

EFFECT OF 20-HYDROXYECDYSONE ON CHITINASE AND β -N-ACETYLGLUCOSAMINIDASE DURING THE LARVAL-PUPAL TRANSFORMATION OF *MANDUCA SEXTA* (L.)

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Abstract—The relationship between ecdysteroid and chitinolytic enzyme activities was investigated using isolated abdomens from fifth instar larvae of the tobacco hornworm, *Manduca sexta* (L.). Low levels of 20-hydroxyecdysone (1 μ g/g live weight) stimulated exo-splitting β -N-acetylglucosaminidase about four-fold in integument while higher hormonal levels (15 μ g/g) which were less effective for β -N-acetylglucosaminidase induced endo-splitting chitinase about 50-fold. Chitinase activity was increased by about ten-fold over the control level in response to injection of 7.5 μ g of 20-hydroxyecdysone/g live weight into abdomens, while β -N-acetylglucosaminidase activity was elevated only about 1.5-fold over control. Apparently, ecdysteroid causes synthesis or activation of chitinolytic enzymes *in vivo* and the major regulatory effect is on the level of the endo-splitting chitinase.

Key Word Index: Molting, chitin, chitinase, β -N-acetylglucosaminidase, ecdysteroid, 20-hydroxyecdysone, tobacco hornworm, integument, *Manduca sexta*

INTRODUCTION

Chitin depolymerization by chitinolytic enzymes is one of the major ecdysial events in insect cuticular morphogenesis. Those enzymes have been purified from various insects and characterized, and studies using isolated enzymes *in vitro* have revealed that chitin degradation is initiated by a randomly attacking chitinase (endochitinase) followed by hydrolysis of oligosaccharide intermediates by an exo-splitting β -N-acetylglucosaminidase (exochitinase) with the overall rate of depolymerization limited by the endo-splitting chitinase (Kramer *et al.*, 1985; Fukamizo and Kramer, 1985a,b). Insect molting is triggered by prothoracicotropic hormone from the brain which in turn stimulates the prothoracic gland to secrete molting hormone (Bollenbacher *et al.*, 1975; Riddiford, 1985). Although a number of studies on the physiological roles of ecdysteroids have been performed, little is known about any direct relationship between the molting hormone and chitinolytic enzyme activities. An association of high ecdysteroid titer with chitinolytic enzymes *in vivo* has been observed in several arthropods (Spindler, 1983; Kramer *et al.*, 1985). Kimura (1973a) performed the first biochemical investigations about how the overall chitinolytic activity is induced by ecdysteroid injected into iso-

lated larval abdomens of *Bombyx mori*, an experimental system devoid of the prothoracic glands. A juvenoid administered in the diet of *Ephesia cautella* was used to indirectly suppress titers of both ecdysteroid and chitinolytic enzymes (Spindler-Barth *et al.*, 1986). In the present study, we have investigated the effect of 20-hydroxyecdysone on the activities of individual chitinolytic enzymes including chitinase and β -N-acetylglucosaminidase in the integument of isolated larval abdomens of the tobacco hornworm, *Manduca sexta* (L.). The data suggest that 20-hydroxyecdysone modulates the induction of chitinolytic enzymes *in vivo* and that the major regulatory effect is on the level of the endo-splitting chitinase.

MATERIALS AND METHODS

Insects and materials

Eggs of *Manduca sexta* (L.) were a gift from the Metabolism and Radiation Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Fargo, North Dakota U.S.A. Larvae were reared on an artificial diet according to Bell and Joachim (1976) at $27 \pm 1^\circ\text{C}$. Glycol chitin, the substrate for chitinase, was prepared by the method of Yamada and Imoto (1981). *p*-Nitrophenyl-2-deoxy- β -D-glucopyranoside, the substrate for β -N-acetylglucosaminidase and 20-hydroxyecdysone were purchased from Sigma Chemical Co.

Ligation and injection of larvae

Larvae at the beginning of the wandering stage were chilled on ice and ligated with thread between the second and the third abdominal segments (Nijhout, 1975; Reinecke *et al.*, 1980). The anterior portion of the larvae was excised and the ligated area rinsed with 70% ethanol. 20-Hydroxyecdysone was dissolved in 10% isopropanol and

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sterilized by passing it through 0.2 μm membrane filter (Metricel Membrane GA-8, Gelman Sciences, Inc.). Antibiotics were dissolved in deionized water and filter sterilized. The abdomens were injected with 20-hydroxyecdysone or solvent into an abdominal proleg at two and three days after ligation.

Enzyme preparation

Extraction of chitinolytic enzymes from integument was performed as follows: isolated abdomens at the appropriate stage were dissected and the integument with attached tracheae and muscles was separated from the other tissues. The integument was homogenized in 50 mM sodium phosphate buffer pH 7.0 (0.3 g tissue wet weight/ml) containing 1 mM phenylmethanesulfonyl fluoride (PMSF) and 1 mM phenylthiourea (PTU) to prevent the activity of proteases and tyrosinase, respectively. After centrifugation at 17,400 g for 20 min at 4°C, the supernatant was dialyzed against the same buffer as described above and employed for assays of chitinolytic enzymes.

Assay of chitinolytic enzyme activities

Endo-splitting chitinase activity was determined by the method of Koga *et al.* (1983). Supernatant (0.05–0.1) was mixed with glycol chitin in 50 mM sodium phosphate buffer, pH 6.4 (0.5 mg/ml; total volume, 1.5 ml) at 32°C and incubated for several hours. The production of reducing end groups was measured colorimetrically at 420 nm after addition of 2 ml of the potassium ferrirocyanide reagent (Imoto and Yagishita, 1971). For measurement of exo-splitting β -N-acetylglucosaminidase activity, supernatant (0.02 ml) was mixed with 1 ml *p*-nitrophenyl-2-acetamido-2-deoxy- β -D-glucopyranoside (*p*NpGlcNAc, 1.2×10^{-4} M) dissolved in 0.1 M sodium phosphate, 0.05 M citric acid, pH 5.6 at 25°C. Production of *p*-nitrophenol was monitored continuously at 337 nm on a Cary 118 spectrophotometer (Dziadik-Turner *et al.*, 1981).

Protein determination

Protein assays were performed using the BioRad assay method based on the Coomassie brilliant blue dye binding procedure of Bradford (1976). Bovine serum albumin served as the standard protein in this assay.

RESULTS AND DISCUSSION

When *M. sexta* larvae were collected during early wandering behavior and ligated between the second and third abdominal segments, the isolated abdomens did not molt, while the anterior portion molted about five days after ligation, and pupated with the cuticle appearing to tan normally. If the production of molting enzymes is controlled by ecdysteroid levels, then abdomens injected with molting hormone should show an increase in those enzyme activities. We determined the time course for molting enzymes that degrade cuticular chitin, chitinase and β -N-acetylglucosaminidase, in integument from isolated abdomens that were injected with 20-hydroxyecdysone or solvent only. In control abdomens integumentary chitinase remained at very low levels during the five days following ligation (Fig. 1A). β -N-acetylglucosaminidase increased about two-fold from about 45 to 100 nmol *p*NpGlcNAc hydrolyzed/min per mg protein (Fig. 1B). However, in abdomens receiving injections of 20-hydroxyecdysone [7.5 μg total/g live weight at two (5 μg) and three days (2.5 μg) after ligation], chitinase activity increased sharply by approximately ten-fold on the fourth day after ligation while β -N-acetylglucos-

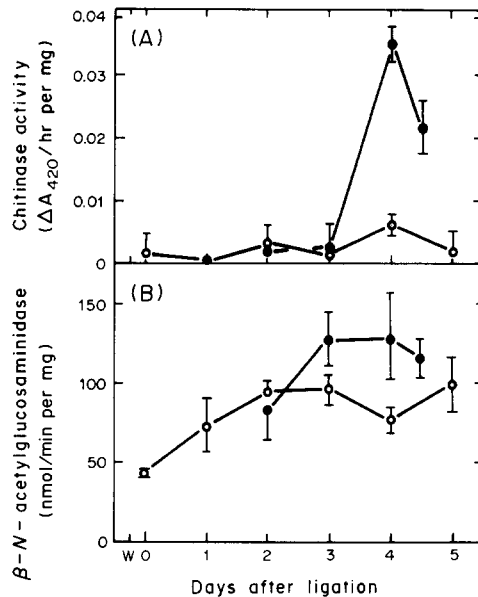


Fig. 1. Effect of 20-hydroxyecdysone injection on chitinolytic enzymes levels in integument of isolated abdomens from wandering stage larvae (W) of *Manduca sexta*. (A) Chitinase. (B) β -N-acetylglucosaminidase. Hormone injected (●); solvent injected (○). Mean value \pm SEM, $n = 3$. 20-Hydroxyecdysone (7.5 $\mu\text{g}/\text{g}$ body weight) was injected at two and three days post ligation.

aminidase was elevated less than two-fold relative to solvent-injected abdomens. Dorsal pigmentation and molting fluid secretion of the cuticle was observed to occur concomitantly with chitinase induction (Reinecke *et al.*, 1980). The treated abdomens pupated on the fifth day after ligation. These results demonstrated that in *M. sexta* ecdysteroid induces chitin hydrolysis *in vivo* and that the major stimulating effect is on the endo-splitting chitinase.

The extent of induction of chitinolytic enzymes in *M. sexta* integument was dependent on the amount of 20-hydroxyecdysone injected into the isolated abdomens (Fig. 2). β -N-Acetylglucosaminidase was increased four-fold from a basal level of approx. 80 enzyme units to 320 units after injection of 1.5 μg 20-hydroxyecdysone/g of ligated abdomen. Higher hormone levels (7.5–75 μg live weight) were less effective in inducing exoenzyme activity. Chitinase exhibited a higher threshold response to molting hormone than did β -N-acetylglucosaminidase with the highest activity induced at 15–75 $\mu\text{g}/\text{g}$ live weight. Chitinase was increased as much as 50-fold over basal levels by the hormone. Little or no chitinase activity was detected before or after injection of 0.15 to 1.5 μg 20-hydroxyecdysone. These results demonstrated that β -N-acetylglucosaminidase was present in integument at low levels before administration of exogenous ecdysteroid to the ligated abdomens and was elevated by relatively low ecdysteroid concentrations that did not stimulate chitinase activity. Chitinase was absent in integument until it was induced by a hormone level that was approximately an order of magnitude greater than that which increased β -N-acetylglucosaminidase activity. Apparently, the rate limiting enzyme, chitinase, in the binary chit-

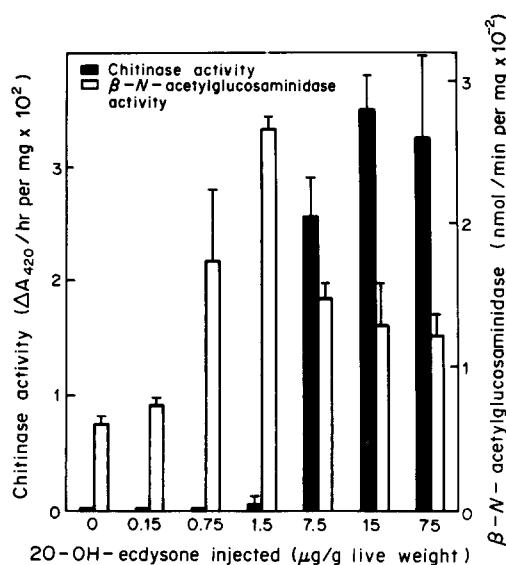


Fig. 2. Dose-response for 20-hydroxyecdysone injection on chitinase and β -N-acetylglucosaminidase in integument of ligated abdomens of *M. sexta*. Mean value \pm SEM, $n = 3$. The total amount injected was divided between two injections at two days (two-thirds of total dose) and three days (one-third of total dose) post ligation. Enzyme activities were measured one day after the second injection of 20-hydroxyecdysone.

inolytic enzyme system of *M. sexta* is the last of the two enzymes to appear as a result of hormone treatment.

There is good evidence for a positive causal relationship between ecdysteroid titer and chitinolytic enzyme production. Chitinolytic enzyme activity has been observed around the time of apolysis in *B. mori* (Kimura, 1973a,b), *M. sexta* (Bade, 1975; Dziadik-Turner *et al.*, 1981; Koga *et al.*, 1983), *Leucophaea maderae* (Zielkowski and Spindler, 1978), *Musca domestica* (Singh and Vardanis, 1984) and *E. cautella* (Spindler Barth *et al.*, 1986). We have extended the studies of *M. sexta* by demonstrating a differential response of individual chitinolytic enzymes to 20-hydroxyecdysone in isolated larval abdomens. At low ecdysteroid titer, β -N-acetylglucosaminidase activity was stimulated approximately four-fold but no chitinase activity was detected. At higher hormonal levels, chitinase activity was increased more than ten-fold and the exoenzyme level by only two-fold.

We propose the following temporal relationship between ecdysteroid and chitinolytic enzymes. Pupation is preceded by two releases of ecdysteroid (Bollenbacher *et al.*, 1981). The first release occurs during the fourth day after ecdysis to fifth stadium larvae and triggers the cessation of feeding, purging of the gut contents, initiation of wandering behavior (Dominick and Truman, 1984) and probably the elevation of β -N-acetylglucosaminidase activity. The exoenzyme was already present at substantial levels in the integument when larvae were ligated at a time subsequent to the first release of ecdysteroid. A second larger amount of ecdysteroid is released two days later that probably increases both chitinase and β -N-acetylglucosaminidase activities. Thus, as the ecdysteroid titer increases prior to apolysis, the

exoenzyme probably appears first, followed by the endoenzyme. Chitin degradation would not occur until after the second pulse of ecdysteroid because chitinase is required to initiate the depolymerization process. This proposal of hormonal regulation is in accord with results from kinetic studies of chitinolytic enzymes *in vitro* that demonstrate how the end-splitting chitinase catalyzes the rate-limiting steps in chitinolysis (Fukamizo and Kramer, 1985a,b).

The stimulatory effect of 20-hydroxyecdysone on β -N-acetylglucosaminidase in *M. sexta* was manifested at relatively low doses ($< 1 \mu\text{g/g}$ live weight) while a ten-fold higher amount caused chitinase activity to appear. Perhaps the rise in ecdysteroid together with its subsequent decline are necessary for chitinolysis *in vivo* since the peak of chitinase activity appears to coincide with tanning of the dorsal abdomen. In *Manduca* pupal tanning does not begin until ecdysteroid titer declines *in vivo* and *in vitro* (Mitsui and Riddiford, 1976). However, digestion of the old cuticle may precede tanning of the new one such that only the ecdysteroid rise is necessary for chitin catabolism. In preliminary mRNA isolation and translation experiments using epidermis from whole animals, chitinolytic enzyme mRNA has been found *in vivo* at developmental times which are analogous to those times when enzyme activity is detected in hormone treated isolated abdomens (Corpuz L., Kramer K. J. and Muthukrishnan S., unpublished data). β -N-Acetylglucosaminidase mRNA was present in epidermal tissue during and subsequent to the fifth larval instar wandering period while chitinase mRNA did not appear until the middle to late prepupal stage.

In epidermal cells ecdysteroid stimulates *in vivo* the secretion of molting fluid which contains chitinolytic, proteolytic and esterolytic enzymes (Hepburn, 1985; Riddiford, 1985). Molting fluid is secreted in hormone treated abdomens of *M. sexta* three to four days after ligation. This time course corresponds to that of chitinolytic enzyme stimulation in the integument. The hormone induces several other hydrolytic enzymes including acetylcholinesterase and β -galactosidase from an embryonic cell line of *Drosophila melanogaster* (Best-Belpomme and Courgeon, 1980) as well as fat body tyrosine glucoside hydrolase, which liberates tyrosine and glucose for cuticle tanning in *M. sexta* isolated abdomens (Ahmed *et al.*, 1983). DOPA decarboxylase is a non-hydrolytic enzyme also induced by ecdysteroid *in vivo* and *in vitro* in certain flies (Sekeris and Fragoulis, 1985). The induction of chitinase in *M. sexta* abdomens was detectable at approx. $20 \mu\text{M}$ 20-hydroxyecdysone. Concentrations of molting hormone that are about five-fold lower have been measured in pupating *M. sexta* (Bollenbacher *et al.*, 1981). However, when 20-hydroxyecdysone is administered by injection, a large proportion is expected to be catabolized rapidly. It is not surprising that relatively high levels of hormone are required for such experiments.

As a next step in the investigation of how morphogenetic hormones regulate chitin catabolism during the larval-pupal transformation of *M. sexta*, we are presently studying the transcription of chitinolytic enzyme mRNAs in integument from both whole animals and ecdysteroid-treated isolated abdomens.

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