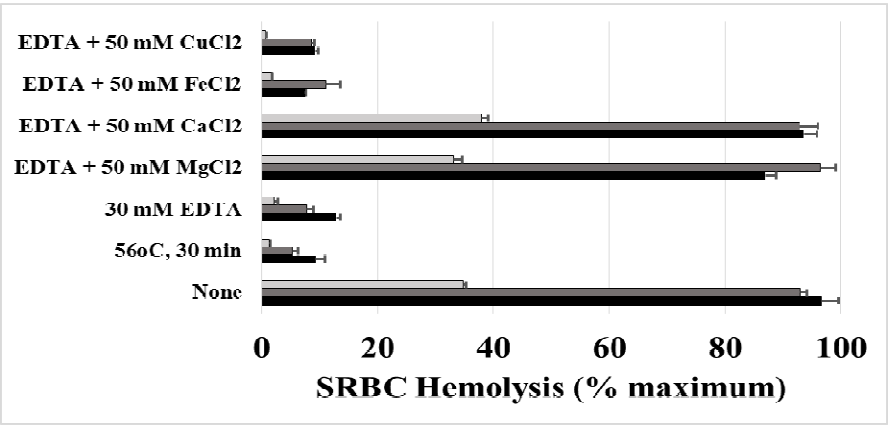

Figure 4. Effects of EDTA and mild heat treatment on the hemolysis of SRBCs by plasma from *C. acutus*, *C. moreletii*, and *Ca. crocodilus*. Crocodilian plasma was heated to 56°C for 30 min, or treated with 30 mM EDTA, and then incubated with 1% (v/v) SRBCs at different at different temps for 30 min. The hemolysis was measured spectrophotometrically at 540 nm as described in Material and Methods. The data represent the means ± sd for four independent determinations.

4. Discussion

Serum complement evolved as an early form of immunological host defense, and can be found in virtually all vertebrates, and many ancient invertebrates [12-14]. Serum complement is known to be a critical component of innate immunity, and human patients that exhibit complement deficiencies are prone to recurring infections [15-16]. It has many protein components, and three primary components of activation: classical [17], alternative [18], and lectin-mediated [19]. Recent studies have shown that crocodilians exhibit potent and broad-acting serum complement activities [20-23]. Studies with the American alligator (*Alligator mississippiensis*) have shown that the alternative [24] and lectin-mediated [25] activation pathways of complement activity are functional. The CH_{50}, or the volume of plasma that will cause the lysis of 50% of SRBCs, is similar to those measured in other crocodilian species (Figure 1).

The kinetic efficiencies with which the serum complement proteins hemolyzed the SRBCs is similar to other crocodilian species. For instance, SRBC hemolytic activities were maximal at approximately 30-45 min for *Caiman latirostris* [23]. However, other crocodilians exhibit more rapid complement action, as maximal activity is achieved in the plasma of *Alligator mississippiensis* at 20 min [24], and at 15 -20 min for *C. porosus* and *C. johnstoni* [21].

Because crocodilians are ectothermic vertebrates, their biochemical and physiological processes are largely affected by external temperatures. In 2010, Cupul and coworkers [26] reported that four *C. acutus* individuals showed thermal preferences from 28.8-30.7°C. In addition, *C. moreletii* exhibits a thermal preference of approximately 30°C (Marco Lopez, personal communication), and *Ca. crocodilus* thermoregulates to 29.9-34.8°C, depending on the size of the animal [27]. These temperature preferences are in the range of the maximum serum complement activities observed in this study (Figure 3). It is reasonable to expect that optimal temperatures for major physiological functions (digestion, respiration, immunity, etc.) would fall within the temperature ranges to which these animals thermoregulate. The maximum serum complement activity observed for all three species occurred at 30°C, although the activity for *Ca. crocodilus* was substantially lower ($P < .01$) than for *C. acutus* and *C. moreletii*. In addition, the complement activities are noticeably suppressed at lower temperatures (5-15°C), and thus during the colder winter months, the depressed temperatures would potentially lead to reduced abilities to fight infection, However, these crocodilians are less active during these months, and thus few to no territorial disputes occur during this time. In addition, microbial growth occurs much more slowly at lower temperatures, and thus the need for a highly active immune system during the winters becomes less important.

The hemolysis of SRBC be crocodilian plasma could be due to a variety of proteins that exhibit activities that can compromise the integrity of plasma cell membranes. For instance, phospholipase A_2, an enzyme that cleaves fatty acids from the *sn*-2 position of membrane phospholipids, is known to be expressed in crocodilian serum [28-30]. This enzyme is known to have the ability to compromise the integrity and function of plasma membranes. To determine the heat stability of the molecules responsible for the hemolytic activity, the plasma was subjected to mild heat treatment. Serum complement proteins are known to be very heat labile. This heat sensitivity (Figure 4) rules out the possibility that the hemolytic activity was due to the presence of antimicrobial peptides expressed by crocodilian leukocytes [31]. In addition, it is known that several serum complement enzyme components require divalent metal ions [32-33], and thus the sensitivity to EDTA (Figure 4) provides further evidence of an active crocodilian

serum complement system. The specific requirement for either Mg^{2+} or Ca^{2+}, but not both, is a characteristic of complement activity observed in other crocodilian species [21-24].

5. Conclusions

Crocodilians exhibit territorial aggression, and sometimes cause severe injuries. Because these animals live in aqueous environments in which a rich diversity of potentially infectious microbes live, they have put an enormous evolutionary pressure on themselves to develop an immune defense that can prevent serious infection in these wounds. Some adaptive immune components of crocodilians seems less developed relative to other higher vertebrates [34-35]. However, the complement activity is potent compared to other vertebrates. In addition, the crocodilian complement system exhibits a broader spectrum of activities than those of higher vertebrates [20]. This phenomenon has also been observed in teleost fish [36-37]. Thus, it reasonable to postulate that crocodilians followed the evolutionary path of a well-developed innate immunity, potentially at the expense of adaptive immunity. The results of this study show that *C. acutus* and *C. moreletti* exhibit potent, rapid, and temperature-dependent serum complement activities. However, the hemolytic activity displayed by *Ca. crocodilus* is much less active than that of the aforementioned. Several studies have documented the aggressive natures of both *C. acutus* [38] and *C. moreletii* [39-41]. However, *Ca. crocodilus* does not grow as large as the other two species [42], and also is not as aggressive, and these facts might account for the lower serum complement innate immunity of this species.

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