Purification and Characterization of a Juvenile Hormone Binding Protein from Whole Body Homogenates of the Wax Moth, *Galleria mellonella* Final Instar Larvae

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Abstract – A juvenile hormone binding protein (JHBP) has been isolated from the whole body homogenate of *Galleria mellonella* final instar larvae by gel filtration. The isolated protein is homogenous as judged by column chromatography and gel electrophoresis in the presence and absence of denaturing agent. The JHBP has a relative molecular weight of 32 k by denaturing gel electrophoresis and 28 k by gel filtration. The protein exhibits a dissociation constant of $3.9 \times 10^{-7}$ M for JH III.

Key Words – Juvenile hormone binding protein, *Galleria mellonella*, Gel filtration, Gel electrophoresis

초 록 – 꼴벌부체명나방 종령유충의 whole body에서 gel filtration 방법으로 유악호르몬 결합 단백질을 분리, 정제하였다. 분리된 단백질은 column chromatography법과 전기영동법에 의해 동가 성을 확인하였다. 이 결합단백질은 전기영동법에 의해 32 K, gel filtration에 의해 28 K의 상대적 분자량을 나타냈다. 또한, JH III에 대한 해리도는 $3.9 \times 10^{-7}$ M로 확인되었다.

검색어 – 유악호르몬 결합단백질, 꼴벌부체명나방, 질여과정, 전기영동법

Insect growth and development as well as reproductive function are, in part, under the control of the juvenile hormones (JH). In lepidopterous larvae there are three natural hormones representing a homologous series of acyclic sesquiterpene derivatives (Gilbert et al., 1977). The hormones are synthesized in the corpora allata and secreted into hemolymph, in which most JHs form a complex with a specific binding protein.

Since a JH-specific binding protein (JHBP) was observed in the hemolymph of *Manduca sexta* (Goodman and Gilbert, 1974; Kramer et al., 1974), JHBPs have been confirmed over 30 species of insects and both their characteristics and general roles have been studied (Goodman and Chang, 1985). The hormone binding proteins of the circulatory system are involved not only in the mediation of hormone dispersion to distant target tissue but also in the conservation of hormone titers in the plasma at the proper physiological levels.

These proteins exert their regulatory effects on circulating hormone levels by: (1) facilitating the passage of hydrophobic hormones from the site of synthesis into the circulatory system, (2) protecting the hormone from

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enzymatic attack, (3) preventing the rapid loss of hormone due to excretion, (4) reducing non-specific binding, and (5) serving as a reservoir from which the hormone can be transferred to the target site (Westphal, 1980). From a general point of view, their basic properties of the ligand must be considered in the protein-hormone interaction: electrostatic charge, polarity of molecule, and the spatial-chemical configuration. Since the JHs are not charged, electrostatic effects may be dismissed. The second property of the ligand is of central importance to the JH–protein interaction. Not only must three-dimensional characteristics of the binding site. The addition or deletion of key substituents, or the modification of the geometrical or stereochemical configuration, will result in marked differences in the binding affinity. The hormone binding proteins can be conveniently divided into two classes based on their affinity for, and capacity to bind, their respective ligands. An association constant (Ka) of 10^{-6} M is between high-affinity (>10^{-6} M) and low-affinity (10^{-8} M) binding proteins. The two categories of binding proteins are also distinguished by the fact that low-affinity proteins have multiple binding sites while high-affinity binders usually have only one site. Recent studies on JHBP have revealed that a membrane receptor also resides both in the membrane of ovarian follicle cells of Rhodnius prolixus (Flennuck and Davey, 1985, 1987) and in that of Drosophila male accessory glands (Yamamoto et al., 1988). Further studies showed that the tritiated photoaffinity analogs of JH bound to a 38 K cytosolic protein and to an 29 K protein present in 0.5 M KCl extracts of nuclei from both epidermis and fat body of Manduca larvae (Palli et al., 1990).

In this paper, we describe a partial purification procedure which preparation of low molecular mass JHBP from Galleria mellonella homogenates.

Materials and Methods

Chemicals

JHIII. Disopropylfluorophosphate (DFP), paraoxon were purchased from Sigma: {10^{-4}_H(N)}-JHIII(15.5 Ci/mol) was purchased from New England Nuclear Chemicals.

Insect

Galleria mellonella were reared on a commercial arti-

ficial diet (Beck, 1960) under a 29±1°C, LD 0:24, RH 75% conditions and selected the final instar larvae. Insects were homogenized in 0.01 M Tris–HCl buffer (pH 7.2) containing PTU, DFP and paraoxon. The homogenate was then centrifuged at 10,000 g for 20 min at 4°C, and the supernatant was assayed as described later.

Preparation of JH stock solution and glassware

Basic stock solution of JHIII were prepared in benzene : hexane (4:1, v/v). Known amounts of tritium-labeled and unlabeled JHIII were mixed, the solvent was evaporated in stream of nitrogen, and the residue dissolved in anhydrous ethyl alcohol to give a concentration 5 x 10^{-6} M and radioactivity 7,000–8,000 CPM/µl. Glassware which could come into contact with the aqueous solution of JH was coated with polyethylene glycol (PEG) of 20,000 according to Kramer et al. (1976).

Binding analysis

Column separations were routinely monitored by preincubating the sample with 1–20 ng of labeled JHIII. After separation, 100 µl aliquots of the resulting fractions were radioassayed according to the method described by Goodman et al. (1976). When it was necessary to avoid preincubation, 100 µl aliquots of the resulting fractions were incubated with labeled JHIII (8,000 CPM/µl) and assayed for binding activity by the dextran-coated charcoal technique. Ten minutes after the addition of dextran-coated charcoal, the assay solution was centrifuged for 2 min at 10,000 g to remove unbound JH with the charcoal.

The supernatant was used for determination of JH binding activity of the protein by measuring radioactivity with a liquid scintillation counter (Rackbeta, Finland).

Determination of equilibrium constants

Increasing amounts of JHIII stock solution dissolved in benzene–hexane, ranging over 0.041–1.4 µM, were added to glass tubes. After evaporation of solvent, 200 µl JHBP fraction in 10 mM Tris–HCl buffer, 100 mM NaCl, 0.1% gelatin, pH 7.2 were added. Then, after a 5 hr incubation at 5°C, a separation of bound and unbound hormone was achieved by the method described above.

Protein determination

The Lowry method (Lowry et al., 1951) was used to measure the total amounts of protein in the homogenate sample. A low concentration of protein was determin
spectrophotometrically at 280 nm. An absorption coefficient of bovine serum albumin was used as a protein standard.

The concentration of protein was determined directly on elution from the columns.

**Electrophoresis**

Sodium dodecyl sulfate (SDS) PAGE was performed according to Laemmli (1970). Samples containing about 20 μg protein in sample buffer 20% (w/v) sucrose and 0.01% bromophenol blue were applied on the top of the gel slab. The separation was carried out at 5°C and 200 V constant. For quantitative analysis, unstained gel was sliced into 2-3 mm segments. The slices were eluted with 300 μl 25% H₂O₂ for 24 hr at 30°C. The samples of this eluent were used for the determination of the JHBP activity.

**Protein separation by gel filtration**

The homogenated sample (800 μg of protein in 10 ml) was applied to a Sephadex G-150 column (2.5 x 100 cm) equilibrated with 0.01 M Tris-HCl buffer containing PTU 10⁻⁴ M, DFP 10⁻³ M and paraxoxn 10⁻⁴ M, pH 7.2. Proteins were eluted with the same buffer at a flow rate of 30 ml/hr and 6 ml fractions were collected. Fractions which contained low molecular mass JHBP with high affinity were collected, concentrated under the nitrogen. The active materials from the column used for electrophoresis.

**Molecular mass determination**

The molecular mass of JHBP fraction in PAGE was compared with that of the standards of known molecular mass. The gel filtration was carried out at 5°C on an Sephadex G-75 column equilibrated with 20 mM Tris-HCl, 100 mM NaCl, pH 7.2. Prior to application of the protein sample, the column was calibrated using standards of known molecular mass.

**Results**

**JHBP fraction**

Gel filtration on Sephadex G-150 showed about 90% of proteins of G. mellonella final instar larvae. The conditions for Sephadex G-150 gel filtration are critical for high yield of the unmodified JHBP. This step results in removal of these undesired enzymes and in separation of low-molecular mass proteins containing JHBP activity from a rather small fraction of JHBP activity associated with high-molecular mass proteins (Fig. 1).

**Molecular weights**

When the JHBP fraction was applied to a Sephadex G-75 column, the binding activity was eluted with a distribution coefficient corresponding to a Mr. of 28,000 Da (Fig. 2, Laurent and Killander, 1964). In sodium dodecyl sulfate - polyacrylamide gel electrophoresis, the JHBP appears as a protein band at 32,000 Da (Fig. 3).
Fig. 3. Determination of molecular mass of JHBP. 20 µg JHBP after gel filtration and standard proteins of known molecular mass were electrophoresed. The molecular weight standards were phosphorylase B (Mr=97,400), BSA (66,000), egg albumin (45,000), bovine carbonic anhydrase (31,000), trypsin inhibitor (21,500) and white lysozyme (14,400).

Equilibrium binding measurements

Binding isotherms for the interaction of JHBP with JH III is presented as Scatchard plot analysis (1949, Fig. 4). The binding parameters obtained from a nonlinear least-squares fit of the data to the equation B/F = B/Kd + Bf/Kd, was Kd JH III = 3.9 x 10^{-7} M; where B is the concentration of bound hormone, F is the concentration of unbound hormone, Bf is the total concentration of binding sites, and Kd is the dissociation constant.

Discussion

Two kind of JHBP with low molecular mass have been purified from the hemolymph of M. sexta (Akama et al., 1975; Kramer et al., 1976; Goodman et al., 1978) and are JHBP with high molecular mass and three different peptide chains from the the hemolymph of G. rufus (Hartmann, 1978). Wing et al. (1984) have analyzed JHBP activities from the hemolymph of 14 species of Lepidoptera including G. mellonella. Based upon this information, we tried to purify the whole body homogenates G. mellonella JHBP. The present results indicate that whole body JHBP of G. mellonella has a low molecular mass as found in M. sexta. Ozyhar and Kochman (1987) showed that the hemolymph JHBP of G. mellonella also has a molecular mass of 32 k. In M. sexta, estimates of molecular mass (Mr) for JHBP range from 25 k to 32 k; 25 k as determined by cDNA-deduced amino acid composition (Lerro and Prestwich, 1990); 28 k by ultracentrifugation, gel filtration and amino acid composition (Kramer et al., 1976; Goodman et al., 1978).

Two forms of molecular mass for JHBP were also observed in G. mellonella by a difference in the methods; 32 k by SDS-PAGE and 28 k by gel filtration chromatography.

The difference of molecular mass between these JHBP may be due to the advances in a purification or electrophoretic conditions as mentioned by Park and Goodman (1993). The dissociation contents for JH homologs represent apparent affinities of the hormone binding, because in most experiments 10R enantiomers of non-radioactive homologs were racemic mixtures. Dissociation contents of hemolymph JHBP have been observed in several insects which have JH I as a natural hormone; the Kd of Estigmene acrea has 7.6 x 10^{-7} M (Wing et al., 1984), those of M. sexta are 1.2 x 10^{-7} M (Goodman et al., 1978), 4.4 x 10^{-7} M (Kramer et al.,
1976) and $8.8 \times 10^{-8}$ M (Koeppe et al., 1984). The Kd of G. mellonella hemolymph for JH III was $4.7 \times 10^{-7}$ M (Ozyhar and Kochman, 1987) but that of whole body JHBP $3.9 \times 10^{-7}$ M in our data. The comparison of the affinities between the purified JHBP from Galleria hemolymph and JHBP from crude extract of the cytosol of Galleria silk glands (Wisniewski, 1984) indicates that is JH bound one other of magnitude weaker to the hemolymph carrier than to the cytosol JHBP. If the difference in the electrostatic interactions in supramolecular organization has been widely recognized. Van Mellaert et al. (1985) analyzed JHBP activities in hemolymph and ovaries of Sarcophaga bullata. They postulated the uptake of JH carrier in the ovary. It has also been suggested that some other hydrophobic hormones may enter the cells in complex with their carrier protein.

**Literature Cited**


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