CHARACTERIZATION OF APYRASE-LIKE ACTIVITY IN OCHLEROTATUS TRISERIATUS, OCHLEROTATUS HENDERSONI, AND Aedes aegypti

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Abstract. The saliva of a blood-feeding insect can facilitate the intake of blood and effect the transmission of a pathogen. Apyrase is a salivary enzyme that inhibits the aggregation of platelets by hydrolyzing the activating molecule ADP. Apyrase also hydrolyzes ATP, which is a signal for neutrophil activation. Investigators have reported that malaria vector species in the Anopheles gambiae species complex and the genus Simulium had more apyrase activity than sibling species that were non-vectors. In this study, salivary gland extracta from sibling species Ochlerotatus triseriatus (Say), vector of LaCrosse virus, and the non-vector Oc. hendersoni Cockerell were examined. Apyrase activity was characterized from both species, but no difference in activities was observed. Differences in days to maximal apyrase activity after eclosion and apyrase levels after a blood meal were detected between Oc. triseriatus and Aedes aegypti L. (Rockefeller strain). These differences indicate that Ae. aegypti may be able to feed sooner and more often than Oc. triseriatus.

INTRODUCTION

Arthropod salivary glands are at the center of the mechanism of virus transmission, and the components of arthropod saliva are under intense scrutiny. The varied compounds that facilitate blood feeding are of special interest. The coagulation cascade is activated by injury to a host’s blood vessels, and whereas coagulation may interfere with taking a blood meal, the blood could also later clot within the arthropod’s digestive tract.1 Anticoagulants and antiblet factors have been identified in the saliva of many arthropods.2 In addition, compounds that may facilitate a blood meal such as vasodilators and antihistamines have been identified.3

Clot formation at the site of an injury involves the activity of platelets. Apyrase is a salivary protein responsible for interfering with platelet aggregation in the blood meal host by hydrolyzing the activating molecule ADP. Apyrase also hydrolyzes ATP, which is a signal for neutrophil activation. Apyrase has been detected in mosquitoes,4,5 bed bugs,6 black flies,7 ticks,8,9 fleas,10 and Culicoides.11 Apyrase dephosphorylates ADP and ATP but not AMP.12 ADP is a signal for platelet aggregation, and ATP is a signal for the activation of neutrophils. In addition, apyrases depend on an ion co-factor, either calcium or magnesium.

The more apyrase present in mosquito saliva, the more easily mosquitoes can feed.13 Vectors in the Anopheles gambiae species complex14 and the genus Simulium7 had more apyrase activity than the consubgeneric species that were non-vectors. Apyrase is also a factor in at least one pathogen-vector relationship. A Plasmodium species in Ae. aegypti causes a reduction in apyrase that results in an increased probing time.15 It is during probing that the pathogen is transmitted.16 Therefore, apyrase in mosquitoes is one important component in the taking of a blood meal and the subsequent transmission of a pathogen.

Despite the interest in Ochlerotatus triseriatus as a vector of LaCrosse (LAC) virus and the extensive knowledge of mosquito salivary proteins, only one study has examined a salivary component in Oc. triseriatus,16 and no study has compared saliva of Oc. triseriatus to that of its sibling species and non-vector Oc. hendersoni. In this study, we examined the activity of an apyrase-like enzyme in Oc. triseriatus and Oc. hendersoni and determined the effect of pH on activity. Since apyrase is a necessary enzyme in the taking of a blood meal, this study also describes the activity of apyrase following eclosion and after a blood meal in relation to the behavior and gonotrophic cycle of Oc. triseriatus.

METHODS

Mosquito collection. Ochlerotatus triseriatus and Oc. hendersoni used in these studies were collected in the field as eggs. Larvae in the fourth instar were identified to species. Ochlerotatus triseriatus and Oc. hendersoni adults were reared from eggs collected in Mahomet, Illinois (Champaign County and Peoria, Illinois (Peoria County). In addition, Ae. aegypti (Rockefeller strain) from the University of Arizona served as a reference strain to compare with literature reports on salivary gland morphology and apyrase activity.4 All mosquitoes were raised in 3.8-liter cages with water and 30% dextrose available on cotton wicks. Mosquitoes were not mated. Except for the experiment to determine the effect of age post-eclosion on apyrase activity, mosquitoes dissected for the experiments were five days post-eclosion. Except for the experiment to determine the effect of blood feeding on apyrase activity, mosquitoes used in the experiments were not blood fed.

Salivary gland dissection. Mosquitoes were anesthetized with CO2 and immobilized in 0.5 mL of 0.15 M NaCl in a depression dish. After removal of the head, salivary glands were removed and placed in 20 μL of 0.15 M NaCl. Four glands were pooled, placed in each tube of saline, and then stored at –20°C in 0.15 M NaCl.

Apyrase assay. Salivary glands were prepared from storage with the addition of 20 μL of 0.1% Triton X-100 to give a final concentration of 0.05%.4 The glands were then thawed, vortexed for 60 seconds, and centrifuged for 30 seconds at 9,000 × g.4 The supernatant was used in all assays. Gland extract was diluted with 0.15 M NaCl to a final concentration of 0.01 gland/μL.

A stock solution was diluted to a final concentration of 50 mM Tris, 100 mM NaCl, 5 mM CaCl2, 20 mM β-mercaptoethanol, and 2 mM substrate (Cupp M, unpublished data) and was added to microtiter plate wells. Salivary gland extract was
added to the stock solution to initiate the reaction so that the equivalent of 0.05% of a gland was tested in each microtiter well. A standard curve was prepared using the reaction stock solution and varying amounts of inorganic phosphate. The phosphate was placed in microtiter plate wells and a 0.02 M ammonium molybdate solution was then added to all wells to give a final concentration of 0.002 M to complex with the phosphate and stop the reaction.

The apyrase activity in salivary glands was measured using the equivalent of 0.05% of a gland in place of the phosphate in microtiter plate wells. The reaction was allowed to proceed for 30 minutes and cold water was then added, followed by ammonium molybdate to stop the reaction. The Fiske-Subbarrow reagent, composed of 1-amino-2-naphthol-4-sulfonic acid, sodium bisulfite, and sodium sulfite, was added to all wells and color was allowed to develop for 20 minutes. The activity was determined colorimetrically using a microtiter plate reader with a light filter of 650 nm.

Activity was compared among Oc. triseriatus, Oc. hendersoni, and Ae. aegypti. The mosquito apyrase activity was evaluated for specificity by testing ATP, ADP, and AMP as substrates. Also, the pH of activity was evaluated by varying the pH of the Tris solution in the standard stock solution. Contribution of an ion cofactor was examined by including MgCl₂, CaCl₂, or EDTA, a chelating agent, in the stock solution.

In addition, salivary glands from mosquitoes previously fed on 30% dextrose were dissected to compare apyrase activity in 1–10-day post-eclosion mosquitoes. Apyrase activity and age of mosquito were compared between Oc. triseriatus and Ae. aegypti. Salivary glands from five days post-eclosion, blood-fed females of Oc. triseriatus and Ae. aegypti (0–5 days post blood-feeding) were also dissected to determine the depletion and recovery of apyrase activity. Only females that took a complete blood meal of human blood, as judged by abdominal expansion, were dissected. One unit of activity is defined as the amount of enzyme that released 1 μmol of inorganic phosphate per minute at 37°C.⁴

**Statistical analysis.** One-way analysis of variance (ANOVA) was used to compare apyrase activities within and among the species for all experiments. With the experimental error set at 5%, an *a posteriori* test (Fisher protected least significant difference test) determined if apyrase activities significantly differed.

**RESULTS**

Substrate had a significant effect on apyrase activity for all three species (Table 1). With an experimental error of 5%, activity was significantly higher with ATP as a substrate versus ADP or AMP (F = 25.42, degrees of freedom [df] = 2, P < 0.0001, by one-way ANOVA). *Ochlerotatus triseriatus* and *Oc. hendersoni* gland extracts also had significantly higher activity with ATP as a substrate compared with ADP or AMP (F = 63.84, df = 2, P < 0.0001 for *O. triseriatus* and F = 121.02, df = 2, P < 0.0001 for *Oc. hendersoni*). There was no significant difference among the three species with respect to activity with ATP (F = 1.37, df = 2, P = 0.2840, by one-way ANOVA), ADP (F = 1.62, df = 2, P = 0.2302), or AMP (no variation).

The ion co-factor affected apyrase activity for all three species. The salivary apyrase had significantly less apyrase activity with ATP and Mg⁺² as the provided ion cofactor than with Ca²⁺ (Table 2) (F = 20.82, df = 2, P < 0.0001 for *Ae. aegypti*, F = 38.88, df = 2, P < 0.0001 for *Oc. triseriatus*, and F = 41.29, df = 2, P ≤ 0.0001 for *Oc. hendersoni*). There was no significant difference among the three species and their activities with CaCl₂ (F = 2.54, df = 2, P = 0.1125), MgCl₂ (F = 0.93, df = 2, P = 0.4164), or EDTA (no variation).

Apyrase activity with ATP was measured with Tris solutions at pH 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, and 10.0 (Figure 1). The apyrase activity in *Ae. aegypti* varied significantly with pH (F = 4.94, df = 6, P = 0.0027). With the experimental error set at 5%, apyrase activity was significantly lower between pH 7.0 and 7.5. Activity then increased to a plateau from pH 8.5 to pH 10.0. Similar patterns occurred with *Oc. triseriatus* and *Oc. hendersoni*. *Ochlerotatus triseriatus* apyrase activity varied significantly with pH (F = 5.81, df = 6, P = 0.0011). With the experimental error set at 5%, activity significantly increased from low levels at pH 7.0–8.0 to a plateau of activity from pH 8.5 to pH 10.0. *Ochlerotatus hendersoni* apyrase also significantly differed with pH (F = 8.17, df = 6, P = 0.0001) and increased from lows at pH 7.0–8.0 to a plateau of activity from pH 8.5 to pH 10.0. There was no significant difference among the three species at any pH.

Apyrase activity with ATP significantly differed with age post-eclosion in both *Ae. aegypti* and *Oc. triseriatus* (F = 4.96, df = 9, P = 0.0027 and F = 12.19, df = 9, P < 0.0001, respectively) (Figure 2). With the experimental error set at 5%, *Ae. aegypti* had significantly lower activity on day 1 post-eclosion. Activity increased two days post-eclosion. Apyrase activities on days 2 through 10 post-eclosion were not significantly different. Apyrase levels in *Oc. triseriatus* were at the lowest level one-day post-eclosion and then significantly increased on day 2 and again on day 4. Activities for *Ae. aegypti* were not significantly different from activities for *Oc. triseriatus* on day 1 post-eclosion, day 2, days 4 through 10. *Aedes aegypti* apyrase activity was significantly greater than *Oc. triseriatus* activity 3 days post-eclosion (F = 15.89, df = 1, P = 0.0163).

Apyrase activity levels with ATP varied significantly with time after a complete blood meal in both *Ae. aegypti* (F = 8.38, df = 5, P = 0.0013) and *Oc. triseriatus* (F = 4.77, df = 5, P = 0.0124) (Figure 3). Apyrase levels significantly increased one day post–blood feeding in *Ae. aegypti* and two days post–blood feeding in *Oc. triseriatus*. Apyrase activity in *Ae. aegypti* and *Oc. triseriatus* did not differ significantly immediately after a blood meal, but activity in *Ae. aegypti* was significantly greater than activity in salivary glands from blood-fed *Oc. triseriatus* one (F = 172.91, df = 1, P = 0.0002), two (F = 14.87, df = 1, P = 0.0182), three (F =

### Table 1

<table>
<thead>
<tr>
<th>Species</th>
<th>ATP</th>
<th>ADP</th>
<th>AMP</th>
</tr>
</thead>
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<tr>
<td><em>Aedes aegypti</em></td>
<td>176.03 ± 23.77⁺</td>
<td>115.62 ± 19.47⁺</td>
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<tr>
<td><em>Ochlerotatus triseriatus</em></td>
<td>144.44 ± 9.26⁺</td>
<td>79.56 ± 12.66⁺</td>
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<td><em>Oc. hendersoni</em></td>
<td>144.95 ± 8.08⁺</td>
<td>94.25 ± 8.30⁺</td>
<td>0.0 ± 0.0⁺</td>
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</table>

* A difference in the letters assigned to an activity level indicates a significant difference as tested by one-way analysis of variance (n = six pools of four salivary glands in *Ae. aegypti* [F = 25.43, degrees of freedom [df] = 2, P < 0.0001], *Oc. triseriatus* [F = 63.84, df = 2, P < 0.0001], and *Oc. hendersoni* [F = 121.02, df = 2, P < 0.0001]). There was no significant difference among the *P* species activity with respect to ATP (F = 1.37, df = 2, P = 0.2840), ADP (F = 1.62, df = 2, P = 0.2302), or AMP (no variation).
149.35, df = 1, P = 0.0003), four (F = 9.52, df = 1, P = 0.0368), and five (F = 26.57, df = 1, P = 0.0067) days after a blood meal.

**DISCUSSION**

The dephosphorylation of ATP and ADP, but not AMP, and the requirement of a bivalent ion co-factor by salivary gland extract from *Oc. triseriatus* (Say) and *Oc. hendersoni* (Cockerell) indicate that an apyrase-like enzyme is active within their salivary glands. Most blood feeding arthropods have an apyrase enzyme in their saliva. Apyrase-like activity has been characterized in *Ae. aegypti*, *Anopheles* species, *Simulium* species, *Cimex* species, fleas, and ticks.4-6,8,10,14 Among the apyrase enzymes, there are differences in substrate preference, ion co-factor preference, and the pH of optimal activity.

The apyrase from *Ae. aegypti*, *Oc. triseriatus* (Say), and *Oc. hendersoni* (Cockerell) had greater activity with ATP than with ADP (Table 1), as did the apyrase from *Ae. aegypti*4 and *Ae. albopictus* (Skuse).5 That there was no significant difference in activity levels among the three mosquito species in this study with either ATP or ADP indicates that *Oc. triseriatus* (Say) and *Oc. hendersoni* (Cockerell) have an apyrase with as much *in vitro* activity as *Ae. aegypti* (Rockefeller). Increased activity with ATP or ADP is not always consistent among closely related species. Cupp and others7 characterized the apyrase of *Simulium* species and found that both anthropophilic species (*S. metallicum* and *S. ochraceum*) had an apyrase with greater activity with ADP than ATP in contrast to other *Simulium* species tested. A preference for ADP over ATP was also found in the apyrase of the tick *Ornithodoros moubata* (Murray).8 The preference of one substrate over another can be dependent on pH as with *C. lectularius* (L.)6 or dependent on the ion co-factor as with *O. savignyi* (Audouin).9

As is the case with *Ae. aegypti* (Rockefeller), the apyrase from *Oc. triseriatus* (Say) and *Oc. hendersoni* (Cockerell) had significantly more activity with ATP and calcium as the ion co-factor than with magnesium (Table 2). *Aedes albopictus* (Skuse), some *Anopheles* species, and *C. lectularius* also have apyrase enzymes that have more activity with calcium what with magnesium.5,6,14 Some arthropod apyrases have equal activity with calcium or magnesium as a co-factor including
the flea apyrase and the apyrase characterized from Culicoides variipennis (Sonorensis). Ion co-factor preference in *O. moubata* depends on the substrate.

The optimal pH ranges for the apyrase of *Oc. triseriatus* (Say) and *Oc. hendersoni* (Cockerell) were 8.5–10.0, whereas the apyrase of *Ae. aegypti* reached its highest activity at the pH range of 8.0–10.0 when ATP was the substrate (Figure 1). Other investigators have found that the apyrase of *Ae. aegypti* peaked in activity at pH 9.0. All arthropod apyropases characterized have an optimal pH between 7.0 and 9.0, with *Ornithodoros* species and fleas found at the low end of the range and *Simulium* and *Aedes* species found at the high end of the range. The pH of human blood is between 7.35 and 7.45, a pH at which mosquito apyrase has less activity, although the activity is sufficient for feeding. The actual pH of the environment in which apyrase operates may not be the pH of blood alone. During feeding, endothelial cells lyse, platelets release granule contents, and neutrophils release inflammatory mediators that may alter the pH of the environment near the site of blood feeding.

In *Ae. albopictus* (Skuse), the synthesis of blood-feeding enzymes is delayed until after emergence from the pupal cuticle. In this study, the apyrase levels in *Ae. aegypti* reached a stable level within one day after eclosion, whereas the apyrase levels in *Oc. triseriatus* (Say) reached a stable level four days after eclosion (Figure 2). It was found that *Ae. aegypti* had a maximum, steady level of apyrase activity two days after eclosion, in contrast to what was found in *Ae. albopictus* (Skuse), which did not reach a stable level until three days after eclosion. *Aedes aegypti* may be more efficient at blood feeding at an earlier age than *Oc. triseriatus* (Say), although a minimal level of apyrase activity in *Oc. triseriatus* (Say) to ensure a successful blood meal has not been determined.

Blood feeding itself depletes salivary gland enzymes. In fact, after a complete blood meal, apyrase activity in *Ae. aegypti* and *Oc. triseriatus* (Say) decreased approximately 75% (Figure 3). After a blood meal, apyrase activity decreased 75% in *Culicoides, 50% in Cimex* and 50% in flea species. *Aedes aegypti* apyrase activity recovered to pre-feeding levels by three days after a meal, whereas *Oc. triseriatus* (Say) apyrase activity did not recover to pre-feeding levels even after five days. At five days after a blood meal, *Oc. triseriatus* (Say) had recovered to approximately 40% of its pre-meal apyrase activity. The gonotrophic cycle of *Ae. aegypti* is shorter than the gonotrophic cycle of *Oc. triseriatus* (Say) by one day in our laboratory. Therefore, *Ae. aegypti* may need to replenish its salivary gland enzymes, including apyrase, faster than *Oc. triseriatus* (Say) to feed again. *Aedes aegypti* also takes incomplete blood meals and feeds more than once in a gonotrophic cycle, and therefore may either sequester salivary enzymes after a blood meal or immediately initiate manufacture of more enzymes.

The inability of *Ochlerotatus triseriatus* (Say) to replenish its apyrase reserves might be an important factor in the LAC virus cycle. The rapid recovery of apyrase activity in *Ae. aegypti* after a blood meal could contribute to its efficient transmission of arboviruses. When *Oc. triseriatus* (Say) is infected with LAC virus, 18–21 days are required for the ingested virus to be orally transmitted (extrinsic incubation time). If *Oc. triseriatus* (Say) has optimal feeding ability only with its first blood meal, it may be an inefficient transmitter of LAC virus. Indeed, transovarial transmission, or infection of the egg by a female, may be essential to the maintenance of LAC virus in a disease-endemic area. Alternatively, lowered apyrase activity may hinder blood feeding, increasing probing time and hosts, therefore enabling *Oc. triseriatus* (Say) to transmit the virus to more hosts.

In summary, because there were no significant differences between apyrase activity of *Oc. triseriatus* (Say) or *Oc. hendersoni* (Cockerell) and *Ae. aegypti* with respect to substrate preference, ion co-factor requirement, or optimal pH, it is logical to assume that there was no significant difference between the more closely related *Oc. triseriatus* (Say) and *Oc. hendersoni* (Cockerell). Although hematophagous arthropods such as ticks, bed bugs, fleas, and flies do not independently evolved components necessary to blood feed at the family level, the evolution of blood feeding in the Culicidae occurred at the subfamily level. The apyrase found in *Aedes* and *Ochlerotatus* mosquitoes probably developed in an ancestor to the genus from a 5'-nucleotidase gene. This occurred many times in other taxa as shown by the number of blood feeding arthropods that have apyrase activity. The 5'-nucleotidases are found on the surface of cells and act to remove phosphate groups from nucleotides so that they can be transported across the cell membrane.

Although more research into the salivary gland escape barrier to LAC virus in *Oc. hendersoni* (Cockerell) is needed, further characterization of salivary gland enzymes of these sibling species is an important component of that work. It has been documented that LAC virus-infected *Oc. triseriatus* (Say) probe longer for a blood meal than non-infected *Oc. triseriatus* (Say). It has also been suggested that LAC virus may inhibit or disrupt the secretion of salivary gland proteins, including apyrase, necessary for a successful blood meal. Further research into the apyrase activity in salivary glands of infected and non-infected *Oc. triseriatus* (Say) could determine if LAC virus can affect the physiologic function of the salivary glands that could in turn affect mosquito blood feeding behavior.

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**Table 2**

<table>
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<th>Species</th>
<th>CaCl₂</th>
<th>MgCl₂</th>
<th>EDTA and no ion</th>
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<td><em>Aedes aegypti</em></td>
<td>189.03 ± 24.61*</td>
<td>127.40 ± 27.14°</td>
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<td><em>Ochlerotatus triseriatus</em></td>
<td>143.44 ± 12.27°</td>
<td>103.38 ± 16.49°</td>
<td>0.0 ± 0.0°</td>
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<tr>
<td><em>Oc. hendersoni</em></td>
<td>203.81 ± 20.51*</td>
<td>144.67 ± 19.44°</td>
<td>0.0 ± 0.0°</td>
</tr>
</tbody>
</table>

* A difference in the letters assigned to an activity level indicates a significant difference as tested by one-way analysis of variance in *Ae. aegypti* (F = 20.82, degrees of freedom [df] = 2, P < 0.0001), *Oc. triseriatus* (F = 38.88, df = 2, P < 0.0001), and *Oc. hendersoni* (F = 41.29, df = 2, P < 0.0001). There was no significant difference among the three species and their activities with CaCl₂ (F = 2.54, df = 2, P = 0.1125), MgCl₂ (F = 0.93, df = 2, P = 0.4164), or EDTA (no variation).
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