SITE OF SYNTHESIS AND PHYLOGENETIC DISTRIBUTION OF A HEMOLYMPH TROPHIC FACTOR OF THE TOBACCO HORNWORM, MANDUCA SEXTA

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SUMMARY

Identification of fifth instar larval Manduca sexta fat body and epidermis as sites of synthesis of a hemolymph protein (hemolymph trophic factor or HTF) was achieved using in vitro 3H-leucine incorporation into protein and subsequent immunoprecipitation of tissue homogenates. Fat body is the primary site of HTF synthesis with a maximal rate on Day 1; epidermis is a secondary site with peak synthesis on Day 0. In vitro radiolabelling followed by TCA precipitation of general protein of fat body and epidermal homogenates suggest that fat body actively elaborates protein on Days 0–5 with peak rates on Days 1 and 4, while epidermis is active on Days 0–5 with a peak rate on Day 3. Based on Anti-HTF ELISA estimates, HTF [500 to 1000 µg/ml] was found in the hemolymph of representatives of the insect orders Blattodea, Hemiptera, Orthoptera, and Lepidoptera and in the class Crustacea, but not in the class Merostomata. These studies suggest a possible fundamental role for HTF among modern arthropods in cuticular deposition involving both epidermis and fat body. The physiological role of HTF is undetermined.

Key words: Manduca sexta; hemolymph proteins; immunoprecipitation; ELISA; fat body; epidermis.

INTRODUCTION

Hemolymph proteins have been the focus of research ever since the 1950's (Shigematsu, 1958) and much information that has become available about hemolymph proteins greatly to our understanding of insect biochemistry and physiology (Wyatt and Pan, 1978; Riddiford and Law, 1983; Levenbook, 1985; and Kanost et al., 1990). Most attention, however, has been given to proteins that are present in high titer (e.g., storage proteins) or are integral to well-studied physiological mechanisms (e.g., transport proteins). This laboratory has focused upon one hemolymph protein (hemolymph trophic factor (HTF)), a molecule found in relatively low titer but which has the ability to enhance in vitro cuticular deposition by the epidersis (Wielgus, 1983). Subsequent studies have demonstrated that HTF is a tetrameric protein of 286 kDa composed of two 69 kDa subunits and two 74 kDa subunits, with a Stokes' radius of 55.3 × 10⁻⁸ cm, an isoionic point of >10, and a relatively low maximum titer of 1.5 mg/ml in larval M. sexta. There is also evidence that HTF is present in epidermal tissue, fat body tissue, and the noncellular cuticle (Wielgus et al., 1990), suggesting that HTF might play a fundamental role in the formation of larval cuticle. However, much more information about this protein must be acquired before concluding statements of significance can be made. Fundamental knowledge that is lacking includes: (1) amino acid composition and sequences of the subunits, (2) posttranslational modifications of the subunits, (3) regulation of synthesis and release of the subunits and/or holoprotein, (4) nature of the chemical interaction of HTF and other components of the cuticle, (5) tissue(s) of synthesis of HTF and, (6) the phylogenetic distribution of HTF. This study investigated the latter two issues and provides evidence that HTF is a widely distributed protein among the arthropods and that it is synthesized both by epidermis and fat body.

MATERIALS AND METHODS

Experimental animals. Nondiapausing Manduca sexta larvae (Lepidoptera: Sphingidae) were reared as previously described (Wielgus and Gilbert, 1978). Synchronous larvae were selected at head capsule slippage from the fourth instar at 14.00-18.00 AZT (Arbitrary Zeitgeber Time). These animals are Day 0 until 14:00 the next day, 9-15 h following the completion of ecdysis. Slower-growing gate II animals were selected as described by Caglayan and Gilbert (1987) to maximize developmental synchrony. Feeding stage larvae, Day 0 through Day 5, were utilized exclusively as tissue donors because cuticle deposition occurs during that period (Wielgus and Gilbert, 1978; Riddiford et al., 1980). Fat body was extirpated from the third and fourth abdominal segments and as previously described (Wielgus, 1983). All tissues were rinsed in three changes of Grace's insect tissue culture medium (Gibco/BRL, Gaithersburg, MD) prior to experimentation.

Hemolymph donors were treated as follows. Last instar Malacosoma americanum larvae (eastern tent caterpillar; class Insecta, order Lepidoptera) and Pseucobotta pennsylvanica nymphs (wood cockroach; class Insecta, order Blattodea) were collected from a wooded area in Rockbridge County, VA, while Oncopeltus fasciatus nymphs (milkweed bug; class Insecta, order Hemiptera) and Acheta domestica adults (common cricket; class Insecta, order Orthoptera) were purchased from Carolina Biological Supply Co., Burlington, NC. Limulus sp. (horsehoe crab; class Merostomata) plasma powder was purchased from Sigma Chemical (St. Louis, MO). Hemolymph from M. sexta and M. americanum was collected as previously
described (Wielgus, 1983). *P. pennsylvaniae*, *O. fasciatus*, and *A. domesticus* hemolymph were obtained by abdominal puncture of chilled animals and use of capillary tubes, while *Homarus americanus* (Maine lobster; class Crustacea) hemolymph was collected by syringing from live, locally purchased animals. All hemolymph was centrifuged at 30 000 xg to remove hemocytes and subsequently stored at −70 °C until assay. *Limulus* plasma powder was dissolved in distilled water to give a final concentration of 50 mg/ml prior to assay.

**In vitro radiolabelling.** Rates of protein synthesis were estimated by incubating 60 mg fat body or a 3–8 mm square piece of integument in 1.0 ml Grace’s medium containing 3.45 nM 3H-leucine (60 Ci/mmol; Research Products International Corp., Mt. Prospect, IL) in 10 × 75 mm sterile glass tubes in a shaking water bath. Optimal conditions of 16 h incubation at 25 °C with 140 nM 3H-leucine were determined by preliminary trials using 60 mg fat body aliquots/ml, varying times of incubation and varying 3H-leucine concentrations followed by 12% TCA (trichloroacetic acid) precipitation of total protein of tissue homogenates and final scintillation counting as below.

**Total protein precipitation.** Total protein of fat body and epidermis was precipitated following radiolabelling as above. Tissues were washed three times with 2.0 ml ice-cold PBS (pH 7.4, buffered saline), air-dried near dryness, and homogenized in 1.0 ml protein solubilizing solution (PSS: 0.1% Triton X-100 in 0.0625M Tris, pH 6.8). Prior to homogenization of epidermis, it was gently scraped from the cuticle and pooled into 60 mg aliquots. Fifty μl aliquots of homogenates were set aside for immunoprecipitation as above: 2.0 ml 12% TCA was added to the remaining homogenate volumes in 15-ml glass centrifuge tubes and allowed to sit on ice for 1 h prior to centrifugation at 15 000 xg for 15 min. The pellet was washed with 2.0 ml ice cold 12% TCA and re-centrifuged as before. Following aspiration of supernatant, the pellet was resuspended in Protosol solubilizer (NEN Research Products, Wilmington, DE) at 90 °C for 30 min prior to transfer to a scintillation vial, and, with 5.0 ml scintillation fluid added, counting on a Packard Tri-Carb CA2200 liquid scintillation analyzer. All data were expressed in dpm/60 mg tissue equivalents.

**Immunoprecipitation of HTF.** Radiolabelled HTF was precipitated selectively from fat body and epidermal homogenates according to the method of Irvine and Jones (1979). Ten μl of freshly washed 10% heat-inactivated *Staphylococcus aureus* Protein A, cell suspension (SAC; Sigma) was added to each 50 μl aliquot of solubilized protein mixture, set aside as above, vortexed and incubated 15 min on ice in a microfuge tube. The tubes were microfuged 5 min at 4 °C and the supernatant transferred to a clean microfuge tube to which was added 25 μl anti-HTF IgG (60 μg/ml PBS). The pellet was washed with 0.025M K phosphate, pH 7.6, 0.1M NaCl, containing 0.001M EDTA (PBS) and incubated at 4 °C for 24 h and the microtiter plate emptied and washed as described in Wielgus et al. (1990). All ELISAs were conducted as follows. All volumes are 100 μl well unless otherwise stated. Materials to be tested were diluted 1:1, 1:10, and 1:100 in phosphate-buffered saline (PBS) and incubated at 4 °C for 24 h and the microtiter plate emptied and incubated with 1% BSA (bovine serum albumin) in PBS for 5 min and again emptied. ANTI-HTF IgG (60 μg/ml PBS) was added to each well for 1 h, followed by washing with 300 μl/well 0.02% Tween in PBS. The plate was incubated for 2 h with secondary antibody conjugate (goat anti-rabbit IgG linked to alkaline phosphatase at a concentration of 1.0 μg/ml in 1% BSA).

After washing as above, substrate (1.0 mg p-nitrophenylphosphate/ml 1.0 M diethanolamine) was reacted until a positive control of 5.0 μg HTF/ml read 0.400 absorbance units at 405 nm (about 30 min); the reaction was stopped with a saturated solution of EDTA. One percent BSA in PBS was used in all assays as a negative control and blank and all solutions and controls were assayed in sextet and the values averaged. The standard curve is previously published (Wielgus et al., 1990).

Potential endogenous phosphatase activity was measured in all samples by diluting each sample 1:100 in PBS and incubating 100 μl aliquots with 100 μl of p-nitrophenylphosphate for 30 min and then determining the absorbance as above. Anti-HTF IgG (60 μg/ml PBS) was also utilized for immunoprecipitation of HTF in the protein radiolabelling experiments.

**RESULTS**

**Radiolabelling.** In this study, rate of incorporation of exogenous leucine into protein was taken to be a measure of the rate of protein synthesis. Because endogenous amino acid pools as well as rates of protein synthesis vary with the physiological state of a cell, initial trials were performed incubating 60 mg M. sexta Day 3 fat body/ml Grace's for varying times and 3H-leucine concentrations to determine optimal parameters for in vitro protein labelling. Time trials with an arbitrarily chosen 100 nM 3H-leucine concentration at 25 °C demonstrated that 16 h incubation resulted in significant incorporation of label into the total precipitable protein (data not shown). As seen in Fig. 1, varying the concentration of precursor under the above conditions reveals that this system saturates above 140 nM 3H-leucine and thus this concentration was used in all subsequent experiments to avoid substrate limiting effects. Because the tissue weights of epidermis were much less per incubation (<10