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Identification and Purification of Juvenile Hormone Binding Protein from *Hyphantria cunea* Drury

In Hee Lee and Hak Ryul Kim

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

We identified juvenile hormone binding protein (JHBP) from last instar larval hemolymph of *Hyphantria cunea* using gel filtration and non-SDS PAGE. Two kinds of JHBP in hemolymph were found at two peaks by gel filtration (Sephadex G-100) and also at Rm values of 0.13 and 0.57 by non-SDS PAGE.

JHBP was partially purified using anion exchange chromatography, preparative gel filteration, and preparative PAGE. Dextran coated charcoal (DCC) binding assay was employed to monitor the location of JHBP in chromatographic profile during the purification process. Purity of JHBP was checked by silver staining of 10% SDS-polyacrylamide.

KEY WORDS: Juvenile Hormone Binding Protein, hemolymph, *Hyphantria cunea*

Insect metamorphosis is controlled mainly by the concentration of juvenile hormone (JH)(Gilbert *et al.*, 1978; Riddiford and Turunen, 1978; De Kort and Granger, 1981). JH is transported to target tissue by juvenile hormone binding protein (JHBP) in hemolymph (Westphal, 1980). It has also been reported that JHBP protects JH from attack by general carboxylesterase present in hemolymph (Goodman and Gilbert, 1978; Sparks *et al.*, 1979).

JHBP was first identified in hemolymph of *Hyalophora cecropia* by analytical gel filtration (Whitmore and Gilbert, 1972) and then purified by ion exchange chromatography, gel filtration and preparative isoelectrofocusing and partially characterized in a few insect species (Goodman et al., 1976; Kramer *et al.*, 1976; Prestwich *et al.*, 1987). Two kinds of JHBP were found in hemolymph; one with a low affinity and large molecular weight(De Bruijn *et al.*, 1986), and the other with a high affinity and small molecular weight protein (De Kort and Granger, 1981; Dillwith *et al.*, 1985). JHBP with high affinity and large molecular weight was also found in *Locusta migratoria* (De Kort *et al.*, 1984) and *Drosophila melanogaster* (Shemshedini and Wilson, 1988).

This work is aimed at identification and purification of small molecular weight JHBP from hemolymph of *H. cunea* using gel filtration and electrophoresis.

Materials and Methods

Insects and hemolymph collection

Larvae of *Hyphantria cunea* were maintained on artificial diet (Dong Bang Co., Ltd.) at 28 ± 1° C with the photoperiod of 16 hr light : 8 hr dark. Hemolymphs were 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 10,000g for 10 min at 4°C to remove hemocytes and cell debris, and the supernatant was stored at -70°C until use.

Identification of JHBP

Gel filtration

Hemolymph was diluted three times with 0.01M Tris, 0.1M NaCl buffer (TN buffer) including 10

10^{-4}M phenylmethylsulfonyl fluoride (PMSF) and 10^{-4}M paraoxon and then incubated with 10^{-9}M [^{3}H] JH III at 4°C for 1hr. After incubation, the hemolymph was eluted from Sephadex G-100 column (1.8 x 55 cm) with TN buffer. Fractions(2 ml) were collected at flow rate of 12 ml/hr. Two hundreds μl per fraction was put into 10 ml scintillation cocktail(2l toluene, 1l triton X-100, 12g omnifluor (NEN)) to measure radioactivity.

Electrophoresis

After dilution with TN buffer followed by incubation with [^{3}H]JH III at 4°C for 1hr, hemolymph was electrophoresed on 6% native polyacrylamide gel. The gel was then sliced to detect the position of a radioactivity in the gel. Each slice was soaked in scintillation cocktail for overnight.

Purification of JHBP

DCC binding assay

DCC binding assay (Engelmann, 1981) was employed to monitor the presence of JHBP during the purifying process. The concentrations of charcoal and dextran each were 0.5% and 0.05%, respectively. One hundred microliter taken from each fraction of purifying step was incubated with [^{3}H]JH III (20,000 cpm) for 1hr, was incubated in DCC solution for 2 min, and was centrifuged at 8,000g for 30 sec to precipitate unbound hormone. The supernatant (100 μl) was mixed with scintillation cocktail and radioactivity was measured using Liquid Scintillation Counter (Beckman LS 100C).

Polyethylene glycol(PEG) precipitation

Hemolymph was mixed with 33% PEG (MW:6,000)(1:4 ratio). The sample was incubated for 1hr at 4°C and centrifuged at 9,000g for 20 min to precipitate protein. The precipitated protein was redissolved in TN buffer.

Preparative ion exchange chromatography

DEAE cellulose (DE 52, Whatman) column was washed with TN buffer over 12hr and hemolymph proteins were precipitated by PEG. Precipitated hemolymph protein was eluted first with 0.01M Tris buffer (pH 8.2, 1 vol. of resin) and then linear gradient of NaCl (0.1-0.5M, 200 ml).

Preparative gel filtration

Fractions eluted from DEAE cellulose column were subjected to DCC binding assay to detect the presence of JHBP. Fractions containing JHBP were concentrated through lyophilization and applied on Sephadex G-50 (27 cm) and Sephadex G-200 (27 cm) column (1.8 × 54 cm). The column was equilibrated with TN buffer and fractions (2.6 ml) were collected at flow rate of 12 ml/hr.

Preparative electrophoresis

Fractions containing JHBP were pooled, concentrated by lyophilization, and incubated with [^{3}H]JH III for 1hr. The mixture was reacted with 0.5% DCC solution for 2 min to remove [^{3}H]JH III unbound to JHBP. The samples removed of the unbound [^{3}H]JH III were loaded on 6% polyacrylamide gel (128mm × 6mm i.d.). After electrophoresis, gel was cut at 3 mm intervals and each of gel slices was put into scintillation cocktail for radioactivity. JHBP was recovered by electroelution from slices of another gel which is equivalent to the one with high radioactivity.

Analytical electrophoresis

Partially purified JHBP was confirmed on 10% SDS-polyacrylamide gel described by Laemmli (1970). Fractions containing JHBP in each step of purification were collected and concentrated. Proteins were electrophoresed and stained with silver nitrate according to Wray *et al.* (1981).

Results

Identification of JHBP

When hemolymph of last instar larvae treated with esterase inhibitor (paraoxon) was incubated with [^{3}H]JH III and subjected to gel filtration, two protein peaks were shown in elution profile (Fig. 1). One hundred microliter of each fraction was used to measure radioactivity and each fraction with high radioactivity was subjected to DCC binding assay (data not shown). Results showed that there were three high radioactivity peaks but

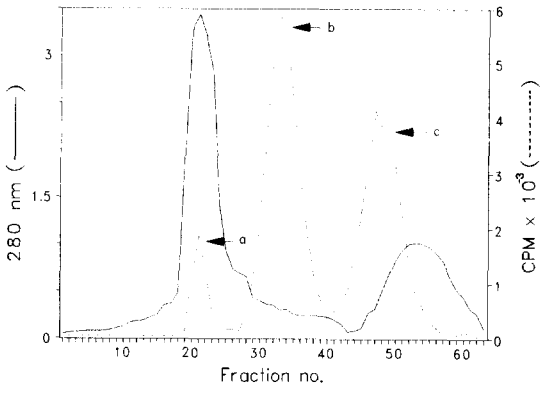

Fig. 1. Gel permeation chromatography of hemolymph on a column of Sephadex G-100. The hemolymph was treated with paraoxon (10^{-4}M) and incubated with [^{3}H] JH III (10^{-9}M) for 30 min. The radioactivity of each fraction (500 μl) was counted in Omnifluor cocktail (NEN) using Beckman LSC 100. This confirmed three peaks of radioactivity on JHBP in hemolymph. It was supposed that first peak (a) was due to low affinity high MW JHBP, second peak (b) to high affinity low MW JHBP (Killwith *et al.*, 1985) and third peak (c) resulys from unbound radioactivity.

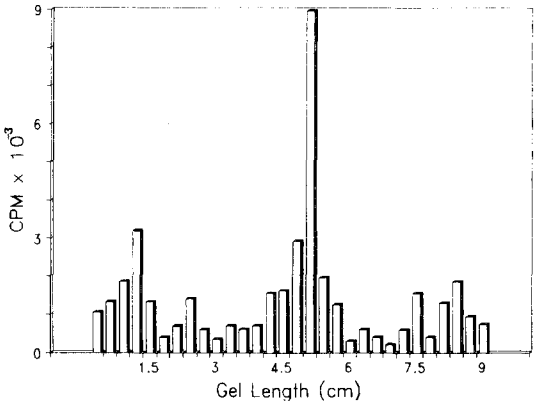

Fig. 2. Distribution of radioactivity in native 6% polyacrylamide gel of hemolymph from last instar larvae. The hemolymph was incubated with [^{3}H]JH III in the presence of paraoxon. Following incubation, unbound radioligand in the sample was removed by DCC. The sample was subjected to PAGE, and then the gel was sliced by 3 mm interval. Each slice was soaked in the scintillation cockail for 10 hours, and counted the radioactivity.

the second peak showed characteristic binding activity.

Hemolymph proteins incubated with [^{3}H]JH III were electrophoresed on 6% native gel and gel was cut into 3mm segments for radioactivity. The result showed that there were two radioactivity peaks at Rm values of 0.13 and 0.57, and the second peak was higher than the first one (Fig.2).

Purification of JHBP

As a first step of purification, hemolymph proteins precipitated by 33% PEG (6,000) was redissolved in TN buffer followed by anion exchange chromatography (DE 52). Four protein peaks were obtained and posterior part of first peak was shown to contain JHBP by DCC binding assay (Fig. 3). Fractions containing JHBP were pooled, concentrated and subjected to gel filtration. Elution profile showed one protein peak and posterior part of peak (7 fractions) was shown to contain the binding activity by DCC binding assay (Fig. 4.). In addition, each fraction was electrophoresed on 10% SDS polyacrylamide gel to compare and confirm the presence of JHBP (Fig. 5). To further purify JHBP fractions from the

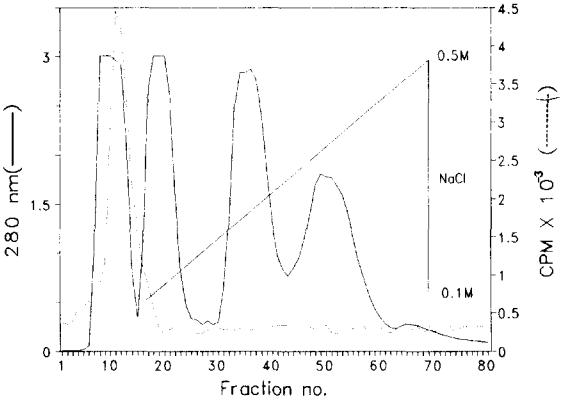

Fig. 3. Ion exchange chromatography of hemolymph proteins precipitated with 33% PEG on DE 52. Unbound proteins were eluted with 0.01M Tris, 0.1M NaCl buffer, pH8.2, and bound proteins were eluted with a gradient of 0.1M to 0.5M NaCl in the same buffer. Fractions were analyzed for protein at 280 nm, and radioactivity in fractions was measured using DCC binding assay. Fractions (9-13) containing JHBP were lyophilized, and used for next step of purification.

gel filtration containing JHBP were incubated with [^{3}H]JH III and electrophoresed. The disc gel was sliced to detect the position with a specific

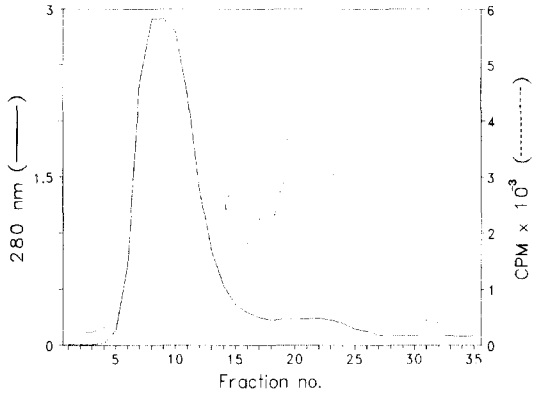

Fig. 4. Gel filtration of Sephadex G-200 and G-50 of radioactive fractions (8-11) from ion exchange chromatography (Fig. 3). By means of DCC binding assay of each fraction, binding activity was confirmed in 20-23 fractions.

radioactivity in the gel. High radioactivity was detected in the range of the same Rm value as in Fig. 2 (Fig. 7). JHBP in the slices was electrophoresed on 10% SDS polyacrylamide gel to check the purity of the protein (Fig. 8). This JHBP was electrophoresed with low molecular weight standard marker to determine M.W. of subunit of JHBP. The molecular weight of JHBP subunit was estimated to be 32kD (data not shown).

Discussion

It has been generally known that two kinds of JHBP are present in hemolymph of insects (Klages and Emmerich, 1979; Turunen and Chippendale, 1981; Lenz *et al.*, 1986). In the

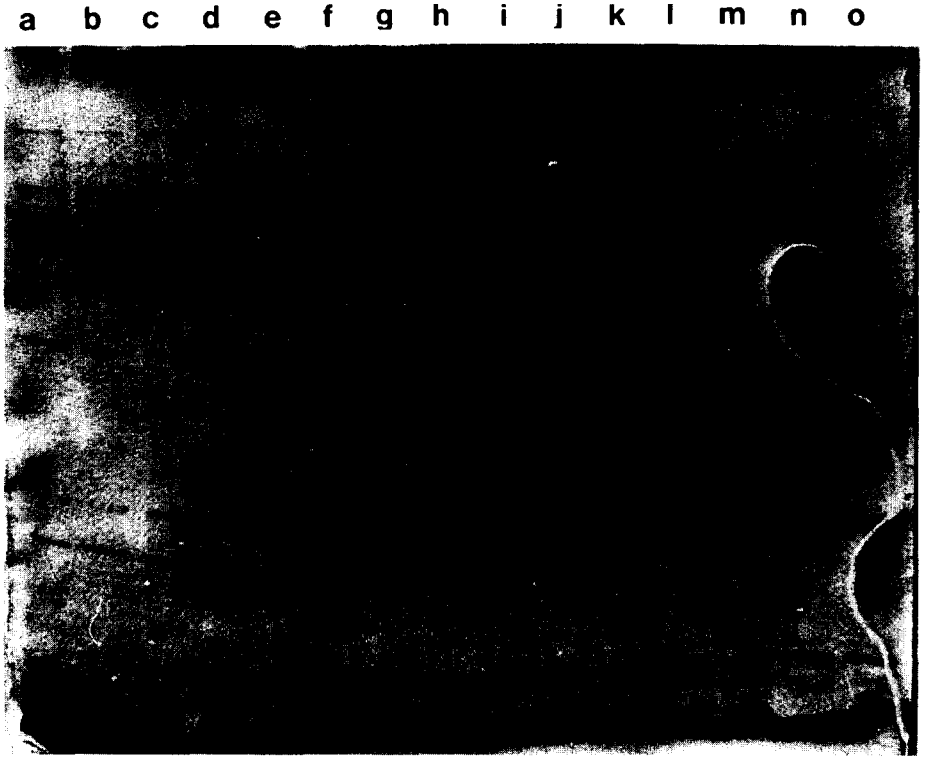

Fig. 5. Fractions (9-23) of gel filtration (Fig. 4) were electrophoresed on 10% SDS-polyacrylamide gel. One μl of protein was loaded on each lane. After electrophoresed, the gel was stained by silver nitrate. Symbols (a-o) showed fractions (9-23), respectively. Arrow indicates JHBP.

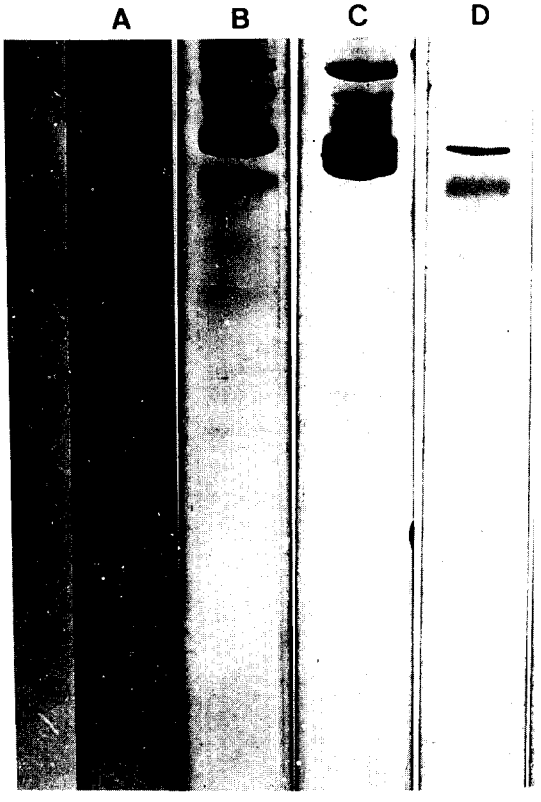

Fig. 6. Steps in the purification of the JHBP in the hemolymph of *H. cunea* monitored using electrophoresis in 6% polyacrylamide gel which stained with coomassie blue. (A) Cell free hemolymph. (B) Hemolymph protein precipitatd with 33% PEG. (C) Fractions (9-13) of Ion exchange chromatography on DE 52. (D) Fractions (20-23) of Gel filtration on Sephadex G-200 and G-50.

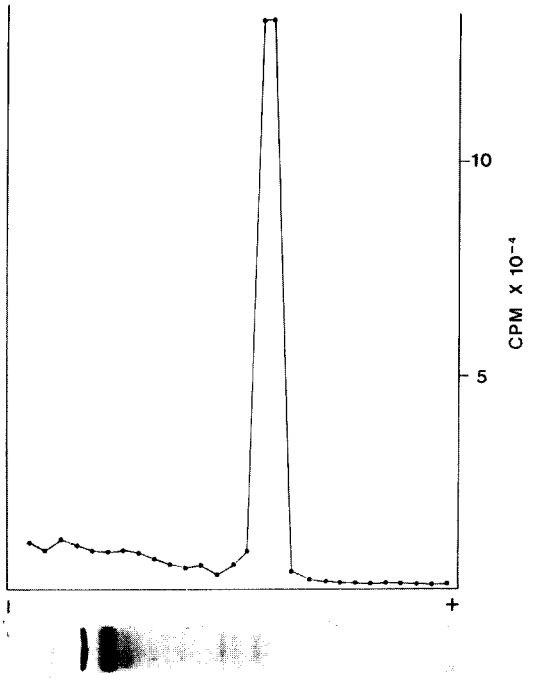

Fig. 7. Distribution of radioactivity in native 6% polyacrylamide gel of radioactive fractions (20-23) from gel filtration. Fractions were incubated with [^{3}H]JH III and unbound radioligand in the samples was remoed by DCC before electrophoresis. The gel was sliced by 3 mm interval and each was counted the radioactivity.

present experiment with *Hyphantria cunea*, JHBP was found in larval hemolymph by gel filtration and electrophoresis. Two kinds of JHBP was shown to exist in hemolymph of *H. cunea* by gel filtration (Sephadex G-100)(Fig. 1). The binding activity of proteins in each fraction was measured by DCC binding assay. We concluded that the third peak (c) in radioactivity profile was due to unbound [^{3}H]JH III (data not shown). Hemolymph proteins were electrophoresed on 6% polyacrylamde gel and the gel was sliced to measure a radioactivity in it. High radioactivity indicated that JHBP was present in hemolymph (Fig. 2). Proteins in the process of purification showed one characteristic radioactivity peak due to the presence of JHBP (Fig. 7). This implies that other proteins except JHBP are removed in great amounts in the course of purification. JHBP could not be identified with coomassie blue staining because the amounts of JHBP in hemolymph was very low (Fig. 6), but *H. cunea* JHBP was able to be shown by silver staining (Figs.5 and 8).

As a first step of purification in the present work, hemolymph proteins were first precipitated with 33% PEG to reduce activities of protease and esterase. To confirm the presence of JHBP during purification, photoaffinity labelling method using [^{3}H]epoxyfarnesyl diazoacetate ([^{3}H]EFDA) has been employed by others (Koeppe *et al.*, 1984; Wang *et al.*, 1989; Prestwich and Atkinson, 1990), but DCC binding assay used in this work was also proved to be effective in monitoring JHBP in each step of purification. Column packed first with Sephadex G-200 and G-50 (1:1) was used to narrow the scope of characteristic

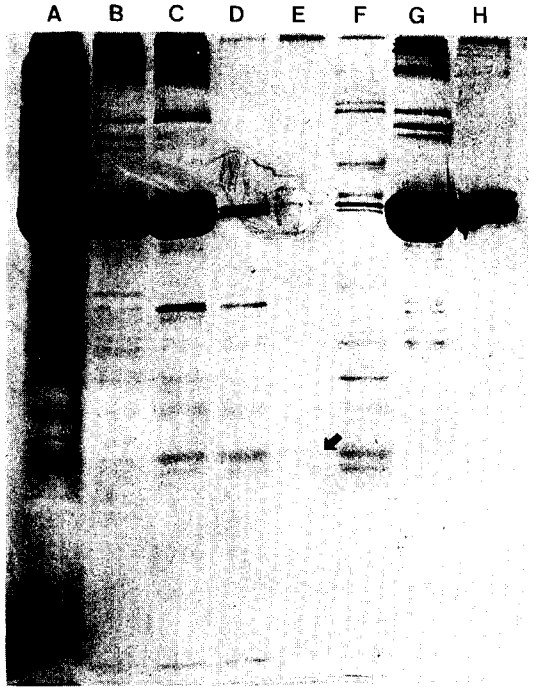

Fig. 8. Electrophoretic profile on 10% SDS-polyacrylamide gel of JHBP during purification. The gel was stained by silver nitrate. The arrow indicates JHBP. A,B,C,D,E,F; fractions containing JHBP. G,H; fractions containing no JHBP. (A) Cell free hemplymph. (B) Hemolymph proteins precipitated with 33% PEG. (C) Fractions (9-13) of Ion exchange chromatography on DE 52. (D) Fractions (20-23) of Gel filtration on Sephadex G-200 and G-50. (E) JHBP isolated by gel slice. (F) Fractions (32-34) in Fig. 1. (G) Fractions (22-24) in Fig. 3. (H) Fractions (8-10) in Fig. 4.

radioactivity due to the presence of JHBP in elution profile.

Molecular weight of hemolymph JHBP have been determined in many insect species including *Diatraea grandiosella* with 30kD (Dillwith *et al.*, 1985), *Leucophaea maderae* with 220kD (Rayne and koeppe, 1988), *Drosophila melanogaster* with 400kD (Shemshedini and Wilson, 1988). JHBP of *Hyphantria cunea* in present work was estimated to have M.W. of 32kD which is similar to that of JHBP of *D. grandiosella*.

This report is a preliminary work to purify JHBP in a large amounts to investigate the property and physiological role of JHBP in *H. cunea*.

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미국흰불나방(***Hyphantria cunea*** D.)의 유충호르몬 결합단백질의 확인 및 정제

이인희 · 김학열(고려대학교 생물학과)

미국흰불나방의 종령유충 혈림프에 존재하는 유충호르몬 결합단백질(JHBP)을 [^{3}H]으로 표지된 유충호르몬(JH)을 사용하여 혈림프의 gel filtration과 전기영동으로써 확인하였다.

JHBP의 정제는 ion exchange chromatography, gel filtration과 전기영동의 방법으로써 수행되었는데 정제과정중의 JHBP의 존재위치는 DCC 결합분석법을 이용하여 확인하였다. 혈림프내에 존재하는 JHBP의 상대량이 매우 적으므로 정제과정중의 JHBP의 확인과 정제된 JHBP의 전기영동상에서의 확인은 silver staining방법으로써 실행하였다.