The Presence of 20-Hydroxyecdysone Acid and Ecdysone Acid in Eggs of the Giant Freshwater Prawn *Macrobrachium rosenbergii*

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High polarity products (HPP) are observed to increase dramatically during the embryogenesis of *Macrobrachium rosenbergii*; in this investigation, their identity was examined employing high performance liquid chromatography (HPLC) and radioimmunoassay (RIA). 20-Hydroxyecdysone, inokosterone, and ecdysone accounted for up to 25% of HPP immunoreactivity, and 20,26-hydroxyecdysone comprised less than 5%. Therefore, as development proceeds, the acidic 26-oic derivatives become the dominant ecdysteroid species. It has been postulated on the basis of this investigation that 26-oic derivative formation is the major route of ecdysteroid inactivation; concomitantly increasing 20-hydroxyecdysone and 20-hydroxyecdysonic acid titres and observed low quantities of 20,26-dihydroxyecdysone suggest the rapid conversion of active hormones to 26-oic derivatives. Conjugate formation may also be a route of ecdysteroid inactivation. These pathways may be important in controlling the titres of active ecdysteroids within the egg, and are possibly manifested by the accumulation of metabolites during embryogenesis.

**Key words:** prawns, ecdysteroid acids, ecdysteroid conjugates, embryogenesis, *Macrobrachium rosenbergii*

Among the ecdysteroids known to have molting activity in Crustacea are 20-hydroxyecdysone in general and ponasterone A in some species of crab. In addition, highly polar ecdysteroids have been demonstrated to exist in the tissues of a number of crustacean species, but whether these “high polarity products (HPP)” possess any biological activity is yet unknown. In insects, highly polar ecdysteroids are a partial representation of the inactivation products of ecdysteroid metabolism. There is much evidence that pathways in which C-26 hydroxylation leads to conversion to 20-hydroxyecdysonic and ecdysone acid, and C-3, C-22 conjugation to the formation of sulfate and phosphate esters in Insecta1,2) exist in Crustacea as well.

We have previously investigated ecdysteroid fluctuations during embryogenesis in the eggs of the prawn, *Macrobrachium rosenbergii* revealing the presence of significant amounts of immunoreactive HPP, particularly towards the end of the embryonic developmental period.3) HPP has been detected in the eggs of another prawn species *Palaemon serratus* and in the crab *Carcinus maenus*, and has been shown, via hydrolytic means, to consist mainly of conjugated 20-hydroxyecdysone in *P. serratus*4) or of ponasterone A in *C. maenus*.5) In the land crab *Gecarcinus lateralis*, 20,26-dihydroxyecdysone has been detected as a metabolic product of ecdysone,6) and in *C. maenus*, metabolism to ecdysone acids has been demonstrated in adult individuals.7)

In a study by Young et al.8) the late-stage eggs of *M. rosenbergii* were reported to contain high levels of hydrolyzable polar conjugates; only minor amounts of ecdysteroid acids and other non-hydrolyzable species were reported to be present. Our previous work has indicated similarities with the above research group regarding overall ecdysteroid concentrations; however, HPP obtained from *M. rosenbergii* eggs in this laboratory was only partially hydrolyzable to free 20-hydroxyecdysone, and ecdysone,5) while over 70% of HPP immunoreactivity was resistant to enzymatic hydrolysis. As our preliminary work on the nature of HPP during embryogenesis in *M. rosenbergii*, which indicated apparently contrasting results with those of the above group, the purpose of the present investigation was to clarify the nature of the highly polar ecdysteroids found in the *M. rosenbergii* employed in this laboratory. HPP immunoreactive fractions obtained from newly-laid, mid-embryogenic and pre-hatchout eggs were subject to analyses.

**Materials and Methods**

**Sampling**

The giant freshwater prawn *Macrobrachium rosenbergii* exhibits and 18-day embryonic period under conditions of 28°C, during which the developing egg mass is brooded via attachment to the reproductive setae. Female spawners were maintained in 361 glass aquaria; each aquarium was equipped with a filtration and aeration unit and divided by a partition into two compartments each containing one prawn. Spawners were maintained *ad libitum* on kuruma prawn pellets. Sampling was conducted on newly-spawned eggs (Days 1-4, eggs vivid orange), metamorphic eggs (Days 10-12, some disappearance of yolk, compound eye

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apparent), and pre-hatchout eggs (Days 15–17, eggs dark gray-brown). Ten to 25 grams early, metamorphic, or pre-hatchout eggs were collected and pooled from at least six individuals for each stage. These were quick-frozen at $-80^\circ$C, and stored at $-30^\circ$C until their use in ecdysteroid extraction procedures.

**Extraction of Ecdysteroids**

Eggs (10–25 g) were homogenized in the appropriate quantity of methanol (from 50–80 ml). Subsequent extraction was performed as previously detailed. Briefly, homogenization was followed by partitioning between chloroform/distilled water and n-hexane/distilled water. Extracts (in water) were processed by Sep-Pak C$_{18}$ purification prior to high performance liquid chromatography (HPLC) injection.

**High Performance Liquid Chromatography**

Ecdysteroids were separated by reverse-phase HPLC (ODS-SOTM, 4.6 x 250 mm, Tosoh Co., Ltd.). A methanol/acetonitrile/water system (system I) was employed to first obtain HPP immunoreactive fractions, and to subsequently analyze ecdysteroids released from the conjugated form after hydrolysis with Helix pomatia preparation. In system I, solvent A (10:15:75) was eluted for 23 min, switching to a linear gradient reaching solvent B (50:5:45) after 32 additional minutes (flow rate 1.0 ml/min, fractions collected at 30 s intervals). This system provided good resolution of 20-hydroxyecdysone and the two isomeric forms of inokosterone, with however, virtually all HPP remaining unresolved at the solvent front. In preparatory analysis (determination of Helix quantity as detailed below), a system of 50% methanol (system II) was employed, which however did not provide resolution of inokosterone from 20-hydroxyecdysone.

A system from Lafont et al. and Modde et al. employing a gradient of 8–40% acetonitrile (60 min) in 20 mM Tris/HClO$_4$, pH 7.5 (flow rate 0.8 ml/min, 1 fraction per 30 s) (system III) provided the resolution of HPP into several peaks, and therefore was utilized in the detection of the ecdysenoic acids, and 20,26-dihydroxyecdysone. Tritiated 20-hydroxyecdysenoic acid, ecdysenoic acid, and 20,26-dihydroxyecdysone, employed here as reference compounds, were the generous gift of Dr. R. Lafont, Ecole Normale Superieure, France.

**Radioimmunoassay**

A radioimmunoassay (RIA) for crustacean ecdysteroids as described previously was employed in this investigation. Briefly, HPLC fractions, or ecdysone standards (2-fold serial dilution, 78–40,000 pg/ml) were incubated with a rabbit anti-20-hydroxyecdysone for 24 hours. This was followed by the addition of tritium-labeled ecdysone, and secondly goat anti-rabbit IgG serum, punctuated by 24 h incubation periods. Lastly, aliquots were centrifuged (3,200 rpm, 30 min, 4°C); the supernatant was discarded by aspiration. Liquiflour scintillator/Triton X (2 : 1) was added to aliquots, and immunoreactivity in HPLC fractions was determined as 20-hydroxyecdysone equivalents. Dr. Nagata, The University of Tokyo, kindly provided the 20-hydroxyecdysone antiserum. This antiserum shows specificity for the ecdysteroid kernel such that side chain modifications do not have much effect on cross-reactivity; ecdysteroid determinations are given in terms of 20-hydroxyecdysone equivalents.

**Enzymatic Hydrolysis of Polar Conjugates**

Firstly, the appropriate quantity of enzyme to be used in subsequent hydrolysis experiments was determined by incubating 45 ng HPP from pre-hatchout eggs in 1.5 ml 50 mM acetate buffer (pH 5.3, 37°C, 24 h, with shaking) in the presence of 0, 25, 50, 100, and 200 mg semipurified sulfatase from the snail, Helix pomatia (Sigma, H-1). The reaction was terminated by the addition of a 5-fold volume of methanol, and the reaction tubes were centrifuged at 10,000 × g to remove particulate matter. The pellet was extracted with 1 ml methanol, and the supernatants were combined and processed for rechromatography on the 50% methanol solvent system. RIA was performed to determine the percentage of HPP immunoreactivity corresponding to conjugates released as free ecdysteroids. Incubation in a buffer under identical conditions without enzyme served as a control.

In subsequent analyses, HPP fractions from early, metamorphic, and pre-hatchout eggs were subject to enzymatic hydrolysis by incubation with 50 mg of enzyme under the conditions described above. Rechromatography was done...
Table 1. Determination of Helix enzyme quantity for use in hydrolysis experimentation

<table>
<thead>
<tr>
<th>Percentage immunoreactivity</th>
<th>0</th>
<th>25</th>
<th>50</th>
<th>100</th>
<th>200*</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPP</td>
<td>99.3</td>
<td>80.1</td>
<td>81.4</td>
<td>81.6</td>
<td>81.4</td>
</tr>
<tr>
<td>20E/Ino</td>
<td>0.5</td>
<td>14.5</td>
<td>13.5</td>
<td>13.3</td>
<td>12.4</td>
</tr>
<tr>
<td>E</td>
<td>0.0</td>
<td>2.4</td>
<td>2.5</td>
<td>2.4</td>
<td>2.5</td>
</tr>
<tr>
<td>LPP</td>
<td>0.2</td>
<td>2.9</td>
<td>2.5</td>
<td>2.6</td>
<td>1.6</td>
</tr>
</tbody>
</table>

* Quantity enzyme (mg).

Percent of total immunoreactivity of high-polarity products (HPP) released as free ecdysteroid, and percent unhydrolyzable HPP remaining after incubation in the presence of 0, 25, 50, 100, and 200 mg enzyme. Other abbreviations are as follows: 20-hydroxyecdysone (20E); inokosterone (Ino); ecdysone (E); low-polarity products (LPP).

on the methanol/water/acetonitrile system (system III).

Results

Isolation of HPP Immunoreactive Fractions

Ecdysteroids were first separated by HPLC with methanol/water/acetonitrile (system I). HPP eluted as two peaks: HPP1, eluting at a retention time of 5 min, containing over 99% of total radioactivity of immunoreactive fractions more polar than 20-hydroxyecdysone; and HPP2, which was present as only a minor peak, eluting at 10 min. HPP2 could not be obtained in a sufficient enough amount to carry out hydrolysis experiments; therefore, only HPP1 fractions could be used in the subsequent hydrolysis experiments. HPLC elution patterns are shown in Fig. 1; the elution positions of 20-hydroxyecdysone, ecdysone, and the 25-R and 25-S isomers of inokosterone, as well as of other ecdysteroid species are indicated.

Determination of Helix Quantity for Enzymatic Hydrolysis

As indicated in Table 1, maximum hydrolysis of HPP from pre-hatchout eggs was achieved by the addition of 25 mg Helix preparation; in the control incubation (0 mg enzyme), the HPP fractions remained unaffected. Fifty mg Helix preparation was subsequently chosen as the quantity to be used in subsequent analysis of HPP from all egg stages.

Analysis of High-Polar Conjugates

HPP1 fractions isolated from early, metamorphic, and pre-hatchout eggs were subjected to Helix (50 mg) digestion, and free ecdysteroids released from conjugates were examined by HPLC-RIA (system I). HPP1 resistant to enzymatic hydrolysis consisted of, respectively, 72.6, 85.8, and 75.1% of total HPP1 immunoreactivity in early, metamorphic, and pre-hatchout eggs (Fig. 2). 20-Hydroxyecdysone was the major hydrolyzed conjugate in early and metamorphic eggs, followed by a less significant peak of inokosterone. In pre-hatchout eggs, the major conjugate corresponded to a peak exhibiting the same retention time as inokosterone. Ecdysone conjugates comprised 1-3% of total immunoreactivity. Low-polarity products (LPP) conjugates with retention times similar to 2-deoxyecdysone and ponasterone A were detectable in trace amounts in metamorphic and pre-hatchout eggs, but undetectable during early embryogenesis. Additionally seen in the middle and latter stages were immunoreactive fractions of a middle polarity. From 2-3% of total immunoreactivity at all stages corresponded to HPP2. This led to the speculation that HPP2 was free 20,26-dihydroxyecdysone, present in the conjugated form as well. Relative percentages of conjugates present (as 20-hydroxyecdysone equiv.) during embryogenesis are summarized in Fig. 2.

Identity of Non-Hydrolyzable High-Polarity Ecdysteroids

HPP1 fractions resistant to enzymatic hydrolysis were rechromatographed with the acetonitrile/tris buffer system (system III). As shown representatively for pre-hatchout eggs (Fig. 3, top), two immunoreactive peaks at 20 min (fr. 40), and 28 min (fr. 56) were detected; these had retention times identical to those of [3H]-20-hydroxyecdysone acid and [3H]-ecdysone acid. HPP2 exhibited the same retention time as authentic [3H]-20,26-dihydroxyecdysone (Fig. 3, bottom); its identity was therefore confirmed as such.

In addition, whole extracts (those not subject to any
Fig. 3. (Top) Rechromatography of unhydrolyzable HPP1 on acetonitrile/tris buffer solvent system.
Two immunoreactive peaks at 20 min (fr. #40) and 28 min (fr. #56) exhibited retention times identical to those of authentic 20-hydroxyecdysoneic acid (20Eoic) and ecdysoneic acid (Eoic).
(Bottom) Rechromatography of HPP2 on acetonitrile/tris buffer solvent system. All observed immunoreactivity showed a retention time (31.5 min, fr. #63) identical to that of 20,26-dihydroxyecdysone (20,26E), thus confirming its identity as such. Patterns are shown representatively for pre-hatchout eggs.

Fig. 4. Elution of whole egg extracts and tritiated reference compounds on acetonitrile/tris buffer solvent system as shown for pre-hatchout eggs (100 ng).
Major immunoreactive peaks corresponded to 20-hydroxyecdysoneic acid (20Eoic) and ecdysoneic acid (Eoic). Other ecdysteroids detected were 20,26-dihydroxyecdysone (20,26E); 2-hydroxyecdysone (20E); inokosterone (Ino); other abbreviations indicate retention times of authentic standards for makisterone A (MaA), ecdysone (E), 2-deoxy-20-hydroxyecdysone (2420E), ponasterone A (PoA), and 2-deoxyecdysone (2dE). Peaks C1, C2, and C3 of immunoreactive material eluting prior to 20Eoic, between 20Eoic and Eoic, and subsequent to Eoic were considered to correspond to conjugated ecdysteroid species.

Discussion

In the giant freshwater prawn *Macrobrachium rosenbergii*, we have observed ecdysteroids to be present in newly-laid eggs at concentrations of 15–20 ng/g; these levels decrease slightly until metamorphosis from the nauplius stage to the zoal stage, and subsequently increase rapidly, with levels of nearly 500 ng/g at hatchout.3) 20-Hydroxyecdysone and HPP are the major ecdysteroid groups: immediately after spawning, this ratio is 1:1, but by metamorphosis, no 20-hydroxyecdysone is detectable. After the appearance of the compound eye, both 20-hydroxyecdysone and HPP increase rapidly; however, HPP reaches levels 5-fold greater than 20-hydroxyecdysone about 2 days prior to hatchout. Thereafter, 20-hydroxyecdysone concentrations decrease, while HPP continues to increase to comprise 99% of the total ecdysteroid immunoreactivity.3)

Through the present investigation, it has been revealed that the majority of HPP is comprised of unhydrolyzable compounds, and that these correspond to 20-hydroxyecdysoneic acid and ecdysoneic acid. Conjugated ecdysteroids are present to some extent, but are much less abundant than the ecdysoneic acids. Lesser amounts of 20,26-dihydroxyecdysone are also detectable. This is the first report to our knowledge in which 20-hydroxyecdysoneic acid and ecdysoneic acid have been detected as major ecdysteroid species during the embryogenesis of a crustacean species. High polarity products have been reported in the eggs of the prawn *Palaemon serratus*,4) and in the crabs *Carcinus maenus*5) and *Achthonyx hululatus*10) However, in contrast to *M. rosenbergii*, HPP in these species consisted almost entirely of conjugated ecdysteroids. In *C. maenus*, HPP hydrolysis yielded primarily ponasterone A, and some 20-hydroxyecdysone.5) Hemolymph HPP in the mysid crustacean, *Siriella armata*, is completely hydrolyzable to 20-hydroxyecdysone and ecdysone.11) Interestingly, in *P. serratus*, even after enzymatic digestion, minor amounts of HPP were still present.4) C-26 hydroxylation to 20,26-dihydroxyecdysone6) and further conversion to ecdysoneic acids7) have been demonstrated as a means of inactivation of ecdysteroid in the adult Crustacea. In *C. maenus*, ponasterone A, the principal ecdysteroid of this species, undergoes C-25 hydroxylation and conversion to 25-deoxy-20-hydroxyecdysoneic acid, or subsequent C-26 hydroxylation and conversion to 20-hydroxyecdysoneic acid; these, along with conjugates, are the excretory products of various tissues.3) In the crab *Gecarcinus lateralis*, 20-hydroxyecdysone and treatment) were run for reference with the acetonitrile/tris buffer system (system III). As shown for pre-hatchout eggs in Fig. 4, immunoreactive peaks, C1, C2, and C3, eluting prior to 20-hydroxyecdysoneic acid, between 20-hydroxyecdysoneic acid and ecdysoneic acid, and prior to 20-hydroxyecdysone were also detected. These were presumed to correspond to the conjugated forms of various free ecdysteroids, but were not present in sufficient quantities to be individually hydrolyzed. In general, the ecdysoneic acids comprised 70–75% of total HPP immunoreactivity; 20,26-dihydroxyecdysone was about 5%, and remaining high polar immunoreactivity (due to conjugates) was 20–25%.

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20,26-dihydroxyecdysone have been reported as metabolites of ecdysone; in addition, a highly-polar substance resistant to enzymatic hydrolysis has been detected, but not further identified. In the lobster, Homarus americanus, significant amounts of non-hydrolyzable HPP are present in hemolymph in addition to an immunoreactive fraction assumed to be 20,26-dihydroxyecdysone. Young et al. have found that HPP in prehatchout eggs is comprised of mostly conjugated ecdysteroids, principally 2,3-diaceetylecdysone 22-phosphate, with only minor amounts of the ecdysteroid acids; reasons for discrepancies between their study and ours are unclear but are perhaps related to the strain of animal used or the conditions of rearing. We have found large amounts of ecdysteroid acids similar in developing ovaries.

In insects, 20-hydroxyecdysone acid and ecdysone are the metabolic products of 20-hydroxyecdysone and ecdysone. Pathways for conversion to these substances exist in Pieris brassicae (Lepidoptera), Locusta migratoria, (Orthoptera), and Calliphora vicina pupae and larvae, and in L. migratoria adults. These pathways, and other metabolic routes leading to acetylation and phosphate ester formation are stated to be similar to those occurring in L. migratoria embryos. 20-Hydroxyecdysone acid and ecdysone acid have additionally been detected in the eggs of S. gregaria.

However, numerous conjugate species exist in insect eggs; the conjugating moieties as well as the ecdysteroid genin change during the course of embryonic development and metabolism. In Locusta migratoria, the 22-adenosinomono-phosphoric ester of 2-deoxyecdysone (of maternal origin) exists in newly-laid eggs, and the 22-phosphate esters of 2-deoxy-20-hydroxyecdysone, 20-hydroxyecdysone, and 20-hydroxyecdysone acetate exist in developing eggs. The latter group constitute products of the conjugative route of ecdysteroid metabolism. Numerous 22-phosphate esters, in addition to the 26-oic acids, are present in newly-laid eggs of S. gregaria, and 26-hydroxyecdysone 2-phosphate has been discovered in the eggs and ovaries of Manduca sexta. Regarding the role of these conjugates, it is generally accepted that some, particularly those of maternal origin, are a reserve of free ecdysteroid, and are hydrolyzed for purposes of embryonic molting, and others are inactivation products. Conjugated ecdysteroids themselves, probably do not have any actual function.

Regarding Crustacea, the metabolism of ecdysteroids probably proceeds along lines similar to Insecta. In M. rosenbergii, titres of 20-hydroxyecdysone gradually decrease after oviposition and become undetectable at the end of the nauplius stage. 20-Hydroxyecdysone acid and ecdysone are the predominant ecdysteroids. It has been postulated here that 20-hydroxyecdysone and possibly ecdysone are utilized during this early developmental period, at the end of which the compound eye, embryonic heart, and appendage-like structures become discernible. It is postulated that 20-hydroxyecdysone and ecdysone are metabolized to 20-hydroxyecdysone acid and ecdysone acid; the presence of 20,26-dihydroxyecdysone appears to reflect this pathway. As in P. serratus, in which the Y-organ becomes capable of ecdysone synthesis midway through embryogenesis, M. rosenbergii ecdysteroid titres begin to increase dramatically, culminating in hatchout levels 20-fold greater than those of early embryogenesis. However, while 20-hydroxyecdysone and the 26-oic acids increase concomittantly, the ratio of 26-oic acids to 20-hydroxyecdysone increases from 2:1 to 5:1; subsequently, 20-hydroxyecdysone titres decline, and 20-hydroxyecdysone acid and ecdysone acid together with conjugates and 20,26-dihydroxyecdysone approach 99% of total immunoreactivity.

Because 20-hydroxyecdysone acid and ecdysone acid are predominant over 20-hydroxyecdysone to this extent, it is necessary to consider that these highly polar ecdysteroids may have a role in embryogenesis. Rather, the authors postulate that the rapid formation of ecdysone acid during embryogenesis is highly important in controlling titres of active steroids. Ecdysone acid formation appears to be irreversible, and similar acidification of the C-26 carbon appears to be a deactivation step in vertebrates as well. In the closed environment of the egg, the acidity of the 26-oic derivatives may facilitate their excretion as metabolites from the embryo, to be held within the egg capsule until hatchout. Additional investigation may reveal further similarities with insects regarding ecdysteroid metabolism.

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References


