Immunochemical similarities among the hemolymph juvenile hormone binding proteins of moths

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Abstract. The hemolymph from various species of moths was analyzed for cross-reactivity with a panel of six monoclonal antibodies made against the hemolymph juvenile hormone binding protein of *Manduca sexta*. With the exception of one antibody, the immunoreactivity was limited to the sphingid family. One monoclonal antibody cross-reacted with a number of lepidopteran species; however, families such as Noctuidae and Pyralidae, known to have high affinity, low molecular weight juvenile hormone binding proteins, did not cross-react. Immunological cross-reactivity with *Manduca sexta* juvenile hormone binding protein in several primitive moth families supports the current model of phylogenetic relationships in the order Lepidoptera.
Key words. Juvenile hormone; *Manduca sexta*; Lepidoptera; immunotaxonomy; monoclonal antibodies; hemolymph.

The juvenile hormones are a series of homologous acyclic sesquiterpenoids central to growth, development and reproduction in insects. The lability and surface-active nature of these molecules preclude transit when unbound, thus dictating transport via hemolymph proteins from the site of synthesis to the target tissue. Three classes of hemolymph transport molecules have been reported: high affinity, high molecular weight proteins; high affinity, low molecular weight proteins; and low affinity, high molecular weight proteins [1].

The high affinity, low molecular weight juvenile hormone binding proteins (JHBP) of the order Lepidoptera have received considerable attention. More than 20 of these molecules have been partially characterized [2, 3], and with the exception of the species from the subfamily Saturniinae, the affinities and molecular weights of these proteins are similar. The molecular weights range from 20 to 40 kD and have dissociation constants in the range of 10^{-7} M. These similarities, in conjunction with a common ligand, suggest that the lepidopteran JHBP structure may have been partially if not fully conserved during evolution. The recent development of a panel of monoclonal antibodies against the JHBP of *Manduca sexta* makes it possible to establish immunological relationships among the JHBPs of Lepidoptera.

Materials and methods

Superfamily classification follows that described by Hodges [4]. Species identifications were confirmed by specialists.

Hemolymph was drawn from field-collected penultimate or ultimate stage larvae, treated with glutathione to prevent melanization and then stored at $-20\,^{\circ}C$ until needed. In most cases hemolymph from more than one individual was collected and pooled. Prior to analysis, hemolymph samples were thawed and centrifuged to remove precipitated protein. Hemolymph samples (2.5 µl) were prepared for polyacrylamide gel electrophoresis (PAGE) and placed in alternate gel wells separated by wells containing sample buffer only. Hemolymph from *Manduca sexta* was placed in at least one well on every gel and served as an internal standard. Hemolymph proteins were separated by either sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 10%) [5] or nondenaturing PAGE (10%) [6]. Proteins of known M_r were employed as markers on SDS-PAGE gels. Proteins were transferred to nitrocellulose sheets (0.45 µm; Bio-Rad, Richmond, CA, USA) [7] and visualized with India ink [8]. Staining with India ink outlined lanes for excision and provided a rapid assessment of the electrophoretic separation. Gels showing poor separation or lanes out of register were discarded. Lanes containing electrophoretically separated hemolymph were cut from the nitrocellulose paper, divided longitudinally into 3 identical portions and prepared for Western blot analysis. Western blot analysis was carried out using a panel of previously characterized monoclonal antibodies (MAbs) [7]. MAb-protein complexes were localized using goat anti-mouse IgG conjugated to horseradish peroxidase (KPL, Gaithersburg, MD, USA) and the substrate 4-chloro-1-naphthol (Sigma, St. Louis, MO, USA). The staining process was quenched when background test strips began to develop color. Proteins stained with India ink appeared gray while JHBP bands appeared purple.

Enzyme immunoassay (EIA) was performed by spotting hemolymph samples (2 µl) on a nitrocellulose sheet and air drying for 10 min. The sheet was then treated with MAb 5 [7]. MAb-protein complexes were localized using goat anti-mouse IgG conjugate to horseradish peroxidase (KPL) and the substrate *o*-phenylenediamine (Sigma). Results were quantified by punching out spots, placing them in wells of a microtiter plate, and analyzing with an automated spectrophotometric plate reader. A standard curve using hemolymph from *Manduca sexta* (fourth stadium, early head capsule slippage stage) was included on each plate. Values were corrected for background using control MAb.

Results

Use of MAbs, in contrast to polyclonal antibodies, requires that the target epitope undergo minimal conformational change during analysis since unfolding of the

Species cross-reactivity with *Manduca sexta* anti-JHBP MAbs. Hemolymph proteins (2 µl) were separated by SDS-PAGE, transferred to nitrocellulose paper and stained with India ink to visualize the profile. The paper was then divided longitudinally into 3 identical strips and Western blotted with anti-JHBP MAbs. Only those proteins with molecular weights between 20 and 40 kD were examined. Positive responses were detected visually. The minimal detection limit for MAb 5 was 0.25 µl of fourth stadium, head capsule slippage stage *Manduca sexta* hemolymph.

Species	Monoclonal antibodies					
	1	2	4	5	8	9
Sphingoidea						
Sphingidae						
Sphinginae						
Manduca sexta	+	+	+	+	+	+
Manduca quinquemaculata	+	+	−	+	+	+
Ceratomia amyntor	+	−	−	+	−	−
Pachysphinx modesta	−	−	−	+	−	−
Macroglossinae						
Eumorpha pandorus	−	−	−	−	−	−
Hyles lineata	+	−	−	+	−	−
Bombycoidea						
Saturniidae						
Citheroniinae						
Eacles imperialis	−	−	−	+	−	−
Saturniinae						
Actias luna	−	−	−	−	−	−
Hyalophora cecropia	−	−	−	−	−	−
Lasiocampidae						
Malacosoma americanum	−	−	−	+	−	−
Malacosoma disstria	−	−	−	+	−	−
Noctuoidea						
Arctiidae						
Pyrrharctia isabella	−	−	−	−	−	−
Euchaetes egle	−	−	−	+	−	−
Noctuidae						
Heliothinae						
Heliothis zea	−	−	−	−	−	−
Plusiinae						
Trichoplusia ni	−	−	−	−	−	−
Pseudoplusia includens	−	−	−	−	−	−
Notodontidae						
Macrurocampa marthesia	−	−	−	−	−	−
Lymantriidae						
Lymantria dispar	−	−	−	+	−	−
Geometroidea						
Geometridae						
Ennomos magnaria	−	−	−	−	−	−
Pyraloidea						
Pyralidae						
Pyraustinae						
Ostrinia nubilalis	−	−	−	−	−	−
Galleriinae						
Galleria mellonella	−	−	−	−	−	−
Tortricoidea						
Tortricidae						
Cydia pomonella	−	−	−	−	−	−
Cossoidea						
Cossidae						
Prionoxystus robiniae	−	−	−	+	−	−
Sesioidea						
Sesiidae						
Synanthedon sp.	−	−	−	−	−	−
Yponomeutoidea						
Plutellidae						
Plutella xylostella	−	−	−	+	−	−
Gelechioidea						
Oecophoridae						
Depressaria pastinacella	−	−	−	+	−	−

protein may disrupt the single antigenic determinant. Assuming that native PAGE would subject putative JHBPs to minimal conformational change, hemolymph samples were first analyzed in this fashion. Although native PAGE does not permit molecular weight estimates, it identifies those proteins that cross-react with the various MAbs. All species were then tested by SDS-PAGE with identical results. To eliminate the possibility that electrophoresis may have generated artifacts, hemolymph from the various species was tested by EIA. Species positive by EIA (at least 3 times background) were also positive by PAGE.

Since *Manduca sexta* is a member of the Sphingoidea, particular emphasis was placed on examining species from this group. MAb 5, which has the broadest degree of cross-reactivity, identified JHBP-like epitopes within the superfamily as well as in a number of other lepidopteran species (table). On the other hand, MAb 4 displayed a surprising degree of specificity, interacting with JHBP from *Manduca sexta* only. As was true with species outside the Sphingidae, the molecular weight of any protein that cross-reacted with the MAb panel was similar to that of *Manduca sexta* ($\sim$ 30 kD). Curiously, hemolymph from one sphingid species in the Macroglossinae, *Eumorpha pandorus*, did not cross-react with any MAb.

The most closely related superfamily, Bombycoidea, yielded surprising results. Although *Hyalophora cecropia*, long known to utilize only high molecular weight JH transport proteins, did not display cross-reactivity, another saturniid in the Citheroniinae, *Eacles imperialis*, proved positive. Representatives from the tent caterpillars, Lasiocampidae, also displayed a cross-reacting protein similar in molecular weight to *Manduca sexta* JHBP. Indeed, with the exception of sphingid species, *Malacosoma americanum* displayed the highest amount of EIA activity of all the species tested.

Excluding the arctiid and lymantriid families, the Noctuoidea displayed no cross-reactivity with anti-JHBP. Considering that hemolymph JHBPs characterized from this group resemble that seen in *Manduca sexta*[2], it was surprising that no activity was recorded by PAGE or EIA. The same negative pattern also appeared in the Pyraloidea, another superfamily that displays JHBPs similar to *Manduca sexta*[2]. We also tested a small number of species from groups that are thought to be the most primitive members of Ditrysia. Although the presence of JHBP has yet to be confirmed by conventional methods, hemolymph from several superfamilies cross-reacted with MAb 5.

Discussion

Excluding the order Hemiptera, the juvenile hormones are distributed throughout the class Insecta[9]. Correspondingly, most species provide a specific hemolymph transport mechanism for the hormones. In Lepidoptera, transport is mediated by hemolymph proteins that are similar in molecular weight and affinity, prompting speculation that at least portions of the JH transporting molecules have been conserved during evolution of the ditrysian Lepidoptera. This study focused on whether functionally similar proteins in this suborder display common antigenic determinants. Precedence for such conservation has been reported for another hemolymph transport protein, lipophorin. In this study, polyclonal antiserum to *Manduca sexta* lipophorin-II displayed cross-reactivity with insect species as evolutionarily distant as the roach, *Periplaneta americana*[10].

Detection of cross-reacting epitopes does not necessarily confirm the presence of a JHBP since other proteins may have identical epitopes that are not associated with the transport molecule. This point is clearly illustrated in the case of MAbs 8 and 9 that cross-react with both JHBP and the blue biliverdin-linked protein, insecticyanin, from the hemolymph of *Manduca sexta*[7]. Yet this problem is compensated for, in large measure, by the use of a panel of MAbs that recognize distinctly different epitopes. This study, in conjunction with our initial findings[7], indicates that each of the MAbs used binds to distinctly different epitopes. Lack of cross-reactivity with 6 distinctly different epitopes implies little or no immunorelationship and suggests that the JHBP must be distinctly different from the JHBP of *Manduca sexta*.

Although there is some taxonomic confusion within the order Lepidoptera, the present study generally confirms the phylogeny based on morphological characteristics as presented by Brock[12] and reviewed by Common[13]. On the basis of morphological traits, it has been suggested that Tineoidea represents the most primitive group in Ditrysia. A tineoid-stock ancestor was thought to give rise to the superfamilies, Yponomeutoidea, Gelechioidea and Copromorphoidea. Although our studies were limited to a single species within each of the families Plutellidae and Oecophoridae, the positive response recorded suggests that the epitope if not the protein itself has been conserved. Unfortunately no confirmatory binding studies have been reported to verify that these species have hemolymph JHBP. The lack of response in the single tortricid species was unexpected in light of the evolutionary scheme proposed by Brock[12]; however, one species presents a limited view of the entire superfamily.

Brock[12] further suggested that the Sesioidea, Pyraloidea, and Cossoidea were derived from a common stem displaying tortricoid-like characteristics. Our single species from the Sesiodea, *Synanthedon* sp., failed to display cross-reactivity. The proposed scheme also indicates a 'vertical relationship' between Pyralidae, Noctuidae and Geometridae. Since many of the species in these groups were previously shown to have high affinity, low molecular weight JHBPs, we hypothesized that cross-reactivity between anti-Manduca JHBP and these species would be prevalent. Surprisingly, only the arctiids and the lymantriids of this group were immunopositive. The hemolymph of the Gypsy moth, *Lymantria dispar*, con-

tained a highly cross-reactive protein between 33–38 kD. JH-photoaffinity labelling has identified a binding protein with the same mobility on SDS-PAGE [14]. Given the polyphyletic nature of the order [12], these proteins may have arisen independently in several different ancestral lines. With the exception of certain arctiids and lymantriids, it is clear that lines arising from pyraloid-like ancestors do not have JHBPs that display immunocross-reactivity with the *Manduca sexta* JHBP.

The relatively close phylogenetic relationship between the superfamilies Sphingoidea and Bombycoidea suggested some degree of cross-reactivity was to be expected. Interestingly, *Hyalophora cecropia* and *Actias luna*, members of the Saturniidae, do not display cross-reactivity while another species in this family, *Eacles imperialis*, does. It should be noted that the subfamily Citheroniinae to which *Eacles imperialis* belongs has until recently been considered a separate family [15]. Strong immunopositive responses were also observed for the lasiocampids, *Malacosoma americanum* and *Malacosoma disstris*.

The cross-reactivity of the MAbs within the family Sphingidae proved unexpected. The cross-reactivity of MAb 4 did not extend to another member of the same genus while several MAbs did not cross-react with members in the same subfamily. Even more striking is the cross-reactivity with the other sphingid subfamily, Macroglossinae. In the case of *Eumorpha pandorus*, no cross-reactivity was detected while in the case of *Hyles lineata*, two MAbs cross-reacted with the JHBP.

In summary, the cross-reactivity of MAb 5 with superfamilies as diverse as Gelechioidea and Sphingoidea indicates that a region of between 5 to 22 amino acids [11] has been conserved in a hemolymph protein of 20–40 kD. Whether this cross-reactivity represents a JHBP or merely an antigenic determinant in a functionally unrelated protein is unknown. Given the importance of the JHBP in hormone protection and transport, it is highly unlikely that normal development in any species would occur if the protein were genetically deleted. Thus, the confirmed absence of cross-reactivity in certain families indicates that these groups have evolved distinctly different types of JHBP.

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1 Goodman, W. G., in: Morphogenetic Hormones of Arthropods, pp. 83–124. Ed. A. P. Gupta. Rutgers University Press, New Brunswick 1990.
2 Goodman, W. G., and Chang, E. S., in: Comprehensive Insect Physiology, Biochemistry and Pharmacology, vol. 7, pp. 491–510. Eds G. A. Kerkut and L. I. Gilbert. Pergamon Press, Oxford 1985.
3 Koeppe, J. K., and Kovalick, G. E., in: Biochemical Actions of Hormones, vol. 13, pp. 266–304. Ed. G. Litwack. Academic Press, Orlando 1986.
4 Hodges, R. W., Checklist of the Lepidoptera of America North of Mexico. E. W. Classey Ltd, London 1983.
5 Lammeli, U. K., Nature *227* (1970) 680.
6 Hames, B. D., in: Introduction to Polyacrylamide Gel Electrophoresis, pp. 1–91. Eds B. D. Hames and D. Rickwood. IRL Press, Oxford 1981.
7 Goodman, W. G., Park, Y. C., and Johnson, J., Insect Biochem. *20* (1990) 611.
8 Hancock, K., and Tsang, V. C. W., Analyt. Biochem. *133* (1983) 157.
9 Schooley, D. A., Baker, F. C., Tsai, L. W., Miller, C. A., and Jamieson, G. C., in: Biosynthesis, Metabolism and Mode of Action of Invertebrate Hormones, pp. 373–383. Eds J. Hoffmann and M. Porchet. Springer-Verlag, Berlin 1984.
10 Ryan, R. O., Smidt, J. O., and Law, J. H., Arch. Insect Biochem. *1* (1984) 375.
11 Laver, W. G., Gillian, M. A., Webster, R. G., and Smith-Gill, S. J., Cell *61* (1990) 553.
12 Brock, J. P., J. nat. Hist. *5* (1971) 29.
13 Common, I. F. B., A. Rev. Ent. *20* (1975) 183.
14 Prestwich, G. D., Science (Wash. D.C.) *237* (1987) 999.
15 Covell, C. V., A Field Guide to the Moths of Eastern North America. Houghton Mifflin, Boston 1984.

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In vitro secretion of ecdysteroids by Y-organs of the crayfish, *Procambarus clarkii*

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Abstract. It was demonstrated that excised Y-organs of the crayfish, *Procambarus clarkii*, synthesize in vitro 3-dehydroecdysone (3-DHE) as the major product, together with small amounts of ecdysone. Both were identified by immunological and spectroscopic methods. The increase of ecdysteroidogenesis in the Y-organs was accompanied by an increase of the major free ecdysteroid, 20-hydroxyecdysone, in the hemolymph. This suggests a physiological role of 3-DHE, the details of which are still to be elucidated.
Key words. Molting hormone; ecdysteroids; 3-dehydroecdysone; Y-organ; crayfish.