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QUARTERLY

Immunoaffinity purification of juvenile hormone-binding protein from *Galleria mellonella* hemolymph*

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Previously described methods of purification of hemolymph juvenile hormone-binding protein (hJHBP) from *Lepidoptera* were tedious and required multiple steps. These methods resulted in low protein yield (Kramer *et al.*, 1976; Goodman *et al.*, 1978; Peterson *et al.*, 1982; Park *et al.*, 1993; Ożyhar & Kochman, 1987). In this report a simple method of purification of hJHBP from *Galleria mellonella* (L.) larvae is described. Monoclonal antibodies against hJHBP were obtained and crosslinked to CNBr-activated Sepharose 4B. The hemolymph of *G. mellonella* was centrifuged and then chromatographed on Sephadex G-200 gel filtration column. Juvenile-hormone-binding activity containing material from Sephadex G-200 column was subjected to purification on an immunoaffinity column. Bound protein was eluted from anti-hJHBP Sepharose 4B gel by lowering pH to 3.0 with 200 mM citric acid 200 mM Na_2HPO_4 buffer. This method resulted in 320-fold purification of *G. mellonella* hJHBP with 56% yield.

Juvenile hormone (JH) has profound effects on insect growth, development, reproduction and behaviour. For instance it prevents larval metamorphosis, is responsible for stimulation of both male and female adult reproductive maturation, inhibits production of storage proteins and also is involved in control of insect diapause, migratory behaviour, etc. (for review see Cymborowski, 1984; Kochman & Wieczorek, 1991; Riddiford, 1994). JH synthesis occurs primarily in *corpora allata* glands although some tissues (e.g. imaginal discs) are capable of its autonomous synthesis in order to prevent their precocious adult development (Wiśniewski *et al.*, 1987).

In the long chain of JH signal transmission from the site of JH biosynthesis to a DNA level, the first hormone carrier is its hemolymph binding protein. In insect hemolymph more than 99.9% JH was found to be bound to the specific hJHBP (Hidayat & Goodman, 1994; Park *et al.*, 1993). This protein protects JH against degradation by non-specific esterases (Sanburg *et al.*, 1975) and is presumably respon-

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Abbreviations: DMSO, dimethyl-d_6-sulfoxide; HAT, hypoxanthine, aminopterine, thymidine; JH, juvenile hormone; JH III, juvenile hormone III, methyl-*cis*-10,11-epoxy-3,7,11-trimethyl-*trans*-*trans*-2,6-dodecadienoate; JHBP, juvenile hormone-binding protein; hJHBP, hemolymph juvenile hormone-binding protein; IgG, immunoglobulin G; PBS, phosphate buffered saline; PEG, polyethylene glycol; TC, tissue culture.

sible for JH recognition by the membrane of the target cells (Kochman & Wieczorek, 1995; Wieczorek, 1994). Several laboratories have focused their interest on elucidation of the structure and function of hJHBP. In *Lepidoptera, Manduca sexta* and *G. mellonella* JHBPs were most extensively studied. Several attempts have been made to purify hJHBP from these two species. *M. sexta* hJHBP was purified for the first time over 20 years ago by Kramer and co-workers (1976), and since then several other procedures of its purification have been published. Generally, these methods involved gel filtration followed by one or more anion exchange chromatographies or hydroxylapatite chromatography and as a final purification step, preparative isoelectric focusing or gel electrophoresis (Goodman *et al.*, 1978; Kramer *et al.*, 1976; Peterson *et al.*, 1982). A recently described method of *M. sexta* hJHBP purification is even more complicated. It involves ammonium sulphate fractionation followed by Sephacryl S-200 gel filtration, selective pressure filtration with Amicon YM-10 membrane, affinity chromatography on a resin containing covalently bound JH III acid and finally HPLC TSK-GEL column chromatography (Park *et al.*, 1993). The above protocol may exemplify the difficulties encountered by the researchers in hJHBP purification and explain the need for a simpler and more efficient method.

For the first time *G. mellonella* hJHBP was purified to homogeneity by a procedure employing gel filtration, phosphocellulose chromatography, chromatofocusing and again gel filtration technique (Ożyhar & Kochman, 1987). This seemingly simple method has a serious disadvantage. The most critical step for obtaining homogenous hJHBP involves setting the conditions for the phosphocellulose column in that way it selectively retards most contaminating proteins and allows hJHBP to pass freely. Different lots of phosphocellulose often required additional experimental adjustment of the above indicated conditions, extending the time necessary for successful purification.

The aim of this work was to elaborate a simple, specific and entirely reproducible method of hJHBP purification. Affinity chromatography methods seem to meet most of these requirements (Jones *et al.*, 1995). There are basically two possible strategies: to use a ligand-bound resin or an antibody against hJHBP coupled to the resin. Since JH analogs may bind all proteins interacting with hormone, including JH esterase, therefore immunoaffinity chromatography was the method of choice.

MATERIALS AND METHODS

Chemicals. (10 *R*, *S*)-JH III, bovine serum albumin, thimerosal and ovalbumin were purchased from Sigma. *N'N'*-bismethyleneacrylamide, polyethylene glycol, TEMED, and gelatin were purchased from Serva. Sephadex G-200 Fine, CNBr-activated Sepharose 4B and DEAE-Sephacryl were purchased from Pharmacia. 10-^{3}H-labelled JH III was purchased from NEN Chemicals. Protein standards for gel electrophoresis were purchased from Boehringer (phosphorylase *b*, 97.4 kDa; bovine serum albumin, 66.2 kDa; aldolase, 39.2 kDa; triose phosphate isomerase, 26.6 kDa; trypsin inhibitor, 21.5 kDa; lysozyme, 14.4 kDa) and Pharmacia (phosphorylase *b*, 97.0 kDa; bovine serum albumin, 67.7 kDa; ovalbumin, 43.0 kDa; carbonic anhydrase, 30.0 kDa; trypsin inhibitor, 20.1 kDa). Dulbecco-PBS was purchased from Gibco. Blueprot protein quantification kit was purchased from Kucharczyk (Gdańsk, Poland).

All other chemicals were of the highest commercially available purity.

Hemolymph collection. *Galleria mellonella* (L.) (*Lepidoptera, Pyralidae*) larvae were reared according to Sehnal & Slama (1966). The hemolymph from late seventh instar larvae was collected and stored at –20°C as described previously (Ożyhar *et al.*, 1983). The pooled samples were thawed, and centrifuged at 10000 × *g* for 15 min, at 4°C.

Immunisation. For animal immunisation, homogeneous hJHBP, from *G. mellonella*, was obtained as previously described (Ożyhar & Kochman, 1987). Six week old male BALB/c mice were given a subcutaneous injection of 100 µg of hJHBP emulsified in Freund's complete adjuvant. Three subsequent immunisations with 50 µg of antigen were given intraperitoneally at two-week intervals. Mice were boosted intravenously with 20 µg of antigen 3 days before cell fusion.

Cell fusion. The modification of Köhler & Milsteins's (1975) procedure described by Dippold

et al. (1980) was applied. Briefly: spleen cells from immunised mice were fused with SP-2/0 cells at 5:1 ratio, using PEG 1500 diluted with Dulbecco-PBS containing 15% (v/v) DMSO. Fused cells were evenly distributed into six 24-well, flat bottom tissue culture (TC) plates prefilled with 1 ml/well of HAT medium and incubated at the 37°C in 5% CO_2 humidified air. When the clones reached the diameter 0.5–1 mm (10–13 days) the samples of media from individual wells were screened for the presence of antibodies by ELISA.

Cloning of hybrid cells and production of ascites. Cells were cloned by limiting dilution, in 96-well, flat bottom TC plates. Mouse peritoneal cells plated at the density 1–3 $\times 10^3$/well were used as feeder layer. Hybrid cells were subcloned at least twice or until culture medium from all wells showed a uniform pattern of reactivity. Cells from the well with the highest antibody activity (clone 104) were grown to a large number *in vitro* and injected intraperitoneally into BALB/c mice primed with 0.3 ml of 2,6,10,14-tetramethylpentadecane for production of ascites fluid.

Preparation of antibodies and crosslinking to Sepharose 4B. Immunoglobulins, containing anti-hJHBP were purified from ascites fluid using ammonium sulphate fractionation and column chromatography on DEAE-Sephacel gel (Dunbar & Schwoebel, 1990). Immunoglobulin preparation was homogeneous in SDS/ /PAGE yielding two protein bands of 25 kDa and 50 kDa representing light and heavy chain, respectively (not shown).

Anti-hJHBPs were covalently bound to CNBr--activated Sepharose 4B as described by the manufacturer (Pharmacia, 1977). Briefly: 6 ml of CNBr-activated Sepharose 4B were washed and mixed with 9 ml of 100 mM $NaHCO_3$, 500 mM NaCl, pH 8.3 (22°C) containing 32.4 mg of purified immunoglobulins. After two hours of incubation at room temperature the suspension was treated with 100 mM Tris/HCl, 500 mM NaCl, pH 8.0 buffer (22°C) and then the gel was washed with 100 mM CH_3COONa, 500 mM NaCl, pH 4.0 (22°C).

Two anti-hJHBP Sepharose 4B gel columns (anti-hJHBP Sepharose 4B columns, diameter to height ratio 1:4), analytical (100 µl) and preparative (6 ml), were prepared. While not used, anti-hJHBP Sepharose 4B columns were stored in 10 mM Tris, 100 mM NaCl, pH 7.3 (at 22°C; stock buffer) containing 1 mM thimerosal, at 4°C.

Preparation of the JH stock solutions and glassware. Stock solution of about 2×10^{-5} M (10 *R, S*) JH III was prepared in redistilled spectroscopy grade hexane. Known amounts of tritium-labelled and unlabelled hormones were mixed; the concentration of unlabelled JH III was determined spectrophotometrically at 220 nm using the molar absorption coefficient of 13 830 M^{-1} cm^{-1} (Trautmann *et al.*, 1974).

Glassware which could come into contact with aqueous solutions of JH was coated with polyethylene glycol according to Kramer and co-workers (1976).

Juvenile-hormone-binding assay. Juvenile-hormone-binding activity was determined by the charcoal assay (Goodman *et al.*, 1978) in the presence of 0.1% gelatin, as described previously (Ożyhar & Kochman, 1987). To a glass tube 1 µl of 2×10^{-5} M JH III (specific activity 10^9 d.p.m./µM) in hexane was added and the solvent was allowed to evaporate. Then, in each hormone-containing tube, a 20 µl sample was mixed with 180 µl 0.1% gelatin in stock buffer. This solution was incubated for 30 min at 5°C and then mixed with 25 µl of freshly prepared charcoal suspension. The charcoal suspension was prepared by adding 15 g/l charcoal and 8 g/l dextran T 70 to stock buffer. After 10 min of incubation at 5°C the content of the tube was centrifuged at 10000 $\times$ *g* for 2 min, at 4°C. The amount of bound hormone was determined from the measurements of radioactivity in 150 µl of the supernatant.

Protein determination. Protein concentration was analysed according to Bradford (1976) with Blueprot kit, using bovine serum albumin as a standard.

Electrophoresis. SDS/PAGE was carried out according to Laemmli (1970), in 4% stacking gel and 15% separating gel. Gels were stained with Coomassie Brilliant Blue R250 (Merril, 1990).

RESULTS

Absorption and release of purified hJHBP from anti-hJHBP Sepharose 4B

Conditions for absorption and release of hJHBP from analytical anti-hJHBP Sepharose

4B column were studied in preliminary experiments, using homogeneous hJHBP preparation obtained according to the previously elaborated method (Ożyhar & Kochman, 1987). 100 μg of hJHBP was found to be almost quantitatively bound by 100 μl of anti-hJHBP Sepharose 4B in stock buffer, pH 7.3 (not shown). To find the conditions for release of bound hJHBP, a step pH gradient with 200 mM citric acid/200 mM Na_2HPO_4 (citrate-phosphate) buffer of decreasing pH was applied. As shown in Fig. 1 small amounts of protein are released at pH 6.0 and pH 5.0. At pH 3.0 almost whole bound protein was quantitatively eluted from the anti-hJHBP column.

Semi-continuous purification of hJHBP

Gel filtration of the hemolymph, on Sephadex G-200 column equilibrated with stock buffer containing 0.25 mM 1-phenyl-2-thiourea, was previously found to separate hJHBP from most of the lipophilic high molecular mass proteins and some proteolytic enzymes (Ożyhar & Kochman, 1986; Ożyhar & Kochman, 1987). In this step hJHBP is purified approx. 12-fold with at least 90% yield. Additionally, fractions eluted from Sephadex G-200 column, containing hJHBP activity, appeared invariably in the descending portion of the second protein peak (Fig. 2A). Therefore we decided to apply the gel filtration on Sephadex G-200 column as a step preceding the purification of hJHBP on immunoaffinity column.

Conditions for quantitative adsorption of hJHBP and its release from anti-hJHBP Sepharose 4B column described above, were used to elaborate a following method for separation of hJHBP from contaminating proteins, after pre-

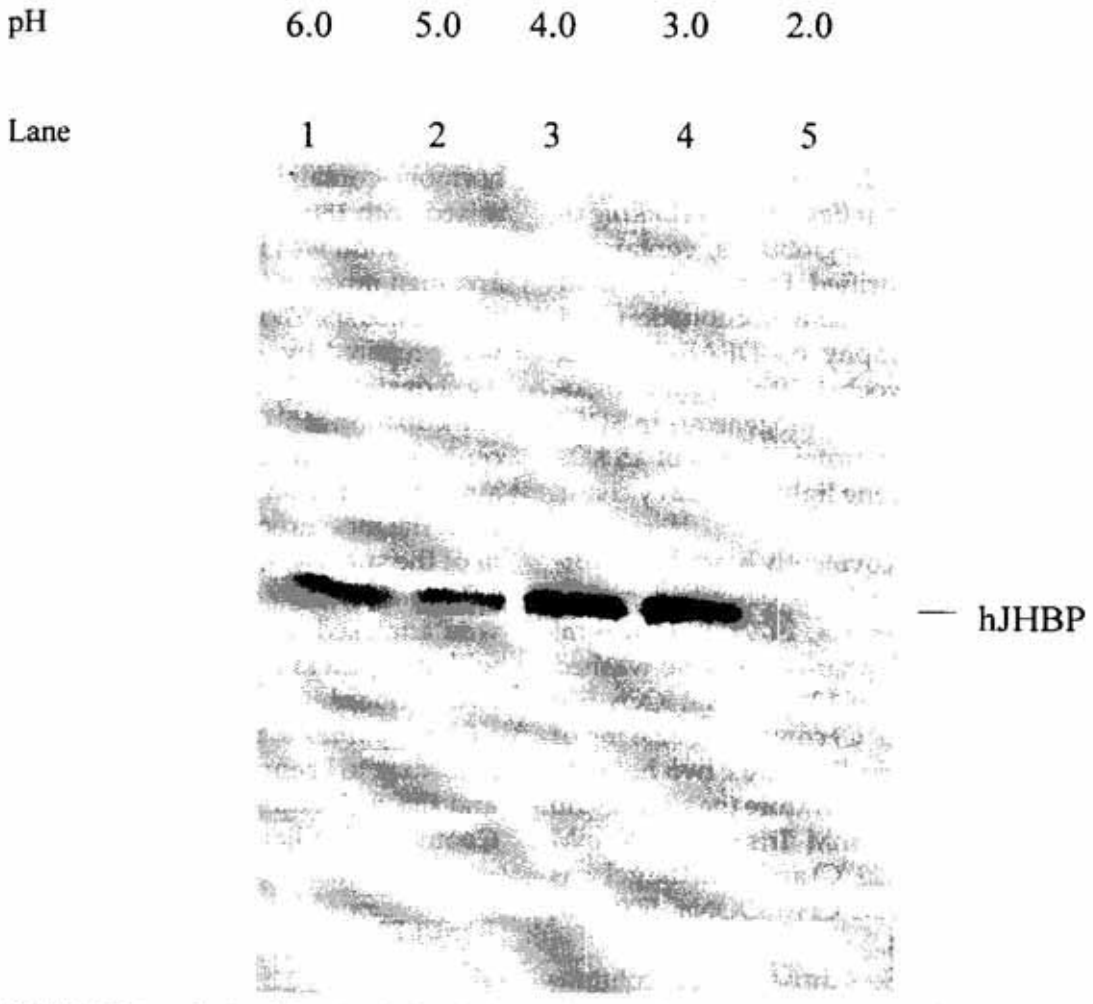
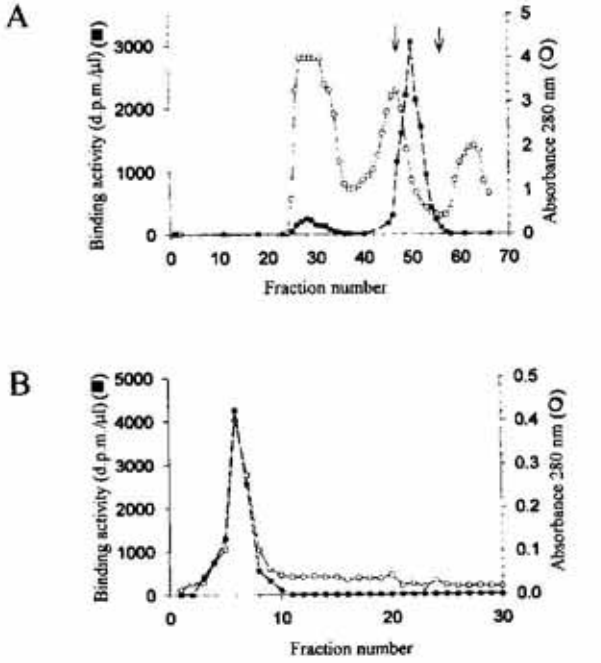


Fig 1. SDS/PAGE analysis of elution of hJHBP bound to anti-hJHBP Sepharose 4B column.
Solution of 100 μg of hJHBP, obtained as described previously (Ożyhar & Kochman, 1987), was applied to analytical anti-hJHBP Sepharose 4B column equilibrated with stock buffer. The column was washed with 500 μl portions of 200 mM citrate-phosphate buffer of decreasing pH and samples (30 μl) of collected eluate were subjected to SDS/PAGE: pH 6.0 (lane 1), pH 5.0 (lane 2), pH 4.0 (lane 3), pH 3.0 (lane 4) and pH 2.0 (lane 5).

liminary purification step on a Sephadex G-200 column. All preparation steps were performed in the cold room (4–6°C).

Frozen hemolymph (7 ml obtained from about 350 larvae) was thawed and then centrifuged at 10000 × *g*, for 10 min, and was applied onto Sephadex G-200 column equilibrated with stock buffer containing 0.25 mM 1-phenyl-2-thiourea. Proteins were eluted with the above buffer (Ożyhar & Kochman, 1987).



Withdrawn small samples of eluate were continuously monitored for absorbance at 280 nm and for juvenile-hormone-binding activity. Effluent was drained into a beaker until the absorbance of the second peak reached the maximum (Fig. 2A). Then the outflow of Sephadex G-200 column was connected to the top of anti-hJHBP Sepharose 4B column equilibrated with stock buffer, pH 7.3. Anti-hJHBP Sepharose 4B column was disconnected from Sephadex G-200 column when absorbance at 280 nm reached minimum between the second and the third absorption peak. Then anti-hJHBP Sepharose 4B column was washed with stock buffer, until A_{280} reached the base line (3–5 column vol.). Afterwards bound protein was released from the gel with 3 column volumes of 200 mM citrate-phosphate buffer, pH 3.0. The effluent was adjusted to pH 7.0 with 3 M Tris and analysed for protein and juvenile-hormone-binding activity. Protein profile obtained from anti-hJHBP Sepharose 4B column is presented in Fig. 2B. SDS/PAGE analysis of hemolymph proteins eluted from Sephadex G-200 column and those released from anti-hJHBP Sepharose 4B column is shown in Fig. 2C. Figure 3 shows the comparison of electrophoretic mobilities of hJHPB obtained with different methods: according to Ożyhar & Kochman (1987) (lane 6)

Fig. 2. Purification of hJHBP from G. mellonella.
The hemolymph from late seventh instar larvae was frozen at –20°C. After being thawed it was centrifuged at 10000 × *g* for 15 min at 4°C, subjected to preliminary purification on a Sephadex G-200 column and then immunoaffinity purification on anti-hJHBP Sepharose 4B column. **(A)** Sephadex G-200 chromatography. The hemolymph (7 ml) was subjected to gel filtration on Sephadex G-200 column (K26/100, Pharmacia) equilibrated and eluted with stock buffer (10 mM Tris, 100 mM NaCl, pH 7.3) containing 0.25 mM 1-phenyl-2-thiourea; 7 ml fractions were collected. Arrows indicate fractions (47–56) applied onto anti-hJHBP Sepharose 4B column. Open circles represent protein concentration, and black squares indicate JHBP activity. **(B)** Immunoaffinity purification on anti-hJHBP Sepharose 4B. Fractions from Sephadex G-200 column were subjected to immunoaffinity chromatography on anti-hJHBP Sepharose 4B column (1.4 cm × 6 cm) equilibrated with stock buffer. Protein was eluted with 200 mM citrate-phosphate buffer, pH 3.0 (for details see Results); 1.2 ml fractions were collected. Open circles represent protein concentration, and black squares indicate JHBP activity. **(C)** SDS-PAGE analysis. Hemolymph (lane 1), pooled fractions (47–56) eluted from Sephadex G-200 column (lane 2), pooled fractions (4–10) eluted from anti-hJHBP Sepharose 4B column (lane 3), Boehringer protein standard (lane 4).

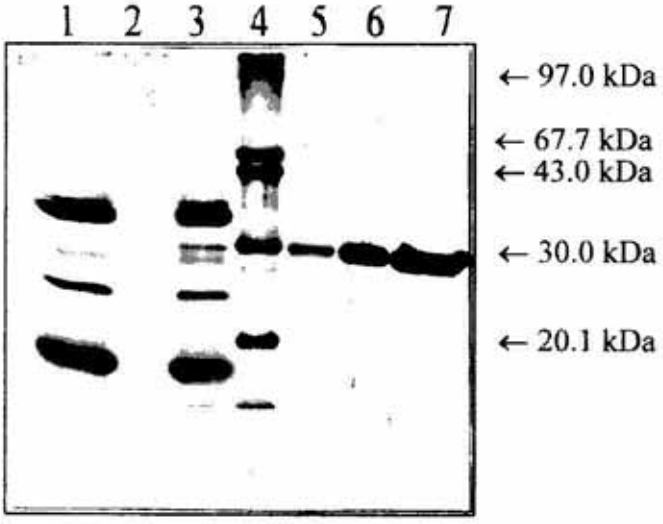


Fig. 3. SDS/PAGE monitoring of hJHBP fraction throughout purification procedure (for details see Results).
Breakthrough peak (lane 1); effluent from anti-hJHBP Sepharose 4B with stock buffer (lane 2); hJHBP eluted from Sephadex G-200 column (lane 3); Pharmacia molecular mass standards (lane 4); effluent from anti-hJHBP Sepharose 4B column (lane 5); hJHBP preparation obtained according to previous method (Ożyhar & Kochman, 1987) (lane 6); overloaded fraction released from anti-hJHBP Sepharose 4B column (lane 7).

or with immunoaffinity method (lanes 5 and 7). Additionally, proteins (Sephadex G-200 eluate) applied on anti-hJHBP Sepharose 4B column (lane 3) and breakthrough peak (lane 1) are shown. A protein band corresponding to electrophoretic mobility of hJHBP, which is barely visible in the fraction obtained from Sephadex G-200 column disappeared in the breakthrough peak of anti-hJHBP Sepharose 4B column (Fig. 3, lane 3 and lane 1, respectively). This indicates quantitative absorption of hJHBP from fractions obtained from Sephadex G-200 column. A scheme of semi-continuous system of hJHBP purification is shown in Fig. 4. The results of the purification are summarized in Table 1.

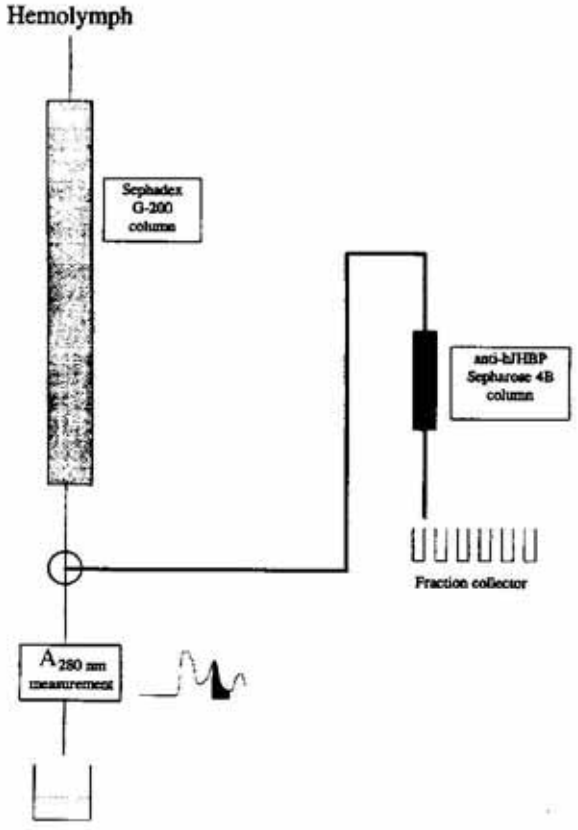


Fig. 4. A scheme of semi-continuous column chromatography system used for hJHBP preparation.
At the detector box, fractions applied to anti-hJHBP Sepharose 4B are shown shaded (see Fig. 2A).

DISCUSSION

The immunoaffinity method presented in this article allows to purify hJHBP 320-fold and with 56% yield (Table 1) in essentially one step (yet two-column) procedure (Fig. 4). The 320-fold purification achieved by this method nicely corresponds to the same range of purification obtained with previous methods for *G. mellonella* hJHBP (305-fold (Ożyhar & Kochman, 1987) and for *M. sexta* hJHBP, i.e. 189- to 320-fold (Kramer *et al.*, 1976; Goodman *et al.*, 1978; Peterson *et al.*, 1982; Park *et al.*, 1993). Our purification yield of 56% is higher than the yields achieved by previous methods which ranged from 5% to 35%, with the most recent results being 24% for *M. sexta* hJHBP (Park *et al.*, 1993) and 31% for *G. mellonella* hJHBP (Ożyhar & Kochman, 1987). Yield reported in this paper is within the range of many recently published immunopurification procedures (Chiao *et al.*, 1995; Gingras *et al.*, 1996; Kwatra *et al.*, 1995 Vannier *et al.*, 1996). For example immunoaffinity purification of complement receptor type 1 (CD35, CR1) from human serum in a two-step procedure, resulted in a yield of 50% (Seya *et al.*, 1996). Results as high as almost 80% (Bhullar & Seneviratne, 1996) and as low as 20% (Van-

Table 1
Purification of hJHBP from Galleria mellonella

Step	Volume	Total protein	Total activity	Specific activity	Yield	Purification
	(ml)	(mg)	(d.p.m. $\times 10^3$)	(d.p.m. $\times 10^3$/mg)	(%)	(-fold)
1. Hemolymph	6.8	1020	14 892	14.6	100	1
2. Sephadex G-200	70	85	13 540	160	91	11
3. Anti-hJHBP-Sepharose 4B	10	1.76	8 293	4 712	55.7	323

nier *et al.*, 1996) were also reported. It is apparent that hJHBP preparations obtained with immunoaffinity method and with the previous procedure (Ożyhar & Kochman, 1987) have identical electrophoretical mobilities and showed molecular mass of 30 kDa (Fig. 3, lanes 5 and 6). However, prolonged electrophoresis of hJHBP purified with immunoaffinity method showed that hJHBP band is slightly diffused which indicated some microheterogeneity of this protein (Fig. 2C, lane 3). The same microheterogeneity was also detected in a preparation obtained according to the previously elaborated method (Ożyhar & Kochman, 1987) (not shown). This might indicate some posttranslational modification of hJHBP.

The described procedure allows to shorten the purification time several fold and is fully reproducible. Essentially it is not even required to monitor hJHBP activity during Sephadex G-200 gel filtration. The reproducibility of the method makes it sufficient to monitor A_{280} and apply the descending part of the second absorbance peak onto anti-hJHBP Sepharose 4B column, in order to select fractions containing hJHBP. In our laboratory we have employed the same immunoaffinity column without loss of its properties nine times during one year period.

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