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

꿀벌부채명나방 종령유충에서 유약호르몬 결합단백질의 정제와 특성

Ki Heung Ahn, Sang Hak Jeon and Kyung Ro Lee
안기흥 · 전상학 · 이경로

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 electrophresis and 28 k by gel filtration. The protein exhibits a dissociation constant of 3.9×10^{-7} M for JH III.

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

초 록 - 꿀벌부채명나방 종령유충의 whole body에서 gel filtration 방법으로 유약호르몬 결합단백질을 분리, 정제하였다. 분리된 단백질은 column chromatography법과 전기영동법에 의해 등가성을 확인하였다. 이 결합단백질은 전기영동법에 의해 32 K, gel filtration에 의해 28 K의 상대적 분자량을 나타냈다. 또한, JH III에 대한 해리도는 3.9×10^{-7}M로 확인되었다.

검색어 - 유약호르몬 결합단백질, 꿀벌부채명나방, 겔여과법, 전기영동법

Insect growth and development as well as reproductive funtion are, in part, under the control of the juvenile hormones (JH). In lepidopterous larvae there are three natural hormones representing a homologus series of acylic 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

Department of Biology, Kon-Kuk University, Seoul 143-701, Korea

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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^{-6} M) binding proteins. The two categories of binding proteins are also distinguished by the fact that low-affinity proteins have multiple binding site while high-affiniy binders usually have only one site. Recent studies on JHBPs have revealed that a membrane receptor also resides both in the membrane of ovarian follicle cells of *Rhodnius prolixus* (Ilenchuck and Davey, 1985, 1987) and in that of *Drosophila* male accessory glands (Yamamoto *et al.*, 1988). Further studies showed that the tritiated photoaffinity analogous 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, Diisopropylfluorophosphate (DFP), paraoxon were purchased from Sigma: [10-^{3}H(N)]-JHIII(15.5 Ci/m mol) was purchased from New England Nuclear Chemicals.

Insect

Galleria mellonella were reared on a commercial artificial 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 ethanol to give a concentration 5 × 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, 100μl 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 5hr 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 determinde

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 applid 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% H_2O_2 for 24hr 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 × 100 cm) equilibratied with 0.01 M Tris-HCl buffer containing PTU 10^{-4} M, DFP 10^{-3} M and paraoxon 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 electroporesis.

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 an 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).

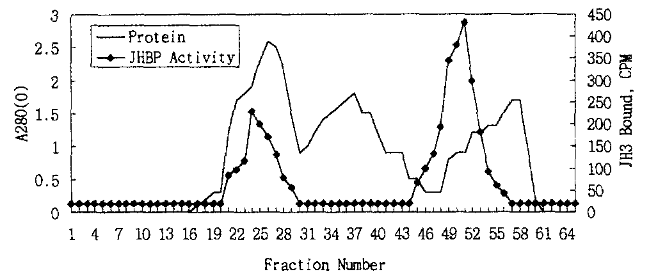

Fig. 1. Gel filtration chromatography on Sepadex G-150. 10 ml of whole body homogenate was applied to a column of Sepadex G-150.

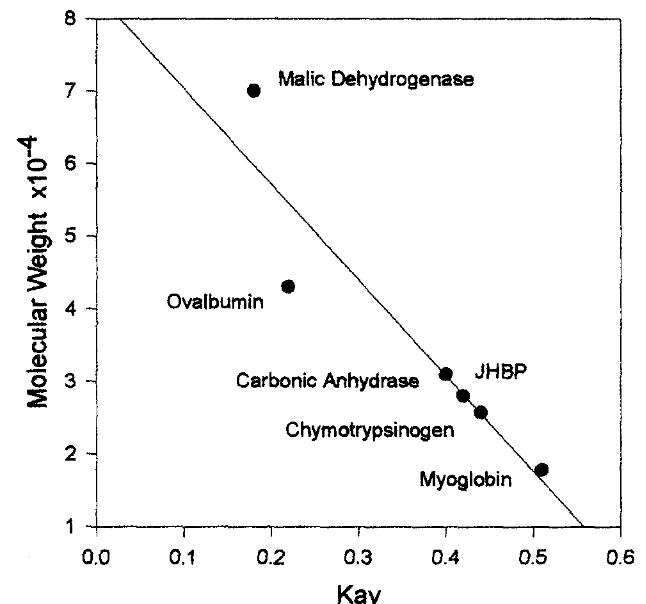

Fig. 2. Measurement of molecular mass of JHBP by gel filtration. The molecular weight standards were malic dehydrogenase (Mr=70,000), ovalbumin (43,000), carbonic anhydrase (31,000), chymotrypsinogen (25,700) and myoglobin (17,800).

Molecular weights

When the JHBP fraction was applied to a Sphadex 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).

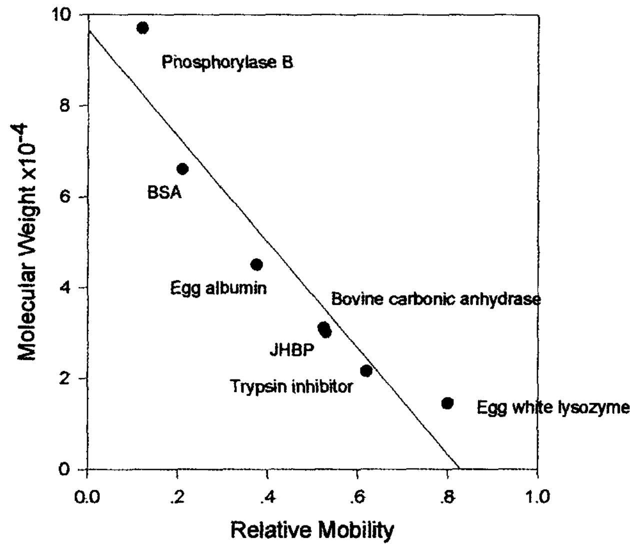

Fig. 3. Determination of molecular mass of JHBP. 20 ug JHBP after gel filtration and standard proteins of known molecular mass were electrophoresed. The molecular weight standards were phosphorylase B (Mr=97,400), BSA (66,000), egg albumin (45,000), bovine carbonic anhydrase (31,000), trypsin inhibitor (21,500) and white lysozyme (14,400).

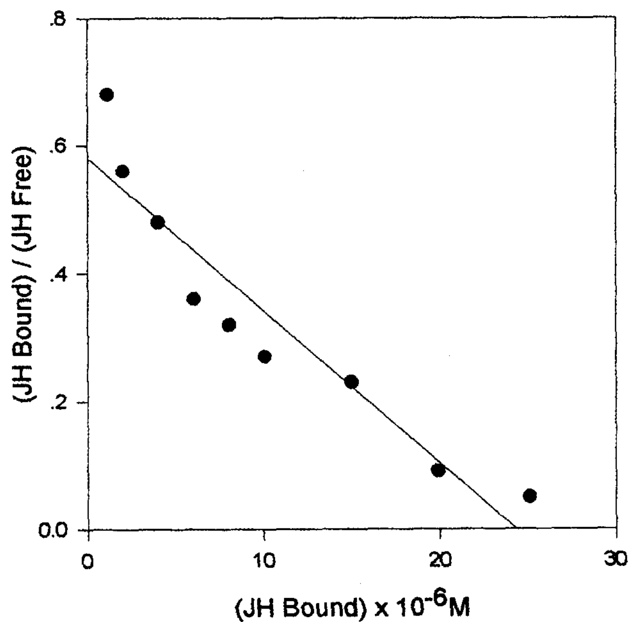

Fig. 4. Scatchard plot analysis of JH-JHBP interaction. The assay was performed by adding increasing amounts of JH III containing 10^{-6} M of labeled JH III to a fixed concentration of 0.25 uM JHBP and incubating at 5°C for 5hr.

Equilibrium binding measurements

Binding isoterms 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 $B/F=-B/Kd+B_T/Kd$, was Kd JH III=3.9×10^{-7} M; where B is the concentration of bound hormone, F is the concentration of unbound hormone, B_T is the total concentration of binding sites, and Kd is the dissociation constant.

Discussion

Two kind of JHBPs with low molecular mass have been purified from the hemolymph of *M. sexta* (Akamatsu *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 ultracentrification, 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 JHBPs may be due to the advances in a purification or electrophoretic conditions as mentioned by Park and Goodmann (1993). The dissociation contents for JH homologs represent appearent 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 7.6×10^{-8}M (Wing *et al.*, 1984), those of *M. sexta* are 1.2×10^{-7} M (Goodman *et al.*, 1978), 4.4×10^{-7} M (Kramer *et al.*,

1976) and 8.8×10^{-8} M (Koeppe *et al.*, 1984). The Kd of *G. mellonella* hemolymph for JH III was 4.7×10^{-7} M (Ozyhar and Kochman, 1987) but that of whole body JHBP 3.9×10^{-7} M in our data. The comparision 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 change between the JH carrier and the cytosol receptor had any physiological significance, one may postulate that these proteins could be formed noncovalent complexes. The role of electrostatic interactions in supramolecular organization has been widley 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.

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