

IMMUNOLOGICAL STUDY OF JUVENILE HORMONE
BINDING PROTEIN FROM HEMOLYMPH OF THE FALL
WEBWORM (LEPIDOPTERA: ARCTIIDAE)

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ABSTRACT

The juvenile hormone binding protein (JHBP) was purified from the hemolymph of *Hyphantria cunea* (Lepidoptera: Arctiidae) using anion exchange, gel filtration, and Mono P FPLC chromatofocusing chromatography. The protein is a single polypeptide (M_r 32,000) with an apparent dissociation constant of 0.43 μM for juvenile hormone III. An antibody developed against hemolymph JHBP (hJHBP) was prepared, and use of it showed that a protein immunologically identical with hJHBP occurs in fat body and ovary. The hJHBP of *H. cunea* was neither immunologically related to the lipophorin from *H. cunea* nor similar to the hJHBP of *Bombyx mori* and *Periplaneta americana*.

Key Words: Hemolymph, hemolymph proteins, insect, immunodetection, juvenile hormone III, lipoprotein, protein purification.

RESUMEN

La proteína de unión de la hormona juvenil (JHBP) fue purificada a partir de la hemolinfa de *Hyphantria cunea*. Para purificar la JHBP fueron usados el intercambio de aniones, la filtración en gel y cromatografías cromatofocales Mono P FPLC. La proteína es un polipéptido simple (M_r 32,000) con una constante de disociación aparente de 0.43 μM para la hormona juvenil III. Fue preparado un antibiótico desarrollado contra la hemolinfa JHBP (hJHBP) y su uso demostró que una proteína inmunológicamente idéntica a la hJHBP está presente en el cuerpo graso y el ovario. La hJHBP de *H. cunea* no estuvo inmunológicamente relacionada con la lipoforina de *H. cunea* ni fue similar a la hJHBP de *Bombyx mori* y *Periplaneta americana*.

Juvenile hormone is synthesized by the corpora allata, released into hemolymph, and transported to target tissues by the juvenile hormone binding protein (JHBP) (Whitmore & Gilbert 1972; Rudnica et al. 1979; Ozyhar et al. 1983). JHBP is synthesized by fat body and released into the hemolymph (Nowock et al. 1976; Ferkovich et al. 1977). Generally, high M_r (over 200,000) and low M_r (20,000-40,000) JHBPs are present in the hemolymph (de Kort & Granger 1981; de Kort et al. 1983). JHBP from hemolymph was first purified from *Manduca sexta* (Kramer et al. 1976) and then from *Diatraea grandiosella* (Dillwith et al. 1985), *Diploptera punctata* (King & Tobe 1988), and *Platyrepia virginalis* (Prestwich & Atkinson 1990). It has been reported that hemolymph juvenile hormone binding protein (hJHBP) is closely related to apoprotein-I of lipophorin (Rayne & Koeppe 1988; Koopmanschap & Dekort 1988).

Proteins with JH affinity were found to be present in ovary and fat body and their characteristics were also reported (van Mellaert et al. 1985; Koeppe et al. 1987; Shem-

shedini & Wilson 1993). However, information on the relationship between hJHBP and JH binding protein in these tissues is still limited, thus additional information is needed for a better understanding of the role of JHBP.

Here, we describe the presence of a JHBP in ovary and fat body which is immunologically identical with hJHBP by using an antibody against low M_r hJHBP from the fall webworm, *Hyphantria cunea* Drury (Lepidoptera: Arctiidae). In addition, the physicochemical characteristics of hJHBP are described.

MATERIALS AND METHODS

Insects

Fall webworms, *Hyphantria cunea*, were reared on artificial diet (Dong Bang Oil Co., Ltd., Seoul, Korea) at $28 \pm 1^\circ\text{C}$ and $70 \pm 5\%$ relative humidity and with a photoperiod of 16:8 (L:D).

Collection of Hemolymph and Extraction of Ovary and Fat Body

Hemolymph was collected into a cold Eppendorf microcentrifuge tube from last instar larvae by cutting the forelegs. A few crystals of phenylthiourea were added to prevent melanization, and the hemolymph was centrifuged at 10,000 g for 10 min at 4°C to remove hemocytes and cell debris. The supernatant was stored at -70°C .

Ovaries were dissected from 5-day-old pupae in cold Ringer's solution (128 mM NaCl, 1.8 mM CaCl_2 , 1.3 mM KCl; pH 7.4). They were used directly for electron microscopic observation or homogenized and centrifuged at 10,000 g for 10 min and the supernatant used as the sample.

Fat body was dissected from last instar larvae in Ringer's solution. These tissues were stored at -70°C until used.

Dextran Coated Charcoal (DCC) Binding Assay

A DCC suspension (0.5% active charcoal and 0.05% dextran, M_r 80,000) in buffer (10 mM Tris, 5 mM MgCl_2 , 50 mM KCl, pH 7.4) was used to separate protein-bound JH from unbound hormone (Engelmann 1981). [^3H]JH III (NEN Corp., Wilmington, DE; specific activity: 440 GBq/mmol; 50,000 cpm) was put into a microcentrifuge tube and the solvent evaporated with a gentle stream of nitrogen gas. Buffer (100 μl ; 10 mM Tris, 5 mM MgCl_2 , 50 mM KCl, pH 7.4), and the sample used for the binding assay, were added to the hormone and incubated for 1 h at 4°C . DCC suspension (100 μl) was added to the tube, the contents incubated for 2 min and then centrifuged at 10,000 g to remove free hormone. An aliquot (10 μl) of supernatant was transferred to 10 ml of scintillation cocktail solution (toluene 2 liters, triton X-100 1 liter, 12 g Omnifluor, NEN) and the radioactivity was measured by liquid scintillation counter (Beckman LS 100 $^\circ\text{C}$, Palo Alto, CA).

Purification of JHBP

Anion exchange chromatography. Hemolymph (10 ml) was diluted 1:1 with TPN buffer [10 mM Tris, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 0.01% NaN_3 , pH 8.2] and dialyzed against the same buffer for 4 h. Dialyzed hemolymph was applied to a DEAE cellulose (DE 52, Whatman, Hillsboro, OR) column (1.8 \times 20 cm) and eluted with 1 column volume of TPN buffer. This was followed by a NaCl concentration gradient (0-0.5 M) in buffer (10 mM Tris, 0.01% NaN_3 , 0.1 mM PMSF, pH 8.2, total 300

ml) at a flow rate of 18 ml per h and then monitored for the presence of JHBP by the DCC binding assay. Fractions containing JHBP were pooled, dialyzed against 10 mM Tris buffer (0.01% NaN 0.1 mM PMSF, pH 8.2) and applied to a Sepharose CL-6B (Pharmacia, Piscataway, NJ) column (0.9 × 15 cm). The column was washed with the same buffer using a gradient of 0.05-0.35 M NaCl. Each fraction (2.6 ml) was monitored for JHBP by the DCC binding assay. Fractions containing JHBP were lyophilized before the next purification step.

Gel Filtration. The lyophilized hemolymph sample was dissolved in 2 ml water and applied to a Sephadex G-100 column (1.8 × 55 cm) and eluted with buffer (25 mM bis-Tris, pH 7.2) at a flow rate of 12 ml per h. Each fraction (2.6 ml) was monitored for JHBP by the DCC binding assay. Fractions containing JHBP were pooled, concentrated by lyophilization, and applied to a Sephadex G-75 column (0.9 × 20 cm) and eluted with buffer (25 mM bis-Tris, pH 7.2) at a flow rate of 8 ml per h. Fractions of 1.5 ml were collected.

Mono P FPLC Chromatofocusing Chromatography. Fractions containing JHBP from the Sephadex G-75 column were subjected to FPLC chromatofocusing chromatography on a Mono P column (Mono P HR 5120, 5 × 200 mm, Pharmacia). Free proteins were eluted from the column with 25 mM bis-Tris buffer (pH 7.2, 10 ml), and then Polybuffer (pH 7.4, 52 ml) was used to elute resin-bound proteins. Each fraction was monitored for JHBP by the DCC binding assay.

Determination of Molecular Weight and Isoelectric Point

The molecular weights were determined on sodium dodecyl sulfate-polyacrylamide gel (10%) as described by Weber & Osborn (1969). Standard molecular weight markers were: bovine serum albumin, M_r 66,200; hen egg albumin, M_r 45,000; bovine carbonic anhydrase, M_r 31,000; soybean trypsin inhibitor, M_r 21,500; hen egg white lysozyme, M_r 14,400.

Isoelectric focusing was performed on 5% polyacrylamide gel according to Klages & Emmerich (1979) by using ampholytes in the pH range 3-10 (Sigma, St. Louis, MO).

Production of Antibody Against JHBP

Fractions (0.5 ml) containing JHBP after Mono P FPLC chromatofocusing were mixed thoroughly with Freund's complete adjuvant (0.5 ml) and injected subcutaneously into a rabbit. Injections were repeated every other day for the first week. The fourth injection was conducted 1 week after the third injection, and a booster injection was given 2 weeks later. JHBP-containing fractions (0.5 ml) were mixed with incomplete adjuvant (0.5 ml) for the booster injection. Blood was taken 1 week after the booster injection and centrifuged at 10,000 g for 10 min. Antibody against JHBP was purified by immunoprecipitation of extraneous antibodies using JHBP-free fractions.

Immunodiffusion

Immunodiffusion was conducted using 1% agarose gel containing 0.1% (w/v) sodium azide in veronal buffer (pH 8.6) as described by Ouchterlony (1949). The plates were stained in 1% amido black 10B and destained in 2% acetic acid.

Electrophoresis and Immunodetection

Fractions from each purification step were electrophoresed on 10% SDS-polyacrylamide gel as described by Laemmli (1970) to determine purity. Gels were stained with silver nitrate according to Wray et al. (1981).

Western blotting was used to detect JHBP on SDS-polyacrylamide gels. Samples were electrophoresed as above and transferred to nitrocellulose sheets in Tris-glycine buffer (25 mM Tris, 92 mM glycine, 30% methanol, pH 8.3) at 100 V for 2 h (Towbin et al. 1979). After transfer, the nitrocellulose sheets were equilibrated in TBS (20 mM Tris, 500 mM NaCl, pH 7.5) for 10 min and incubated in blocking solution (3% gelatin in TBS) for 30 min. The sheet was then washed with TTBS (0.05% tween 20 in TBS) twice, each time for 5 min, and incubated for 1 h in a solution containing a 300-fold dilution of the primary antibody against JHBP. This sheet was again washed with TTBS twice and incubated for 1 h in a solution containing a 3000-fold diluted secondary antibody (goat antirabbit-horseradish peroxidase conjugated IgG). After incubation, the sheet was again washed with TTBS twice and submerged in horseradish peroxidase color development reagent containing 4-chloro-1-naphthol in 20 ml ice-cold methanol + 0.015% H₂O₂ in 100 ml TBS for development of purple color.

Electron Microscopic Observation by Immunogold Labeling

Ovaries and fat body were dissected from pupae in Ringer's solution and prefixed in 2.5% glutaraldehyde for 2 h at 4°C. Tissues were washed with 0.1 M phosphate buffer (pH 7.2) 3 times at 15 min intervals and dehydrated in an ethanol series. Dehydrated tissues were then put into propylene oxide and embedded in an Epon-Araldite mixture. Embedded tissues were semithin-sectioned using an ultramicrotome (Sorvall MT-II, Wilmington, DE) and stained in 1% toluidine blue and attached to a grid. The thin section-attached grid was washed with TBS. This grid was reacted with the primary antibody solution 30-fold diluted with antibody buffer and again washed with TBS 3 times to remove nonspecifically attached antibody. This grid was then reacted with antirabbit IgG combined with protein-A gold particles (30 nm in diam) for 40 min, washed with TBS and distilled water 3 times each, stained in 2% uranyl acetate and observed under JEOL JEM 100 CX-II electron microscope at 80 kV.

RESULTS

Purification of JHBP from Hemolymph

The DCC binding assay for each fraction showed that JHBP was eluted from the DE-52 column with a linear NaCl gradient (0.06-0.1 M) (Fig. 1A). Fractions containing JHBP were subjected to a second anion exchange chromatography treatment (Sephacel CL-6B). JHBP was found to be present in the backside of the first peak (0.1 M NaCl) (Fig. 1B). Fractions containing JHBP from the second ion exchange chromatography run were subjected to gel filtration (Sephadex G-100) and fractions containing radioactivity were located (Fig. 1C) and pooled. The pooled fractions were applied to Mono P FPLC chromatofocusing columns and 4 peaks isolated by using A₂₈₀ (Fig. 1D). The fourth peak exhibited high binding activity for [³H]JH III (Fig. 1D, inset). Because the JHBP fractions were contaminated with other hemolymph proteins after Mono P chromatofocusing, gel filtration on Sephadex G-75 was necessary (data not shown). Fractions were applied to a 10% SDS gel and completely purified JHBP was confirmed (Fig. 2A).

Characterization of hJHBP

JHBP was electrophoresed with a low molecular weight standard marker to determine the M_r of the JHBP subunit. The M_r was estimated to be 32,000 (Fig. 2B). Also,

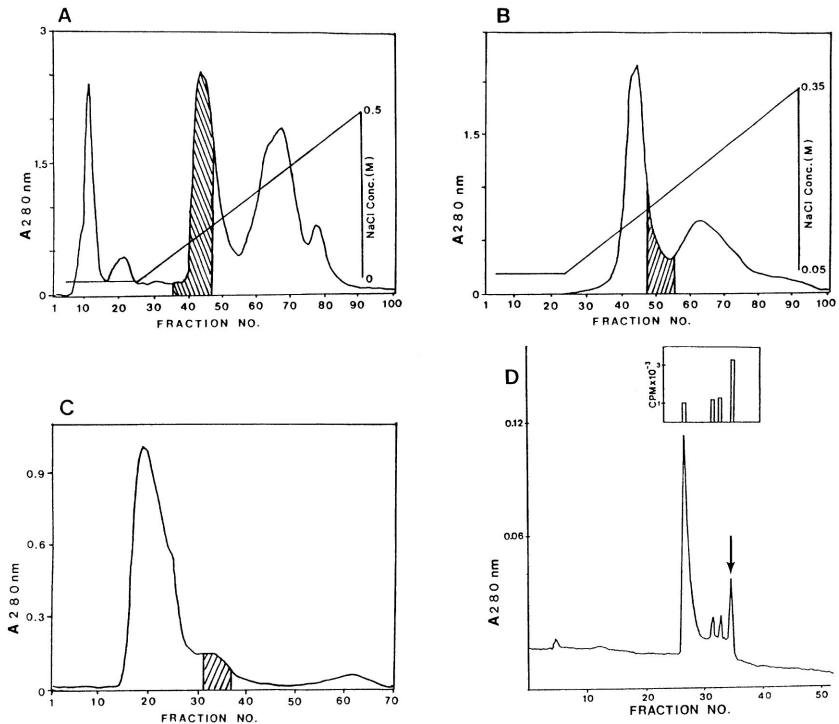


Figure 1. Chromatography elution profiles for purification of *H. cunea* hemolymph JHBP. A, the first anion exchange chromatography of dialyzed hemolymph proteins on DE52. Hatched region (35-46) indicates fractions containing JHBP; B, the second anion exchange chromatography on Sepharose CL-6B. Fractions (47-55, hatched region) showing radioactivity in DCC binding assay were pooled and lyophilized for the next purification step; C, gel (Sephadex G-100) filtration of partially purified JHBP. JHBP containing fractions were confirmed (hatched region, 31-37); D, Mono P FPLC chromatofocusing column. Four major protein peaks were resolved and collected (fractions 26-29, 32, 35, 37-38). Each peak was also analyzed by DCC binding assay (inset). Arrow indicates peak containing JHBP.

the pI was determined to be 5.3 (data not shown). Saturation analysis of the purified hJHBP revealed a high affinity for JH III (K_D of 0.43 μ M) (Fig. 3).

Immunological Studies

Antibodies were made against fractions containing JHBPs (Fig. 4A, lane b) that had been separated by Mono P FPLC chromatofocusing. Extraneous antibodies, i.e., other than antibody against hJHBP, were precipitated with JHBP-free fractions (Fig. 4A, lane a). The resulting supernatant from this precipitation showed pure hJHBP antiserum (Fig. 4B).

A protein that is immunologically identical with hJHBP was found in ovaries by immunodiffusion and western blotting (Fig. 5). However, the hJHBP of *H. cunea* was neither immunologically related to the lipophorin from *H. cunea* nor was it similar to the hJHBP of *Bombyx mori* and *Periplaneta americana* (Fig. 5). The western blots

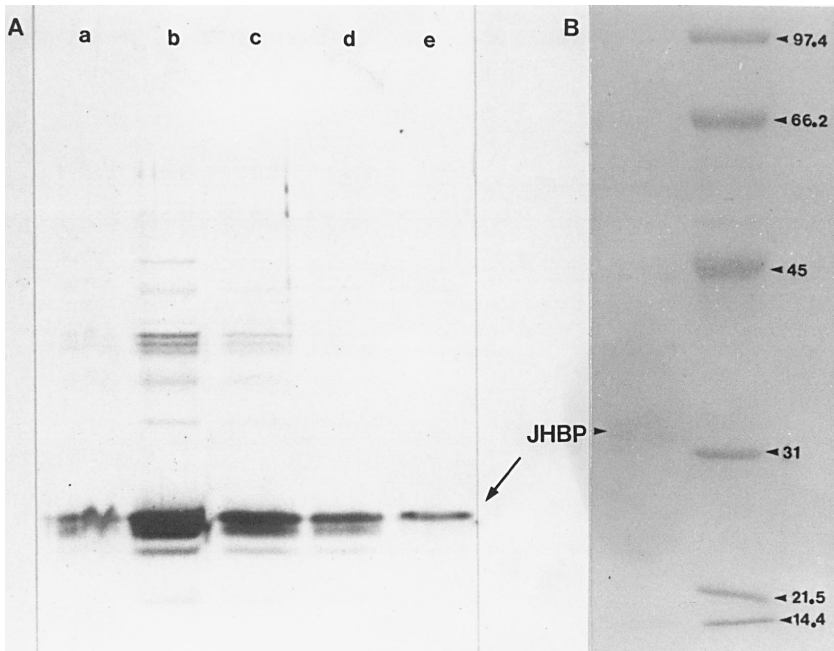


Figure 2. A, electrophoretic profile of fractions from final purification step (gel filtration on Sephadex G 75). Anterior portions of the chromatographic profile still contain other proteins in addition to JHBP (A: a, b, c, d). Completely purified JHBP from the posterior portion of the chromatography was confirmed on lane e. The gel (10%) was stained with silver nitrate. B, SDS-polyacrylamide gel (15%) electrophoresis to determine the molecular weight of JHBP. The gel was stained with coomassie brilliant blue. Right lane contains the low molecular weight standard markers and the arrow in left lane indicates JHBP.

shown in Figure 5 (left) show a crossreaction for the *H. cunea* hemolymph, ovarian extracts, and JHBP with the antibody against hJHBP. Likewise, the immunodiffusion test (Fig. 5, right) showed an immunoprecipitation line extending from the JHBP well through the ovarian extract and ending at the *H. cunea* hemolymph well, thus corroborating the western blot data.

Electron microscopic observations showed that gold particles accumulated in ovarian protein bodies as well as the fat body (Fig. 6). These results clearly indicate protein that is immunologically identical with hJHBP is present in protein bodies of ovary.

DISCUSSION

The DCC binding assay was employed to purify a low M_r JHBP. Fractions showing radioactivity according to the DCC binding assay were electrophoresed and silver stained and found to be a single polypeptide with an M_r 32,000. The protocols employed to purify hemolymph JHBP from *H. cunea* are similar to those used to purify JHBP of *Diatraea grandiosella* (Dillwith et al. 1985) and *Manduca sexta* (Kramer et al. 1976). The hJHBP of *H. cunea* has a pI of 5.3 (data not shown) and can be effectively separated from other hemolymph proteins with chromatofocusing. Low M_r

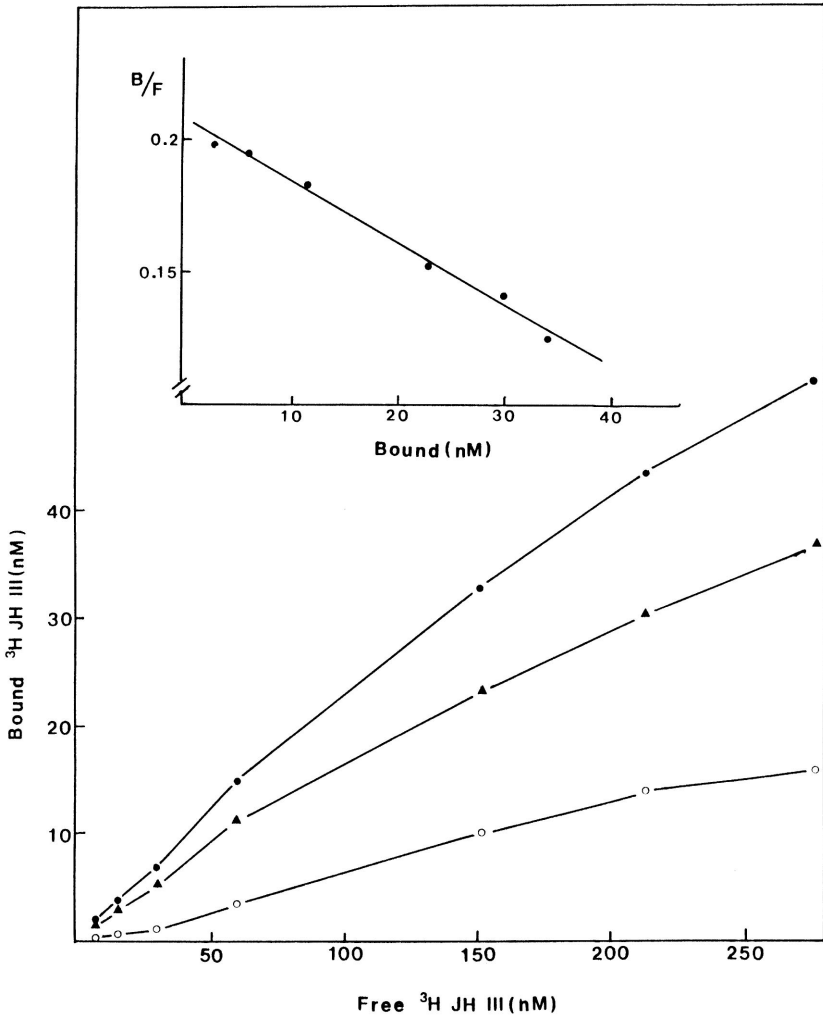


Figure 3. The saturation analysis (Scatchard plot) of the binding of JH III to the purified hJHBP. •••, total binding; ∞∞, nonspecific binding; ▲▲▲, specific binding. The specific binding was used for the Scatchard analysis (inset).

JHBP has an M_r range of 20,000-40,000 in several insects (de Kort & Granger 1981; de Kort et al. 1983).

The dissociation constant of *H. cunea* hJHBP was estimated to be 0.43 μ M. This value is similar to those of pyralid moth's 0.08-0.28 μ M (Lenz et al. 1986), *D. grandiosella* 0.31 μ M, (Dillwith et al. 1985), and *M. sexta*, 0.44 μ M (Kramer et al. 1976). The JHBP purified from hemolymph of *H. cunea* is of low M_r and high affinity.

Thus far, most JHBP antibody production employed large M_r proteins. Preparation of antibodies for the low M_r JHBP was difficult because of the small amount of

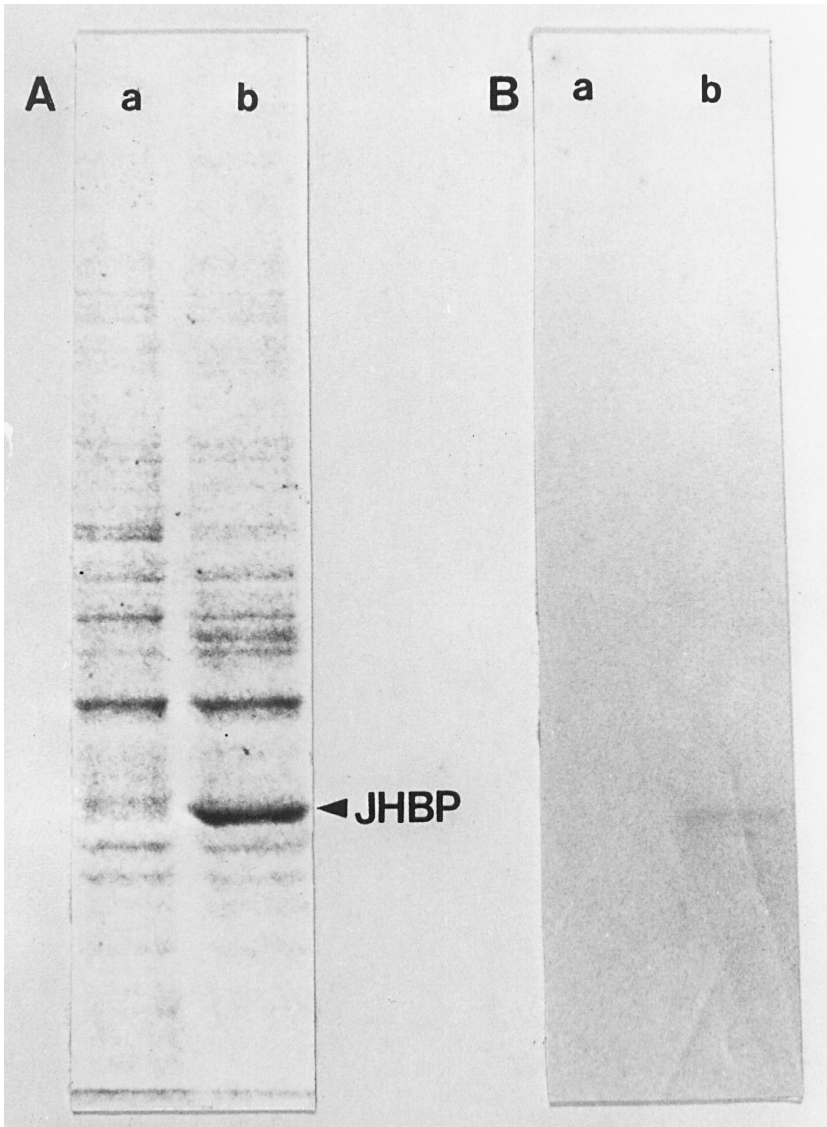


Figure 4. Western blot of purified JHBP. The JHBP band was detected by enzyme immunoassay using antibody against JHBP (B,b). Left panel (A) shows the electrophoretic patterns of fractions from the Mono P FPLC chromatofocusing chromatography. The JHBP-containing fractions (the fourth peak that the arrow indicates in Fig. 1D, lane b) were used for the production of antibody and fractions (anterior three peaks in Fig. 1D, lane a) that lack JHBP were used for the immunoprecipitation to remove other antibodies except antibody against JHBP.

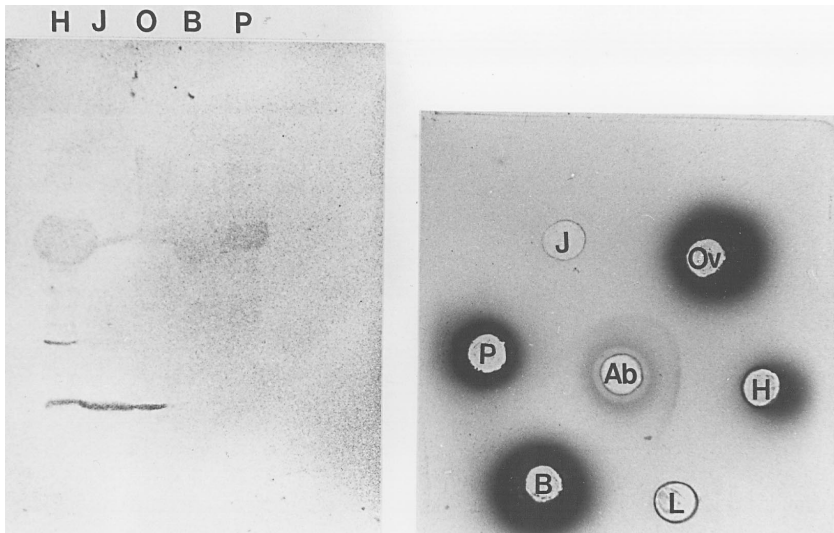


Figure 5. Left panel: Western blots of proteins from the hemolymph (H), JHBP (J), ovarian extracts (O) of *H. cunea*, the hemolymph (B) of *B. mori*, the hemolymph (P) of *P. americana*. The JHBP bands were detected by enzyme immunoassay using antibody against the hemolymph JHBP. In three lanes (H,J,O), a single M, 32,000 protein corresponding to the JHBP was detected by the antibody. Right panel: Immunodiffusion test to determine the relationship of several antigens (J, JHBP, 6 μ g protein; Ov, ovarian extracts, 1.8 mg protein; H, hemolymph of *H. cunea*, 1 mg protein; B, hemolymph of *B. mori* 1.4 mg protein; L, lipophorin of *H. cunea*, 20 μ g protein; P, hemolymph of *P. americana*, 1.2 mg protein; Ab, antibody against JHBP in hemolymph of *H. cunea*).

material in hemolymph. In the present work with *H. cunea*, immunoprecipitation was very effective in purifying antibody against low M, JHBP from other antibodies.

hJHBP was confirmed to be lipophorin in *Leucophaea maderae* (Koeppel et al. 1988) and *Locusta migratoria* (Koopmanschap & de Kort, 1988). Immunodiffusion studies conducted here indicate that the hJHBP was neither immunologically related to lipophorin in *H. cunea* nor the hJHBP of *Bombyx mori* and *Periplaneta americana*. The hJHBP of *H. cunea* appeared to be species-specific.

hJHBP is synthesized by fat body and released into hemolymph. Protein with JH affinity (JH binding protein) is also present in cytosol and nucleus of fat body (Shemshedini & Wilson 1993). A JH binding protein has been reported in the ovary (van Mellaert et al. 1985), but it is not known how it is related to hJHBP. We found a protein that is immunologically related to hJHBP that appears in fat body and the cytosol of ovarian protein body of *H. cunea*. This protein is assumed to be a JHBP. More information on the relationship between hJHBP and JHBP in the cytosol is required to better understand the transport of JH from secretory cells to target tissues and receptors.

ENDNOTE

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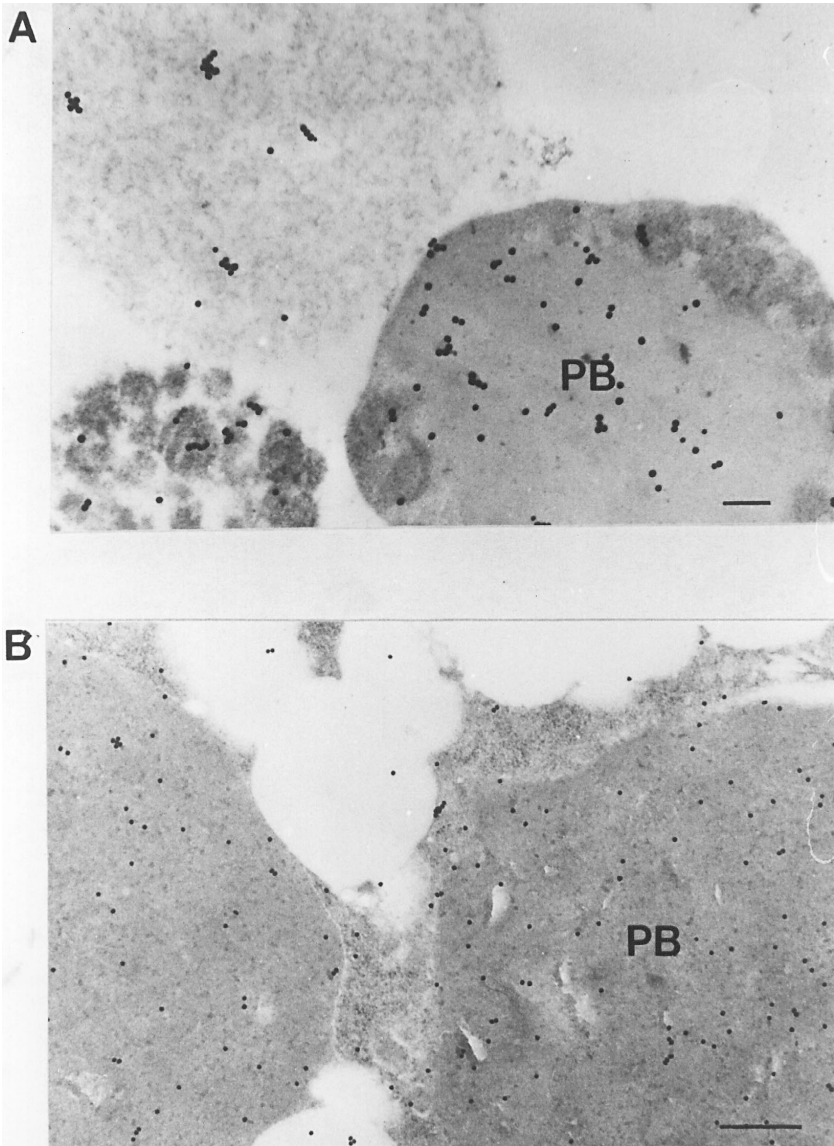


Figure 6. Immunoelectron micrographs of fat body (A) and ovary (B) from *H. cunea*. Immunogold particles were observed in large protein bodies (PB). Bar length = 0.2 μ m (A), 0.5 μ m (B).

REFERENCES CITED

- DE KORT, C. A. D., AND N. A. GRAGER. 1981. Regulation of the juvenile hormone titer. *Annu. Rev. Entomol.* 26: 1-28.

- DE KORT, C. A. D., M. G. PETER, AND A. B. KOOPMANSCHAP. 1983. Binding and degradation of juvenile hormone III by hemolymph proteins of the Colorado potato beetle: a re-examination. *Insect Biochem.* 13: 481-487.
- DILLWITH, J. W., S. D. MANE, AND G. M. CHIPPENDALE. 1985. High affinity juvenile hormone binding protein of the hemolymph of the southwestern corn borer, *Diatraea grandiosella*. Characteristics and relation to diapause. *Insect Biochem.* 15: 233-246.
- ENGELMANN, F. 1981. The identification of juvenile hormone binding protein in the fat bodies of *Leucophaea maderae*, pp. 263-270 in G. E. Pratt and G. T. Brooks [eds.], *Juvenile Hormone Biochemistry*, Elsevier North Holland Biomedical, New York.
- FERKOVICH, S. M., H. OBERLANDER, AND R. R. RUTTER. 1977. Release of juvenile hormone binding protein by fat body of the Indian meal moth, *Plodia interpunctella*, in vitro. *J. Insect Physiol.* 23: 297-302.
- KING, L. E., AND S. S. TOBE. 1988. The identification of an enantioselective JH III binding protein from the hemolymph of the cockroach, *Diploptera punctata*. *Insect Biochem.* 18: 793-805.
- KLAGES, G., AND H. EMMERICH. 1979. Juvenile hormone binding protein in the hemolymph of third instar larvae of *Drosophila hydei*. *Nature* 286: 282-285.
- KOEPPE, J. K., R. C. RAYNE, M. D. SHEARIN, D. J. CARVER, E. A. WHITSEL, AND R. C. VOGLER. 1988. Synthesis and secretion of a precursor hemolymph juvenile hormone-binding protein in the adult female cockroach *Leucophaea maderae*. *Insect Biochem.* 18: 661-666.
- KOEPPE, J. K., R. C. RAYNE, E. A. WHITSEL, AND M. E. POOLER. 1987. Synthesis and secretion of juvenile hormone-binding protein by fat body from the cockroach *Leucophaea maderae*: protein a immunoassay. *Insect Biochem.* 17: 1027-1032.
- KOOPMANSCHAP, A. B., AND C. A. D. DE KORT. 1988. Isolation and characterization of a high molecular weight JH III transport protein in the hemolymph of *Locusta migratoria*. *Arch. Insect Biochem. Physiol.* 7: 105-118.
- KRAMER, K. J., P. E. DUNN, R. C. PETERSON, H. L. SEBALLOS, L. SANBURG, AND J. H. LAW. 1976. Purification and characterization of the carrier protein for juvenile hormone from the hemolymph of the tobacco hornworm *Manduca sexta* Johannson (Lepidoptera: Sphingidae). *J. Biol. Chem.* 251: 4979-4985.
- LAEMMLI, U. K. 1970. Cleavage of structural proteins during assembly of head of bacteriophage T4. *Nature* 227: 680-685.
- LENZ, C. J., J. W. DILLWITH, AND G. M. CHIPPENDALE. 1986. Comparison of some properties of the high juvenile hormone binding protein from the larval hemolymph of pyralid moth. *Arch. Insect Biochem. Physiol.* 3: 61-73.
- NOWOCK, J., B. HAMMOCK, AND L. I. GILBERT. 1976. The binding protein as a modulator of juvenile hormone stability and uptake, pp. 354-372 in L. I. Gilbert [ed.], *The Juvenile Hormone*, Plenum Press, New York.
- OUCHTERLONY, O. 1949. Antigen-antibody reactions in gels. *Acta Path. Microbiol. Scan.* 26: 507-515.
- OZYHAR, A., J. R. WISNIEWSKI, F. SEHNAL, AND M. KOCHMAN. 1983. Age dependent changes in the binding and hydrolysis of juvenile hormone in the hemolymph of last instar larvae of *Galleria mellonella*. *Insect Biochem.* 13: 435-441.
- PRESTWICH, G. D., AND J. K. ATKINSON. 1990. Rapid purification and N-terminal amino acid sequence of a photoaffinity-labeled juvenile hormone binding protein from an arctiid moth larva, *Platyprepia virginialis*. *Insect Biochem.* 20: 801-807.
- RAYNE, R. C., AND J. K. KOEPPE. 1988. Relationship of hemolymph juvenile hormone binding protein to lipophorin in *Leucophaea maderae*. *Insect Biochem.* 18: 667-673.
- RUDNICA, M., F. SEHNAL, V. JAROLIN, AND M. KOCHMAN. 1979. Hydrolysis and binding of the juvenile hormone in the hemolymph of *Galleria mellonella*. *Insect Biochem.* 9: 569-575.

SHEMSHEDINI, L., AND T. G. WILSON. 1993. Juvenile hormone binding proteins in larval fat body nuclei of *Drosophila melanogaster*. *J. Insect Physiol.* 39: 563-569.

TOWBIN, H., T. STAHELIN, AND J. GORDON. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* 76: 4350-4355.

VAN MELLAERT, H., S. THEUNIS, AND A. DE LOOF. 1985. Juvenile hormone binding proteins in *Sarcophaga bullata* hemolymph and vitellogenic ovaries. *Insect Biochem.* 15: 655-661.

WEBER, K. AND M. OSBORN. 1969. The reliability of molecular weight determination by dodecyl sulfate-polyacrylamide gel electrophoresis. *J. Biol. Chem.* 244:4406-4412.

WHITMORE, E., AND L. I. GILBERT. 1972. Haemolymph lipoprotein transport of juvenile hormone. *J. Insect Physiol.* 18: 1153-1167.

WRAY, W., T. BOULIKAS, V. P. WRAY, AND R. HANCOCK. 1981. Silver staining of proteins in polyacrylamide gels. *Anal. Biochem.* 118: 197-203.

