



Molecular characterization of the insect immune protein hemolin and its high induction during embryonic diapause in the gypsy moth, *Lymantria dispar*

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Received 3 October 2001; received in revised form 11 April 2002; accepted 12 April 2002

Abstract

During the embryonic (pharate first instar) diapause of the gypsy moth, *Lymantria dispar*, a 55 kDa protein is highly up-regulated in the gut. We now identify that protein as hemolin, an immune protein in the immunoglobulin superfamily. We isolated a gypsy moth hemolin cDNA and demonstrated a high degree of similarity with hemolins from three other moth species. Hemolin mRNA levels increased at the time of diapause initiation and remained high throughout the mandatory period of chilling required to terminate diapause in this species, and then dropped in late diapause. This mRNA pattern reflects the pattern of protein synthesis. These results suggest that hemolin is developmentally up-regulated in the gut during diapause. Diapause in this species can be prevented using KK-42, an imidazole derivative known to inhibit ecdysteroid biosynthesis, and gypsy moths treated in this manner failed to elevate hemolin mRNA. Conversely, this diapause appears to be initiated and maintained by the steroid hormone, 20-hydroxyecdysone, and the addition of 20-hydroxyecdysone to the culture medium elevated hemolin mRNA in the gut. Our results thus indicate a role for 20-hydroxyecdysone in the elevation of hemolin mRNA during diapause. Presumably, hemolin functions to protect the gypsy moth from microbial infection during its long, overwintering diapause. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: Diapause; Hemolin; Immune protein; Gypsy moth; 20-Hydroxyecdysone; Midgut; KK-42; Pharate first instar larvae

1. Introduction

Diapause, the developmental arrest used commonly by insects to circumvent adverse seasons, is characterized at the molecular level by both a major shut-down in the expression of many genes as well as the up-regulation of a selective set of genes (reviewed by Denlinger, 2002). Among the genes that are up-regulated during diapause are several, but not all, the genes that encode heat shock proteins (Yocum et al., 1998; Rinehart et al., 2000; Rinehart and Denlinger, 2000). Additional diapause up-regu-

lated genes encode an apurinic/apyrimidinic (AP) endonuclease that is possibly involved in DNA repair (Craig and Denlinger, 2000), a defensin-like protein known to be involved in fighting microbial infections (Palli et al., 2001), and a drosomycin-like protein that is likely to be involved in antifungal defense (Daibo et al., 2001). Thus, several of the diapause up-regulated genes that have been identified so far encode proteins that may be involved in stress responses or may contribute to the protection of the insect during its long period of dormancy.

In the gypsy moth, *Lymantria dispar*, a conspicuous 55 kDa protein is evident in the midgut and hindgut during diapause (Lee and Denlinger, 1996, 1997). This species enters an obligate overwintering diapause as a pharate first instar larva: embryonic development has been completed but development is halted before the pharate larva completely consumes the yolk (Leonard, 1968; Masaki, 1956). Upon the termination of diapause

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in the spring, the pharate larva consumes the remaining yolk and breaks through the chorion to begin its larval life. The 55 kDa gut protein is abundantly synthesized in early diapause and is evident for approximately the first 100 days of diapause. Interestingly, a decline in the synthesis of this protein after 100 days correlates with the end of a mandatory chilling period required for the termination of diapause. Eggs chilled at 5 °C for less than 100 days fail to hatch, while those chilled for more than 100 days have the capacity to terminate diapause when they are transferred to 25 °C (Bell, 1989; Tauber et al., 1990). Although the 55 kDa protein serves as a good marker for diapause its identity and role have not been determined.

In this study, we report that the 55 kDa gut protein in the diapausing gypsy moth is hemolin, as evidenced by its N-terminal amino acid sequence similarity to other hemolins, insect immune proteins belonging to the immunoglobulin superfamily (IgSF) (Faye and Kanost, 1997; Mendoza and Faye, 1999). We also isolated a gypsy moth hemolin cDNA and determined that its expression pattern is consistent with the synthesis of the 55 kDa protein. This diapause is induced and maintained by ecdysteroids, the insect growth hormones (Lee and Denlinger, 1997; Lee et al., 1997, 1998), and in the present study we report that the hemolin gene is up-regulated by ecdysteroids, an observation consistent with the up-regulation of hemolin during diapause. The hemolin identity of this 55 kDa gut protein suggests that it functions in protecting the gut from microbial infection during diapause.

2. Materials and methods

2.1. *Insects*

Gypsy moth larvae and pupae were obtained from the Otis Methods Development Center, APHIS, USDA, Otis, MA and kept in an environmental chamber at 25 °C (16L:8D) until adult eclosion. Pairs of newly emerged adults were mated in separate containers one day after eclosion. Females deposited egg masses 1–2 days after mating. Newly deposited eggs were kept at 25 °C for 30 days to allow complete embryogenesis and the entry into diapause as pharate first instar larvae (Bell, 1989). Egg masses were then chilled for 160 days at 5 °C. Chilled egg masses were transferred to 25 °C for the completion of postdiapause development.

2.2. *Protein purification*

After diapause initiation (20–30 days postoviposition) pharate larvae were separated from the chorion and rinsed in a saline solution (Ephrussi and Beadle, 1936) to remove extra-embryonic yolk. Approximately 2000 guts

were dissected in a saline solution and transferred to a microcentrifuge tube containing 20 mM Tris–HCl buffer (pH 8.6). Gut tissues were homogenized and centrifuged for 15 min at 12,000g at 4 °C. Supernatants and pellets were stored at –70 °C until use. Dissected guts $n = 10$ from same age groups of pharate larvae were transferred to microcentrifuge tubes containing 5 μ l Grace's medium without methionine (GIBCO) and pulse-labeled with 10 μ Ci 35 S-methionine (1175 Ci/mmol, NEN) for 1.5 h at 25 °C. After incubation, tissues were homogenized, centrifuged as described previously, and the supernatants and pellets of the 35 S-labeled samples were stored at –70°C.

The supernatants of both the non-radiolabeled and radiolabeled samples were mixed and diluted with buffer A (20 mM Tris–HCl, pH 8.6, 10 mg/ml CHAPS) to a final volume of 2 ml. Proteins in the gut supernatants were fractionated by Fast Protein Liquid Chromatography (FPLC) using a mono-Q column (Pharmacia) in buffer A. After injection of a 2 ml aliquot, the column was developed with a 1% per min linear gradient of NaCl at a flow rate of 0.5 ml/min at 4 °C. After fractionation, the amount of incorporation of 35 S-methionine into protein in 5 μ l of each fraction was measured by trichloroacetic acid (TCA) preparation (Mans and Novelli, 1961). Radioactivity on the filters was measured using a liquid scintillation counter.

Proteins in each fraction were analyzed by SDS-PAGE (Laemmli, 1970). Aliquots of fractions containing high radioactivity were filtered with a YM-10 membrane disk (Amicon) and concentrated using a Savant speed-vacuum dryer. A portion of the concentrated samples and an original sample were run on an 8% SDS-polyacrylamide gel. The gel was stained with Coomassie Brilliant Blue R-250, destained with glacial acetic acid:methanol:water (10:45:45). The gel was dried on filter paper and exposed to X-ray film.

2.3. *Protein sequence analysis*

The entire concentrated sample of fraction 11, containing the radiolabeled 55 kDa protein, was run on 8% SDS-polyacrylamide gels with an upper buffer containing 0.1 mM thioglycolate. After separation, proteins were electroblotted to PVDF membrane (Biorad) in Towbin buffer (25 mM Tris, 192 M glycine, 20% methanol). After transfer, the membrane was rinsed three times (5 min each) with deionized water and stained with 0.025% Coomassie Blue R-250 in 40% methanol for 5 min and destained in 50% methanol for 10 min. The membrane was air-dried and exposed to X-ray film at –70 °C. The protein band of interest was excised from the membrane and the N-terminal sequence was determined by automated Edman degradation using an Applied Biosystems Model 477A pulsed liquid-phase sequencer equipped with an on-line HPLC for PTH-amino acid

analysis. The N-terminal sequence was used to search the NCBI database using the BLASTP algorithm (Altschul et al., 1990). The computation for the homology comparison of the determined partial amino acid sequence was performed at the National Center for Biotechnology Information (NCBI) using the BLAST network service.

2.4. Cloning of hemolin cDNA

Total RNA was extracted from homogenized eggs (25 days old) using the SV total RNA isolation system (Promega). Poly A⁺ RNA was selected using the PolyA-Tract mRNA Isolation System III (Promega). Reverse transcriptase (RT) reactions were performed with 0.6 µg poly A⁺ RNA as a template for cDNA synthesis (Horodyski et al., 2001). A sense primer, 5'-AA(AG)GA(CT)CA(AG)CC(ACT)GC(ACGT)GA-3' was designed from a portion of the determined N-terminal sequence, KDQPAE. An antisense primer, 5'-TACATGCA(GA)TA(TGA)ATCAT-3' was designed from an amino acid sequence MIYCMY that is highly conserved among insect hemolins corresponding to residues 231–236 of *Manduca sexta* hemolin (Sun et al., 1990; Wang et al., 1995). A PCR reaction was performed with 0.1 µg of cDNA as a template in PCR buffer (Stratagene) containing 3.5 mM MgCl₂, 0.2 mM dNTPs and 0.4 µM of each primer in 50 µl. A hot start was performed by adding 2 units of Taq2000 (Stratagene) in 10 µl PCR buffer into the reaction tubes when the thermal cycler reached 94 °C during the 4 min initial denaturation step. The mixtures were then overlaid with 75 µl mineral oil and amplified in a thermal cycler (Perkin-Elmer 480) for 4 cycles (94 °C, 1 min; 65 °C, 1 min; 72 °C, 1 min), followed by 4 cycles (94 °C, 1 min; 60 °C, 1 min; 72 °C, 1 min), followed by 4 cycles (94 °C, 1 min; 55 °C, 1 min; 72 °C, 1 min), followed by 35 cycles (94 °C, 1 min; 52 °C, 1 min; 72 °C, 1 min), followed by a 5 min final extension at 72 °C. The PCR products were analyzed on a 3% NuSieve-GTG agarose (FMC BioProducts) gel. The 680 bp PCR product was excised from the gel, reamplified using the identical PCR conditions, purified using the Wizard PCR preps DNA purification system (Promega) and subcloned into pCRII-TOPO (Invitrogen).

2.5. Rapid amplification of cDNA ends (RACE)

The following gene-specific non-degenerate primers were designed based on the sequence of the RT-PCR product:

3'-RACE primer:
5'-CAGGATTACTCTGTCACCTGATG-3',
5'-RACE primer:
5'-CTCAGGTATTGAGGTTCCAGCTC-3'.

The RACE reactions were performed according to the instructions of the Marathon cDNA amplification kit (Clontech). Double-stranded cDNA was synthesized from whole egg poly A⁺ RNA, ligated to the Marathon adaptor (Clontech), and used as template for 3'- and 5'-RACE reactions with Adaptor primer 1 (Clontech) and the above gene-specific primers. The resulting RACE products were separated on a 1% low melting point (LMP) agarose gel. A 3'-RACE product (0.9 kb) and a 5'-RACE product (0.7 kb) were excised from the gel and subcloned into pCRII-TOPO (Invitrogen).

2.6. DNA sequence analysis

The sequences of the RT-PCR and RACE products, cloned in pCRII-TOPO, were determined using the Big Dye Terminator Cycle Sequencing Kit (PE Applied Biosystems) and analyzed at the Ohio University Sequencing facility. Computer analysis of DNA sequences was done using DNASIS version 2.1 (Hitachi). Databases were searched using the BLASTP algorithm (Altschul et al., 1990) and amino acid sequences were aligned using CLUSTAL W (Thompson et al., 1994) available by internet access.

2.7. Northern hybridization

Total RNA was prepared from whole eggs or dissected gut tissues. For gut extraction, dehaired eggs were dissected in sterilized saline solution and guts were collected into RNAlater (Ambion). RNA was extracted using the SV total RNA isolation system (Promega). RNA was separated by 1% agarose formaldehyde gel electrophoresis, stained with ethidium bromide and transferred onto a Hybond-N nylon membrane (Amersham). The hemolin RT-PCR clone, pHId-1, was used as a template to synthesize an antisense RNA probe. Probes were labeled with ³²P to a specific activity of > 10⁹ cpm/µg using the MAXIscript in vitro transcription kit (Ambion) and ³²P-UTP (3000 Ci/mmol, New England Nuclear). Northern blots were hybridized to the probes at 2 × 10⁶ cpm/ml at 60 °C overnight in a solution containing 50% (vol/vol) formamide, 5× SSC (0.15 M NaCl, 0.015 M Na citrate, pH 7.0), 2.5× Denhardt's solution (0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% BSA), 50 mM sodium phosphate (pH 7.0), 0.1% SDS, 200 µg/ml sheared and denatured herring sperm DNA, 200 µg/ml yeast tRNA, and poly(A) at 500 µg/ml. Membranes were washed for 2 h in four changes of 0.1× SSC/0.1% SDS at 65 °C. As a control for RNA loading, the ethidium bromide-stained gel was photographed before blotting to visualize the rRNA.

2.8. Physiological analysis

For experiments with the imidazole derivative, KK-42 (1-benzyl-5-[(E)-2,6-dimethyl-1,5-heptadienyl] imidazole)

dehatched eggs were treated topically with acetone or an acetone solution containing KK-42 obtained from Kuwano, Kyushu University, Japan (Kuwano et al., 1991). Treated eggs were incubated for 5 days at 25 °C (90–98% RH). After incubation whole guts were dissected from pharate first instar larvae in sterile saline solution and total RNA was extracted as described previously. For experiments examining the effect of 20-hydroxyecdysone (20E) eggs were first treated with KK-42 as described previously. After 5 days of incubation, pharate larvae were carefully removed from the chorion and extra-embryonic yolk was washed away using saline solution. Pharate larvae were incubated individually in a 5 µl hanging drop of Grace's medium containing 1 µg/ml 20E (Sigma) for an additional 24 h at 25 °C (90–98% RH) in an environmental chamber. Pharate larvae in hanging drops of medium containing 10% isopropanol were used as controls. The whole of Grace's medium contained 0.2 µg/ml of the antibiotic gentamycin (Sigma). After incubation guts were dissected from the pharate larvae and total RNA was extracted as described previously.

3. Results

3.1. Purification and sequencing of the 55 kDa diapause-associated gut protein

When pharate first instar larvae initiate diapause at 15–20 days after oviposition, the rate of total protein synthesis is greatly reduced, and only a few proteins are actively synthesized in the gut. One such gut protein (approximately 55 kDa in SDS-PAGE), mostly present in the supernatant (Fig. 1A), was abundantly synthesized during diapause (Lee and Denlinger, 1996).

To isolate this 55 kDa protein, the supernatant mixture of radiolabeled and non-radiolabeled gut samples was first fractionated by mono-Q FPLC and the fraction containing the radiolabeled 55 kDa protein was then determined by TCA precipitation. The highest peak of radioactivity was present in fraction 11 (Fig. 1B). To determine whether the 55 kDa protein was present in fraction 11, proteins of fractions 9–15, whole supernatant and whole gut samples were analyzed with both Coomassie Blue staining and autoradiography in an SDS-PAGE gel (Fig. 1C). Autoradiography indicated that the 55 kDa protein was mostly present in fraction 11 (Fig. 1C, bottom). A few bands of lower molecular weight were also detected in fraction 11, but the incorporation rates were lower than that of the 55 kDa protein. In the Coomassie blue stained gel we detected the 55 kDa protein band that corresponded to the radiolabeled protein. After confirming the presence of the 55 kDa protein, the remainder of fraction 11 was used to determine its N-terminal amino acid sequence. The portion of the membrane con-

taining the 55 kDa protein was excised, and the N-terminal sequence was determined to be DSPVLLPVLKDQPAE. This sequence was used to search the non-redundant NCBI database using the BLASTP algorithm (Altschul et al., 1990). The greatest identity was with the N-terminal regions of the immune protein, hemolin, from three lepidopteran species, *Manduca sexta*, *Hyalophora cecropia*, and *Hyphantria cunea*. (Shin et al., 1998; Sun et al., 1990; Wang et al., 1995).

3.2. Isolation and characterization of the gypsy moth hemolin cDNA

To obtain cDNA fragments encoding portions of hemolin two sets of degenerate primers for RT-PCR were designed. A sense primer was based on a region of the N-terminal portion of the purified protein. An antisense primer was based on a region that is highly conserved among insect hemolins, MIYCMY (corresponding to residues 231–236 of the *Manduca* hemolin sequence) (Wang et al., 1995). An abundant RT-PCR product of 680 bp was obtained, which is similar to the expected sizes of DNA fragments encoding the corresponding portion of lepidopteran hemolins. The deduced translation of the DNA sequence of the RT-PCR product is highly similar to known hemolins of *H. cunea*, *M. sexta* and *H. cecropia* (Shin et al., 1998; Sun et al., 1990; Wang et al., 1995). To obtain the full-length cDNA sequence, we designed gene-specific primers from this DNA fragment to use for amplification of the 3'- and 5'-ends (RACE) (Fig. 2).

Sequence analysis of full-length cDNA revealed a single open reading frame (ORF) of 1266 nucleotides (Fig. 2A), beginning with a methionine at residue 18. A second methionine was present in the frame at residue 24. The sequence flanking this matches more closely with a consensus translation initiation site (Cavener and Ray, 1991), but since we cannot be certain which site is used to initiate protein synthesis, the first methionine residue is designated as residue 1. A hydrophobic sequence is present at the N-terminus of the ORF, typical of secreted or membrane-bound proteins. The most likely site of signal peptide cleavage is following Ser-21, but a potential cleavage site is also present following Ser-26 (Nielsen et al., 1997). The deduced sequence of *Lymantria* hemolin contains 4 immunoglobulin (Ig)-like domains. This is a characteristic feature of other known hemolin sequences as well as proteins involved in axon pathfinding and cell adhesion (Djabali et al., 1990; Kidd et al., 1998). The sizes of the four Ig-like domains between the conserved cysteine residues that flank the domains varies from 44 residues (domain IV) to 58 residues (domain II) (Fig. 2A and B). The amino acid sequence that we determined by N-terminal sequencing matches exactly with the deduced sequence, confirming that the cDNA clone encodes gypsy moth hemolin.

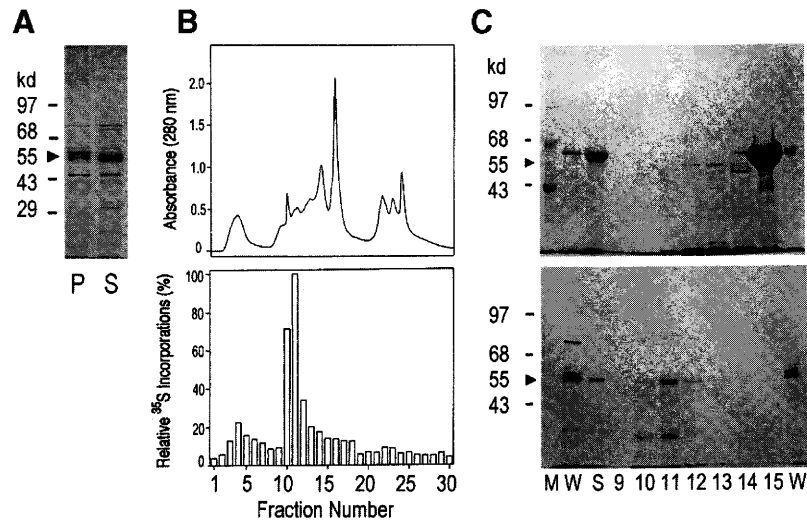


Fig. 1. Purification of the 55 kDa gut protein by FPLC using mono-Q anion-exchange chromatography and autoradiography. A. Comparison of gut protein synthesis in the pellet (P) and supernatant (S). Radiolabeled guts were homogenized and separated from supernatant and pellet by centrifugation. Equal amounts of ^{35}S -incorporated radioactivity were run on 10% SDS-PAGE gels. B. Separation of supernatant by FPLC using mono-Q chromatography (top) and the pattern of ^{35}S -incorporated proteins (bottom) in each fraction. Gut supernatant was separated by passage through a mono-Q column equilibrated with 0.1 M Tris (pH 8.6). The amount of ^{35}S -methionine incorporation into protein was measured by TCA precipitation. C. Identification of the 55 kDa protein in the Coomassie Blue stained gel (top) and in the autoradiography (bottom). FPLC fractions from 9 to 15, molecular weight markers (M), whole gut (W) and supernatant (S) were run on an 8% SDS-PAGE gel and examined for the presence of the 55 kDa protein.

However, the likely signal peptide cleavage site(s) are upstream from the N-terminus that we determined. The fact that the obtained N-terminal sequence follows a lysine (K) residue suggests that there is a trypsin-like protease involved. Therefore, it is likely that additional processing events are implicated in formation of the mature protein.

A similarity search using the BLASTP algorithm (Altschul et al., 1990) revealed that *Lymantria* hemolin is most similar to hemolins from *H. cunea* (66%) (Shin et al., 1998; accession number, AF023276), followed by *M. sexta* (47%) (Wang et al., 1995; U11879) and *H. cecropia* (45%) (Sun et al., 1990; S65948). There is also a high degree of similarity with numerous proteins of the IgSF, including neuroglians (35% identity) from *Drosophila melanogaster* (Bieber et al., 1989; AF050085) and *M. sexta* (Chen et al., 1997; U50719), *D. melanogaster* roundabout (robo) (30%) (Kidd et al., 1998; AE003458), leech tractin (26%) (Huang et al., 1997) and human cell adhesion molecule, L1 (26%) (Djabali et al., 1990). Phylogenetic relationships between the four hemolins were conducted using MEGA version 2.1 program (Kumar et al., 2001). The four hemolins were divided into two subgroups: one for *L. dispar* and *H. cunea*, the other for *M. sexta* and *H. cecropia* (data not shown). The alignment of *Lymantria* hemolin with three other hemolins, and the extra-cellular domains of two neuroglians, robo and L1 reveals that all Ig-like domains of hemolin are flanked by conserved cysteine residues, and the greatest similarity is observed in the regions flanking

these residues that form the Ig-like domain folds by the formation of disulfide bonds. (Fig. 3).

Potential sites for *N*- and *O*-linked glycosylations were localized. Hemolin in *L. dispar* contains three putative *N*-glycosylation sites (Asn-X-Thr/Ser); there are two such sites in *H. cunea* and *H. cecropia*, and one in *M. sexta*. (Shin et al., 1998; Sun et al., 1990; Wang et al., 1995). Among the three putative *N*-linked glycosylation sites of *Lymantria* hemolin, the site located in domain III was highly conserved between the hemolins of the four species. Another *N*-linked glycosylation site in domain II was conserved with *Hyphantria* hemolin and also with neuroglians of *Drosophila* and *Manduca*. However, an additional *N*-linked glycosylation site between domains II and III was unique to *L. dispar*. Putative *O*-linked glycosylation sites were identified by computer analysis (Hansen et al., 1997) in serine or threonine residues of hemolins (without signal sequences) of four species: four in *L. dispar*, two in *H. cunea*, one in *H. cecropia* but none in *M. sexta*. The site located in domain II of *Lymantria* hemolin was only conserved with *Hyphantria* hemolin, but the other sites were variable between species (Figs. 2 and 3).

There is a discrepancy in the apparent size of purified gypsy moth hemolin (55 kDa) and the predicted size from the deduced amino acid sequence from hemolin cDNA sequence. Assuming that the signal peptide is cleaved after Ser-21, the predicted size of the mature protein deduced from cDNA is 401 residues, with a predicted mass of 44.9 kDa (Fig. 2). If we assume that the

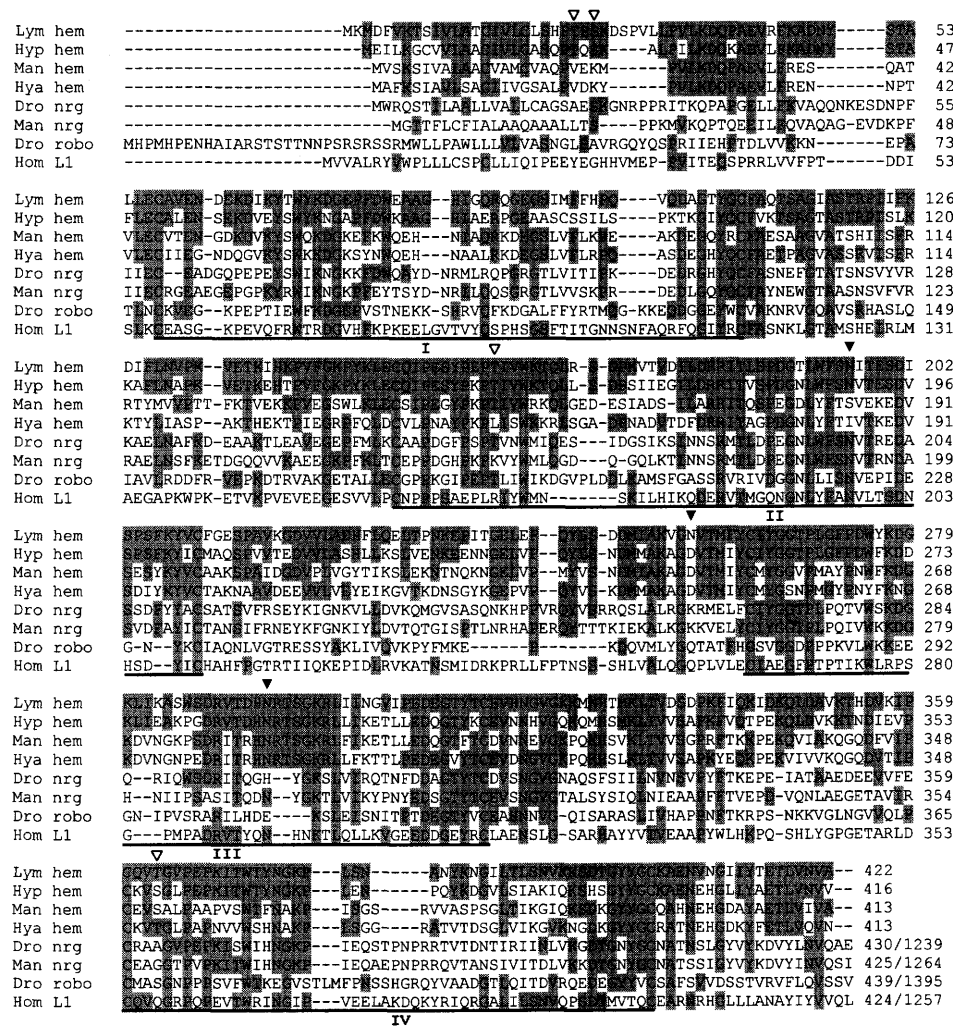


Fig. 3. Alignments between *Lymantria* hemolin and their related homologs. Alignments of the deduced amino acid sequence of *Lymantria* (Lym) hemolin (hem) with three other hemolins of *H. cunea* (Hyp), *M. sexta* (Man) and *H. cecropia* (Hya), and two neuroglians (nrg) of *Drosophila* (Dro) and *Manduca*, and *Drosophila* roundabout (robo) and human (Hom) L1. Identical residues are highlighted in gray. The four Ig-like domains are underlined in bold between the conserved cysteine residues. Three potential sites for N-linked glycosylation are marked by reversed dark triangles. Four potential sites for O-linked glycosylation are marked by reversed open triangles. The complete C-termini of neuroglians, robo and L1 are not shown, and their sizes are designated.

30. Diapause can be terminated when the eggs are returned to 25°C after prolonged chilling (>100 days) at 5 °C.

To determine the expression profile of hemolin mRNA in relation to diapause, RNA samples from whole eggs from several stages of prediapause, diapause and postdiapause gypsy moths were compared by Northern blot analysis (Fig. 4). The pattern of hemolin mRNA levels changed considerably during this time. A major 1.7 kb mRNA band, was detected with the hemolin probe from day 4 and its levels greatly increased from days 10–15 (just before entry into diapause). The level of the 1.7 kb hemolin mRNA remained high until day 40 (early diapause) which coincides with 10 days of chilling at 5 °C. However, this transcript was not detectable following prolonged chilling periods (days 70 and 130) or following the postdiapause period (4 days at 25

°C after chilling). Another major RNA, approximately 1.4 kb, was present with similar intensity to that of 1.7 kb RNA. At the present time, we do not know the relationship between these two mRNAs. We believe that the lower band is not a degradation product, since in all cases, a discrete band of ribosomal RNA was observed in the ethidium bromide-stained gel (Fig. 4). This band most likely represents either a second mRNA transcribed from the hemolin gene or an artifact caused by comigration of hemolin mRNA with ribosomal RNA. At days 15 and 20, a faint band of approximately 2.8 kb was also detected with the hemolin probe. A genomic Southern blot hybridized to the hemolin cDNA clone yielded 1 or 2 bands of varying intensity, which may indicate the presence of a cross-hybridizing region or the interruption of the hemolin gene by introns (data not shown). Regardless, the developmental changes in the intensity of hemo-

References

- Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic logical alignment search tool. *Journal of Molecular Biology* 215, 403–410.
- Andersson, K., Steiner, H., 1987. Structure and properties of protein P4, the major bacteria-inducible protein in pupae of *Hyalophora cecropia*. *Insect Biochemistry* 17, 133–140.
- Bell, R.A., 1989. Respiratory activity during embryonic development in a diapausing and a selected non-diapausing strain of the gypsy moth, *Lymantria dispar* L. *Comparative Biochemistry and Physiology* 93A, 767–771.
- Bettencourt, R., Assefaw-Redda, Y., Faye, I., 2000. The insect immune protein hemolin is expressed during oogenesis and embryogenesis. *Mechanisms of Development* 95, 301–304.
- Bettencourt, R., Gunne, H., Gastinel, L., Steiner, H., Faye, I., 1999. Implication of hemolin glycosylation and Ca⁺⁺-binding on homophilic and cellular interactions. *European Journal of Biochemistry* 266, 964–976.
- Bettencourt, R., Lanz-Mendoza, H., Lindquist, K.R., Faye, I., 1997. Cell adhesion properties of hemolin, an insect immune protein in the Ig superfamily. *European Journal of Biochemistry* 250, 630–637.
- Bieber, A.J., Snow, P.M., Hortsch, M., Patel, N.H., Jacobs, J.R., Traquina, Z.R., Schilling, J., Goodman, C.S., 1989. *Drosophila* neuroglian: a member of the immunoglobulin superfamily with extensive homology to the vertebrate neural adhesion molecule L1. *Cell* 59, 447–460.
- Cavener, D.R., Ray, S.C., 1991. Eukaryotic start and stop translation sites. *Nucleic Acids Research* 19, 3185–3192.
- Chen, C.L., Lampe, D.J., Robertson, H.M., Nardi, J.B., 1997. Neuroglian is expressed on cells destined to form the prothoracic glands of *Manduca* embryos as they segregate from surrounding cells and rearrange during morphogenesis. *Developmental Biology* 181, 1–13.
- Craig, T.L., Denlinger, D.L., 2000. Sequence and transcription patterns of 60S ribosomal protein P0, a diapause-regulated AP endonuclease in the flesh fly, *Sarcophaga crassipalpis*. *Gene* 255, 381–388.
- Daibo, S., Kimura, M.T., Goto, S.G., 2001. Upregulation of genes belonging to the drosomycin family in diapausing adults of *Drosophila triauraria*. *Gene* 278, 177–184.
- Denlinger, D.L., 2002. Regulation of diapause. *Annual Review of Entomology* 47, 93–122.
- Djabali, M., Mattei, M.G., Nguyen, C., Roux, D., Demengeot, J., Denizot, F., Moos, M., Schachner, M., Goridis, C., Jordan, B.R., 1990. *Genomics* 7, 587–593.
- Ephrussi, B., Beadle, G.W., 1936. A technique of transplantation for *Drosophila*. *American Naturalists* 70, 218–225.
- Faye, I., Kanost, M., 1997. Function and regulation of hemolin. In: Brey, P., Hultmark, D. (Eds.), *Molecular Mechanisms of Immune Responses in Insects*. Chapman and Hall, London, pp. 173–188.
- Hansen, J.E., Lund, O., Rapacki, K., Brunak, S., 1997. O-glycbase version 2.0—A revised database of O-glycosylated proteins. *Nucleic Acids Research* 25, 278–282.
- Horodyski, F.M., Bhatt, S.R., Lee, K.-Y., 2001. Alternative splicing of transcripts expressed by the *Manduca sexta* allatotropin (Mas-AT) gene is regulated in a tissue-specific manner. *Peptides* 22, 263–269.
- Huang, Y., Jellies, J., Johansen, K.M., Johansen, J., 1997. Differential glycosylation of tractin and leechCAM, two novel Ig superfamily members, regulates neurite extension and fascicle formation. *Journal of Cell Biology* 138, 143–157.
- Kidd, T., Brose, K., Mitchell, K.J., Fetter, R.D., Tessier-Lavigne, M., Goodman, C.S., Tear, G., 1998. Roundabout controls axon crossing of the CNS midline and defines a novel subfamily of evolutionarily conserved guidance receptors. *Cell* 92, 205–215.
- Kumar, S., Tamura, K., Jakobsen, I.B., Nei, M., 2001. MEGA2: Molecular Evolutionary Genetics Analysis software. Arizona State University, Tempe, Arizona, USA.
- Kuwano, E., Fujisawa, T., Suzuki, K., Eto, M., 1991. Termination of egg diapause by imidazoles in the silkworm, *Antheraea yamamai*. *Agricultural and Biological Chemistry* 55, 1185–1186.
- Ladendorff, N.E., Kanost, M.R., 1990. Isolation and characterization of bacteria induced protein P4 from hemolymph of *Manduca sexta*. *Archives of Insect Biochemistry and Physiology* 15, 33–41.
- Ladendorff, N.E., Kanost, M.R., 1991. Bacteria induced protein P4 (hemolin) from *Manduca sexta*; a member of the immunoglobulin superfamily, which can inhibit hemocyte aggregation. *Archives of Insect Biochemistry and Physiology* 18, 285–300.
- Laemmli, U.K., 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* 227, 680–685.
- Lee, K.-Y., Denlinger, D.L., 1996. Diapause-regulated proteins in the gut of pharate first instar larvae of the gypsy moth, *Lymantria dispar*, and the effect of KK-42 and neck ligation on expression. *Journal of Insect Physiology* 42, 423–431.
- Lee, K.-Y., Denlinger, D.L., 1997. A role for ecdysteroids in the induction and maintenance of the pharate first instar diapause of the gypsy moth, *Lymantria dispar*. *Journal of Insect Physiology* 43, 289–296.
- Lee, K.-Y., Valaitis, A.P., Denlinger, D.L., 1997. Further evidence that diapause in the gypsy moth, *Lymantria dispar*, is regulated by ecdysteroids: a comparison of diapause and nondiapause strains. *Journal of Insect Physiology* 43, 897–903.
- Lee, K.-Y., Valaitis, A.P., Denlinger, D.L., 1998. Activity of gut alkaline phosphatase, proteases and esterase in relation to diapause of pharate first instar larvae of the gypsy moth, *Lymantria dispar*. *Archives of Insect Biochemistry and Physiology* 37, 197–205.
- Leonard, D.E., 1968. Diapause in the gypsy moth. *Journal of Economic Entomology* 61, 596–598.
- Lowe, J.B., 2001. Glycosylation, immunity, and autoimmunity. *Cell* 104, 809–812.
- Mans, R.J., Novelli, G.D., 1961. Measurement of the incorporation of radioactive amino acids into protein by a filter-paper disk method. *Archives of Biochemistry and Biophysics* 94, 48–53.
- Masaki, S., 1956. The effect of temperature on the termination of diapause in the egg of *Lymantria dispar* Linné (Lepidoptera: Lymantriidae). *Japanese Journal of Applied Zoology* 21, 148–157.
- Mendoza, H.L., Faye, I., 1999. Physiological aspects of the immunoglobulin superfamily in invertebrates. *Developmental and Comparative Immunology* 23, 359–374.
- Nielsen, H., Engelbrecht, J., Brunak, S., von Heijne, G., 1997. Identification of prokaryotic and eukaryotic signal peptides and prediction of the cleavage sites. *Protein Engineering* 10, 1–6.
- Palli, S.R., Kothapalli, R., Feng, Q., Ladd, T., Perera, S.C., Zheng, S.-C., Gojtan, K., Pang, A.S.D., Primavera, M., Tomkins, W., Retnakaran, A., 2001. Molecular analysis of overwintering diapause. In: Denlinger, D.L., Giebultowicz, J., Saunders, D.S. (Eds.), *Insect Timing: Circadian Rhythmicity to Seasonality*. Elsevier, Amsterdam, pp. 133–144.
- Rasmuson, T., Boman, H.G., 1979. Insect immunity-V. Purification and some properties of immune protein P4 from haemolymph of *Hyalophora cecropia* pupae. *Insect Biochemistry* 9, 259–264.
- Rinehart, J.P., Denlinger, D.L., 2000. Heat-shock protein 90 is down-regulated during pupal diapause in flesh fly, *Sarcophaga crassipalpis*, but remains responsive to thermal stress. *Insect Molecular Biology* 9, 641–645.
- Rinehart, J.P., Yocum, G.D., Denlinger, D.L., 2000. Developmental upregulation of inducible hsp70 transcripts, but not the cognate form, during pupal diapause in the flesh fly, *Sarcophaga crassipalpis*. *Insect Biochemistry and Molecular Biology* 30, 515–521.
- Rudd, P.M., Wormald, M.R., Stanfield, R.L., Huang, M., Mattsson, N., Speir, J.A., DiGennaro, J.A., Fetrow, J.S., Dwek, R.A., Wilson, L.A., 1999. Roles for glycosylation of cell surface receptors

- involved in cellular immune recognition. *Journal of Molecular Biology* 293, 351–366.
- Russell, V., Dunn, P.E., 1996. Antibacterial proteins in the midgut of *Manduca sexta* during metamorphosis. *Journal of Insect Physiology* 42, 65–71.
- Shin, S.W., Park, S.-S., Park, D.-S., Kim, M.G., Kim, S.C., Brey, P.T., Park, H.-Y., 1998. Isolation and characterization of immune-related genes from the fall webworm, *Hyphantria cunea*, using PCR-based differential display and subtractive cloning. *Insect Biochemistry and Molecular Biology* 28, 827–837.
- Su, X.-D., Gastinel, N.L., Vaughn, D.E., Faye, I., Poon, P., Bjorkman, P.J., 1998. Crystal structure of hemolin: a horseshoe shape with implications for homophilic adhesion. *Science* 281, 991–995.
- Sun, S.-C., Lindstrom, I., Boman, H.G., Faye, I., Schmidt, O., 1990. Hemolin: an insect-immune protein belonging to the immunoglobulin superfamily. *Science* 250, 1729–1732.
- Tauber, M.J., Tauber, C.A., Ruberon, J.R., Tauber, A.J., Abrahamson, L.P., 1990. Dormancy in *Lymantria dispar* (Lepidoptera: Lymantriidae): analysis of photoperiodic and thermal responses. *Annals of Entomological Society of America* 83, 494–503.
- Thompson, J., Higgins, D., Gibson, T., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties, and weight matrix choice. *Nucleic Acids Research* 22, 4673–4680.
- Trenczek, T., 1998. Endogenous defense mechanisms of insects. *Zoology* 101, 298–315.
- Wang, Y., Willot, E., Kanost, M.R., 1995. Organization and expression of the hemolin gene, a member of the immunoglobulin superfamily in an insect, *Manduca sexta*. *Insect Molecular Biology* 4, 113–123.
- Yocum, G.D., Joplin, K.H., Denlinger, D.L., 1998. Upregulation of a 23kDa small heat shock protein transcript during pupal diapause in the flesh fly *Sarcophaga crassipalpis*. *Insect Biochemistry and Molecular Biology* 28, 677–682.
- Yu, X.-Q., Kanost, M.R., 1999. Developmental expression of *Manduca sexta* hemolin. *Archives of Insect Biochemistry and Physiology* 42, 198–212.