Rapid communication

Identification of a juvenile hormone esterase gene by matching its peptide mass fingerprint with a sequence from the Drosophila genome project

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Abstract

Juvenile hormone esterase (JHE, EC 3.1.1.1) from whole Drosophila melanogaster prepupae has previously been purified by selective precipitations, isoelectric focussing and two column chromatography steps. JHE bands from dried silver-stained SDS–PAGE gels of that material were digested with trypsin. The masses of the tryptic digest peptides were determined by MALDI–TOF mass spectrometry. Only one predicted gene product (CG8425) from the D. melanogaster genome matches the JHE tryptic fingerprint with high confidence. This predicted JHE sequence includes features that are conserved among all active members of the serine carboxylesterase multigene family as well as features peculiar to JHEs from other species. Also we show that this JHE can be purified by an alternative method using anion exchange chromatography followed by trifluoromethylketone affinity chromatography. A cDNA encoding this JHE was isolated using 3’ and 5’ RACE. This sequence is in agreement with the Drosophila genome project’s prediction except that the sixth predicted intron is not removed; instead there is a stop codon followed by a polyadenylation signal and a polyA tail. © 2001 Elsevier Science Ltd. All rights reserved.

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Precisely timed changes of juvenile hormone (JH) titre are essential for the regulation of many aspects of insect development and reproduction. One key event is the clearing of JH that generally precedes the moult from the last larval stage to the pupal stage of holometabolous insects. The very low JH titre at this time is generally achieved by the combined effect of reduced JH synthesis and scavenging by JH degrading enzymes (Roe and Venkatesh, 1990). The main JH degrading activity at this time in Drosophila melanogaster is juvenile hormone esterase (JHE, EC 3.1.1.1) which hydrolyses JH, an α/β unsaturated methyl ester, to the corresponding carboxylic acid (Campbell et al., 1992). An enzyme with JHE activity has been purified from D. melanogaster prepupae and it has a very low $K_m$ (89 nM) and a high specificity constant ($6.8 \times 10^6 \text{M}^{-1} \text{s}^{-1}$) for JH, consistent with a role in scavenging JH at low concentrations (Campbell et al., 1998). Most data suggest that there is only one major JHE in D. melanogaster prepupae but there is some evidence for more than one form in adults and another Drosophila species (Campbell et al., 1992; Khlebodarova et al., 1996).

Full or partial cDNA or amino acid sequences of JHEs are available from several lepidopterans and two Coleopterans (Thomas et al., 2000) but none from D. melanogaster or any other dipteran. We had pure JHE from D. melanogaster prepupae (Campbell et al., 1998) remaining as silver-stained bands in dried SDS–PAGE gels. We were able to determine a tryptic peptide mass fingerprint for this archived material and show that it matches a predicted gene product (CG8425) in the datab-
2. Materials and methods

JHE was purified from prepupae of *D. melanogaster*, strain 12111.2, eight years ago by the classical methods of selective precipitation and isoelectric focusing, followed by anion exchange and gel permeation chromatography (Campbell et al., 1998). The only remaining JHE from this purification was contained within silver-stained SDS–PAGE gels that had been stored dry between sheets of Promega gel drying film, one for 8 yr and another for 2 yr. The two bands were processed separately as follows to obtain two peptide mass fingerprints. The bands were rehydrated with 10 µl of water, and the film was removed. In-gel digestion of the protein was performed with reduction and carbamidomethylation of any cysteine residues. Gel pieces were incubated with 2.8 mM dithiothreitol in ammonium bicarbonate solution (160 µl, 100 mM) for 30 min at 60°C. Iodoacetamide (10 µl, 5.9 mM final conc.) was added and the reaction was kept for a further 30 min at room temperature in the dark. The gel pieces were washed twice with 50% acetonitrile/0.1% trifluoroacetic acid, dried under vacuum, rehydrated with 150 ng of trypsin (Promega sequencing grade) in 10 µl ammonium bicarbonate (100 mM), incubated overnight at 37°C, then extracted twice with 50% acetonitrile/0.1% trifluoroacetic acid in a sonating water bath. For each gel piece, the extracts were pooled and dried in a vacuum centrifuge, redissolved in 2 µl of matrix solution (10 mg/ml of α-cyano, 4-hydroxy, cinnamic acid in 50% acetonitrile/0.1% trifluoroacetic acid), and dried on the sample plate of a Voyager Elite MALDI–TOF mass spectrometer (Perseptive Biosystems). Peptides in a trypsin digest of bovine serum albumin (927.4940 and 2045.0285) were used for a close external calibration of the JHE digest. The mass list from the JHE digest was used to search the NCBI nr database (29/3/2000, *Drosophila* only) using ProFound software (Version 4.8.5, Rockefeller University). The largest and smallest masses from the best matching sequence were used for internal calibration of the mass spectrum and the search was repeated with a mass tolerance of 30 ppm.

JHE from prepupae of *D. melanogaster* was also purified by an affinity method. Prepupae of a wild type *D. melanogaster* strain 12111.2 were staged by the flotation method to 12±6 h after bubble formation, the time of maximum JHE activity (Campbell et al., 1992). Prepupae were stored at −80°C. Frozen prepupae (50 g) were homogenised on ice with 250 ml of 10 mM KOH adjusted with acetic acid to pH 4.5 and a few milligrams of phenylthiourea (Campbell et al., 1998). The homogenate was clarified by centrifugation at 13,000g, 4°C for 20 min followed by filtration through glass wool. The pH of this solution (170 ml) was then raised to 7.5 with the addition of Tris-base and water to a final volume of 340 ml. An aliquot (90 ml) was absorbed with gentle agitation (4°C, 2 h) onto DEAE Sepharose CL6B (6 ml, Pharmacia) that had been equilibrated with 20 mM Tris/HCl, pH 7.5. The DEAE Sepharose was collected in a column and washed four times with 6 ml of 20 mM Tris/HCl, pH 7.5 at room temperature. The column was eluted by sixteen stepwise additions of 1 ml of increasing NaCl concentrations (0.05–1 M) in 20 mM Tris/HCl, pH 7.5. JHE activity was monitored as described previously (Campbell et al., 1992) in the eluting fractions (1 ml). The active fractions were pooled and stored at −80°C.

Details of the MBTFP trifluoropropanone affinity gel and octylthiotrifluoropropanone (OTFP) used to elute the gel are given by Abdel-Aal and Hammock (1986). A settled bed of affinity gel (1.5 ml) was washed with a gradient from ethanol to water, followed by 5 ml of 140 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4, pH 7.3 (PBS), and then 3 ml of lysozyme (10 mg/ml) in PBS to block any non-specific protein binding sites, and the column was again washed extensively with buffer. The active material from the DEAE column was diluted 10-fold in PBS and passed slowly through the affinity column (20 ml in approx. 3 h at 4°C) until JHE activity was detected in the eluate and the column was assumed to be saturated. The column was washed with lysozyme in PBS again, then washed slowly (overnight) with PBS (40 ml) containing 0.1% CHAPS detergent, 5% sucrose, 1 mM EDTA and 0.02% NaN3 (wash buffer). The column containing the affinity gel was sealed with extra wash buffer (2.6 ml final vol.) and 26 µl of OTFP solution (10 mM in ethanol, 100 µM final conc.) and shaken gently (150 rpm, 4°C, 3 h). The column was replaced upright and the excess fluid (1.5 ml) and a wash with 100 µM OTFP in wash buffer (3 ml) were collected from the column outlet. This was designated fraction 1. The affinity gel was shaken again overnight with a little extra OTFP/wash buffer and 1 ml of eluate was collected (fraction 2). Finally, a more stringent elution was performed as for fraction 1 except that the incubation with OTFP/wash buffer was overnight.
and the concentrations of OTFP and CHAPS were 2- and 10-fold greater, respectively (fraction 3). Aliquots of the three affinity fractions (16 µl) were assessed by 10% SDS–PAGE (Laemmli, 1970) using a mini Protein II SDS–PAGE apparatus (Bio-Rad) with silver staining (BioRad silver stain kit).

Aliquots of fractions 1 and 3 (100 µl) were diluted to 1 ml with wash buffer containing 0.5 mg/ml of bovine serum albumen (BSA) and dialysed at 4°C against 11 of PBS changed four times in 48 h and daily thereafter. Aliquots were removed and assayed for JHE activity after 4, 8, and 16 days.

Aliquots of fractions 1 and 3 were concentrated by electrophoresis or precipitation, respectively. An aliquot of fraction 1 (300 µl) was heated with Laemmli (1970) sample buffer (95°C, 5 min) and electrophoresed through a funnel-shaped SDS–PAGE apparatus that concentrates the proteins into a small piece of polyacrylamide gel (Dainese Hatt et al., 1997). The concentrated protein was localised with Fast Blue Stain (Zoion) according to the manufacturer’s instructions. The stained gel band was excised, shrunk with 50% acetonitrile, and rehydrated to its original volume with SDS–PAGE sample buffer. The gel piece was left at room temperature overnight to allow the proteins in the gel piece to dissolve fully. Proteins from the gel piece were then separated in a 10% resolving gel, as above, and stained with Zoion Fast Blue. An aliquot of fraction 3 (1 ml) was concentrated to about 300 µl in a vacuum centrifuge. Cysteine residues were reduced overnight with 1 µl of β-mercaptoethanol then reacted with iodoacetamide (33 µl of 0.5 M) for two hours. The proteins were then precipitated for 30 min on ice with the addition of 50% trichloroacetic acid to a final concentration of 10%. The proteins were sedimented (15000 g, 20 min) and the pellet was washed with cold 90% methanol. The pellet was air-dried, resuspended and heated with Laemmli (1970) sample buffer (95°C, 5 min) and separated in a 10% SDS–PAGE resolving gel. Protein bands at about 66 kDa were resolved and heated with Laemmli (1970) sample buffer (95°C, 5 min) and separated in a 10% SDS–PAGE gel, as above, and stained with Zoion Fast Blue. An aliquot of fraction 3 (1 ml) was concentrated to about 300 µl in a vacuum centrifuge. Cysteine residues were reduced overnight with 1 µl of β-mercaptoethanol then reacted with iodoacetamide (33 µl of 0.5 M) for two hours. The proteins were then precipitated for 30 min on ice with the addition of 50% trichloroacetic acid to a final concentration of 10%. The proteins were sedimented (15000 g, 20 min) and the pellet was washed with cold 90% methanol. The pellet was air-dried, resuspended and heated with Laemmli (1970) sample buffer (95°C, 5 min) and separated in a 10% SDS–PAGE resolving gel. Protein bands at about 66 kDa were excised from the gels of the two concentrated fractions and both bands were digested with trypsin as above.

The mRNA sequence of the JHE gene was determined using 3′- and 5′-Rapid Amplification of cDNA Ends (RACE). Primers were chosen so that 3′- and 5′-RACE would yield overlapping products and therefore the complete cDNA sequence of the JHE gene. Total RNA was isolated from homogenates of whole D. melanogaster prepupae, strain 12I11.2, aged as above for peak JHE activity (Campbell et al., 1992). Approximately 50 mg of prepupae yielded 3.4 µg of purified mRNA using the QuickPrep Micro mRNA Purification Kit (Amersham Pharmacia Biotech). This mRNA (1 µg) was used as the template for the 5′RACE System (Gibco BRL). Two antisense primers, (5′-GTT–CTG–ATC–CTT–TAG–GCC–3′) and (5′-GGC–CAA–AGT–TAC–CAG–ACA– TCA–3′), were designed for nested PCR from the pre-
dicted D. melanogaster cDNA sequence CG8425, and synthesised by GeneWorks. The 5′RACE product was cloned using the pGem-T Easy Vector System (Promega) and sequenced with pUC/M13 forward and reverse universal vector primers (Bresatec).

For 3′RACE, first strand cDNA was synthesised from 1 µg of D. melanogaster prepupal mRNA using AMV Reverse Transcriptase (Promega), and 0.48 µg of a poly-T adaptor primer (5′-GCG–GCC–GCT–TGA–ATT– CCA–ACT–TTT–TTT–TTT–TTT–TTT–T-3′) according to Promega Technical Bulletin No. 502, with the addition of fresh DTT (20mM final concentration). Second strand synthesis and PCR amplification of cDNA used 50 pmoles of adaptor primer (5′-GCG–GCC–GCT– TGA–ATT–CCCA–AC-3′) and a CG8425-derived primer (5′-GTT–GTC–TAC–GGC–GAT–GAG-3′), 1 mM MgCl$_2$, 0.2 mM dNTP mix, 1X Taq DNApolymerase reaction buffer and 10u Taq DNApolymerase (Gibco BRL), and 1 µl of a 1:20 dilution of the first strand cDNA/mRNA mixture, in a total volume of 50 µl. The following amplification conditions were used: 3 min at 94°C for initial denaturation followed by 40 cycles of 94°C for 45 s, 50°C for 1 min, and 72°C for 2 min. Final extension conditions were 72°C for 7 min. A second, hemi-nested round of PCR was performed using 1 µl of the first reaction mixture (purified using the QIAquick PCR Purification Kit, Qiagen), 50 pmoles each of adaptor primer and another CG8425-derived primer (5′- CGC–ACT–TGT–AAT–GTT–CTA–3′). Conditions and other reagents were as above except that the annealing was performed at 57°C. The gel purified product (QIAquick PCR Purification Kit, Qiagen) was cloned into the pSTBlue-1 vector (Perfectly Blunt Cloning Kit, Novagen) and sequenced. All incubations and PCR cycling was performed on either a PerkinElmer GeneAmp PCR System 9700 or 2400. Sequencing was performed by the ABI Prism BigDye terminator method (Perkin-Elmer).

3. Results and discussion

The tryptic digest fingerprints of classically purified JHE from D. melanogaster prepupae (Campbell et al., 1998) unambiguously match CG8425, one of the predicted gene products deposited in the databases by Adams et al. (2000). Tryptic digest peptides were recovered from two archived SDS–PAGE gels containing classically purified D. melanogaster prepupal JHE. The peptide mass spectra from the two gels were virtually identical and one is shown in Fig. 1. The peptide masses were used to search Drosophila sequences after the masses of a trypsin autolysis peptide (842.5) and several peptides derived from human keratin were excluded. Only one predicted gene product (CG8425) was a close match to the submitted mass list (Z=2.36, P<0.05). Thir-
Fig. 1. Tryptic digest fingerprints of classically purified D. melanogaster prepupal JHE and affinity enriched JHE. The upper panel shows a mass spectrum of peptides from a trypsin digest of classically purified D. melanogaster prepupal JHE. The sample was recovered from a dried, silver-stained SDS–PAGE gel. The lower panel shows the mass spectrum of peptides from a trypsin digest of trifluoromethyl ketone affinity purified material (‘Fraction 1’). The sample was recovered from an SDS–PAGE band stained with Fast Blue (Zoion) showing the expected mobility for D. melanogaster prepupal JHE (about 66 KDa). Peaks matching the masses predicted from the CG8425 gene product are marked ‘J’ for JHE. Peaks matching esterase 6 are marked ‘6’. A peak due to trypsin autolysis is marked ‘T’.

Teen peptides derived from JHE matched the theoretical masses for CG8425 within 25 ppm (Fig. 1). These peptides were distributed throughout the N-terminal two thirds of the predicted product with 31% coverage of the sequence to residue 515. That region of the CG8425 sequence can be aligned readily with other carboxylesterase sequences. The predicted mass of the CG8425 product is 91 kDa, about 50% larger than is typical for carboxylesterases, and there were no peptides matching the C-terminal third of the predicted product (residues 516–803) which does not show similarity to carboxylesterases.

The affinity column fractions were not homogeneous but were enriched for bands at about 66 kDa, the mobility previously observed for D. melanogaster prepupal JHE (Fig. 2, Campbell et al., 1998). The 66 kDa zone from fraction 1 contains predominantly the CG8425 product as is clear from the similarity of its peptide fingerprint with that of JHE isolated by classical methods (Fig. 1). Searching with the mass list from this digest again indicates CG8425 (Z=2.23, P<0.05) with only one CG8425-matching peptide not previously identified (MH+=853.4, residues 228–234). Esterase 6 was identified as the next best ‘hit’ when the search was repeated with the nine CG8425-matching masses excluded. Esterase 6 is a well characterised D. melanogaster protein that does not have JHE activity (Myers et al., 1993). The 66 kDa zone from fraction 3 contained predominantly esterase 6 (data not shown). Thus it appears that the different conditions of elution for fractions 1 and 3 afforded a partial separation of the two esterases.

Shevchenko et al. (1998) and Matsumoto and Komori (1999) reported protease digest fingerprints from SDS–PAGE material stored dry at room temperature for nearly 5 or 17 yr, respectively. In both cases the proteins were stained with Coomassie dye which causes little risk of covalent modification of proteins. On the other hand silver staining protocols of the type we used (Biorad silver stain kit) include reagents that risk modifications (Shevchenko et al., 1996). We saw no evidence of this in the close similarity of the mass spectra from the two
Fig. 2. SDS–PAGE of fractions from trifluoromethylketone affinity chromatography. Aliquots (16 μl) of fractions 1, 2, and 3 and molecular weight marker proteins were separated by SDS–PAGE on a 10% gel and stained with silver. A band (L) present in all fractions is lysozyme. Two bands were digested with trypsin (after concentration—see text). Band J (for JHE) showed predominantly the CG8425 product with esterase 6 also detected (Fig. 1) while band 6 showed predominantly esterase 6.

JHE preparations (silver-stained and dried with room temperature storage for 8 yr versus Fast Blue-stained with prompt processing, Fig. 2) and the presence of only one major peak (951.5) that was not matched to the unmodified CG8425 sequence or known contaminants.

A cDNA corresponding to the Drosophila genome project’s predicted product CG8425 was isolated by overlapping 3’ and 5’ RACE and sequenced (Fig. 3, GenBank accession no. AF304352). The cDNA confirms the predicted CG8425 product except that the sixth predicted intron was not removed; instead there is an in-frame stop codon immediately after the predicted splice site followed by a polyadenylation signal and a polyA tail. This is consistent with finding only digest peptide masses to match the N-terminal two-thirds of CG8425 as it was originally submitted. Furthermore, the predicted molecular weight and pI of the mature protein encoded by our mRNA sequence (61.8 kDa and 5.9) are in reasonable agreement with experimental values for D. melanogaster JHE (66 kDa and 5.4, Campbell et al., 1998). Within the coding region of the mRNA are only six nucleotide differences from the CG8425 sequence and all are silent.

The amino acid sequence of JHE from D. melanogaster prepupae matches consensus sequences for type B carboxylesterases (residues 106–116 and 200–215, not shown). Conserved features of carboxylesterases found in this JHE include a catalytic triad composed of Ser213, His471 and Glu346 (Fig. 3). Also conserved is an additional Ser (239 in JHE) that is found in the active site of all catalytically active serine esterases and is proposed to have an essential function (Thomas et al., 1999). Putative oxynion hole residues are conserved (Gly132, Gly133 and Ala214), as are a pair of Cys residues (90 and 108) forming a disulphide linkage, and residues forming salt bridges (Arg67 and Glu106, Arg163 and Asp185). There is a predicted signal peptide (cleaving after Ala20) consistent with the assumption that this JHE is secreted into the haemolymph of prepupae (Campbell et al., 1992).

An alignment (Fig. 4) of JHE from D. melanogaster prepupae with the full-length sequences of JHE from the lepidopterans Heliothis virescens and Choristoneura fumiferana (Feng et al., 1999) shows 29% identity overall. A portion of the protein (residues 123–241) including the oxynion hole residues and the catalytic serine residue shows 54 or 57% identity with the two lepidopteran sequences and 60% identity with the corresponding region from a partial sequence of JHE from the coleopteran Tenebrio molitor (Thomas et al., 2000). The comparisons reveal two features shared among these JHEs. The sequence in the vicinity of the catalytic Ser in most esterases and lipases is GESAG; an alternative, GQSAG, is uncommon but found in the D. melanogaster, lepidopteran and T. molitor JHEs. The GQSAG motif occurs only once more among about 38 carboxylesterase sequences in the D. melanogaster genome (Rubin et al., 2000, and see below). Thomas et al. (1999) propose that Gln rather than Glu indirectly shortens the hydrogen bond between the His and Glu residues of the catalytic triad and that might partially account for the very low $K_m$s of JHEs for their substrates. Thomas et al. (1999) also identified a series of basic amino acid residues along one face of an amphipathic helix as a characteristic of lepidopteran JHEs. This feature (Lys184, Arg191 and Arg195) is also found in D. melanogaster JHE while T. molitor has an amphipathic helix with both acid and basic residues in the corresponding positions.

We have found no evidence to suggest there are more than one JHE in prepupae of D. melanogaster (Campbell et al. 1992, 1998) but there may be more than one form of JHE in adults of D. melanogaster and other Diptera (Campbell et al., 1992) and two JHE activities were separated from prepupae of Drosophila virilis (Khlubodarova et al., 1996). We have analysed the phylogenetic relationships of all the carboxylesterase genes of D. melanogaster (not shown). Candidates for
Fig. 3. Sequence and translation of a *D. melanogaster* cDNA encoding JHE. Peptides corresponding to the masses indicated in Fig. 1 are underlined. Catalytic triad members (Tri) and an additional serine residue (Add’l Ser) are conserved among this sequence, other JHEs and esterases in general. Sequence comparisons also suggest salt bridges and a disulphide bond at the indicated positions. The GQSAG motif (bold) is also found around the active serine residue of lepidopteran JHEs. Lepidopteran JHEs are predicted to have an amphipathic helix that includes basic residues at positions homologous to those indicated here.

Other JHEs include CG8424, CG7529 and CG9858 (Fig. 4). CG8424 has the most similar sequence to CG8425 (40% identity) although it does not have the GQSAG motif. It is the gene immediately 5′ of CG8425 in the genomic DNA and presumably the result of gene duplication. CG7529 shows about 30% sequence identity with CG8425 in common with many other esterases but is noteworthy as the only other *D. melanogaster* esterase to share the GQSAG motif. Finally, CG9858 also shows about 30% amino acid sequence identity with CG8425 and maps to the same chromosomal region as *cricket*, a gene that appears to have a role in the JH system (Shirras and Bownes, 1989; Claudianos et al., 2000). The roles of such candidate JHEs are yet to be demonstrated but might include site-specific JH degradation (Rauschenbach et al., 1996), or different specificities for the three forms of JH that are produced in the higher Diptera, methyl farnesoate, JHIII and JHIII bisepoxide (Yin et al., 1995).
Fig. 4. Alignment of D. melanogaster JHE with other esterase sequences. The sequence of D. melanogaster JHE is aligned with JHES from lepidopterans, H. virescens (Hvir) and C. fumiferana (Cfum), the coleopteran, T. molitor (Tmol), and three other esterases from D. melanogaster (CG numbers). Shading indicates identical and similar residues shared among the sequences. Two features associated with JHE sequences are indicated. Firstly, a Gln residue adjacent to the catalytic Ser (JHE–Gln) is conserved among the JHE sequences. Secondly, an amphipathic helix (JHE-helix) that generally has basic residues in the indicated positions is also conserved among the JHEs. The probable positions of salt bridges and a disulfide bond are indicated by lines joining the relevant residues. Catalytic triad members (Tri) and an additional serine residue (Add’l Ser) proposed to be essential for esterase activity (Thomas et al., 1999) are conserved among the JHE sequences. The three CG numbered sequences share some features with the JHE sequences. CG7529 has the GQSAG motif but not the amphipathic helix and its catalytic triad is the less common Ser–His–Asp rather than Ser–His–Glu. In contrast CG8424 and CG9858 lack the GQSAG motif but more closely resemble the amphipathic helix pattern of the JHES and share the common Ser–His–Glu catalytic triad. CG8424 appears to lack the additional serine residue (Add’l Ser) but the aligned threonine residue might substitute or the sequence might be misaligned with the additional serine residue placed two positions closer to the N-terminus.
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