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Characterization of High Affinity Juvenile Hormone Binding protein in the Hemolymph of *Bombyx mori* L.

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Hemolymph JHBP (hJHBP) was partially purified from last instar larvae of *Bombyx mori* by gel filtration and their optimal reaction conditions of dextran coated charcoal binding assay were determined. Dissociation constant (K_D) of hJHBP for JH III was calculated to be 1.45×10^{-7} M at 4°C. The molecular weight of hJHBP was estimated to be 30 kDa by gel filtration on a calibrated Sephadex G-100 column and 33 kDa by SDS-PAGE. These results indicate that hJHBP consists of a single polypeptide chain. Isoelectric point of hJHBP was found to be pH 5.1 and 19 of the first 20 amino acid residues were determined from N-terminus of purified hJHBP.

KEY WORDS: Juvenile Hormone, Juvenile Hormone Binding Protein, Hemolymph, *Bombyx mori*

Juvenile hormone (JH) plays a very important role in development and reproduction of insects. The concentration of JH in hemolymph is controlled by various factors including juvenile hormone binding protein (JHBP) (de Kort and Granger, 1981). JHBP prevents JH from being hydrolyzed by general esterases by combining with JH specifically (Sanburg *et al.*, 1975) and also transports JH from synthetic place to various target cells. JHBP is also presumed to prevent JH concentration from drastic dropping due to excretion and acts as reservoir directly available to target cells (Goodman and Chang, 1985). It has been known that high affinity low molecular weight JHBP is present in hemolymph together with low affinity high molecular weight JHBP in lepidopteran larvae including *Manduca sexta* (Kramer *et al.*, 1976a; Lenz *et al.*, 1986), *Galleria mellonella* (Rudnicka *et al.*, 1979), *Diatraea grandiosella* (Turunen and Chippendale, 1979; Dillwith *et al.*, 1985), *Diatraea crambidoides, Diatraea saccharalis, Ostrinia nubilalis* and *Homoeosoma electellum* (Lenz *et al.*, 1986). However, high affinity high molecular weight (over 100 kDa) JHBP was found in some other insects such as *Diploptera punctata* (King and Tobe, 1988), *Periplaneta americana* (de Kort *et al.*, 1984), *Leucophaea maderae* (Engelmann *et al.*, 1988; Koeppe *et al.*, 1988), *Locusta migratoria* (Koopmanschap and de Kort, 1988), *Drosophila melanogaster* (Shemshedini and Wilson, 1988) and *Sarcophaga bullata* (Mellaert *et al.*, 1985). In Orthoptera, lipophorin performs the role of JHBP and its binding site for JH is known to be apolipophorin I (Koeppe *et al.*, 1988; de Kort and Koopmanschap, 1989; King and Tobe, 1992). Lipophorin is also present in lepidopteran larvae, but its affinity for JH is very weak that it is close to nonspecific binding. Therefore, JH binding is absolutely due to high affinity low molecular weight JHBP in lepidopteran insects.

The present work is to characterize JHBP in

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hemolymph of *Bombyx mori* as basic data to elucidate the physiological role of JHBP in hemolymph and in the other organs.

Materials and Methods

Chemicals

All reagents used in the present work were of reagent grade and purchased as follows: [^{3}H]JH III (specific activity 13 Ci/mmol) (New England Nuclear); unlabeled JH III, diethyl p-nitrophenyl phosphate (paraoxon), polyethylene glycol 20,000, dextran, pharmalyte (pH 3-10), Sephadex G-100, low molecular weight protein standards for gel filtration, sodium dodecyl sulphate, 2-mercaptoethanol, phenylthiourea, acrylamide, bis-acrylamide, glycine (Sigma Chemical Co.); low molecular weight protein standards for SDS-PAGE (BioRad); activated charcoal (Merck); Omnifluor (Dupont)

Insects and collection of hemolymph

Eggs of silkworm, *Bombyx mori* (Baekokjam, Jam 123 × Jam 124), were obtained from Sericultural Experiment Station and reared on an artificial diet (Dong Bang Co., Ltd.) at 26 ± 1°C and 70 ± 10% R.H. under the photoperiod of 16L : 8D. Hemolymph was collected from one day old fifth instar larvae by cutting prolegs and put it into a cold test tube containing a few crystals of phenylthiourea. The hemolymph was centrifuged at 10,000 g for 10 min and the supernatant was stored at −70°C until used.

DCC binding assay

Dextran coated charcoal (DCC) binding assay was carried out as described by Kramer *et al.* (1976b) with slight modification. Activated charcoal was washed with 1 N HCl, distilled water and 1% (w/v) $NaHCO_3$ and then with distilled water until neutral. The washed charcoal was completely dried and coated with dextran by dissolving 0.5% dextran in 100 ml of 10 mM Tris/1.5 mM EDTA/3 mM NaN_3 (pH 7.3). One gram of dried charcoal was added to this solution and stirred at 4°C overnight. This was washed with above buffer solution five times and diluted two-fold for use. DCC assay was conducted in the following order. Appropriate amount of [^{3}H]JH III dissolved in ethanol was put into the test tube and evaporated under a stream of nitrogen gas. One hundred μl of protein solution was added to the test tube, vortexed and incubated at 4°C for 30 min. One hundred μl of the charcoal solution was again added to this mixture with constant stirring for 2 min and centrifuged at 10,000 g for 1 min. One hundred μl was taken from the supernatant containing unbound hormone and the radioactivity was measured in 10 ml of scintillation cocktail (toluene 2 l/Triton X-100 1 l/Omnifluor 12 g) in a liquid scintillation counter (Beckman LS 100C) at 50% counting efficiency.

Polyethylene glycol coating

Since JH and hJHBP were easily absorbed to hydrophobic surfaces, all glasswares were coated with 1% (w/v) polyethylene glycol to prevent their attachment.

Gel filtration

Hemolymph (1 ml) of fifth instar larvae (day 1) was mixed with the equal volume of 10 mM Tris-HCl/50 mM NaCl (pH 7.3) containing 2×10^{-4} M paraoxon and equilibrated for 1 hr at 4°C and then incubated with 10^{-7} M [^{3}H]JH III for 30 min. This sample was eluted from calibrated Sephadex G-100 column (1.9 × 95 cm). Fractions (3 ml) were collected at the flow rate of 20 ml/hr. Absorbance of the eluate was monitored at 280 nm and 100 μl of each fraction was mixed with 10 ml of scintillation cocktail for the measurement of radioactivity. Protein standards used for the calibration of the column were bovine serum albumin (66,000), carbonic anhydrase (29,000) and cytochrome c (12,400). Blue dextran 2000 was used to determine the void volume.

Polyacrylamide gel electrophoresis

Samples were mixed with sample buffer and boiled at 100°C for 2.5 min. Electrophoresis was carried out on a 10% gel at constant current of 1 mA/well for 2 hrs using discontinuous buffer system as described by Laemmli (1970). Following electroporesis, gels were stained with Coomassie brilliant blue R-250 and destained with the mixture

of 70 ml of acetic acid, 930 ml of methanol and 1,000 ml of distilled water.

Isoelectric focusing

Isoelectric point of hJHBP was determined with 20 mM sodium hydroxide in anode and 10 mM phosphoric acid in cathode according to the method of Wrigley (1968). Hemolymph and partially purified JHBP were incubated with [^{3}H] JH III for 1 hr and the focusing was carried out on a 5.5% gel. After focusing had been completed, sample and control gel were cut into 5 mm segments. Each segment was mixed with 10 ml of scintillation cocktail (sample gel) or with 2 ml of distilled water (control gel) and left with constant shaking for 20 hrs for the measurement of radioactivity or pH.

N-terminal sequence analysis

N-terminal amino acid sequence of purified JHBP was analyzed using Milligen 6600B sequencer.

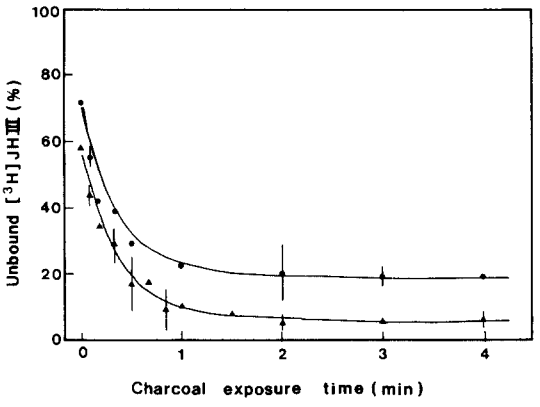

Fig. 1. Binding of [^{3}H]JH III by charcoal as a function of incubation time. After [^{3}H]JH III was equilibrated with buffer alone (▲—▲) and diluted hemolymph (●—●) for 30 min at 4°C, the charcoal suspension was added and pelleted by centrifugation at the given time intervals. One hundred μl was taken from the supernatant containing unbound [^{3}H]JH III for the measurement of radioactivity. Individual data points are the means of triplicate determinations. Vertical bars represent SEM when larger than the size of data points.

Results

Optimization of DCC binding assay conditions

If charcoal exposure time is too long in DCC binding assay, hormone combined with JHBP is detached but too short, unbound hormone remained in large amounts. Therefore, to determine proper charcoal reaction time, DCC solution was put into the test tube containing hemolymph and [^{3}H]JH III-dissolved buffer and centrifuged to pellet charcoal particle and the radioactivity of the supernatant was measured. Hormone was absorbed to charcoal rapidly until 30 sec but slowly thereafter (Fig. 1). Therefore, DCC exposure time was fixed to 2 min in all the binding assay experiments. High molecular weight low affinity binding proteins are present in hemolymph of lepidopteran larvae and so hemolymph was subjected to gel filtration to reduce the influence of these proteins and then used for the binding study (Fig. 2). Partially purified JHBP was serially diluted and reacted with appropriate amounts of [^{3}H]JH III. Specific

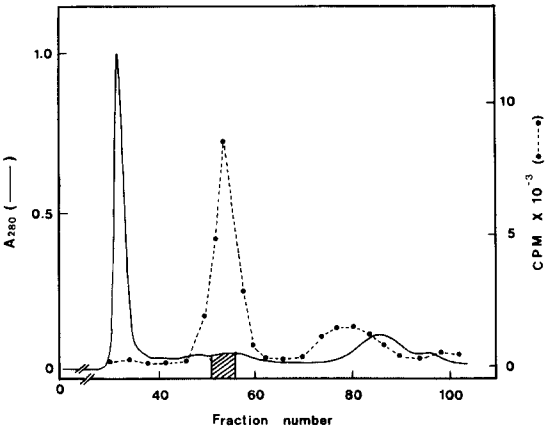

Fig. 2. Column chromatographic separation of hemolymph on a Sephadex G-100 column (1.9 × 95 cm). Hemolymph treated with 10^{-4} M paraoxon was diluted with 10 mM Tris/50 mM NaCl (pH 7.3) by two-fold and then incubated with 10^{-7} M [^{3}H]JH III. JH binding was measured directly by counting of 100 μl aliquots of each fraction (●—●). Hatched lines represent radioactive fractions containing JHBP.

binding increased at constant level at up to the protein concentration of 0.1 mg/ml but decreased slowly (Fig. 3). To fit optimal reaction condition, partially purified JHBP was properly diluted so that 40-50% of total [^{3}H]JH III was bound. Binding of JH to JHBP reached a maximum within 10 min and was stable for 3 hrs (Fig. 4). Therefore, reaction time was set to 30 min. To confirm whether the binding of JH to JHBP is influenced by pH, sample was reacted at various pH of reaction buffer. The result was that JHBP lost most of the binding capacity at strong acid condition (pH 3-4) but maintained high binding capacity above pH 5 (Fig. 5).

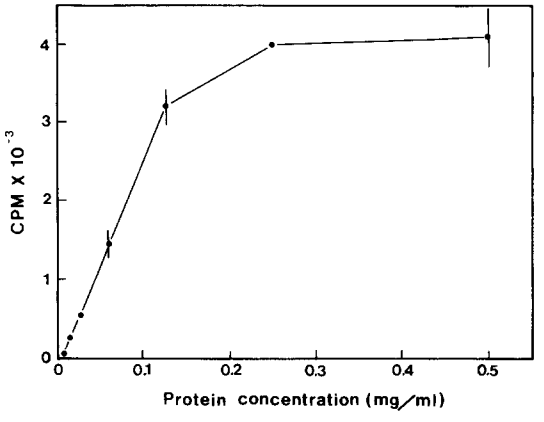

Fig. 3. Determination of saturating amounts of JHBP. Fractions containing JHBP from gel filtration (Fig. 2) were collected, diluted serially and assayed for [^{3}H]JH III binding. Individual data points are the means of triplicate determinations. Vertical bars represent SEM when larger than the size of data points.

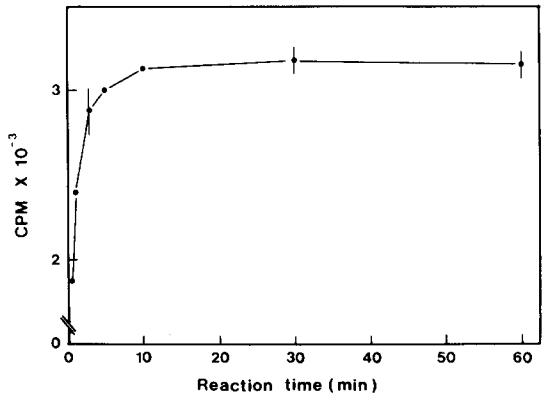

Fig. 4. Relationship between JH binding activity of JHBP and reaction time. Individual data points are the means of triplicate determinations. Vertical bars represent SEM when larger than the size of data points.

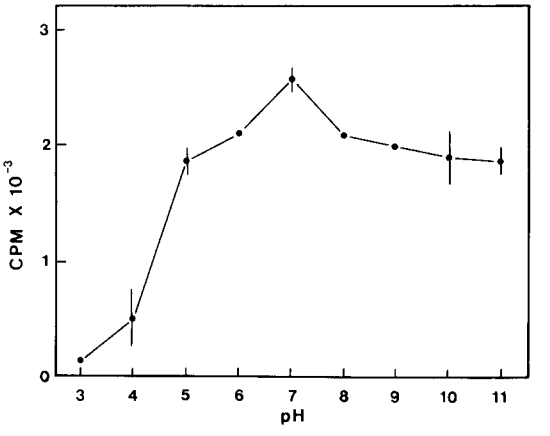

Fig. 5. Effect of pH on the binding of JH to JHBP. Buffers used were; citric acid - NaOH (pH 3.0 - 5.0), phosphate (pH 6.0 - 8.0) and glycine - NaOH (pH 9.0 - 11.0).

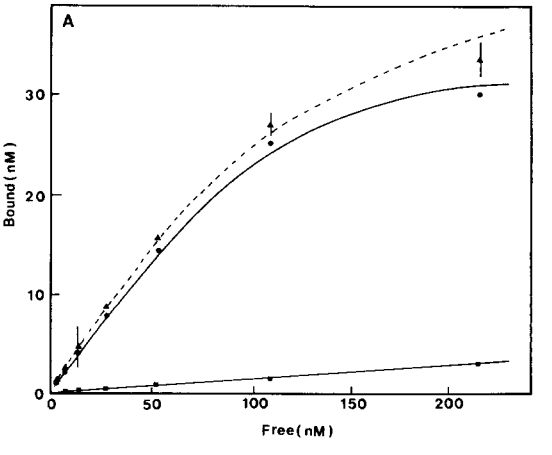

Fig. 6A. Binding of [^{3}H]JH III by larval hemolymph as a function of [^{3}H]JH III concentration. Partially purified JHBP from hemolymph was incubated with various concentrations of [^{3}H]JH III for 30 min at 4°C and the bound hormone was determined by liquid scintillation counting. Specific binding (●—●) was the difference between total (▲—▲) and nonspecific binding (■—■). Individual data points are the means of triplicate determinations. Vertical bars represent SEM when larger than the size of data points.

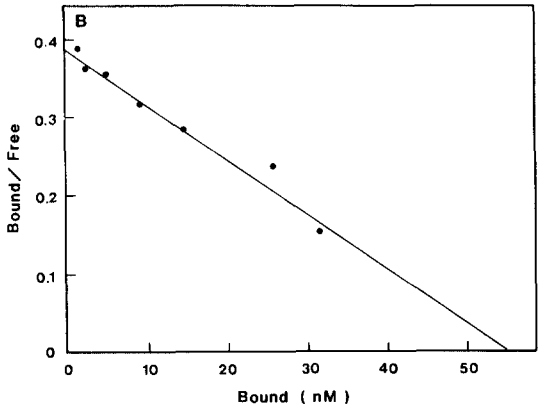

Fig. 6B. Scatchard analysis of specific binding data. Data for specific binding from Fig. 6A were used for this analysis. Dissociation constant (K_D) and total binding capacity (R_t) were determined from the slope and χ intercept, respectively.

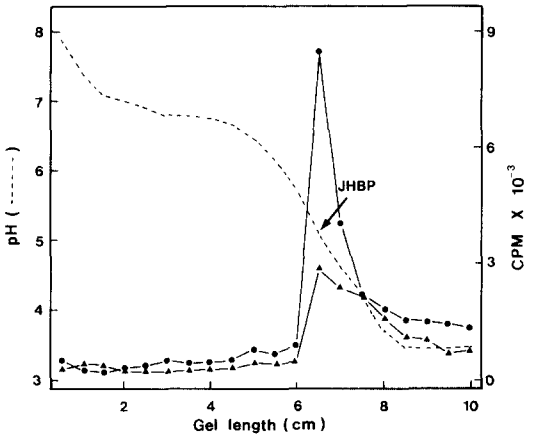

Fig. 7. Determination of isoelectric points of JHBP from larval hemolymph (▲—▲) and radioactive fractions from gel filtration (●—●).

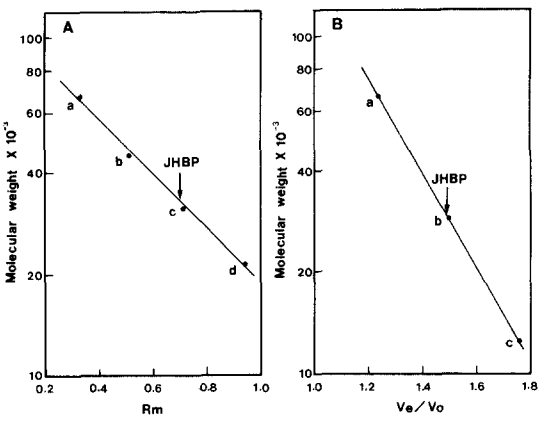

Fig. 8. Determination of molecular weight of JHBP by SDS-PAGE on a 10% gel (A) and by gel filtration on a column of Sephadex G-100 (B). Protein standards used for SDS-PAGE were: a, bovine serum albumin (66.2 kDa); b, ovalbumin (45 kDa); c, carbonic anhydrase (31 kDa); and d, soybean trypsin inhibitor (21.5 kDa). The column was calibrated with a, bovine serum albumin (66 kDa); b, carbonic anhydrase (29 kDa) and c, cytochrome c (12.4 kDa).

Binding affinity and properties of JHBP

The specific binding was saturated at the concentration of above 200 nM [^{3}H]JH III. Also, nonspecific binding increased constantly with the increase of hormone concentration but below 10% of total binding (Fig. 6A). When the data for specific binding were transformed into a Scatchard plot (Scatchard, 1949), they were placed on a single straight line, indicating that JHBP had one kind of binding site. Also, it was calculated that dissociation constant was 1.45×10^{-7} M and binding capacity was 55.9 nM (Fig. 6B).

Hemolymph and partially purified JHBP were reacted with [^{3}H]JH III and then isoelectric-focused. Two sample solutions showed radioactive peaks at pH 5.1 (Fig. 7). Purified JHBP was electrophoresed on a 10% SDS gel with molecular weight protein standards, indicating that molecular weight of JHBP was 33 kDa (Fig. 8A). The molecular weight of JHBP was estimated to be 30 kDa by calibrated gel filtration (Fig. 8B). Also, N-terminal amino acid sequence of purified JHBP was determined and compared with those of other insects (Table 1).

Discussion

In the present work DCC binding assay was used to investigate binding affinity of JHBP in hemolymph for JH. Charcoal was coated with dextran to reduce protein adsorptive sites. Dextran coated charcoal could obtain more reproducible results than uncoated charcoal by more efficiently

Table 1. N-terminal amino acid sequences of JHBPs from three lepidopterous larvae X represents the absence of an identifiable PTH derivative

	1	2	3	4	5	6	7	8	9	10
Bombyx mori	Asp	Gly	Asp	Ala	Leu	Leu	Lys	Pro	X	Lys
**Manduca sexta*	Asp	Gln	Gly	Ala	Leu	Phe	Glu	Pro	Cys	Ser
***Platyprepia virginalis*			Glu	Thr	Leu	Phe	Asp	Pro	Cys	Ser
	11	12	13	14	15	16	17	18	19	20
Bombyx mori	Leu	Gly	Asp	Met	Gln	Lys	Leu	Ser	Ser	Ala
**Manduca sexta*	Thr	Gln	Asp	Ile	Ala	Cys	Leu	Ser	Arg	Ala
***Platyprepia virginalis*	Thr	Gln	Asp	Ile	Lys	Lys	Val	Gly	Val	

*Taken from Lerro and Prestwich (1990).
**Taken from Prestwich and Atkinson (1990).

precipitating tendency (Goodman *et al.*, 1976). The unbound hormone was absorbed by dextran coated charcoal within a few seconds whereas the bound hormone remaind in the supernatant of the incubation medium. Therefore, specific binding could be estimated by measuring radioactivity of the supernatant. However, if charcoal is added to the reaction solution and leave for long time, equilibrium state become broken down because charcoal competes with JHBP for JH and so JH is detached from JHBP (Engelmann, 1981). Therefore, it is important to take reaction time to such extent that unbound hormone is maximally absorbed to charcoal and at the same time equilibrium of hormone-binding protein complex is not broken. Reaction time was fixed to 2 min as a result of Fig. 1. For the binding assay experiment, hJHBP was partially purified through gel filtration to reduce nonspecific binding maximally by high molecular weight low affinity binding proteins present in hemolymph (Fig. 2). Appropriate amount of [^{3}H]JH III was reacted with partially purified JHBP at different concentrations, indicating that specific binding increased constantly but saturated at the protein concentration of 0.2 mg/ml (Fig. 3). Binding equilibrium between JH and JHBP occured within 10 min after the addition of JH and maximum binding was maintained until 3 hrs (Fig. 4), indicating that these bindings were greatly stable. In other insects, binding equilibrium occured within 5 min after the addition of JH in *Diploptera punctata* (King and Tobe, 1988) and within 10 min in *Galleria mellonella* (Ozyhar *et al.*, 1983). Binding capacity of JHBP was very low at strong acid condition below pH 4 but was not greatly influenced by pH change above pH 5 (Fig. 5). In *Manduca sexta*, binding between nuclear suspension of larval integument and JH reached an equilibrium state within 50 min after the addition of JH and optimal binding occurred when pH of the reaction solution was 7.5 (Osir and Riddiford, 1988).

In lepidopteran insects including *B. mori*, JH I and JH II were natural homologs but in the present work JH III was used for the binding study because radioactive form of JH I and JH II were not available. de Kort *et al.* (1984) investigated JH binding ability of JHBP present in hemolymph of six kinds of insects. The affinity of JHBP for JH III was very high in *Leptinotarsa decemlineata, Locusta migratoria, Periplaneta americana* and *Apis mellifera* in which JH III was a principal homolog but very low in *Pieris brassicae* and *Oncopeltus fasciatus* in which JH I and JH II were natural homologs. However, from the result of saturation binding analysis for JH I, JH II and JH III Lenz *et al.* (1986) reported that hemolymph JHBPs of 5 kinds of lepidopteran larvae tested had almost identical binding affinities for these three kinds of homologs. These reaults suggested that the number of carbon atoms of JH molecule

did not influenced the specificity of the binding between JH and JHBP. In the present work JHBP partially purified from hemolymph has high affinity for JH III (K_D = 1.45 × 10^{-7} M) which is similar to those of JHBP in hemolymph of other lepidopteran larvae (Kramer *et al.*, 1976a; Lenz *et al.*, 1986; Abdel-Aal and Hammock, 1988). High affinity JHBPs present in hemolymph of lepidopteran insects have similar characteristics in addition to K_D. That is, JHBP has molecular weight of approximately 30 kDa and also isoelectric point of pH 4.6 - 5.4 (Kramer *et al.*, 1976a; Turunen and Chippendale, 1981; Dillwith *et al.*, 1985; Lenz *et al.*, 1986; Prestwich *et al.*, 1987; Lerro and Prestwich, 1990). In the present work molecular weight of JHBP was estimated to be 30 kDa as measured by gel filtration and 33 kDa by SDS-PAGE and isoelectric point was pH 5.1 which were similar to above. Also, N-terminal sequence of JHBP purified previously through column chromatography was investigated and compared with other insects. It had 40% identity (8/20 identical residue) with that of *M. sexta* (Lerro and Prestwich, 1990) but 24% (4/17 identical residue) with *P. virginalis* (Prestwich and Atkinson, 1990). It was reported that JHBP of *M. sexta* had no immunological reaction with those of *P. virginalis* and *G. mellonella* but had sequence identity above 30% with above insects (Lerro and Prestwich, 1990).

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누에나방 혈림프의 high affinity 유약호르몬 결합단백질의 특성

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누에나방(*Bombyx mori* L.)의 종령 유충의 혈림프 JHBP를 gel filtration을 통해 부분정제한 후 여러 가지 반응조건하에서 [^{3}H]JH Ⅲ와 결합시켜 dextran coated charcoal binding assay의 최적 반응조건을 구하였다. 부분정제된 JHBP의 JH Ⅲ에 대한 dissociation constant(K_D)는 4°C에서 1.45×10^{-7} M이었다. JHBP의 분자량은 calibrated Sephadex G-100 column으로 혈림프를 gel filtration한 결과 30 kDa 였고 순수정제된 JHBP를 SDS-PAGE한 결과 분자량이 33 kDa로 나타남으로써 JHBP가 단일 폴리펩티드 사슬로 이루어져 있음을 알 수 있었다. JHBP의 등전점은 pH 5.1이었다. 또한 순수정제된 JHBP의 N-말단으로부터 20개의 아미노산 중 19개의 아미노산 잔기의 서열을 조사하였다.