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Identification and Isolation of Juvenile Hormone Binding Protein from Hemolymph of *Lymantria dispar* L.

In Hee Lee and Hak Ryul Kim

Department of Biology, Korea University, Seoul 136-701, Korea

Juvenile hormone binding protein (JHBP) was identified in the last instar larval hemolymph of *Lymantria dispar* using dextran coated charcoal (DCC) binding assay and gel filtration. The pI value of JHBP was estimated to be 5.3. JHBP was partially purified by polyethylene glycol (PEG) precipitation, DEAE-cellulose ion-exchange chromatography and gel filtration, and was confirmed by DCC binding assay.

KEY WORDS: JHBP, Hemolymph, *Lymantria dispar*

Insect metamorphosis is mainly controlled by the concentration of Juvenile hormone (JH)(Gilbert *et al.*,1978; Riddiford and Truman, 1978; De Kort and Granger, 1981). JH is synthesized by corpora allata and released into hemolymph and then transported to target tissues by Juvenile hormone binding protein (JHBP)(Westphal, 1980).

JHBP was first confirmed in hemolymph of *Hyalophora cecropia* (Whitmore and Gilbert, 1972) and then characterized and purified (Goodman *et al.*, 1976; Kramer *et al.*, 1976; Prestwich *et al.*, 1987). Two kinds of JHBP were found in hemolymph; that is, one is the protein with low affinity and large molecular weight, while the other is a high affinity and small molecular weight protein (De Kort and Granger, 1981). However, JHBP with high affinity and large molecular weight was also found in a few insects (De Kort *et al.*, 1984).

JHBP protects JH from being degraded by general carboxylesterase present in hemolymph (Goodman and Gilbert, 1978; Sparks *et al.*, 1979). JHBP was purified by gel filtration, ion exchange chromatography and isolectric focusing under the inactivation of all esterase present in hemolymph and also affinity chromatography using AH-Sepharose 4B (Pharmacia) was newly employed for purification of JHBP (Goodman and Goodman, 1981).

The present work aims to identify and partially purify JHBP present in the last instar larval hemolymph of *Lymantria dispar*.

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Materials and Methods

Chemicals

Racemic [10-^{3}H] JH III (sp. radioact. 444 GBq/mmol or 12.0 Ci/mmol) was purchased from New England Nuclear Corp. DEAE cellulose was obtained from Whatman and Sephadex from Sigma. All chemicals used were of analytical grade and obtained from various commercial sources.

Insect

Larvae of *Lymantria dispar* were reared on artificial diet (ICN Biochemicals) at 27 ± 1°C under the photoperiod of 16 hr L: 8 hr D.

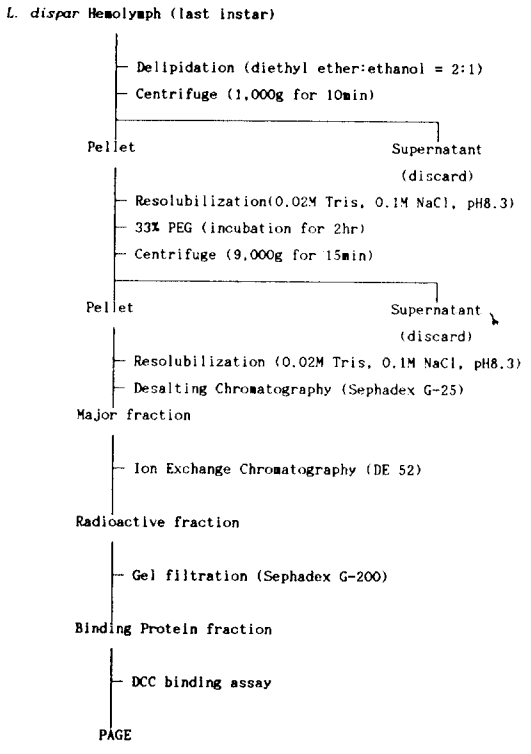

Fig. 1. Procedure of JHBP Purification.

Hemolymph collection

Hemolymph was obtained from the last instar larvae by cutting proleg. A few crystals of phenylthiourea were added to pooled hemolymph to prevent melanization. The hemolymph was centrifuged at 10,000 g for 10 min at 4°C to remove hemocytes and cell debris, and the supernatant was stored at −70°C until use.

Polyethylene glycol (PEG) precoating

Since JH and JHBP have the property to be attached to the hydrophobic surface (Goodman *et al.*, 1976), all glasses were pre-coated with PEG and then submerged in 4% PEG (MW: 20,000, 10% Methanol) for 2 hrs and washed with D.D.W.

Dextran coated charcoal (DCC) binding assay

DCC was employed to separate bound and unbound hormones. Concentrations of charcoal and dextran were 0.5% and 0.05%, respectively as described by Engelmann (1981). After organic solvent in [^{3}H] JH III (2,980 cpm) was evaporated under N_2, the hormone was redissolved in 50 μl of 0.01 M Tris buffer (5 mM $MgCl_2$, 0.15 M KCl, pH 7.4, TMK buffer). The radiolabelled JH III was incubated with certain amount of hemolymph and 0.25% γ-globulin (in TMK buffer). One hundred μl DCC was added to the hemolymph and mixed vigorously for 3 minutes and then centrifuged at 8,000 g for 2 min to precipitate unbound JH. The radioactivity of the supernatant was measured using liquid scintillation counter (Beckman LS 100 C).

Gel filtration

Hemolymph (400 μl) was diluted five times with 0.01 M Tris buffer (0.11 M NaCl, 2 mM PMSF, 0.1 mM NaN_3. 0.1 mM p-Hydroxymercuribenzoic acid (pHMB), pH 7.2) and then the hemolymph was incubated with 2.4×10^{-9} M [^{3}H] JH III (about 50,000 cmp) at 4°C for 8 hrs. After incubation, the hemolymph was eluted through a Sephadex G 100 column (1.8 × 50 cm) with 0.01 M Tris buffer (pH 7.2). Flow rate was 12 ml/hr and 4 ml per fraction was collected. Five hundred μl per fraction was put into 6 ml of scintillation cocktail (2 l Toluene, 1 l Triton X-100, 12 g omnifluor (NEN Corp.)) and the radioactivities were measured.

Isoelectric focusing

Ten μl of hemolymph was incubated with [^{3}H] JH III and polyacrylamide gel (5.5%) electrophoresis was done as described by Wrigley (1968). Two gels each were sliced at 3 mm intervals. One slice was used for the determination of pH while other gel was put into the scintillation cocktail, followed by overnight incubation and then their radioactivities were measured (Klages and Emmerich, 1979).

Gel electrophoresis

Electrophoresis was conducted on 5.5% polyacrylamide gel at the constant current of 3 mA per gel according to Davis (1964). For disc gel electrophoresis, crude hemolymph (3 μl) and the fractions from ion exchange chromatography were applied on the gel. The latter samples were con-

centrated by ultrafiltration before electrophoresis.

Isolation of JHBP

Delipidation of hemolymph — hemolymph was diluted more than 7 times with cold diethylether: ethanol (2:1, v/v) and stirred for 1 hr to remove lipid present in hemolymph. Proteins were precipitated by centrifugation at 1,000 g for 10 min at 4°C (Klages and Emmerich, 1979). The precipitate was redissolved in 0.02 M Tris buffer (0.1 M NaCl, 0.16 mM EDTA, 0.1 mM PMSF, pH 8.3) and centrifuged at 3,000 g at 4°C for 20 min, and the resulting supernatant was obtained.

Protein precipitation by PEG — Delipidated protein in buffer was mixed 1:1 (v/v) with 33% PEG (MW; 4,000) in 0.02 M Tris buffer, vortexed and incubated for 2 hrs at 4°C. This solution was centrifuged at 9,000 g for 15 min and the supernatant was discarded (Koeppe *et al.*, 1981).

Desalting chromatography — The precipitate was redissolved in 0.02 M Tris buffer and eluted through Sephadex G-25 column with the same buffer to obtain protein from PEG-protein complex (King and Tobe, 1988).

Ion-exchange chromatography — The major peaks eluted through Sephadex G-25 column were incubated with [^{3}H] JH III (50,000 cpm) for 30 min and applied to DE-52 (Whatman) column pre-equilibrated with 0.02 M Tris buffer (0.1 M NaCl, pH 8.3). The column was washed with 100 ml of the same buffer and the proteins were eluted with a linear NaCl gradient (0.1-0.5 M) in same buffer. One ml from each fraction was added to scintillation cocktail and then their radioactivity was measured.

Gel filtration — Radioactive fractions were dialyzed against distilled water to remove NaCl and concentrated using Amicon ultrafiltration. After ultrafiltration, the resulting sample was applied to a Sephadex G-200 column. Flow rate was adjusted to 8 ml/hr and fractions of major peak were used for DCC binding assay (Engelmann, 1981) to confirm the presence of JHBP.

Results

When hemolymph and [^{3}H] JH III were incubated at 4°C, binding equilibrium was reached at 8 hr after incubation (Fig. 2). Also, the optimum incubation time of charcoal solution for DCC maximum binding of free hormone to charcoal was determined to be 3 min (Fig. 3). In case hemolymph and [^{3}H] JH III (about 50,000 cpm) were incubated and applied to gel filtration, there were two peaks for proteins while one peak for radioactivity and second protein peak was over-

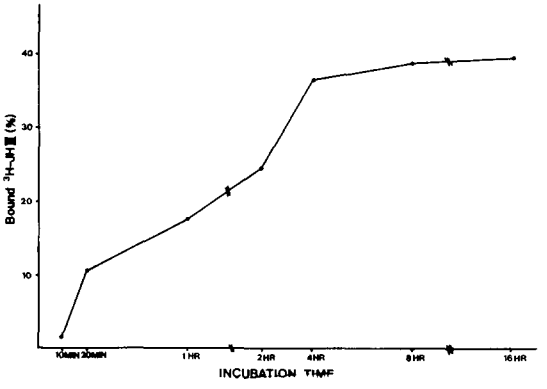

Fig. 2. The effect of incubation time on binding of [^{3}H] JH III (2,980 cmp) to hemolymph. As is seen, binding of the JH to hemolymph protein has reached equilibrium after 8 hr.

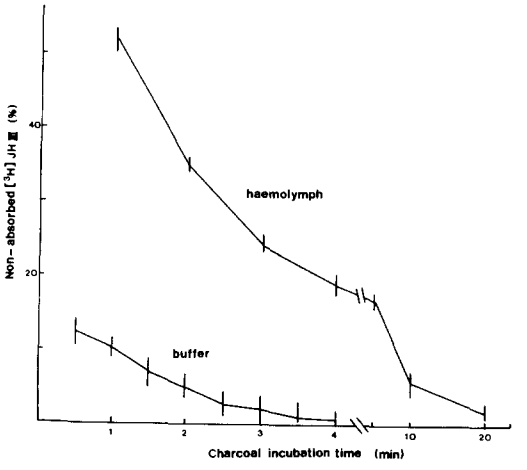

Fig. 3. Adsorption of [^{3}H] JH III by charcoal. Following the incubation in buffer alone or hemolymph (2 μl) for 90 min the charcoal suspension was added and removed at the given time intervals. In all cases, 20,000 cpm as [^{3}H] JH III had.

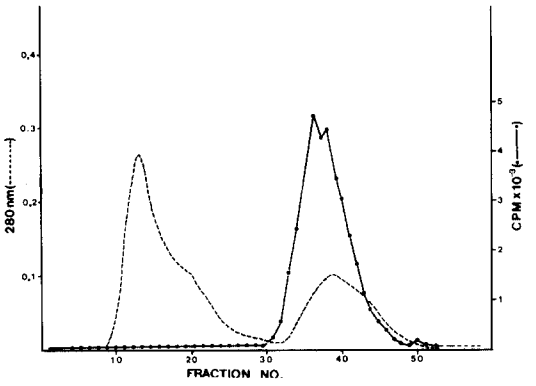

Fig. 4. Elution pattern on Sephadex G-100 of hemolymph proteins of *Lymantria dispar*. This shows one major peak of [^{3}H] JH III-protein complexes following *in vitro* incubation of [^{3}H] JH III with the hemolymph of last larvae.

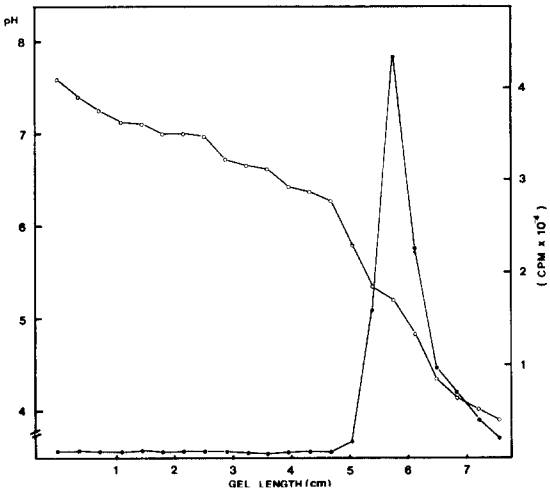

Fig. 5. Isoelectric focusing in a 5.5% polyacrylamide gel of 10 μl hemolymph with a pH 3-10 gradient. The hemolymph was incubated with 1.2×10^{-9} M [^{3}H] JH III. ○—○ : pH profile ●—● : radioactivity.

lapped with radioactivity peak (Fig. 4). This result indicates that second peak contains JHBP. Hemolymph proteins were isoelectrically focused and the radioactivity of each slice was determined (Fig. 5). This result shows that isoelectric point of JHBP is 5.3.

When hemolymph proteins were precipitated by PEG, one major peak was obtained from the precipitate by desalting chromatography (Fig. 6). This

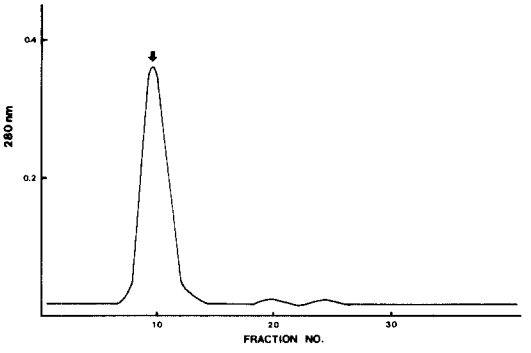

Fig. 6. The elution profile of desalting chromatography. The resolubilized plasma protein was added to an equal volume of 33% PEG 6,000. After the mixture was vortexed, it was centrifuged at 9,000 g for 15 min. And then the precipitate was applied to Sephadex G-25 column. The arrow indicates the first preparation to isolate JHBP.

sample was then applied to DEAE-cellulose ion-exchange chromatography and eluted with NaCl gradients in buffer. Third peak showed a high radioactivity (Fig. 7). These proteins were concentrated and applied to Sephadex G-200 column. One major peak was obtained through gel filtration (Fig. 8) and the proteins of the peak showed binding activity by DCC binding assay (Fig. 9).

Discussion

The presence of JHBP in insect hemolymph was mainly confirmed by binding assay and gel filtration (Emmerich and Hartmann, 1973; De Kort *et al.*, 1983). This protein was known to consist of two types with different characters (Westphal, 1980). One type has high affinity to JH and small molecular weight, while the other bears low affinity to JH and large molecular weight. In the present work with *L. dispar*, one JHBP was confirmed in larval hemolymph but its characteristics were not yet determined. According to Klages and Emmerich (1979), pI values of JHBP in hemolymph were determined to be 5.1 and 8.9 $\pm$ 0.2, respectively. The pI value of JHBP in *L. dispar* was found to be 5.3.

Since the amount of JHBP in hemolymph is relatively small and binding activity is unstable

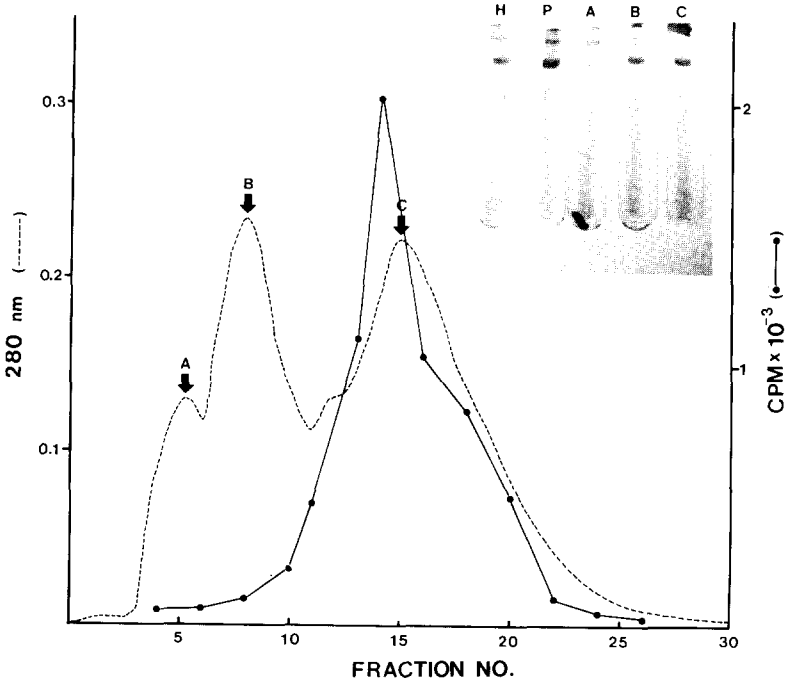

Fig. 7. DEAE-Cellulose anion exchange chromatographic elution profile. Dotted line, absorbance at 280 nm; solid line, JH-binding protein. The inset represents polyacrylamide gel electrophoretic profiles of: (H) hemolymph; (P) hemolyph proteins precipitated with PEG; (A) fractions 3-5; (B) fractions 7-9; (C) fractions 16-18.

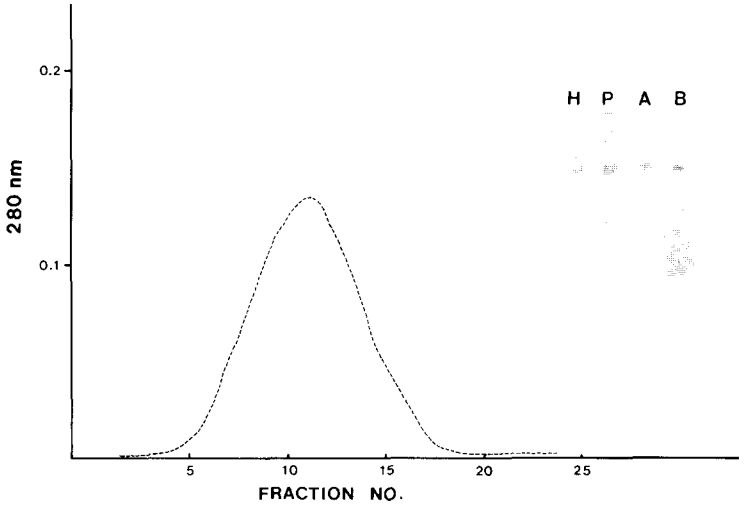

Fig. 8. Sephadex G-200 separation of concentrated partially purified JHBP from an ion exchange chromatography. Fractions (9-13) were used for DCC binding assay. The inset represents polyacrylamide gel profiles of: (H) hemolymph; (P) Hemolymph proteins precipitated with PEG (33%); (A) JHBP fraction from ion exchange chromatography; (B) JHBP fraction (10-12) from Sephadex G-200 column.

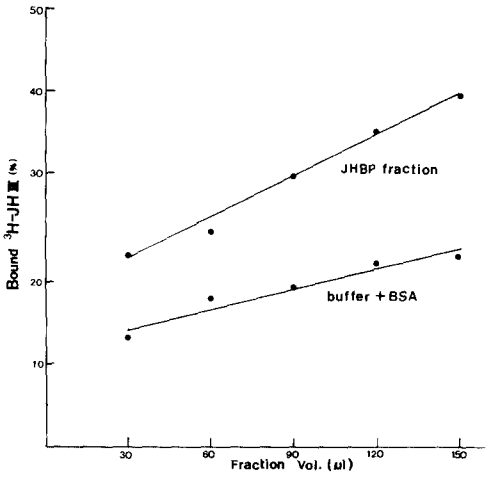

Fig. 9. Binding of [^{3}H] JH III (2,980 cpm) to proteins in fraction from Sephadex G-200 gel filtration. The fraction contained 5 μg of proteins per ml. Buffer contained 2.5 mg γ-globulin per ml.

during purification, large insects such as larvae of *M. sexta* (Kramer *et al.*, 1976) and *L. migratoria* (Koopmanschap and De Kort, 1988) were used for large amount of hemolymph collection, and lipid was extracted from hemolymph and inhibitors (PMSF and pHMB) were added to the delipidated hemolymph proteins for purification of JHBP. Thirty three % PEG was found to have binding capacity to all proteins including JHBP (Koeppe *et al.*, 1981; King and Tobe, 1988). In the purification of JHBP from *L. dispar*, the plasma proteins were first precipitated by 33% PEG. DCC, PEG and hydroxylapatite (HAP) assays were mainly used for binding assay of JHBP. The charcoal effectively competes with the binding proteins of the hemolymph for JH and so can strips the JH from JHBP-JH complex. In the present work, DCC assay (Engelmann, 1981) was adopted and charcoal incubation period was determined by directly measuring the extent charcoal is binding [^{3}H] JH III in buffer containg hemolymph and [^{3}H]JH III in buffer alone. Charcoal insufficiently bound free hormone for short period of incubation (less than 3 min) but stripped the JH from JHBP-JH complex for long period of incubation (more than 5 min). Accordingly, we determined 3 min for the standard charcoal incubation period (Fig. 3).

For the molecular characteristics and detailed physiological function of JHBP in *L. dispar*, further purification is required.

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매미나방(***Lymantria dispar***)에서 Juvenile Hormone Binding Protein(JHBP)의 확인 및 정제

이인희 · 김학열(고려대학교 이과대학 생물학과)

매미나방 종령유충 혈림프내에 존재하는 JHBP을 Dextran Coated Charcoal(DCC) binding assay와 gel filtration에 의해서 확인하였고, JHBP의 pI값은 5.3으로 밝혀졌다. JHBP의 정제는 혈림프단백질을 먼저 PEG로 침전시킨 후 ion exchange chromatography와 gel filtration 방법을 통하여 행하였다. 정제된 fraction의 JH에 대한 binding activity는 [^{3}H] JH-III의 radioactivity 측정과 DCC binding assay를 통해 확인하였고, 정제된 단백질의 순수도는 각 정제단계에 따라 전기영동을 하여 확인하였다.