Pergamon

Insect Biochemistry and Molecular Biology 30 (2000) 307–314

Insect Biochemistry and Molecular Biology

www.elsevier.com/locate/ibmb

Isolation and characterization of juvenile hormone esterase from hemolymph of *Lymantria dispar* by affinity- and by anion-exchange chromatography

Christa Nussbaumer [a,*], Andrew C. Hinton [b], Axel Schopf [a], Andrea Stradner [a], Bruce D. Hammock [b]

[a] *University of Agricultural Sciences, Institute of Forest Entomology, Hasenauerstrasse 38, A-1190 Vienna, Austria*
[b] *University of California Davis, Department of Entomology, Davis, CA 95616, USA*

Received 16 April 1999; received in revised form 20 December 1999; accepted 20 December 1999

Abstract

Juvenile hormone esterase (JHE), which catalyzes the hydrolysis of juvenile hormone, was isolated from the hemolymph of 5th instars of *Lymantria dispar* by two different procedures. One procedure was based on affinity chromatography and the other on anion-exchange chromatography. The material from both purifications showed bands of approximately 50 kDa when analyzed by SDS-PAGE. Isoelectric focusing (IEF) gels in combination with enzyme activity assays indicated two isoelectric forms with the same pI values (pH 5.1. and 5.3) from affinity purification and from anion-exchange chromatography. Amino acid sequencing of several internal peptides from the 50 kDa band following affinity purification and alignment of these sequences with JHEs from previously purified lepidopteran species (*Heliothis virescens, Manduca sexta*) showed high homology of these enzymes.

The isolated JHE, at least in the stage of insect used, was different from the enzyme reported earlier [Valaitis, A.P., 1991. Characterization of hemolymph juvenile hormone esterase from *Lymantria dispar*. Insect Biochemistry 21, 583–595] to hydrolyze JH in the hemolymph of gypsy moth, based on molecular weight and amino acid sequence.

Keywords: *Lymantria dispar*; Juvenile hormone esterase; Affinity purification; Anion-exchange purification; Isoelectric focusing; Amino acid sequencing

1. Introduction

Lymantria dispar L. defoliates forest, shade and fruit trees over much of the northern temperate regions of the world. It originated in Europe and was accidentally introduced into north-eastern USA in 1869. The lack of natural enemies has resulted in disastrous outbreaks (Doane and McManus, 1981). As the gypsy moth gradually spreads to the southern and western regions of the US, it poses a threat to the commercial hardwood forests. Costly efforts to eradicate the pest with chemical (e.g., DTT) and microbial (*Bacillus thuringiensis*, nucleopolyhedrosis virus) pesticides have failed so far. Moreover, importation and establishment of many parasites and predators from the gypsy moth's native range have not succeeded in regulating gypsy moth populations in the infested areas of the US.

A novel approach in integrated pest management is the use of insect growth regulators similar to juvenile hormones to control pests. Juvenile hormones (JHs) are known to play a major role in the regulation of development in insects (reviewed by DeKort and Granger, 1981). Complementing changes in the rates of biosynthesis of JH by the corpora allata, JH selective enzymes are responsible for reduction of JH titers at critical times of insect development (reviewed by Hammock, 1985; Venkatesh et al., 1990). Thus, investigations on the mechanisms of JH degradation will help to gain insight into regulation of insect development, which is indispensible in order to successfully implement new strategies of insect control.

* Corresponding author. Tel.: +43-1-36-86-352-25; Fax: +43-1-36-86-352-97.
E-mail address: nussbaum@ento.boku.ac.at (C. Nussbaumer).

0965-1748/00/$ - see front matter
PII: S0965-1748(00)00002-3

During the past several years, JH degrading enzymes such as juvenile hormone esterases (JHEs) have been characterized from the hemolymph of several insect species. Full or partial cDNA or amino acid sequences are currently available for JHE of *H. virescens* (Hammock et al., 1988; Hanzlik et al., 1989), *Manduca sexta* (Venkatesh et al., 1990; Hinton, unpublished work), *Trichoplusia ni* (Venkatamaran et al., 1994) and *Leptinotarsa decemlineata* (Vermunt et al., 1997b). Until now, only JHE from *H. virescens* has been cloned and expressed in a baculovirus system. The expressed protein showed the expected characteristics of JH hydrolysis and was proven to be identical to JHE from the hemolymph of *H. virescens* (Hanzlik et al., 1989). The complete DNA sequence and thus also the complete amino acid sequence of JHE from *H. virescens* are known. Several peptides from JHE of *M. sexta* have been sequenced by Hinton (unpublished work). Furthermore, a full length clone of the DNA sequence of JHE from *M. sexta* (Hinton, unpublished data) has been translated into amino acid sequence. High homology of amino acid sequences exists between the JHEs of *H. virescens* and *M. sexta*, even though these species belong to different lepidopteran families.

The importance of juvenile hormone esterase activity for JH degradation in the hemolymph of *L. dispar* was demonstrated earlier (Schopf et al., 1996; Tanaka et al., 1989). Valaitis (1991) purified a putative JHE from *L. dispar* using a classical approach including RP-HPLC, but did not find any homology with the amino acid sequence from the JHE previously isolated and cloned from *Heliothis virescens* (Hanzlik et al., 1989).

In the present study, JHE from the hemolymph of *L. dispar* was purified by the affinity purification system described by Abdel-Aal and Hammock (1986), which employs transition state mimics as ligands to bind JH esterolytic activity. Furthermore, partial purification of the same enzyme using the traditional technique of anion-exchange chromatography was performed. Identification of JHE was done by (1) comparison of protein bands separated by SDS-PAGE in material obtained from both purification methods, (2) activity analysis after native isoelectric focusing, and (3) amino acid sequencing of the enzyme isolated by affinity purification. The sequences of HPLC-purified internal peptide fragments of JHE obtained via Edman degradation were compared to amino acid sequences of JHEs from *H. virescens* and *M. sexta*.

2. Materials and methods

2.1. Insects

Gypsy moth eggs were obtained from a laboratory culture of the gypsy moth rearing facility from the USDA (OTIS Cape Cod, MA). Hatched larvae were reared on artificial diet (modified tobacco hornworm diet, Bell et al., 1981) under long day conditions (16 h light:8 h dark) at 20°C. Hemolymph was collected from larvae at the beginning of the 5th instar (L5D2, L5D3), diluted 1:2 with purification buffer (50 mM phosphate buffer, pH 7.4, containing 5% sucrose (Fisher Scientific), 0.02% sodium azide (Fisher Scientific), 0.01% phenylthiourea (PTU, Sigma)) and was frozen at −20°C until further use.

2.2. Enzyme and protein assays

JHE activity in the hemolymph was measured by the partition assay method of Hammock and Sparks (1977), using 5 μM 10^{-3}H(N)-JH III (New England Nuclear Research Products, Boston, MA; 17 Ci/mmol) with a final concentration of 5×10^{-6} M as substrate. Enzyme preparations diluted in purification buffer (100 μl) were incubated with the substrate solution in a waterbath at 30°C. Incubation times (15 or 30 min) and enzyme dilutions (1:100 to 1:500) were selected so that hydrolysis occurred in the linear range of the reaction (10–40% of JH was metabolized). After the extraction of the aqueous phase, the samples, each assayed in duplicate, were counted in a liquid scintillation counter for 2 min.

Protein concentration was determined by the dye-binding method of Bradford (1976) as modified by BioRad^{R}. Diluted dye samples (200 μl; 1:5) were incubated at room temperature for 30 min with 10 μl of sample or standard solution. Bovine serum albumin fraction V (BSA, Sigma) was used for generating a standard curve.

2.3. Purification of JHE by affinity chromatography

The ligand 3-[(4′-mercapto)butylthio]-1,1,1-trifluoropropan-2-one (MBTFP) was prepared after the method of Abdel-Aal and Hammock (1986), attached to epoxy-activated Sepharose and stored in 100% ethanol. An aliquot (300 μl) of this MBTFP-resin was washed on a sintered glass filter (Pyrex 40M) with 50 ml of each of the following ethanol dilutions: 95%, 75%, 50%, 25%, followed by 50 ml each of milliQ-water and purification buffer (50 mM sodium phosphate buffer, pH 7.4 with 5% sucrose, 0.02% sodium azide, 0.01% phenylthiourea).

Frozen hemolymph (40 ml) of 5th instar gypsy moth larvae was thawed on ice and diluted with purification buffer 1:5. The reducing agent β-mercaptoethanol (β-ME) was added to give a concentration of 1 mM. The first batch of this solution was loaded on to the resin in a 50 ml plexiglass column (Econocolumn, i.d.=2.5 cm, l=15 cm, BioRad). The column was placed on a rotating wheel and tumbled at 4°C. Every other hour 100 μl aliquots were taken from the supernatant and assayed for

JHE activity by partition assay to monitor the binding of the JHE to the resin. As soon as the JHE activity came to a low level, the hemolymph solution was eluted and replaced by another batch of 50 ml diluted hemolymph. When maximum loading was achieved, the supernatant was eluted and the resin washed with 100 ml of purification buffer without PTU, containing β-ME (1 mM) and 1-octyl-β-D-glucopyranoside (OG, 0.01%, Amresco). The enzyme was eluted from the affinity gel by gentle shaking in 4 ml of purification buffer with the inhibitor 3-octylthio-1,1,1-triflouropropan-2-one (OTFP; synthesized as described in Hammock et al., 1984) at a final concentration of 300 μM. The supernatants were taken off every 12 h for three days and replaced by purification buffer with fresh β-ME and OTFP. All the eluates were analyzed by 10% SDS-PAGE. Aliquots of the OTFP-inhibited enzyme solutions were reactivated by dialysis. Samples of purified enzyme in purification buffer were put in dialysis tubes (Spectra, MWCO 30 kDa) and dialyzed against the same buffer without OTFP which was changed every day for six days.

2.4. Purification of JHE by anion-exchange chromatography

Anion-exchange chromatography was performed using the strong anion-exchanger Q-Sepharose (Pharmacia, 15 ml), which was equilibrated with 2×100 ml 50 mM Tris–PO_4-buffer (pH 8.5). Hemolymph which was previously diluted 1:5 in phosphate buffer was concentrated by ultrafiltration (Ambion ultrafiltration device using a membrane with 30 kDa MWCO) and then diluted 1:10 in 50 mM Tris–PO_4-buffer (pH 8.5). The diluted hemolymph (100 ml) was batch loaded onto the gel in a 100 ml plexiglass column (Econocolumn, i.d.=2.5 cm, l=30 cm, BioRad) and tumbled for 30 min at 4°C. After eluting the supernatant, the gel was washed twice with 100 ml each of Tris–PO_4-buffer (wash 1 and 2). Elution of the enzyme started with wash 3 using the same Tris–PO_4-buffer (pH=8.5) but adding NaCl. Following salt concentrations were used: 50 mM NaCl for 15 min in wash 3 and wash 4, 100 mM NaCl for 15 min in wash 5 and wash 6, and 200 mM NaCl for 1 h in wash 7. Each fraction was analyzed for JHE activity using the partition assay. Higher salt concentrations (100 and 200 mM NaCl) were found to increase JHE activity by several percent. This has to be considered when comparing the yield of activity from anion-exchange purified material to the material from affinity chromatography. Protein concentration was determined, and the purity of the enzyme was checked by SDS-PAGE.

2.5. Electrophoresis

Protein preparations before and during purification were analyzed by SDS-PAGE (10%) according to the method of Laemmli (1970). The samples were diluted in loading buffer (containing 5% fresh β-ME (Sigma) and Bromophenol Blue (Amresco)) and denatured at 65°C before loading onto the 6% stacking gel.

Analytical isoelectric focusing was performed using an LKB Multiphor apparatus (Pharmacia Biotech) and precast polyacrylamide gels (wide range pH 3–10 and narrow range pH 3.5–6). An aliquot of each native protein sample was applied 20 mm from the cathode. All samples were run in duplicate. After the run, one part of the gel was cut off and silver-stained. Duplicate lanes were cut in 5×5 mm pieces which were soaked in 500 μl purification buffer (pH 7.4) at 4°C and analyzed for JHE activity on the next day. For the determination of the pH profile across the gel, 5 mm squares were cut out of a blank lane of the gel. These gel pieces were soaked in 20 mM KCl for an hour before the pH was measured. A control experiment, where hemolymph was incubated in buffers of different pH values for 1 h and then checked for JHE activity, showed that the pH in the range of 3.5–6 had no marked effect on JHE activity compared to the activity at pH 7.4.

Two dimensional SDS-PAGE was performed after running affinity-purified material on a IEF, excising the two bands which were visible after Coomassie stain at pI 5.1 and 5.3. The gel slices were soaked in loading buffer, heated to 65°C for 10 min and then loaded into two slots next to a lane loaded with affinity purified material. After the run, the gel was silver stained.

2.6. Peptide sequencing

Aliquots of samples purified by affinity chromatography were prepared for internal peptide sequencing using in-gel digestion according to a modified method of Hwang et al. (1996). After the samples were separated on SDS-PAGE, the JHE band was cut out of the Coomassie-stained gel, put into a polycarbonate tube (Falcon) filled with 15 ml milliQ-water and put on a shaker for 10 min. The water was discarded and the procedure was repeated four more times. The gel slices were diced with a sterile razor blade, put into Eppendorf tubes and dried down in a rotating vacuum concentrator. The samples were rehydrated in 50 μl rehydration buffer (0.1 M Tris–HCl pH 9.0, 0.05% SDS) containing 0.25 μg Lys-C endopeptidase (Wako) and were incubated at 30°C overnight. Supernatants were transferred to new Eppendorf tubes. Further extraction of the peptides was performed with 50 μl milliQ-water at 30°C for another 2 h, followed by extraction with 50 μl 0.1% triflouroacetic acid (Sigma) in 70% acetonitrile (Fisher Scientific) and incubated at 30°C for 1 h (repeated once). Supernatants were pooled, dried down in a vacuum concentrator and stored at −20°C until shortly before analysis by HPLC.

The dehydrated protein was dissolved in 25 μl 6 M guanidine–HCl (Sigma), 0.4 M Tris (Fisher Scientific)

pH 8.2, 18 mM dithiotreitol (DTT, Sigma) and incubated at 50°C for 45 min. After the samples had cooled down to room temperature, 2 μl of iodoacetamide acid (500 mM, Sigma) were added, and the reaction was overlaid with nitrogen and incubated at room temperature for 15 min. Samples were diluted to a final concentration of 1.5 M guanidine–HCl, 0.1 M Tris. After centrifugation supernatants were transferred into new tubes and digested with 0.1 μg Lys-C overnight at 30°C. The digested peptides were injected into a microbore HPLC (ABI 172 Model 140B) and separated on a C18 column (Vydac, 1×150 mm). A linear gradient running from 5% solvent A (0.1% TFA in water) to 70% solvent B (0.075% TFA in water) in 115 min was used for elution. Peaks with volumes of 20–100 μl were collected by hand and several of the peptides were chosen for amino acid sequencing. The sequencing was done at the Protein Structure Laboratory at the University of California, Davis (Applied Biosystems 470A Sequencer) using a method described by Rice et al. (1996).

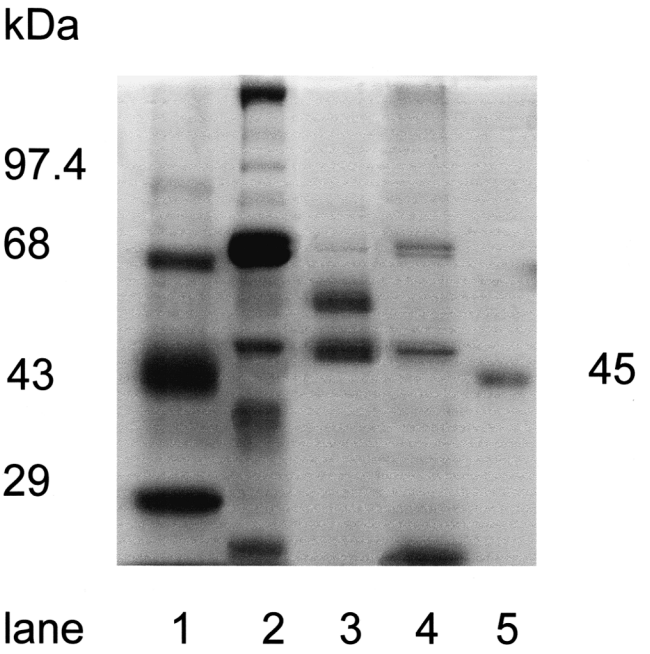

Fig. 1. SDS-PAGE (10%) of sample aliquots from purified JHE of *L. dispar* stained with Coomassie Blue. Lanes 1 and 5 show molecular weight markers. Lane 2–4 were loaded with plasma (90 μg, lane 2), sample after affinity purification (38 μg, lane 3) and sample after anion-exchange chromatography (30 μg of wash 6, lane 4).

3. Results

The results of the affinity purification process of *L. dispar* are summarized in Table 1. About 90% of the JHE activity of each batch was bound to the affinity gel after 4 h. Following elution from the affinity column with OTFP, two major bands were evident on SDS-PAGE: one diffuse band at about 50 kDa and one band at about 60 kDa (Fig. 1). IEF analysis of aliquots of eluted material also showed two bands, at pH 5.1 and 5.3, after silver staining (Fig. 2, part B). Slices of an identical lane that were assayed for JHE activity (Fig. 2, part A) confirmed that the major silver stained bands at pH 5.1 and 5.3 correspond to the majority of JH hydrolyzing activity.

A third peak of JHE activity in *L. dispar* occurred at a pI of 4.5. This isoform of JHE was found in the material purified by affinity chromatography but not in the material subjected to anion-exchange chromatography. Further analysis of the sample by 2D SDS-PAGE suggests that the bands at pH 5.1. and 5.3 both derived from the 50 kDa protein. Fig. 3 shows the 50 kDa bands from the two gel slices from IEF, running at the same Rf as the JHE from affinity purified material. Due to the low amount of protein, no band was seen on 2D SDS-PAGE from the material with a pI of 4.5. Analysis of crude hemolymph by wide (pH 3–9) and narrow (pH 3.5–6) range IEF showed that the JHE activity of the starting material focused at the same pIs observed with the affinity purified material.

Following digestion of the 50 kDa protein for peptide sequencing, four peptides were selected. Two of these peptides were found to have amino acid sequences similar to those of *M. sexta* and *H. virescens* (Table 2). None of the peptides from digestion of the 60 kDa band could be aligned with JHE from *H. virescens*.

The low yield allows one to argue that other proteins could contribute to JHE activity in the hemolymph. Therefore, we also purified the JHE activity by Q-

Table 1
Summary of the purification of juvenile hormone esterase from hemolymph of *Lymantria dispar* by affinity and anion-exchange chromatography

	Total protein (mg)	Total activity (nmol JH III min^{-1})	Specific activity (nmol JH III min^{-1} mg $protein^{-1}$)	Yield (%)	Purification factor
Affinity chromatography					
Plasma	80	880	11	100	1
OTFP eluate 1	0.03	21	700	2	64
Anion-exchange chromatography					
Plasma	42	416	10	100	1
Wash VI (100 mM salt)	0.9	395	439	95	44

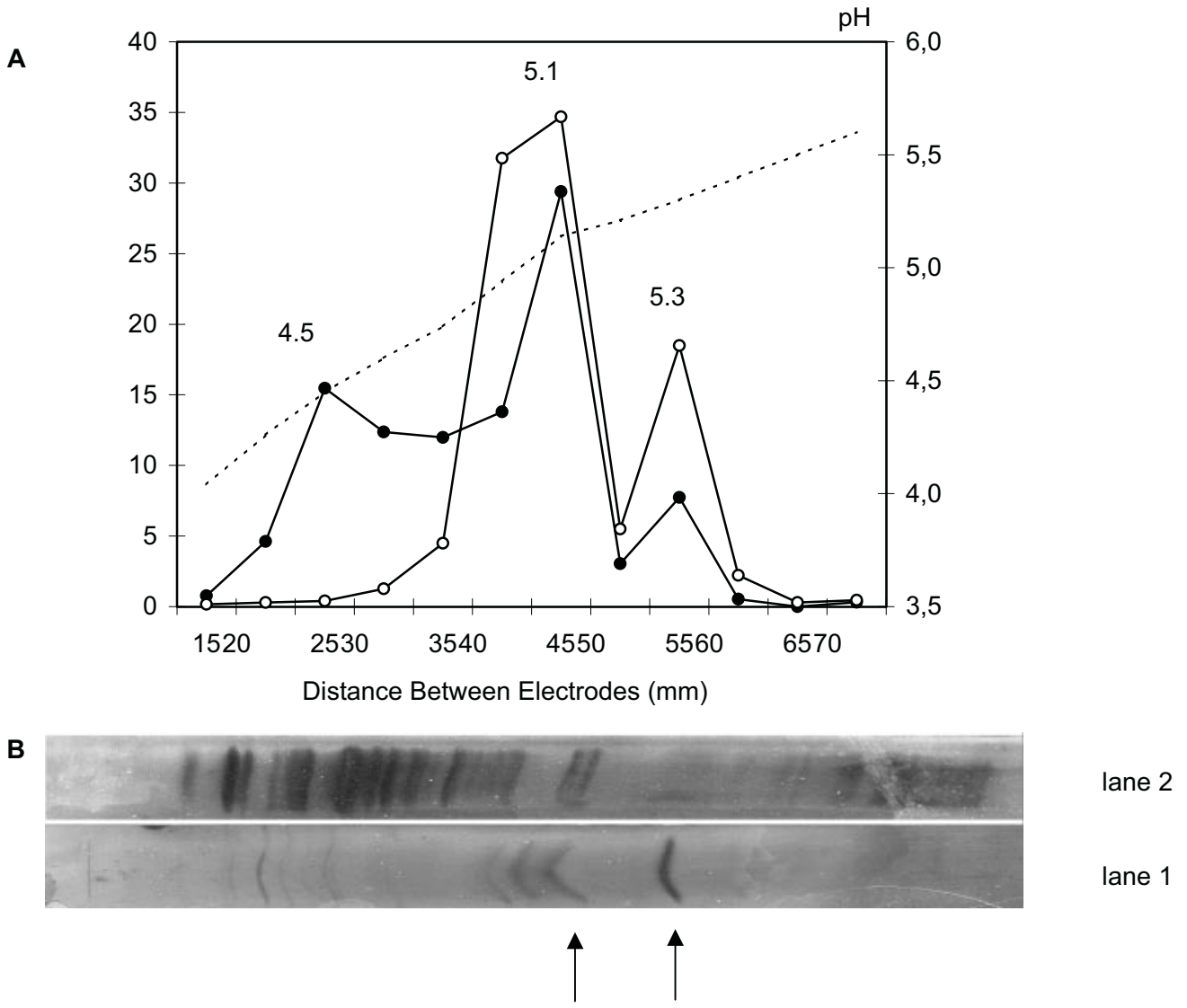

Fig. 2. Isoelectric focusing of JHE from *L. dispar*. A. Aliquots from samples after affinity purification (solid symbols) or after anion-exchange chromatography (open symbols) were run on an ampholine gel (pH 3.5–6). JH hydrolytic activity was measured after slicing and eluting the focused proteins from the gel. Numbers above the peaks indicate the pIs. No additional peaks of activity were obtained from wide range gels of either purification protocol or of crude hemolymph; 56% or 28% of the loaded activity from affinity purified material or material after anion-exchange chromatography, respectively, were recovered from the gel. B. Silver-stained proteins from samples after affinity purification (lane 1, 58 μg protein) or after anion-exchange chromatography (lane 2, 100 μg). Arrows indicate the bands at pI 5.1 and 5.3.

Table 2
Amino acid sequences of peptides from JHE of *Lymantria dispar* purified by affinity chromatography and the alignments of these sequences with JHE from *Heliothis virescens* and *Manduca sexta*

peptide 1:	
L. dispar	L L F T T A P N
H. virescens	S Y F T T S P L
M. sexta	S A F T T N P V
peptide 2:	
L. dispar	N A Q A F G G D P D N N T L A A N
H. virescens	N A K N F G G D P D S D I T A G Q
M. sexta	N A H F F G G G P D D V T L M G Q

Sepharose chromatography. This purification is based on different principles and showed a much higher yield (95% in wash 6). While JHE bound very efficiently to this anion-exchange resin at pH of 8.5, a NaCl concentration of only 50 mM at the same pH was sufficient to start the elution. A step gradient of 50, 100 and 200 mM NaCl was used to recover JHE activity from the column. This one-step purification by ion-exchange chromatography gave partially purified but active JHE (Table 1 and Fig. 1). While the recovery of activity here was much higher, the purification factor was even lower than after affinity purification. All different fractions were analyzed for purity on SDS-PAGE, for protein concentration by Bradford assays and for JHE activity by par-

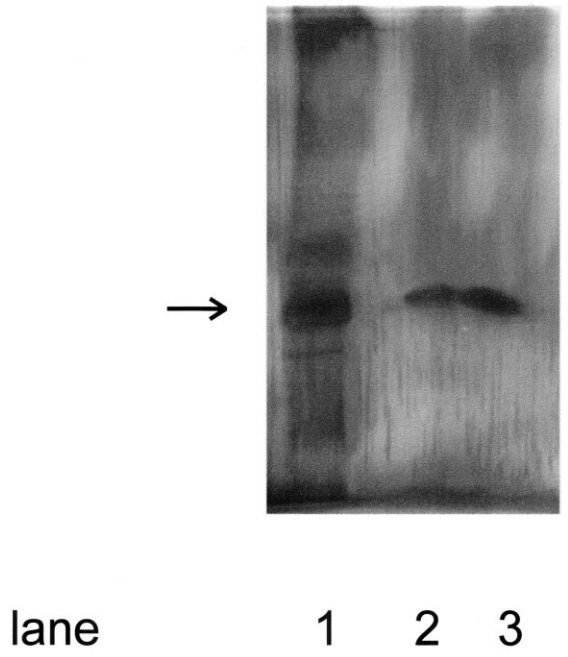

Fig. 3. 2D SDS-PAGE of affinity purified material which was first run out on an IEF gel. The Coomassie stained protein bands at pI 5.1 and 5.3 were cut out, run on SDS-PAGE next to affinity purified material and silver stained. Lane 1 was loaded with affinity purified material, lane 2 and 3 were loaded with gel slices showing bands at pI 5.1 or 5.3, respectively. All three lanes show bands of a MW of 50 kDa (arrow).

tition assays. The highest enzyme activity occurred in wash 5 and wash 6 (100 mM NaCl) with a specific activity of 45 nmoles$*$min$^{-1}*$mg^{-1} (wash 5) or 439 nmoles$*$min$^{-1}*$mg^{-1} (wash 6), respectively. On SDS-PAGE, these two fractions showed a band at around 50 kDa, which probably represents the protein purified by affinity column (Fig. 1, lane 4). The purification data of the purest wash (wash 6) are summarized in Table 1. Analysis of gel slices from narrow range IEF gave the same isoelectric points (pH 5.1 and pH 5.3, see Fig. 2, part A) as observed with the affinity purified material. Data shown on Table 1 summarize the facts from the purest wash (wash 6, 100 mM NaCl).

Fig. 2 compares the different isoforms of JHE obtained following affinity purification or anion-exchange purification. Fig. 2A shows the two or three peaks of JHE activity according to the different pIs, while Fig. 2B shows the corresponding ranges of the silver stained IEF gel and the markedly purer material from affinity purification in lane 1, compared to lane 2.

4. Discussion

Since several JHE sequences (one from *L. dispar)* have been reported recently which have little homology to enzymes previously sequenced and having JH-selective hydrolyzing activity (Valaitis, 1991; Vermunt et al., 1997a,b), it was important to be very careful in establishing which protein(s) contribute to JHE activity in *L. dispar*. Thus, the JHE of *L. dispar* was purified using an affinity purification technique previously shown to be selective for esterases and used in the purification of JHEs of several species (Abdel-Aal and Hammock, 1986; Hammock et al., 1987; Shiotsuki et al., 1994). All three pIs obtained for the JHE of *L. dispar* occur in the same ranges as pIs of other JHEs, which varied between pH 4.2 and pH 6 (Hanzlik and Hammock, 1987; Hanzlik et al., 1989; Venkatesh et al., 1990). Multiple forms of JHE, due to differences in extent of glycosylation, have been found in several other species (Hanzlik and Hammock, 1987; Venkatesh et al., 1990; Vermunt et al., 1997a).

Compared to previous affinity purifications of *H. virescens* (Abdel-Aal and Hammock, 1986) and *Manduca sexta* JHEs (Abdel-Aal and Hammock, 1986; Venkatesh et al., 1990), the purification of JHE from gypsy moth showed a low purification factor of only ×64—as opposed to ×1,120 in *H. virescens* and ×962 or ×780 in *M. sexta*, respectively. This rather low increase of specific activity in the reactivated purified sample and also the low rate of recovery of activity (2%) might be due to inefficiency of reactivation of JHE by dialysis. OTFP is a very strong and tightly-binding inhibitor, known to be difficult to remove from the enzyme (Abdel-Aal and Hammock, 1985). Shiotsuki et al. (1994) also mentioned the problem of reactivation of JHE inhibited with OTFP. The reason this strong inhibitor was used, despite the problems with reactivation, was the fact that OTFP is very efficient in removing JHE from the affinity column. Our main objective was to obtain a sufficient amount of clean protein for peptide sequencing, rather than large amounts of highly active material, in order to be able to characterize the JHE of *L. dispar*.

The partially purified but highly active material from anion-exchange purification showed the same 50 kDa bands as seen with affinity purification but no 60 kDa band was observed on SDS-PAGE of the JHE active fractions. The high yield suggests that all of the JHE activity in this stage of insect is accounted for by the 50 kDa band on SDS-PAGE.

Valaitis (1991) also used a classical approach to purify the JHE of *L. dispar*, including several steps of precipitation, ion-exchange chromatography, gel filtration and RP-HPLC. More than 300 amino acid residues from a 62 kDa protein were obtained. Attempts to align these peptide sequences with the sequence of JHE of *H. virescens* proved difficult. The two proteins were found to have very different primary structures, which seems rather surprising considering that the enzymes share the same function. However, there might be differences in the structures of JHEs from different gene families. Ver-

munt et al., 1997a,b) purified and cloned a putative JHE from a coleopteran species, *Leptinotarsa decemlineata*, with differences in both primary and quarternary structure from the JHE of *H. virescens*. In the present study the alignment of the amino acid sequences of the peptides from *L. dispar* with the sequences of *H. virescens* and *M. sexta* revealed a strong similarity at least in parts of the amino acid sequences and showed that newly purified enzyme is different from the 62 kDa protein isolated by Valaitis (1991).

In our study, JHE activity eluting after affinity chromatography and corresponding to a protein with a molecular weight of approximately 50 kDa was the primary contributor of the JH catalytic activity in hemolymph and thus undoubtedly represents the JHEs of *L. dispar*, at least at this developmental stage. Furthermore, the experiment suggests that this JHE has a common genetic origin to the JHEs of *H. virescens* and *M. sexta*. Our hypothesis that the isolated 50 kDa protein is responsible for JH hydrolysis in *L. dispar* is supported by the following facts:

The enzyme was purified by affinity chromatography using a highly specific resin developed for purification of JHEs. The same method was successfully used to purify JHE from *H. virescens, M. sexta, Bombyx mori, Heliothis zea* (Abdel-Aal and Hammock, 1986; Hammock et al., 1987) and *T. ni* (Hanzlik and Hammock, 1987). Comparison of the amino acid sequences of JHEs from *H. virescens, M. sexta* and *L. dispar* (from the present study) demonstrate that these enzymes have highly conserved regions and are similar in their primary structure. The ultimate proof of the identity of the 50 kDa protein could be obtained from a cDNA clone and expression of active recombinant protein. Until now, only a partial clone of JHE from *L. dispar* (620 bp) has been isolated (Nussbaumer, 1998). The translated amino acid sequence from this cDNA clone contained the sequence of two of the peptides analyzed by amino acid sequencing and thus strongly suggest that the 50 kDa protein purified by affinity chromatography or by anion-exchange chromatography is the JHE that is responsible for JH degradation in the hemolymph of *L. dispar*. The results of our study show that at least within the order Lepidoptera, the differences in primary structures of JHEs might be smaller than supposed so far. Based on the information from this work, a full length clone of this enzyme will be isolated and sequenced yielding greater insight on the degree of homology of JHEs within Lepidoptera.

Acknowledgements

The authors would like to thank Gary Bernon for providing gypsy moth egg masses, Beth Ann Thomas and Haim Moskowitz for their expertise in protein purification, Don Stoutmire for synthesizing the MBTFP ligand and the inhibitor OTFP, Young Moo Lee for expertise in peptide digestion and for amino acid sequencing, Christian Stauffer and Tonya Severson for helpful comments on an earlier version of the manuscript. Financial support of C.N. given by the Austrian Federal Ministery of Science and Transport (BMWV) is greatly acknowledged. Supplies were provided by the US Department of Agriculture (grant number 97-35302-4406).

References

Abdel-Aal, Y.A.I., Hammock, B.D., 1985. Apparent multiple catalytic sites involved in the ester hydrolysis of juvenile hormones by the hemolymph and by an affinity-purified esterase from *Manduca sexta* (Lepidoptera: Sphingidae). Arch. Biochem. Biophys. 243, 206–219.

Abdel-Aal, Y.A.I., Hammock, B.D., 1986. Transition state analogs as ligands for affinity purification of juvenile hormone esterase. Science 233, 1073–1076.

Bell, R.A., Owens, C.D., Shapiro, M., Tardif, J.R., 1981. Development of mass-rearing technology. In: Doane, C.C., McManus, M.L. (Eds.), The Gypsy Moth: Research toward Integrated Pest Management. U.S.D.A. Tech. Bull., vol. 1584, pp. 599–633.

Bradford, M.J., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Analyt. Biochem. 72, 248–254.

DeKort, C.A.D., Granger, N.A., 1981. Regulation of the juvenile hormone titer. A. Rev. Entomol. 26, 1–28.

Doane, C.C., McManus, M.L., 1981. The gypsy moth: research toward integrated pest management. U.S.D.A. Tech. Bull. 1584.

Hammock, B.D., 1985. Regulation of juvenile hormone titer: degradation. In: Kerkut, G.A., Gelbert, L.I. (Eds.) Comprehensive insect physiology, biochemistry and pharmacology, vol. 5. Pergamon Press, New York, pp. 431–472.

Hammock, B.D., Abdel-Aal, Y.A.I., Mullin, C.A., Hanzlik, T.N., Roe, R.M., 1984. Substituted thioflouropropanones as potent selective inhibitors of juvenile hormone esterase. Pesticide Biochem. Physiol. 22, 209–223.

Hammock, B.D., Sparks, T.C., 1977. A rapid assay for insect juvenile hormone esterase activity. Analyt. Biochem. 82, 573–579.

Hammock, B.D., Abdel-Aal, Y.A.I., Hanzlik, T.N., Croston, G., Roe R.M., 1987. Affinity purification and characteristics of juvenile hormone esterase from Lepidoptera. In: Law, J.H. (Ed.), Mol. Entom. 49, UCLA Symposium on Molec. Cell. Biol., New Series, Alan R. Liss, Inc., New York, pp. 315–328.

Hammock, B.D., Harshman, L.G., Philpott, M.L., Székács, A., Ottea, J.A., Newitt, R.A., Wroblewski, V.J., Halarnkar, P.P., Hanzlik, T.N., 1988. Strategies for the discovery of insect control agents: exploitation of biomechanisms regulating insect development. In: Steffens, G.L., Rumsey, T.S. (Eds.) Biomechanisms Regulating Growth and Development. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 137–173.

Hanzlik, T., Hammock, B.D., 1987. Characterization of affinity purified juvenile hormone esterase from *Trichoplusia ni*. J. Biol. Chem. 28, 13584–13591.

Hanzlik, T., Abdel-Aal, Y.A.I., Harshman, L.G., Hammock, B.D., 1989. Isolation and sequencing of cDNA clones coding for juvenile hormone esterase from *Heliothis virescens*. J. Biol. Chem. 264, 12419–12425.

Hwang, B.Y., Smith, A.J., Chu, G., 1996. Internal sequence analysis of proteins eluted from polyacrylamide gels. J. Chromatogr. B686, 165–175.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Nussbaumer, C. (1998) Biology of the solitary braconid, *Glyptapanteles porthetriae* and its influence on the juvenile hormone metabolism of its host *Lymantria dispar*. PhD thesis, University of Vienna, Austria.

Rice, R.H., Mehrpouyan, M., Qin, Q., Lee, Y.M., 1996. Identification of phosphorylation sites in keratinocyte transglutaminase. Biochem. J. 320, 547–550.

Schopf, A., Nussbaumer, C., Rembold, H., Hammock, B.D., 1996. Influence of the braconid *Glyptapanteles liparidis* on the juvenile hormone titer of its larval host, the gypsy moth *Lymantria dispar*. Arch. Insect Biochem. Physiol. 31, 337–351.

Shiotsuki, T., Huang, T.L., Uematsu, T., Bonning, B.C., Ward, V.K., Hammock, B.D., 1994. Juvenile hormone esterase purified by affinity chromatography with 8-mercapto-1,1,1-triflouro-2-octanone as a rationally designed ligand. Protein Expression and Purification 5, 296–306.

Tanaka, S., Chang, M.T., Denlinger, D.L., Abdel-Aal, Y.A.I., 1989. Developmental landmarks and the activity of juvenile hormone and juvenile hormone esterase during the last stadium and pupa of *Lymantria dispar*. J. Insect Physiol. 35, 897–905.

Valaitis, A.P., 1991. Characterization of hemolymph juvenile hormone esterase from *Lymantria dispar*. Insect Biochem. 21, 583–595.

Venkatamaran, V., O'Mahony, P.J., Manzcak, M., Jones, G., 1994. Regulation of juvenile hormone esterase gene transcription by juvenile hormone. Devl Genet. 15, 391–400.

Venkatesh, K., Abdel-Aal, Y.A.I., Armstrong, F.B., Roe, R.M., 1990. Characterization of affinity purified juvenile hormone esterase from plasma of the tobacco hornworm *Manduca sexta*. J. Biol. Chem. 265, 21727–21732.

Vermunt, A.M.V., Vermeesch, A.M.G., DeKort, C.A.D., 1997a. Purification and characterization of juvenile hormone esterase from hemolymph of the colorado potato beetle. Arch. Insect Biochem. Physiol. 35, 261–277.

Vermunt, A.M.V., Koopmanschap, A.B., Vlak, J.M., De Kort, C.A.D., 1997b. Cloning and sequence analysis of cDNA encoding a putative juvenile hormone esterase from the colorado potato beetle. Insect Biochem. Molec. Biol. 27, 919–928.