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Generation of multiple molecular forms of juvenile hormone binding protein from *Galleria mellonella* hemolymph[1]

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Summary. The hemolymph fraction containing juvenile hormone binding protein (JHBP) has been shown to generate multiple molecular forms of JHBP under given experimental conditions. They differ in affinity toward ionic exchanger and in pI values, but exhibit unchanged Stokes radius, sedimentation coefficient and K_d for the hormone. This process is inhibited by phenylmethylsulfonyl fluoride or high ionic strength and presumably reflects an initial step of proteolytic modification of the JHBP.
Key words. Galleria mellonella; juvenile hormone; juvenile hormone binding protein; hemolymph; proteolysis.

It is generally accepted that juvenile hormone (JH) is transported in the hemolymph of insects complexed with binding proteins. At present, two general classes of hemolymph JH binding proteins can be distinguished on the basis of binding affinity: 1) low affinity, low specificity, high capacity, high molecular weight proteins, and 2) high affinity, high specificity binding proteins. Based on molecular weight the latter class can be divided into two groups, those greater than 100,000, and those below 30,000[2]. The only example of such a protein with a mol.wt below 30,000, which has been purified to apparent homogeneity, is JHBP from the hemolymph of *Manduca sexta*[3-5]. Kramer and coworkers[3] obtained two preparations of JHBP with isoelectric points 4.95 and 5.25 from the hemolymph of mid-fifth instar larvae of *Manduca sexta*. It was shown that purified JHBP, CP-α, with pI 4.95 has the same pI as JHBP in the hemolymph subjected to Sephadex G 100 chromatography. The second component, CP-β, of pI 5.25 which can be partially converted to CP-α form, was claimed to be an artifact of unknown origin.

During our attemps to isolate JHBP from the hemolymph of *Galleria mellonella* we observed the generation of new JHBP molecular forms[6]. In this paper the effect of salt concentration and prolonged incubation of the JHBP crude sample on the generation of new molecular forms of the protein is studied. The developmental period of maximal JHBP concentration (the 5th day of the last instar larvae) was used for the collection of the hemolymph[7].

Materials and methods. DEAE Sephacel and Sephadex G 200 were obtained from Pharmacia. Phenylmethylsulfonyl fluoride (PMSF) was obtained from Sigma. JH homologs JH II and JH III were purchased from Sigma and radiolabeled (10-^{3}H) JH II and (10-^{3}H) JH III was purchased from NEN Chemicals. The concentration of JH solution was determined at 220 nm using extinction coefficient of 13,830 M^{-1} cm^{-1} according to Trautmann[8].

The *Galleria mellonella* (Lepidoptera, Pyrylidae) larvae were reared under standard conditions[9]. The hemolymph from last instar larvae (5th day) was collected and stored as described previously[7].

The crude sample of low mol.wt JHBP was isolated using Sephadex G 200 column chromatography[10]. The centrifuged hemolymph (3.6 ml) was applied to a Sephadex G 200 gel column (K 16/100, Pharmacia) equilibrated with 10 mM Tris-HCl buffer containing 100 mM NaCl and 0.25 mM 1-phenyl-2-thiourea, pH 7.3. Proteins were eluted at 5 °C with the same buffer at a flow rate of 12 ml/h; 2-ml fractions were collected. Low mol.wt fractions containing JHBP activity were combined, and then concentrated and equilibrated with 10 mM Tris-HCl, pH 8.5 by ultrafiltration (YM 10 membrane, Amicon).

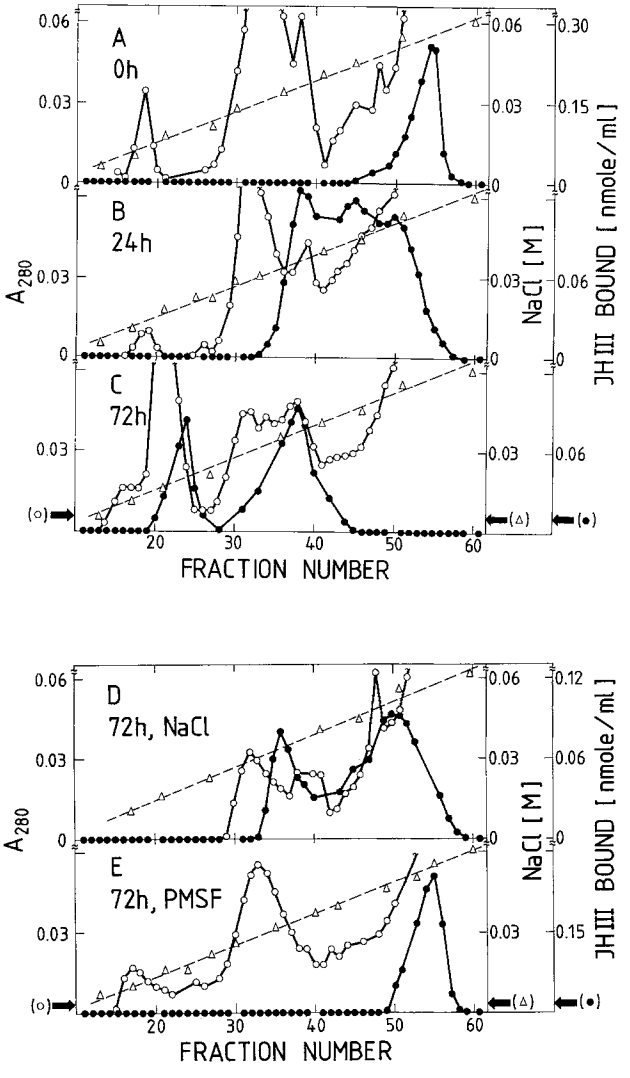
Chromatofocusing was performed according to Sluterman and Elgersma[11] using the Pharmacia chromatofocusing kit. JH binding activity was measured as described previously[7], with slight modifications. To a glass tube coated with polyethylene glycol, 1 µl of 6.4×10^{-5} M JH III in hexane (18,000 dpm/µl) was added. After solvent evaporation, 200 µl of protein sample was added to each tube and carefully vortexed; after 30 min of incubation at 5 °C, 25 µl of freshly prepared charcoal suspension was added (0.15 g charcoal and 0.08 g dextran T 70 were added to 10 ml of 10 mM Tris-HCl buffer containing 100 mM NaCl, pH 8.5). This solution was incubated for 10 min at 5 °C and then centrifuged at 10,000 × g for 2 min also at 5 °C. The amount of bound hormone was determined by measuring the radioactivity in 150 µl of the supernatant.

Equilibrium constants, K_d, were determined as follows; increasing amounts of JH II ranging from 4.1×10^{-8} M to 1.4×10^{-6} M were added to glass tubes coated with polyethylene glycol. After evaporation of solvent, 200 µl of the JHBP sample was added. Then, after 5 h incubation at 5 °C, a separation of bound and unbound hormone was achieved using the charcoal method as described above. Results obtained were analyzed according to Scatchard[12].

JH esterase activity was measured as described previously[7]. Molecular weight was determined by gel filtration[13] using a Sephadex G 100 column (K16/100, Pharmacia) equilibrated with 10 mM Tris-HCl, 100 mM NaCl, pH 7.3, and using standards of known mol.wts (MS II, Serva).

Sedimentation coefficients were determined using ovalbumin as the external standard. 100 µl of protein sample was layered on top of a linear 5–20% sucrose gradient prepared in 10 mM Tris-HCl, pH 8.5 and centrifuged at 45,000 rpm at 5 °C in a SW 50 rotor for 21 h.

Results and discussion. It has previously been shown that a low mol.wt JHBP fraction can be completely resolved from high mol.wt JH binding activity using Sephadex G 200 chromatography[10]. In this paper, the low molecular weight fraction obtained from Sephadex G 200 column chromatography was used for further experimentation. When this fraction was transferred into 10 mM Tris-HCl, pH 8.5, and immediately applied to a DEAE Sephacel column, a single, slightly asymmetrical, peak of JHBP activity was eluted (fig. A). The prolonged incubation of a crude JHBP sample at 5 °C in the above low ionic strength buffer resulted in formation of new molecular forms of JH binding activity, with decreasing affinity for the DEAE Sephacel column



DEAE Sephacel chromatography of crude JHBP sample incubated in low and high ionic strength media. Fractions from Sephadex G 200 column chromatography containing JHBP were pooled, concentrated, and equilibrated with 10 mM Tris-HCl, pH 8.5 (A, B, C) or with 10 mM Tris-HCl containing 100 mM NaCl, pH 8.5 (D), or with 10 mM Tris-HCl, pH 8.5, 0.1 mM PMSF (E). JHBP samples containing 14 mg of protein in 3.0 ml, with a specific activity of 0.23 nmole JH III bound/mg, were incubated for 0 h (A), 24 h (B), and 72 h (C, D, E) at 5 °C and applied to a DEAE Sephacel column (0.6 × 49 cm) previously equilibrated with 10 mM Tris-HCl, pH 8.5. The JHBP sample incubated in 100 mM NaCl (D) was desalted by ultrafiltration prior to application to the DEAE Sephacel column. After the column had been washed with two column vols of 10 mM Tris-HCl, pH 8.5, absorbed proteins were eluted with a linear gradient from 0 to 100 mM NaCl in 10 mM Tris-HCl, pH 8.5. Total volume of gradient was 100 ml, flow rate 10 ml/h, fraction volume 1.5 ml.
JHBP activity was determined as described in 'Materials and methods'. No JHBP activity was eluted before gradient application. Note different scales for JHBP activity in panels A, E and B, C, D. ○—○, Protein; ●—●, JHBP activity; △—△, NaCl concentration.

(fig. B, C). Under the above experimental conditions, JHBP activity is separated from JH esterase activity which can be eluted above 150 mM NaCl (not shown). The process of the formation of new JHBP activities is slowed by the addition of 100 mM NaCl to the incubation buffer (fig. D).

We found that all of the JH binding activities described in this communication exhibited identical dissociation constants for the natural[14] JH homolog (JH II); $K_d = 1.1 \times 10^{-7}$ M at pH 8.5. Neither gel filtration nor sedimentation velocity techniques distinguished different molecular weights for these derivatives. The mol.wt for the derivatives is about 30,000. This mol.wt corresponds to that found for the hemolymph low mol.wt JHBP. A sedimentation coefficient of 2.3 S was found for JHBP crude samples incubated for either 0 or 72 h in 10 mM Tris-HCl, pH 8.5 (not shown). However, the incubation of JHBP in 10 mM Tris-HCl, pH 8.5 shifts the isoelectric point. The starting material, containing JHBP activity, appears as a single peak of pI 8.65 after chromatofocusing, whereas a crude sample of JHBP incubated for 72 h in 10 mM Tris-HCl, pH 8.5 was resolved into two peaks of pI 8.65 and 8.50, respectively (not shown).

Generation of multiple JHBP molecular forms is associated with partial inactivation of the carrier, since 20 and 60% of JHBP activity was lost after 24 and 72 h incubation, respectively. Addition of 0.1 mM PMSF to the incubation medium prevents the generation of new molecular forms of JHBP (fig. E).

The above results indicate that formation of new molecular forms of JHBP is associated with an enzymatic process and most likely with proteolysis. Either a small polypeptide chain is released from JHBP, and this does not affect the Stokes radius and the sedimentation coefficient of the main JHBP chain, or proteolytic cleavage is not associated with the dissociation of the peptide from the core of the JHBP molecule. The above observations are important for two reasons. Firstly, they show that an enzymatic modification of JHBP is the main source of heterogeneity of JHBP observed during the purification procedure of this protein from *Galleria mellonella*, and possibly from other sources. We found that overloading Sephadex G 200 column with hemolymph increased the rate of formation of new JHBP forms (not shown), and this may indicate that in such a case, gel filtration results in decreased resolution of JHBP from a modifying protein(s). Secondly, the observed modification certainly reflects some physiological process which may occur in the hemolymph or peripheral cells.

The proteolytic cleavage of various proteins is often indicative of highly regulated enzymatic systems, and complex processes which have been the subject of several recent reviews[15,16].

No direct information concerning the proteolytic enzymes in *Galleria mellonella* hemolymph are available. It is well known, however, that at the late stage of the last instar, the hydrolytic activity in the hemolymph of many insects is increased[17]. Moreover, just before ecdysis, most of the JHBP is removed from *Galleria mellonella* hemolymph[7] and the titer of JH is extremely low[18]. Thus it is conceivable that new molecular forms of JHBP represent products of initial steps of this protein degradation. It is difficult to know whether the new JHBP forms, which are derived from incubation of low mol.wt JHBP, have any physiological significance. However, one can also speculate that these forms may represent further steps of JHBP processing which, by causing a conformational change, may allow the binding of JHBP to the membrane of target cells. The existence of a membrane receptor for JHBP has recently been postulated[2].

The above considerations should be taken tentatively because the observed rate of transformation of JHBP to new forms is rather slow in the hemolymph.

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Virus-like particles with host protein-like antigenic determinants protect an insect parasitoid from encapsulation

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Summary. Virus-like particles from several species of parasitoid wasps are known to interfere with encapsulation. We raised vertebrate antibodies against the virus-like particles from the wasp *Venturia canescens* and used them to show that antigenic determinants of the particles display similarity to a protein component of the host. When particles on the egg surface are in turn covered with particle-specific antibodies, their protective function is lost, and antibody-treated eggs suffer encapsulation upon injection into larvae of the host, *Ephestia kuehniella* (Lepidoptera).
Key words. Virus-like particles; parasitoid; defence reaction; antigenic determinants.

Foreign particles, for instance parasitoid eggs, brought into the body cavity of insect larvae are usually encapsulated by hemocytes, which form an inactivating coat. Several wasp species are known successfully to circumvent this defense reaction of the host. In some wasp species virus-like particles have been shown to interfere with the host's defense reaction. The particles were recently characterized as a family called polydnaviridae[10]. They are synthesized in the nuclei of the ovarial calyx gland and, in the case of *Venturia canescens*, are secreted on to the surface of the meiotically arrested eggs as they pass through the gland[12]. It is known that eggs are encapsulated if they are depleted of particles or if the particles are experimentally inactivated before injection into the larvae[2,6,8]. In the parthenogenetic ichneumonid *Venturia*, electron microscopic analysis of calyx cells indicates that the particles produced in the nuclei consist of an electron-dense core surrounded by membrane-like structures. On being passaged through the cytoplasm the particles carry an additional membrane, probably from the nuclear envelope, which is left behind when they leave the cell. Particles found on the surface of the egg still appear to have the membrane-like structure acquired inside the nucleus, and therefore this structure seems to constitute an essential part of the particle.

Material and methods. Proteins from tissues. Tissues of *Ephestia* and *Venturia* were dissected manually and homogenized in ice-cold PBS (138 mM NaCl, 2.68 mM KCl, 7.30 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 0.91 mM $CaCl_2$, 0.49 mM $MgCl_2$, pH 7.3).

Virus preparation. Venturia ovaries from 100–150 wasps were dissected. Oocytes were taken from reservoirs of the lateral oviducts and transfered with a Pasteur pipette into a $CsCl_2$ solution to the final concentration of 402.4 mg $CsCl_2$ per ml PBS. After centrifugation in a SW 65 rotor (40 h, 50,000 rpm, 4 °C) the particle band was removed with a drawn-out Pasteur pipette. The particles band at a density of 1.263 g/cm^3. They were diluted 10-fold with cold PBS and pelleted in a Ti 50 rotor (3 h, 35,000 rpm, 4 °C). The pellet was resuspended in 100 µl PBS, and purified particles were stored in liquid nitrogen.

Immunization of rabbits. Antiserum against particles was obtained from rabbits by injecting 2 ml of particles (about 100 µg protein) from a single preparation together with Freund's adjuvant. After 4 weeks the immunization was repeated with the same amount, and 10 days after the second injection 35 ml of blood were collected for serum preparation; the serum was stored in aliquots at −80 °C.

Western blotting. Proteins were electrophoretically separated on 10% polyacrylamide gels as described[5,13]. The separated proteins were transferred to nitrocellulose filters by electroblotting as described[14] using an electrophoresis buffer containing 0.025 M Tris-HCl, pH 7, 0.192 M NaCl and 20% methanol. After preincubation of the filters in blocking buffer (20 mM Tris-HCl, pH 7.5, 0.5 M NaCl 0.05% Nonidet P-40 and 3% bovine serum albumin (BSA)) at room temperature they were incubated overnight at 4 °C in BSA solution containing the antiserum in a 1:100 dilution. The filters were washed for 45 min in blocking buffer without BSA and then incubated for 2 h at room temperature in

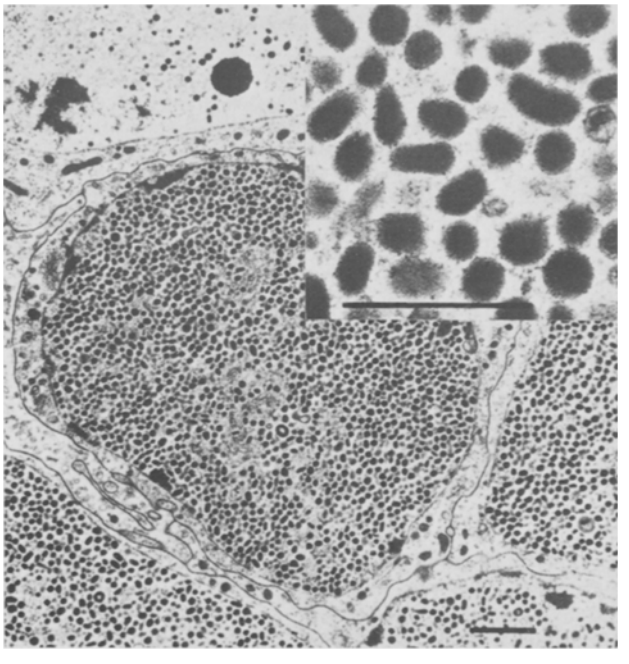

Figure 1. Virus-like particles in the calyx gland of *Venturia canescens*. Electron-dense particles are located predominantly in the nuclei of gland cells. Section shown is from adult wasps. Virus-like particles are already present in calyx cells from pupal stages[12]. Bar represents 1.0 µm (inset 0,5 µm).