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Short Communication

Ecdysone 20-monooxygenase activity during embryogenesis of the tobacco hornworm, *Manduca sexta*

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ABSTRACT

The conversion of ecdysone (E) to 20-hydroxyecdysone (20E), the active arthropod molting hormone, via the cytochrome P450-dependent enzyme, ecdysone 20-monooxygenase (E20M), initiates the metabolic and physiological events that occur during molting and metamorphosis throughout insect postembryonic development. The present study used an *in vitro* radioassay to examine E20M enzyme activity throughout the 120-hour period of embryogenesis in the tobacco hornworm, *Manduca sexta*. E20M enzyme activity increases significantly at two points during embryogenesis, at 12 hours and 72 hours into embryogenesis. At 72 hours, E20M activity achieves its maximal level of activity observed during embryogenesis, subsequently decreasing to basal levels for the rest of the period. Interestingly, the 72-hour peak in enzyme activity corresponds to the time at which the accumulation of the 20-hydroxylated ecdysteroid observed in embryogenesis is at its peak, and this is the period between the secretion of the first and second embryonic cuticle.

KEYWORDS: ecdysone 20-monooxygenase, ecdysone, embryogenesis, *Manduca sexta*, steroid hydroxylase, ecdysteroids, 20-hydroxyecdysone

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ABBREVIATIONS

ecdyone 20-monooxygenase, E20M; ecdysone, E; 20-hydroxyecdysone, 20E; 26-hydroxyecdysone, 26E; 20,26-dihydroxyecdysone, 20,26E

INTRODUCTION

During insect postembryonic development the conversion of the arthropod molting pre-hormone, ecdysone (E), to its physiologically more active metabolite, 20-hydroxyecdysone (20E), is carried out by a cytochrome P450-dependent steroid hydroxylase, viz., ecdysone 20-monooxygenase (E20M; E.C. 1.14.99.22) [1, 2]. In both *Drosophila melanogaster* and *Manduca sexta*, this enzyme is transcribed by the mRNA *shade* [2, 3]. The activity and gene expression of this enzyme has been shown to vary in a tissue and stage specific fashion throughout the postembryonic development of several insects including *M. sexta* [2-5]. These variations in enzyme activity inherently control the proportion of circulating E and 20E during postembryonic development. This proportional control is known to be vital to the regulation of the biochemical and physiological events that are elicited during both metamorphic and non-metamorphic molts in insects [6-8].

Several lines of evidence indicate that ecdysteroids play key roles during insect embryogenesis, i.e., regulation of cuticle deposition, epidermal line elongation, vitellogenesis, etc. [7-10]. A number of studies also revealed that the ecdysteroids available during embryogenesis are maternally

derived and that these ecdysteroids are packaged into embryos mostly in the form of polar conjugates (i.e., as phosphate, lipid, and amide conjugates) [9-15]. Once embryonic development begins, the conjugated ecdysteroids are hydrolyzed, thereby causing the level of free ecdysteroids to increase. The resulting accumulated ecdysteroids are then further metabolized to a 20-hydroxylated "active" form, as in the E to 20E sequence observed during postembryonic development [1, 16, 17]. The 20-hydroxylated product, which in the case of *M. sexta* is 20,26-dihydroxyecdysone (20,26E), is then used to control the development of the embryo [11, 13, 16, 18].

In several insect species such as *Bombyx mori* [19-22], *Aedes aegypi* [9], *Oncopeltus fasciatus* [23], *Blaberus craniifer* [24], and *Nauphoeta cinerea* [25], clear correlations between molting events in the embryo and peaks in molting hormone bioactivity or ecdysteroid immunoreactivity have been noted. These studies suggest that the activation of ecdysteroids is necessary for the progression of insect embryonic development to proceed properly.

In *M. sexta*, changes in E20M enzyme activity and expression have been extensively studied in most developmentally important periods i.e., during larval to larval, larval to pupal, and pupal to adult transitions [2, 4]. Yet the changes in enzyme activity during embryogenesis have been largely unexamined. Therefore, the present study evaluated the changes in E20M enzyme activity and related these variations in enzyme activity to previously established changes in ecdysteroid content as well as physiological events that occur during embryogenesis of *M. sexta*.

MATERIALS AND METHODS

Animals

Eggs (containing embryos) of the tobacco hornworm, *Manduca sexta*, were collected from a breeding stock of adults maintained under non-diapausing conditions (16L:8D, 25°C, 60% relative humidity). Eggs were obtained by placing a tomato or pepper plant free of eggs into a breeding chamber with the breeding stock of male and female adult *M. sexta* 1 hour prior to the beginning of the light phase. The plant remained in the breeding chamber for 2 hours, and then was immediately removed from the breeding chamber and the eggs collected manually from the plant. After collection, eggs were stored under the non-diapausing conditions noted above. Eggs collected over the 2 hour time period (0-2 hour) were considered to have been laid at 1 ± 1 hour.

Ecdysteroids and chemicals

[23, 24 ^{3}H]-E (stocks of 45 and 70 Ci/mmol), which served as the substrate for the radioassays, was purchased from New England Nuclear, Boston, MA. Ecdysteroid standards were purchased from Fluka Chemical Corp., Ronkonkoma, NY. NADPH and scintillation fluid (Ultima Gold) were purchased from Sigma Chemical Co., St. Louis, MO. Salts and solvents were purchased from Fischer Scientific Co., Cleveland, Ohio.

Tissue preparation

Whole *M. sexta* embryos (in the form of intact eggs) were homogenized in a sodium phosphate buffer (50 mM, pH 7.5, containing 250 mM sucrose) at a concentration of 1 egg per 10 µl of homogenization buffer. Homogenizations were carried out using a Potter-Elvejhem tissue grinder with a motor driven Teflon pestle (275 rpm, 25 strokes, 0-4°C).

E20M assay

An established radioassay was used to measure E20M activity in *M. sexta* embryos [4, 26]. E20M activity was assayed at 12 hour intervals starting from 1 ± 1 hour past egg deposition (considered the start of embryogenesis), and ending with 120 ± 1 hours past egg deposition. For the assays, aliquots of 0.05 ml (containing 5 egg equivalents) of homogenate were added to 0.05 ml aliquots of 0.05 M sodium phosphate buffer, pH 7.5, containing 1000 pg [23, 24 $^{-3}$H] E, and NADPH (1.6 mM assay concentration). Assays were incubated for 2 hours at 30°C with constant agitation. All assays were run in duplicate with zero-time controls. E20M activity for each time point in the 120-hour period was the mean of at least 6 and as many as 20 determinations in duplicate. Following incubation, assays were terminated via addition of 1.5 ml of ethanol. The assay tubes were centrifuged at 8,000xg for 10 minutes to remove precipitated protein. After centrifugation, 0.15 ml aliquots of assay supernatant were added to 200 µg each of cold carrier E and 20E, and the mixture evaporated to

dryness. Residual assay supernatant and cold carrier were then resuspended in 50 µl of methanol, and streaked onto thin layer chromatography (TLC) plates (0.25 mm silica gel 60, F-254; E. Merck Darmstadt, Germany). The plates were developed in a solvent system of chloroform: 95% ethanol (4:1, v/v), and the E and 20E bands visualized under short wavelength UV light. The visualized bands were scraped into scintillation vials, resuspended in scintillation fluid, and radioactivity counted using a Beckman model 3801 scintillation counter (^{3}H counting efficiency, 60%; Beckman Instruments, Irvine, CA). E20M activity was expressed as pg of 20E formed per minute per gram of eggs (± SEM).

Statistical analyses

Significant differences between mean E20M activities for each of the time points of the developmental profile were found by ANOVA analysis of variance, followed by pairwise comparisons of mean activity using Tukey's-HSD post-hoc analysis. All significant differences in mean values have a P value ≤ 0.05.

RESULTS AND DISCUSSION

Throughout embryogenesis of *M. sexta*, the titers of E and 20E are low and are consequently, not considered to be the active hormones which elicit molting of the first embryonic cuticle to make way for the second embryonic cuticle [16, 17]. Previous studies have shown that during *M. sexta* embryogenesis, 26-hydroxyecdysone (26E), released via hydrolysis of maternally derived 26E-phosphate conjugates, is hydroxylated at carbon 20 to form 20,26E. 20,26E then appears to direct molting during embryogenesis [16, 17]. 20,26E titers peak 72 hours into embryogenesis, a time which lies between secretion of the first and second embryonic cuticle (Figure 1) [16, 17]. In turn, bioassays have demonstrated that 20,26E is capable of directing molting and metamorphosis. Thus, it has been hypothesized that 26E is the precursor molting hormone that is hydroxylated to 20,26E to form the active molting hormone in *M. sexta* embryonic development [16, 27]. Despite this hypothesis the process by which 20,26E titers increase in embryogenesis of *M. sexta* remains largely uninvestigated. The data presented here show that E20M activity changes during *M. sexta* embryogenesis and that these changes correlate with changes in 20,26E titers.

Under the rearing conditions used, the time period from egg deposition (considered the beginning of embryogenesis i.e., 0 hours) to eclosion of first

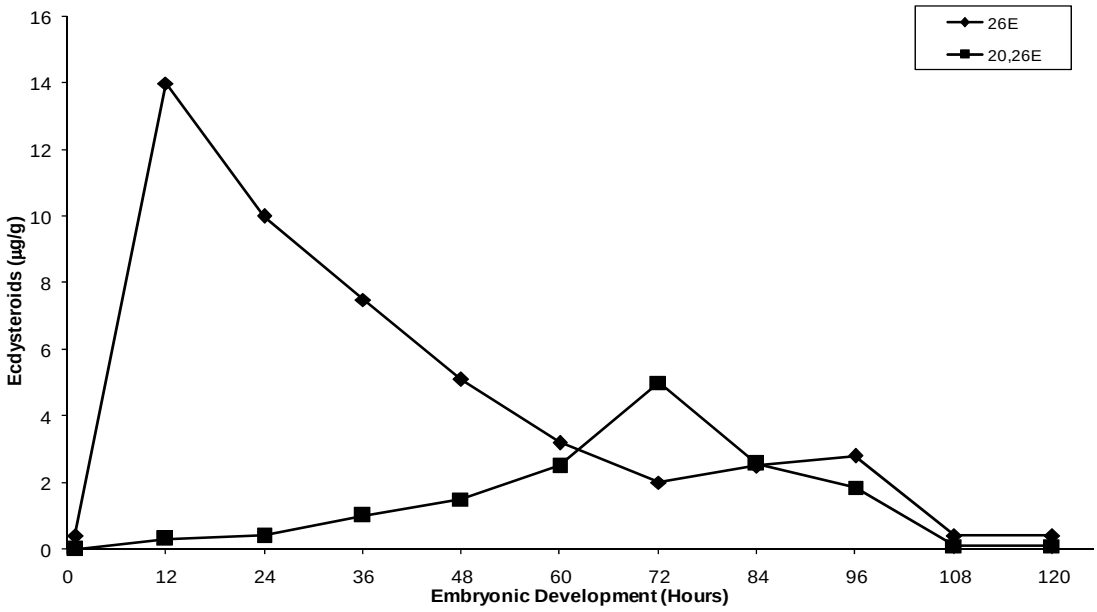

Figure 1. Ecdysteroid concentrations during *M. sexta* embryogenesis. -♦- indicates titer of 26E; -■- indicates titer of 20,26E during the 120-hour period of embryonic development [16].

instar larvae was 120 hours (±4 hours). Measurements of E20M activity were carried out at 12-hour intervals throughout embryogenesis. During the initial stages of embryogenesis, E20M activity was 4.36 ± 0.49 pg 20E/min/g 1 ± 1 hour after egg laying (Figure 2). By 12 ± 1 hours after the beginning of embryogenesis, E20M activity increased significantly to 8.03 ± 1.07 pg 20E/min/g (Figure 2). Over the next 48 hours (i.e., 12 ± 1 hours to 60 ± 1 hours), E20M enzyme activity did not show any significant changes. This 48-hour period is one in which secretion of the serosa and other early embryonic events are occurring [17]. Additionally, this is a period when the polar form of 26E is being hydrolyzed, free 26E is peaking, and the gradual accumulation of 20,26E is initiated (Figure 1). 72 hours into embryogenesis, E20M enzyme activity reaches its peak at, 12.20 ± 1.81 pg 20E/min/g (Figure 2). This peak in enzyme activity corresponds to the time which lies between the secretion of the first and second embryonic cuticle and is coincident with the peak in 20,26E titer (Figure 1) [16, 17]. This is of interest because studies on the regulation of E20M enzyme activity in *M. sexta* fifth larval instar show that increases in enzyme activity can be elicited by its substrate E and to a lesser extent 20E [28]. It would be interesting to determine if the same holds true for E20M during embryogenesis. Figure 1 shows that during the first 12 hours of embryogenesis, the 26E titer increases nearly 30-fold. Meanwhile, the current study found that during the first 12 hours of embryogenesis a significant increase in E20M activity occurs. The increase in 26E in conjunction with the increase in E20M activity suggests that as in postembryonic development, increases in substrate for E20M may cause increased enzyme activity. Conversely, one might expect a pulse of 26E to be necessary for the significant increase in E20M activity observed between 60 to 72 hours, yet there does not appear to be such a peak (Figure 1). Clearly, more investigation into the factors which elicit E20M activity in embryogenesis is necessary.

Interestingly, during the period directly following 72 hours into embryogenesis, the titer of 20,26E begins to decline and E20M enzyme activity drops significantly to 0.92 ± 0.20 pg 20E/min/g. E20M activity then remains at this low level until just before eclosion of the first instar larvae (i.e., 120 ± 1 hours) when activity increases significantly to 3.07 ± 0.58 pg 20E/min/g (Figure 2). The correlation between decreasing titers of 20,26E (Figure 1) and the significant drop in E20M enzyme activity (Figure 2) suggest that the enzyme is the only source of 20-hydroxylation for 26E during embryogenesis. The rapidity of the decrease in

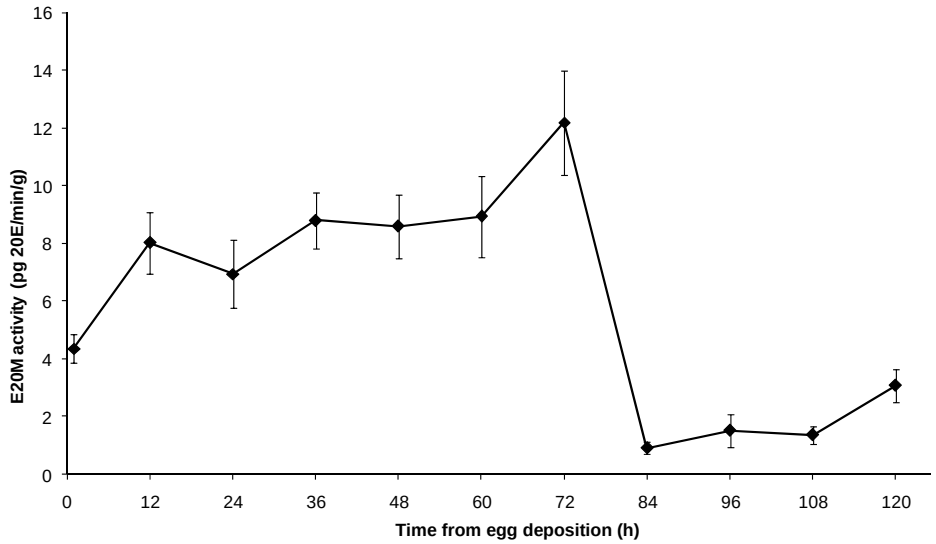

Figure 2. E20M enzyme activity throughout *M. sexta* embryogenesis. Significant changes in enzyme activity are noted between 1 and 12 hours; 60 and 72; 72 and 84 hours; and finally between 108 and 120 hours of embryogenesis. Error bars indicate ± SEM. N = 6 to 20 determinations in duplicate with zero time controls.

E20M activity suggests that the rapid regulation of the enzyme is necessary for accurate developmental events to occur. Finally, the additional significant peak in E20M activity that is observed just prior to eclosion of the first instar larvae suggests a possible role in eliciting egg hatching. However, the latter assumption remains to be validated.

Prior to the current study, few groups sought to determine the role and level of E20M activity during embryogenesis of *M. sexta*, with only one group [4] suggesting that E20M activity was present in eggs. To our knowledge, only one other group, utilizing the silk moth, *Bombyx mori*, biochemically characterized E20M activity during embryogenesis [19-22]. Clearly, detailed studies on *M. sexta* E20M enzyme activity during embryogenesis should be carried out utilizing both biochemical (e.g., using selective inhibitors, determining binding affinities and rates of conversion, etc.) and molecular techniques (i.e., measuring changes in levels of *shade* expression) to fully characterize E20M and evaluate its role in embryogenesis. These studies would allow definitive evidence that E20M is indeed catalyzing the hormonal conversion of 26E to 20,26E and will give a better understanding of the regulation of this developmentally important enzyme. This is necessary in order to determine if this enzyme works in a similar manner to that known for postembryonic regulation [1, 3, 4]. This initial report of E20M enzyme activity throughout *M. sexta* embryogenesis provides valuable initial data on which future studies can be based.

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