

TIME PATTERN OF APPEARANCE AND DISAPPEARANCE OF ACTIVE MOLTING CHITINASE IN *MANDUCA* CUTICLE. THE ENDOGENOUS ACTIVITY

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Localization of insect molting chitinase in the old cuticle of pharate *Manduca* pupae has recently been reported from this laboratory [1]. It was shown that highly active chitinase is held tenaciously in the cuticle so that its activity remains by comparison low in molting fluid and epidermal extracts. Cuticle chitinase preparations may release over 200 μg *N*-acetylglucosamine per milligram cuticle protein per hour at the pH 8.2, of enzymatically active *Manduca* molting fluid, and were shown to utilize endogenous cuticle chitin preferentially in presence of exogenous colloidal chitin prepared from *Manduca* cuticles. At the same time, however, endogenous chitinase activity in newly synthesized cuticle was found to be absent. It was of interest, therefore, to determine at what time endogenous chitinase activity arises in insect cuticle relative to the molt. Specific endogenous cuticle chitinase activity rises sharply about 30 hr before pupal ecdysis; it disappears as abruptly about 12 hr later. A similar but lower endogenous peak in old cuticle chitinase activity occurs just prior to the preceding larval molt.

Larvae of *Manduca sexta*, the tobacco hornworm, were reared on an artificial diet from eggs obtained through the courtesy of Dr R. A. Bell of the U.S.D.A. Metabolism and Radiation Research Laboratory, Fargo, North Dakota. The larvae were observed carefully as the 4th larval molt approached, timed with 10 min of ecdysis, and harvested at various times following ecdysis. As previously reported [2], larval ecdysis of *Lepidoptera* is associated with a circadian clock. However, animals from the same hatch do not all develop at the same rate, as already described by Truman [2]. The larvae used in the experiments here reported were chosen from the most rapidly developing group, i.e. from those belonging to Gate 1 in

Truman's terminology. An additional check to assure that the pharate pupae analyzed were developing at comparable rates was afforded by the fact that events late in the pupal molt, i.e. within the final 32 hr preceding ecdysis, can be timed approximately by observation of the extent of tanning in the new cuticle which at that stage is visible through the old cuticle that still overlies it (Bade, unpublished). Pharate pupae, the cuticles of which were used in analysis of cuticle chitinase activity, were chosen such that they would have undergone pupal ecdysis at about the same time. Larvae used to assay specific endogenous chitinase activity in the last larval molt were timed from the preceding (3rd to 4th instar) larval molt and analyzed if outward appearance was consistent with their being in the pre-molt or molting stage (Bade, unpublished).

To measure chitinase activity, the same abdominal segment was removed from each CO₂-anesthetized larva at the time of analysis. The old cuticle was freed of all adhering tissue and new cuticle, if any, rinsed in ice-cold 0.85% KCl, and immersed in 2% ascorbate (pH 5.5). The segment of old cuticle to be analyzed in the pupal molt was cut in two in the mid-dorsal region and each half incubated at 37°C for 60 min with stirring in the following incubation mixture: 36 mM Tris-HCl buffer (pH 8.2) and 0.2 mg β -glucosidase (EC 3.2.1.21) in 1.0 ml final reaction mixture. Cuticle chitinase activity in the last larval molt was assayed similarly, but some of the larvae were frozen when harvested and each incubation was carried out with old cuticle from one whole abdominal segment. At the beginning and end of the incubation period, an aliquot of the supernatant was analyzed for *N*-acetylglucosamine by a modification of the method of Elson and Morgan [3]. Following the end of incubation, the cuticle segments

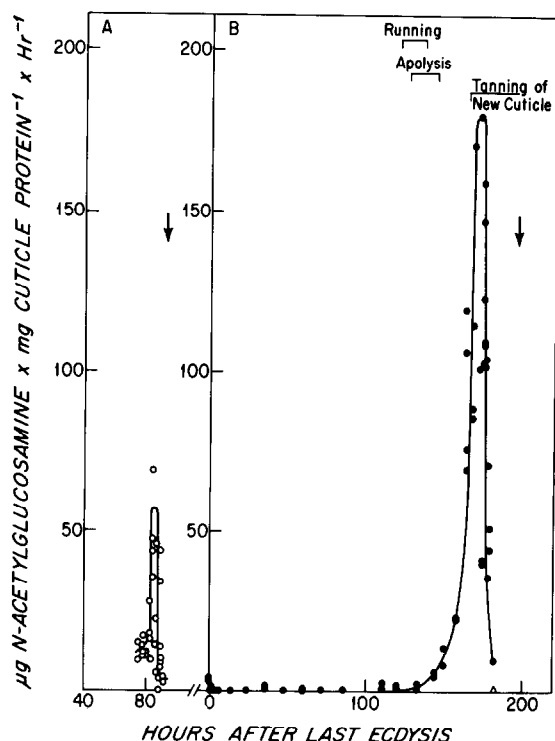


Fig. 1. Specific activity of cuticle chitinase relative to the molt cycle. Arrows indicate ecdysis. A) Molt at end of fourth instar. (○—○—○) activity in 4th instar (larval) cuticle B) Molt at end of fifth instar. (●—●—●) activity in 5th instar (larval) cuticle (Δ—Δ—Δ) activity in new (pupal) cuticle.

were transferred to tubes containing 2.0 ml N/1 NaOH. The tightly closed tubes were boiled for 1 hr. After cooling, aliquots of the alkaline supernatants were analyzed for protein by a slightly modified procedure for the alkaline copper method as described by Lowry et al. [4]. Protein concentrations were read from a standard curve constructed with bovine serum albumin.

The results are plotted in fig. 1; each point was obtained in one determination of specific chitinase activity, i.e. there are two points per larva analyzed. The first appearance of endogenous cuticle chitinase activity in the pupal molt (fig. 1B) coincides approximately with the mature larva leaving the food and burrowing in search of a suitable place to pupate, a stage designated on fig. 1 as 'running'. A few hours later, apolysis begins in the dorsal region of the abdominal segments (as determined by ease of tissue

removal from cuticle in dissections). In the space of several hours it progresses down the sides of the larva and finally encompasses the prolegs. The eventual abrupt rise in endogenous chitinase activity seems to occur after apolysis is complete. Still later, specific endogenous cuticle activity falls again as abruptly as it rose. A similar but much lower rise and fall is seen in the preceding larval molt (fig. 1A); it is completed in a shorter time period than that associated with the pupal molt.

These observations refine those of Jeuniaux [5], who reported a sharp peak in total chitinolytic activity in epidermal extracts from *Bombyx mori* during larval and pupal molts, and extend the work of Kimura [6] who also found higher chitinase activity in cuticular extracts of *Bombyx* following apolysis than before; in addition, he reported a shift of chitinase activity from extracts and subcellular fractions to 'cellular debris' following apolysis. The disappearance of endogenous chitinase activity here reported presumably is a consequence of the previously diminishing chitin content of old cuticle during latter stages of molting [7], i.e. the enzyme or enzymes are presumed to run out of available endogenous substrate. It has not yet been determined what triggers the sudden appearance of high cuticle chitinase activity but the trigger is assumed to be related to hormonal changes which accompany molting [2,8]. The chitinase presumably arises de novo in epidermal cells at some time in the molting cycle. However, it is not yet clear whether what is transferred out of the epidermal cells through new cuticle and molting fluid to old cuticle is active enzyme, or whether one or more proenzymes are synthesized and transported and later activated within the old cuticle. The precipitous rise in enzyme activity within old cuticle following apolysis during the pupal molt, which is here reported, makes the latter hypothesis more attractive.

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