Identification of *Drosophila melanogaster* yellow-f and yellow-f2 proteins as dopachrome-conversion enzymes

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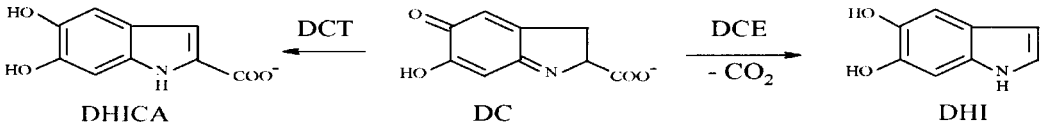
This study describes the identification of *Drosophila* yellow-f and yellow-f2 as dopachrome-conversion enzymes responsible for catalysing the conversion of dopachrome into 5,6-dihydroxyindole in the melanization pathway. *Drosophila yellow*-y gene and *yellow*-b, -c, -f and -f2 genes were expressed in an insect cell/baculovirus expression system and their corresponding recombinant proteins were screened for dopachrome-conversion enzyme activity. Among the *yellow* and *yellow*-related genes, the *yellow*-f and *yellow*-f2 genes were identified as the genes coding for *Drosophila* dopachrome-conversion enzyme based on the high activity of their recombinant proteins in catalysing the production of 5,6-dihydroxyindole from dopachrome. Both yellow-f and yellow-f2 are capable of mediating a decarboxylative structural rearrangement of dopachrome, as well as an isomerization/tautomerization of dopamine chrome and dopa methyl ester chrome. Northern hybridization revealed the transcription of *yellow*-f in larvae and pupae, but a high abundance of mRNA was observed in later larval and early pupal stages. In contrast, yellow-f2 transcripts were present at all stages, but high abundance of its mRNA was observed in later-stage pupae and adults. These data indicate that yellow-f and yellow-f2 complement each other during *Drosophila* development and that the yellow-f is involved in larval and pupal melanization, and yellow-f2 plays a major role in melanization reactions in *Drosophila* during later pupal and adult development. Results from this study provide the groundwork towards a better understanding of the physiological roles of the *Drosophila yellow* gene family.

Key words: 3,4-dihydroxyphenylalanine (dopa), dopa methyl ester, dopamine, melanization.

INTRODUCTION

In insects, dopachrome-conversion enzyme (DCE) catalyses the conversion of L-dopachrome (DC) into 5,6-dihydroxyindole (DHI) in the melanization pathway (see Scheme 1) and significantly accelerates insect melanization reactions. DCE activity has been detected in several insect species [1–6], but there has been limited information regarding its molecular characteristics. Recently we isolated a DCE cDNA (AaDce1) from a mosquito (*Aedes aegypti*) pupal library [7], and subsequently evaluated its chemical characteristics [8]. The amino acid sequence of the *A. aegypti* DCE shares no similarity to those of mammalian dopachrome tautomerase, which uses the same substrate (although their products are different, see Scheme 1) and is involved in the same melanization pathway [9–15]. However, the AaDce1 primary sequence is 16–40% similar to the *Drosophila* yellow proteins (see Figure 1).

The *Drosophila yellow* gene (NCBI accession no. P09957) is related to wing and cuticle pigmentation, and mutation of the *yellow* gene leads to the formation of a yellow-coloured cuticle instead of a black cuticle [16–18]. This classical *yellow* gene, discussed in 509 references between 1916 and 2000 (FlyBase report, http://flybase.bio.indiana.edu/.bin/fbidq.html?FBgn0004034), is one of the most extensively studied genes. Soon after the completion of the *Drosophila* genome-sequencing project in 2000, several genes were classified as *yellow* genes. Since then the *Drosophila yellow* gene family has grown, and now a total of 14 new genes have been annotated as members of the *yellow* gene family [19]. These genes are grouped with the classical *Drosophila yellow* gene (termed *yellow*-y to distinguish it from other *yellow* genes) based on their sequence identity. Members of the *Drosophila yellow* gene family share essentially no sequence identity with proteins from bacteria to humans, except for



Scheme 1 Dopachrome tautomerase (DCT)-mediated isomerization/tautomerization of DC to 5,6-dihydroxyindole-2-carboxylic acid (DHICA) and DCE-mediated decarboxylative structure rearrangement of DC to DHI

Abbreviations used: DC, L-dopachrome; DCE, dopachrome-conversion enzyme; DHI, 5,6-dihydroxyindole; DHICA, 5,6-dihydroxyindole-2-carboxylic acid; L-dopa, 3,4-dihydroxyphenylalanine; dopamine, 3,4-dihydroxyphenylethylamine; HPLC-ED, HPLC with electrochemical detection; HTS, high-titre viral stocks; ORF, open reading frame; MOI, multiplicity of infection; MALDI-TOF, matrix-assisted laser-desorption ionization–time-of-flight.

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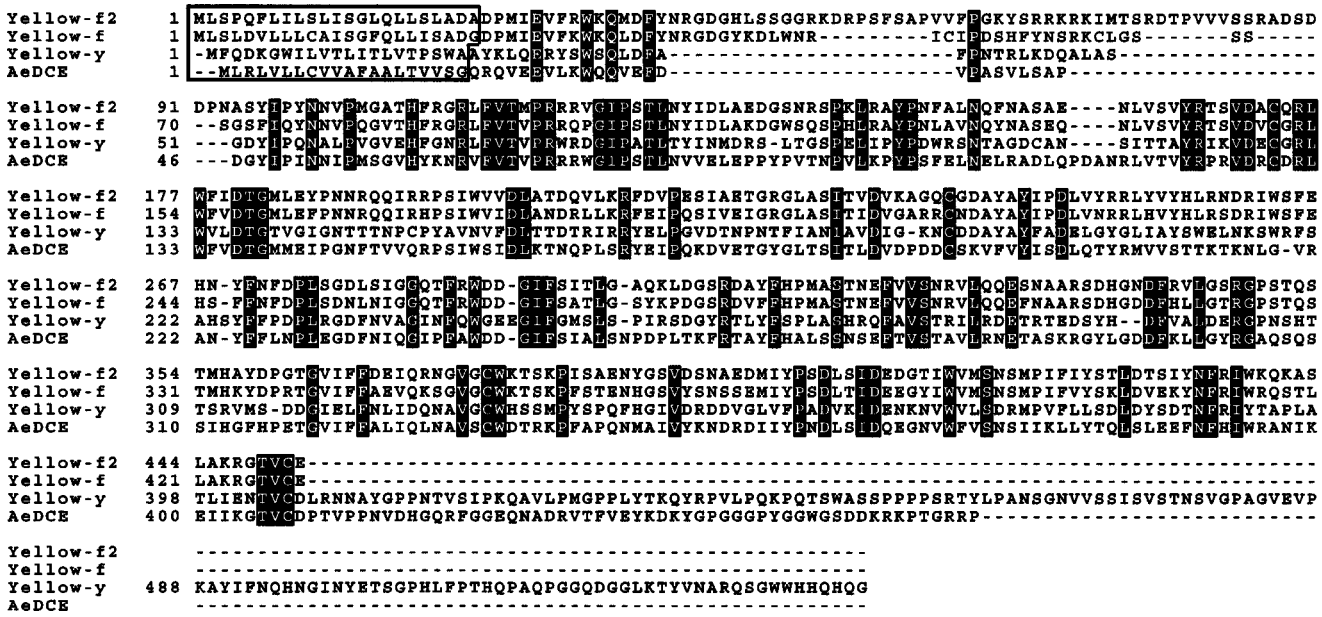


Figure 1 Sequence alignment of *Drosophila* yellow-f, yellow-f2, yellow-y and AaDce1 (AeDCE)

Drosophila yellow-f shares 73%, 41% and 36% sequence identity with yellow-f2, AaDce1 and yellow-y, respectively. The boxed sequences show the predicted signal peptides.

some similarity with the major royal jelly protein from the honeybees [20–22], and several yellow-like proteins from other insects [23,24]. Recent studies suggest that the temporal and spatial profiles of *Drosophila yellow*-y and *ebony* gene together determine the pattern and intensity of melanization [25], but the mechanism by which the *yellow*-y gene promotes the formation of pigments is poorly understood. Nothing is known regarding the physiological function of the rest of the *yellow* gene family.

The sequence similarity between the mosquito DCE and the *Drosophila* yellow-y and other yellow proteins, in conjunction with the positive relationship between yellow-y expression and wing or cuticle melanization, suggests that either yellow-y and/or other members of the yellow protein family may function as *Drosophila* DCE. In this study, we expressed *Drosophila yellow*-y and other *yellow*-related genes, including *yellow*-b, -c, -f and -f2, in an insect cell/baculovirus expression system and screened the recombinant proteins for DCE activity. Herein, we provide data that demonstrate that yellow-f and yellow-f2 from the *Drosophila* yellow protein family function as *Drosophila* DCE and that these two proteins likely play important physiological roles in melanization reactions. Because both the *yellow*-f and *yellow*-f2 share considerable sequence homology with the other members of the *Drosophila yellow* gene family, results from this study may provide some insight towards elucidating the overall physiological functions of the *yellow* gene family and also might serve as a useful reference to study other insect DCEs.

EXPERIMENTAL

Chemicals and reagents

3,4-Dihydroxyphenylalanine (L-dopa), 3,4-dihydroxyphenylethylamine (dopamine), dopa methyl ester, α-methyl dopa, sodium periodate, PMSF, β-mercaptoethanol and rabbit liver esterase were obtained from Sigma (St. Louis, MO, U.S.A.). The insect cell/baculovirus expression system was purchased from Invitrogen (Carlsbad, CA, U.S.A.).

Drosophila melanogaster rearing and maintenance

Both wild-type and *yellow* mutant *Drosophila* used in this study were obtained from the Carolina Biology Supply Company (Burlington, NC, U.S.A.) and reared on a ready-to-use *Drosophila* medium from the same company. Both phenotypes were maintained at 25 ± 0.5 °C and under a 16 h:8 h light/dark cycle.

Construction of recombinant transfer vectors

An insect cell line/baculovirus expression system (Invitrogen) was used for the expression of *D. melanogaster* yellow-y and yellow-y-related proteins. The coding sequences of the *yellow*-y gene (NCBI accession no. P09957), *yellow*-b (accession no. AAF53564), *yellow*-c (accession no. AAF53432), *yellow*-f (accession no. AAF54884), *yellow*-f2 (accession no. AAF54885) and also a truncated *yellow*-y gene lacking the coding sequence for the 81 C-terminal residues were obtained by reverse transcriptase PCR amplification of *Drosophila* cDNA synthesized from mRNA extracted from 5-day-old larvae. The forward primers, containing a *Xba*I or a *Pst*I restriction site, and the reverse primer, containing a *Hin*dIII restriction site, were designed based on the open reading frames (ORFs) of the *yellow*-y and *yellow*-y-related genes (Table 1). The PCR products were cloned into a PCR2.1-TOPO TA cloning vector and then subcloned into a baculovirus transfer vector pBlueBac4.5 (Invitrogen) between the *Xba*I (or *Pst*I for *yellow*-b) and *Hin*dIII restriction sites. All recombinant transfer vectors were sequenced and confirmed that the inserted DNA sequences were in frame.

Table 1 Oligonucleotide primers used for amplification of *Drosophila yellow* genes

The underlined nucleotide sequences represent the introduced restriction sites.

Gene	Strand	Nucleotide sequence	Restriction site
yellow-y	Sense	5′-GGTCTAGATGTTCCAGGACAAAGGG-3′	*Xba*I
	Antisense	5′-CCAAGCTTAACCTTGATGCTGATG-3′	*Hin*dIII
yellow-y (truncated)	Sense	5′-GGTCTAGATGTTCCAGGACAAAGGG-3′	*Xba*I
	Antisense	5′-GGAAGCTTAATAAGTGCGACTTGGA-3′	*Hin*dIII
yellow-b	Sense	5′-GGCCTGCAGATGCTGCGATTCAGATTA-3′	*Pst*I
	Antisense	5′-CCGAAGCTTAGATTCTAAAGCTCAT-3′	*Hin*dIII
yellow-c	Sense	5′-GGCTCTAGATGAACCCACTAGGAATG-3′	*Xba*I
	Antisense	5′-GGCAAGCTTACATTTCGCATGGTGTAC-3′	*Hin*dIII
yellow-f	Sense	5′-GGTCTAGATGTTATCGCTGGACGTT-3′	*Xba*I
	Antisense	5′-CCAAGCTTACTCACA AACTGTTCC-3′	*Hin*dIII
yellow-f2	Sense 1*	5′-GGCTCTAGATGACCTCCCGCGATACA-3′	*Xba*I
	Sense 2*	5′-GGCTCTAGATGTTATCGCCCCAA-3′	*Xba*I
	Antisense	5′-CGAAGCTTTCACTCACAAACTGTGCC-3′	*Hin*dIII

* The sense 1 and sense 2 primers amplified the partial and full ORFs of *yellow*-f2, respectively.

Production and isolation of recombinant baculoviruses

Individual recombinant pBlueBac4.5 transfer vectors were co-transfected with linearized Bac-N-Blue™ (AcMNPV, *Autographa californica* multiple nuclear polyhedrosis virus) viral DNA in the presence of InsectinPlus™ insect-cell-specific liposomes to *Spodoptera frugiperda* (Sf9) insect cells (Invitrogen). The recombinant baculoviruses were purified by plaque assay procedure. Blue putative recombinant plaques were transferred to 12-well microtitre plates and amplified in Sf9 cells. Viral DNA was isolated for PCR analysis to determine the purity of recombinant viruses. High-titre viral stocks (HTS) for individual recombinant viruses were generated by amplification in Sf9 cell suspension culture.

Recombinant protein expression

High Five insect cells (Invitrogen) were used for protein expression. The cells were cultured at 27 °C in an Ultimate Insect™ serum-free medium (Invitrogen) supplemented with 10 units of heparin/ml (Sigma) in culture spinner flasks with constant stirring at 80 rev./min. When the cell density reached 2×10^6 cells/ml, they were inoculated with the HTS of recombinant baculoviruses at a multiplicity of infection (MOI) of 6 (6 viral particles/cell). At each 24 h interval after viral inoculation, 2 ml of culture medium was withdrawn from individual spinner flasks and centrifuged (1000 ***g*** for 20 min at 4 °C) to separate supernatant from cells. Cell pellets were resuspended in one-quarter of the original volume of 50 mM phosphate buffer (pH 7.0), sonicated to lyse the cells and centrifuged (18000 ***g*** for 10 min at 4 °C) to collect soluble cell protein. Both the culture media supernatants and the soluble cell proteins were assayed for DCE activity.

DC preparation and DCE activity assay

DC was prepared by mixing 1 mM L-dopa prepared in water with an equal volume of 2 mM $NaIO_4$ prepared in 20 mM phosphate buffer (pH 7.0) as described in a previous report [8]. DCE activity was determined spectrophotometrically by recording the absorbance decrease at 475 nm using a Hitachi U-2001 double-beam spectrophotometer. The reaction was initiated by mixing 0.1 ml of culture medium supernatant or soluble cell protein into 0.9 ml of DC solution. The absorbance decrease at 475 nm was continuously monitored for 5 min, and the DCE activity of either culture media supernatants or soluble cell proteins was based on the rate of absorbance decrease at 475 nm following their addition into the DC solution. All the enzyme activity assays were conducted at room temperature.

Purification of recombinant yellow-f and yellow-f2

Our initial tests indicated that recombinant yellow-f and yellow-f2 possessed DCE activity. To isolate recombinant yellow-f and yellow-f2 for characterization, a large-scale expression of yellow-f and yellow-f2 was achieved using High Five insect cells grown in 2 litres of Ultimate Insect™ serum-free medium in spinner flasks. When the cell density reached 2×10^6 cells/ml, they were inoculated with the HTS of the recombinant baculovirus at an MOI of 6 and incubated at 27 °C. At day 3 post-infection, the cell culture was harvested and centrifuged at 1000 ***g*** for 20 min at 4 °C. The supernatant was lyophilized to one-tenth of its original volume, and protein in the concentrated supernatant was precipitated by addition of solid $(NH_4)_2SO_4$ to 40% saturation. Precipitated protein then was dissolved in a minimum volume of 20 mM citrate buffer (pH 5.8) and dialysed against the same 20 mM citrate buffer containing 1 mM PMSF and 10 mM β-mercaptoethanol. The dialysed protein was chromatographed on a Bio-Rad UNO-S column (12 mm × 53 mm). The DCE active fractions from UNO-S column chromatography were concentrated using a Millipore membrane concentrator with a molecular-mass cut-off of 30000 Da and chromatographed on a Bio-Rad gel-filtration column (Bio-Sil SEC 125-5, 1 cm × 25 cm). Protein profiles prior to and after each step of DCE purification were analysed by SDS/PAGE. The DCE active protein was analysed by matrix-assisted laser-desorption ionization–time-of-flight (MALDI-TOF) MS following in-gel trypsin digestion, and its peptide profile was used in a database search (http://129.85.19.192/profound_bin/WebProFound.exe?FORM=1).

Substrate specificity of recombinant yellow-f and yellow-f2

Aminochromes from dopa methyl ester, α-methyl dopa and dopamine were used as substrates to determine the substrate specificity of recombinant yellow-f and yellow-f2. These aminochromes were prepared as described for the preparation of DC. Spectral analysis showed that they had an absorbance peak with a λ_{max} at 475 nm as DC did, so the activity of yellow-f and yellow-f2 towards these aminochromes was based on absorbance decreases at 475 nm following the addition of each protein into the substrate preparation. To determine the absorption coefficient (ϵ) of individual aminochromes at 475 nm, 0.05–0.5 mM of each aminochrome was prepared using 10 mM phosphate buffer (pH 7.0), the absorbance of individual aminochromes (0.05–0.5 mM) was determined using a Hitachi U-2001 double-beam spectrophotometer in a 1.0 ml cuvette with a pathlength (d) of 1 cm, and the ϵ for each aminochrome was calculated by plotting its absorbance values over its concentrations using linear regression in Quattro Pro (Corel). The specific DCE activity was calculated based on absorbance decreases in a 1.0 ml reaction mixture containing 0.5 mM aminochrome and 1 μg of yellow-f or yellow-f2 during a 20 s period and expressed as μmol of substrate consumed/min per mg of protein. Exactly the same reaction mixture, but with the enzyme replaced by the same amount of BSA, was used for the negative control. The above reaction mixtures for DC and dopamine chrome were also analysed by HPLC with electrochemical detection (HPLC-ED) to determine the accumulation of enzymic products.

Kinetic properties of recombinant yellow-f and yellow-f2 with DC and dopamine chrome substrates

Both DC and dopamine chrome are natural substrates, and the action of DCE on these two aminochromes leads to the production of the same compound, i.e. DHI. The absorbance versus substrate concentration for aminochromes was not linear at concentrations above 0.5 mM. To quantify DCE-catalysed conversion of DC and dopamine chrome, DHI standard was synthesized by spontaneous decarboxylation of DC generated *in situ* by ferricyanide oxidation of L-dopa at pH 6.5 [26]. DC and dopamine chrome (0.025–1.0 mM) were prepared as described for the DC preparation. Purified enzyme was mixed with freshly prepared DC or dopamine chrome and the reaction mixture was stopped by same volume of ethanol at 30 s after incubation and injected for HPLC-ED analysis. The amount of DHI produced in the reaction mixture was based on a standard curve generated by injection of increasing amounts of DHI standard.

Northern blot analysis

Total RNA was isolated using Trizol reagent (Life Technologies) from 3- and 6-day-old larvae, newly formed, 0.5-, 1- or 2-day-old pupae, and 1 day-old adults. Total RNA was separated in 1% agarose/formaldehyde gel in 20 mM Mops containing 4 mM sodium acetate and 1 mM EDTA at 5 V/cm for 3 h. RNA was transferred to a positive charge nylon membrane (Ambion) and cross-linked using a Bio-Rad UV cross-linker. The blot was hybridized sequentially with a [^{32}P]dCTP-labelled 320 bp fragment of *yellow*-f cDNA, a 400 bp fragment of *yellow*-f2 cDNA and a 360 bp fragment of a *Drosophila* ribosomal protein 49 (rp49) cDNA as a loading control. After hybridization at 42 °C for 16 h, the blots were washed with increasing stringency [twice in 2 × SSC (where 1 × SSC is 0.15 M NaCl/0.015 M sodium citrate) containing 0.1% SDS at room temperature for 25 min, and twice with 0.1 × SSC containing 0.1% SDS at 68 °C for 30 min], and exposed to X-ray films at −80 °C.

RESULTS

Identification of yellow-f as a *Drosophila* DCE

Beginning at 1 day after viral inoculation, DCE activity became detectable in the supernatant of the culture media from cells inoculated with the yellow-f recombinant virus and increased linearly from 1 to 3 days. The maximum activity was observed on day 4 following viral inoculation (results not shown). At 6 MOI, the number of cells increased slightly 1 day after viral inoculation and stopped multiplication thereafter. More than 95% of cells remained intact 4 days after inoculation, but about 25% of cells disintegrated at 5 days. DCE activity in the soluble cell protein became detectable 1 day after viral inoculation, but no noticeable increase in DCE activity was observed after day 1 post-inoculation. Beginning at 1 day after inoculation, the majority of the DCE activity (> 90%) was present in the culture medium supernatant, indicating that the recombinant yellow-f was secreted into the medium after synthesis, which is in agreement with the presence of a signal peptide (see Figure 1). Under the same conditions, however, no DCE activity was detected in either the supernatants or the soluble cell proteins from cell cultures inoculated with recombinant viruses containing *yellow*-y, *yellow*-b, *yellow*-c or partial *yellow*-f2 genes.

Identification of yellow-f2 as a *Drosophila* DCE isozyme

Among the *yellow* genes, *yellow*-f shares 75% sequence identity with *yellow*-f2 and, although high levels of recombinant yellow-f2 were detected in soluble cell protein, no DCE activity was detected. After further analysis of the *yellow*-f2 ORF in the *Drosophila* database, a total of 219 nucleotides, belonging to the coding sequence at the N-terminus, were determined to be erroneously assigned as an untranslated region. Consequently, the forward primer for *yellow*-f2 was redesigned to include its entire ORF (the ORF was recently corrected by the Berkeley *Drosophila* Genome Project research group, accession no. AAL28276). The construction of the yellow-f2 recombinant transfer vector, the isolation of the recombinant yellow-f2 baculovirus and the expression of recombinant yellow-f2 protein were achieved by the same procedures described for other *yellow* genes. After a HTS of recombinant baculovirus containing the full-length yellow-f2 coding sequence was inoculated into the cultured High Five cells, DCE activity became detectable in the supernatant 1 day after inoculation. Activity showed a linear increase from 1 to 4 days after inoculation (results not shown), which is essentially the same as that observed for yellow-f. Similar to recombinant yellow-f, the majority of the DCE activity was present in the culture medium supernatant, suggesting its rapid secretion after synthesis. These data demonstrate that yellow-f2 is a *Drosophila* DCE isoenzyme and that the enzyme has an ORF of 1359 bp rather than the 1140 bp coding sequence described in the *Drosophila* database.

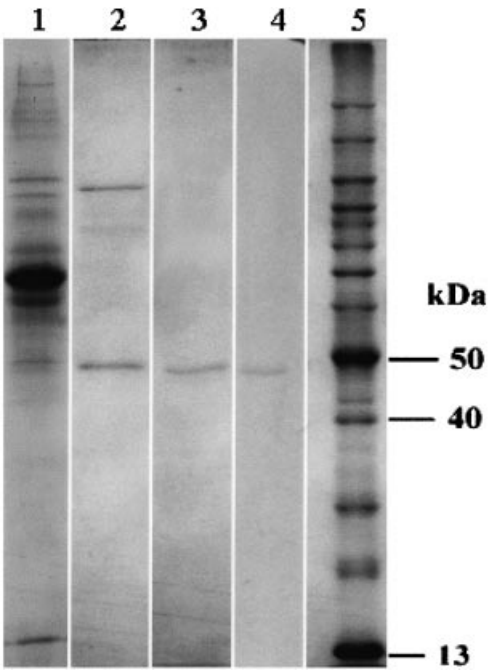


Figure 2 Purification of DCE active protein

Purification of the DCE active protein from culture media supernatant inoculated with the yellow-f recombinant baculovirus was achieved through $(NH_4)_2SO_4$ fractionation, and separation by ion-exchange and gel-filtration chromatographies. SDS/PAGE shows recombinant yellow-f in the culture media supernatant (lane 1), DCE active fractions after UNO-S column chromatography (lane 2) and gel-filtration chromatography (lane 3), and purified recombinant yellow-f2 (lane 4). Lane 5 illustrates the migration of molecular-mass standards under the same electrophoresis conditions.

Isolation of recombinant yellow-f and yellow-f2

Recombinant yellow-f was purified from the culture media supernatant collected 3 days after viral inoculation, a time when the infected cells remained intact. During UNO-S column

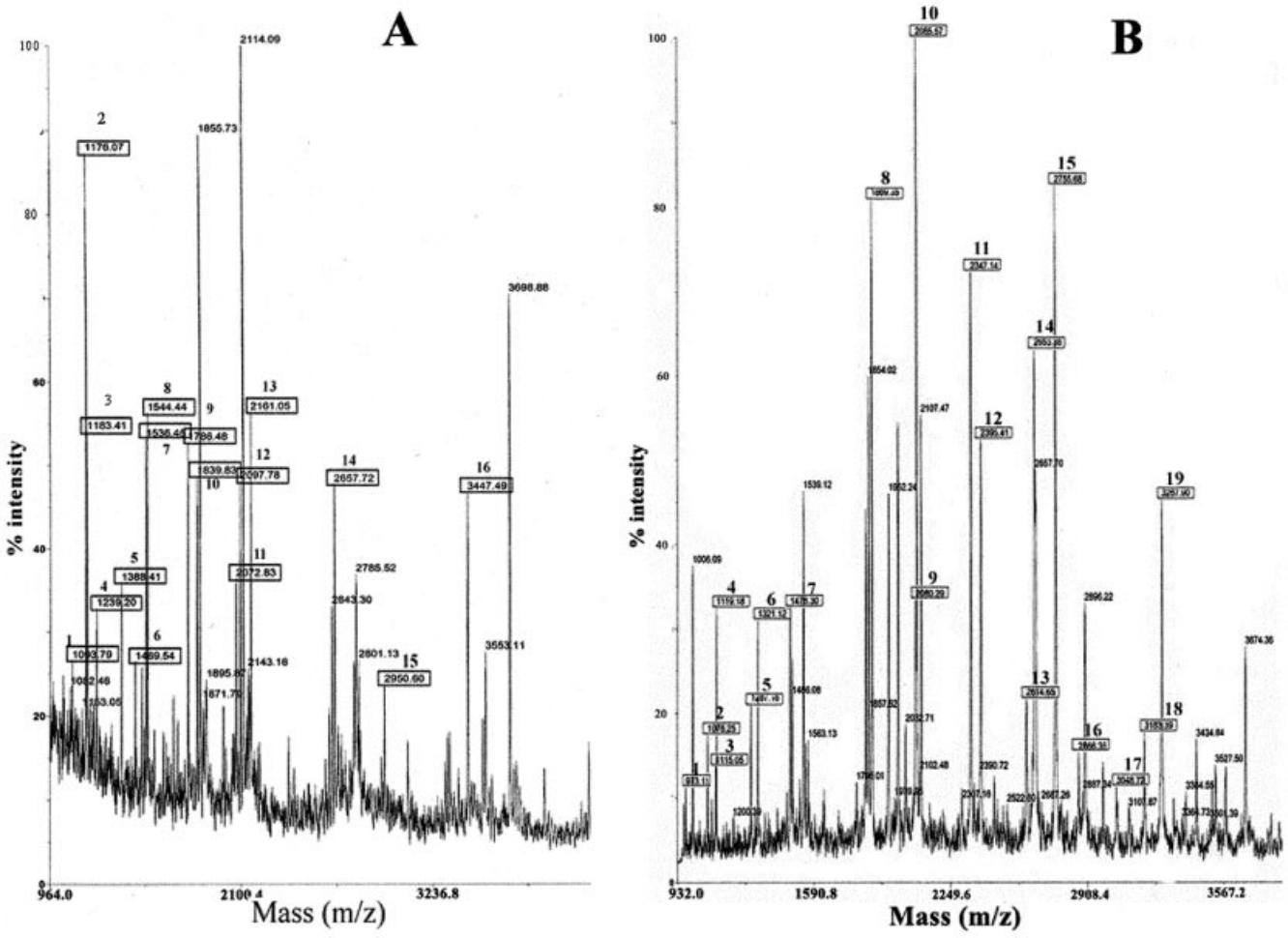


Figure 3 Identification of the DCE active proteins as recombinant yellow-f and yellow-f2

The DCE active proteins with a relative molecular mass of 48000 Da were in-gel digested using trypsin, the tryptic digests were analysed by MALDI-TOF MS, and their protein profiles were used to search databases (see the text for details). The peptides that matched the yellow-f (**A**) and yellow-f2 (**B**) peptide profiles in the database are boxed.

chromatography, the majority of DCE activity was eluted at a NaCl gradient from 150 to 185 mM. Gel-filtration chromatography of the concentrated DCE fractions resulted in the detection of a minor DCE and major DCE active peak with estimated molecular masses of 97400 and 50000 Da, respectively. Figure 2 illustrates the SDS/PAGE protein profiles of the concentrated supernatant prior to purification (Figure 2, lane 1), the DCE active fractions after UNO-S column separation (Figure 2, lane 2) and the DCE active fractions after gel-filtration chromatography (Figure 2, lane 3). The DCE active protein, with a molecular mass of 48000 Da, became the major protein after gel-filtration chromatography and was verified as recombinant yellow-f by MALDI-TOF MS of the protein following its in-gel trypsin digestion (Figure 3A). Recombinant yellow-f2 was purified by the identical procedures and verified by MALDI-TOF analysis (see Figure 2, lane 4, and Figure 3B).

Substrate specificity

Both yellow-f and yellow-f2 were active with DC and aminochromes derived from L-dopa derivatives (dopa methyl ester,

Table 2 Substrate specificity

The specific activity of yellow-f and yellow-f2 was calculated based on the decrease in substrate concentration at 475 nm during a 20 s period of incubation in a reaction mixture containing 0.5 mM aminochrome and 1 μg of yellow-f or yellow-f2 (see the Experimental section). The decreases in substrate concentrations were calculated by the equation $C = (A_1 - A_2)/\epsilon d$, where A_1 is initial absorbance, A_2 is the absorbance 20 s after incubation, ϵ is the molar absorption coefficient at 475 nm, d is equal to 1 cm and C is the decrease in substrate concentration. The total decrease in substrate concentration during the 20 s was then used to calculate the specific activity by multiplying the reaction volume and dividing the incubation time and the amount of protein used in the reaction mixture. The enzyme activity was expressed as substrate decrease (μmol)/min per mg of protein.

	Absorption coefficient		Specific activity (μmol/min per mg)	
Substrate	ϵ ($M^{-1} \cdot cm^{-1}$)	R^2	Yellow-f	Yellow-f2
DC	3245.7	0.9992	90.5 ± 3.4	88.6 ± 3.9
Dopamine chrome	2834.7	0.9998	22.5 ± 1	37.3 ± 2
Dopa methyl ester chrome	2818.3	0.9991	186.4 ± 5.9	754.4 ± 22.7
α-Methyl dopa chrome	3544.4	0.9996	4.6 ± 0.5	8.1 ± 0.6

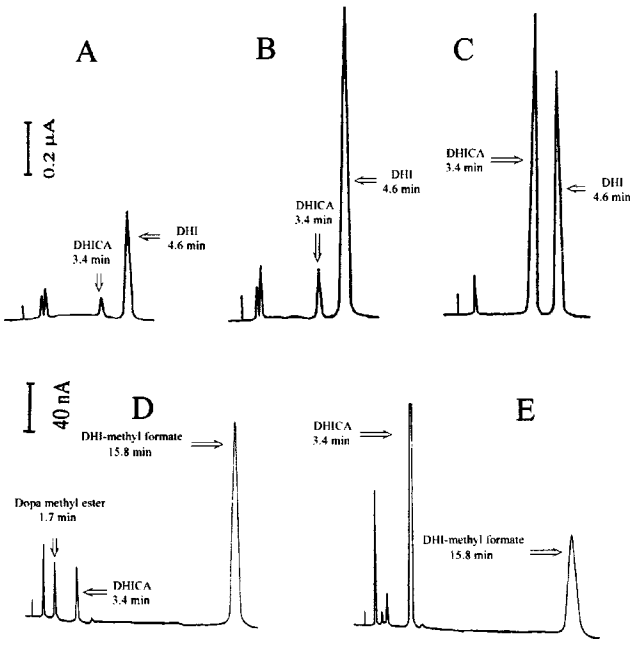


Figure 4 HPLC-ED analysis of product accumulation

Chromatograms (**A**) to (**C**) illustrate the relative amount of DHI in the reaction mixture at 1 (**A**) and 2 min (**B**) after addition of recombinant yellow-f in DC solution (0.5 mM), and a mixture of DHI and DHICA standards (**C**). Chromatogram (**D**) shows the accumulation of DHI-methyl formate in dopa methyl ester chrome solution at 2 min after the addition of recombinant yellow-f. Chromatogram (**E**) illustrates the hydrolysis of DHI-methyl formate to DHICA and methanol by rabbit liver esterase. The dopa methyl ester chrome and yellow-f reaction mixture used to generate chromatogram (**E**) were incubated for 2 min prior to the addition of 10 units of rabbit liver esterase and the sample was incubated for an additional 10 min before injecting for HPLC-ED analysis. Conversion of dopa methyl ester chrome into DHI-methyl formate was essentially complete after 2 min incubation in the presence of recombinant yellow-f. HPLC separation was achieved by reverse-phase separation using an Alltech C_{18} reversed-phase column (30 mm × 70 mm, with 3 μm spherical particles). The mobile phase consisted of 50 mM potassium phosphate (pH 4.8) containing 7.5% acetonitrile with a flow rate of 2 ml/min. A BAS LC-4C detector (Bioanalytic Systems) was used for electrochemical detection.

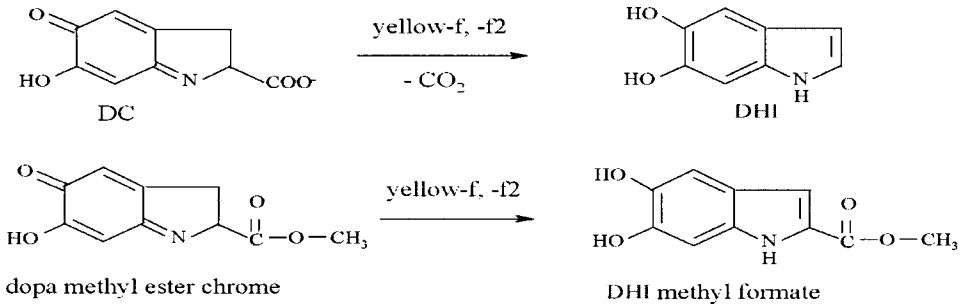
dopamine and α-methyl dopa). The specific activities were shown in Table 2. Figure 4 illustrates the accumulation of DHI in the DC solution or DHI-methyl formate in the dopa methyl ester chrome solution following the addition of recombinant yellow-f. DHI was the major product in the DC and recombinant yellow-f reaction mixture, but low levels of 5,6-dihydroxyindole-2-carboxylic acid (DHICA) were observed in the reaction mixture (Figures 4A and 4B). DHI-methyl formate was the major product in the dopa methyl ester chrome and recombinant yellow-f reaction mixture, but low concentrations of DHICA and trace amounts of DHI were also detected (Figure 4D). The identity of the enzymic product as DHI-methyl formate in dopa methyl ester chrome and recombinant yellow-f reaction mixture was further verified by its rapid hydrolysis to DHICA and methanol after the addition of 10 units of rabbit liver esterase (Figure 4E). These assays show that *Drosophila* yellow-f is capable of mediating both a decarboxylative structural rearrangement of DC and α-methyl dopa chrome as well as an isomerization/tautomerization process of dopa methyl ester chrome and dopamine chrome (see Scheme 2).

Kinetic properties of yellow-f and yellow-f2 to DC and dopamine chrome

Both DC and dopamine chrome are natural substrates in the insect melanization pathway. Kinetic analysis showed that both yellow-f and yellow-f2 were highly efficient in catalysing the DC to DHI pathway, but much less efficient in mediating the dopamine chrome to DHI pathway (Table 3). The V_{max} values of yellow-f and yellow-f2 to DC were similar, but the K_m of yellow-f2 to DC was about one-third that of yellow-f to DC. Therefore, yellow-f2 is more efficient catalytically than yellow-f in the DC to DHI pathway.

Expression of yellow-f and -f2 during development

Northern blot analysis revealed the differential expression of yellow-f and -f2 transcripts in different developmental stages. Low levels of yellow-f mRNA were detected in early larvae (Figure 5A, lane 1), whereas high transcript abundance was observed in later-stage larvae and early pupae (Figure 5A, lanes 2 and 3). Transcript levels of yellow-f decreased in later-stage pupae (Figure 5A, lane 4) and were hardly detectable in adults (Figure 5A, lane 5). The yellow-f2 transcripts were detected at all stages of *Drosophila* development (Figure 5B, lanes 1–5), but, in contrast with yellow-f, high levels of transcript were observed during later pupal and adult stages (Figure 5B, lanes 4 and 5). Data suggest that the expression of yellow-f and yellow-f2 complement each other during *Drosophila* development.



Scheme 2 *Drosophila* yellow-f can mediate a decarboxylative structural rearrangement of DC and α-methyl dopa chrome and an isomerization/tautomerization process of dopa methyl ester chrome and dopamine chrome

Table 3 Kinetic properties

The kinetic parameters were calculated by fitting the experimental data to the Michaelis–Menten equation using the Enzyme Kinetics Module (SPSS Science) based on the respective product concentration for each substrate (0.025–1 mM). The same reaction mixture, but with the enzyme replaced by the same amount of BSA, was used as a control. The activity data are the means ± ranges from two separate experiments.

	Yellow-f				Yellow-f2			
Substrate	K_m (mM)	V_{max} (μmol/min per mg)	k_{cat} (min^{-1})	k_{cat}/K_m ($min^{-1} \cdot mM^{-1}$)	K_m (mM)	V_{max} (μmol/min per mg)	k_{cat} (min^{-1})	k_{cat}/K_m ($min^{-1} \cdot mM^{-1}$)
DC	0.33 ± 0.12	137 ± 5.1	6713 ± 250	20342	0.11 ± 0.05	123 ± 5.3	6273 ± 270	57027
Dopamine chrome	0.29 ± 0.14	32 ± 1.4	1568 ± 69	5407	0.31 ± 0.08	52 ± 2.5	2653 ± 128	8558

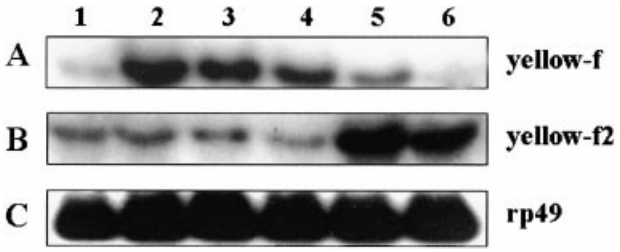


Figure 5 Northern analysis

Total RNA was isolated from *Drosophila* during different developmental stages. About 15 μg of total RNA from individual samples was electrophoresed on 1% agarose/formadehyde gels, blotted on to nylon membrane and sequentially probed with a 320 bp [^{32}P]dCTP-labelled fragment of *yellow*-f cDNA (**A**), a 400 bp probe of *yellow*-f2 cDNA (**B**) and a 360 bp probe of a *Drosophila* ribosomal protein 49 (rp49) cDNA as a loading control (**C**). Total RNA was used from 3-day-old larvae (lanes 1), 6-day-old larvae (lanes 2), 1 h-old pupae (lanes 3), 12 h-old pupae (lanes 4), 24 h-old pupae (lanes 5) and 1-day-old adults (lanes 6).

DISCUSSION

Expression and biochemical characterization of the *Drosophila yellow*-y and *yellow*-y-related genes identified both yellow-f and yellow-f2 as *Drosophila* DCEs. The two DCE isoenzymes are capable of mediating a decarboxylative structural rearrangement of DC and α-methyl dopa chrome as well as an isomerization/tautomerization of dopaminechrome and dopa methyl ester chrome (see Scheme 2). The transcription profiles of yellow-f and yellow-f2 suggest that yellow-f is involved in melanization reactions during larval and early pupal stages, and yellow-f2 plays a role in melanization reactions during later pupal and adult stages. These data suggest the biochemical functions of yellow-f and yellow-f2 and provide a basis for a more complete understanding of these two *Drosophila* enzymes.

We considered the yellow-y protein as the probable candidate for *Drosophila* DCE because its mutation leads to the formation of a yellow-coloured wing and cuticle [16–18]; accordingly, our initial efforts were focused on the expression of the *yellow*-y gene. Surprisingly, no DCE activity was detected in recombinant yellow-y protein. When crude proteins from wild-type strain and yellow mutant of *Drosophila*, respectively, were assayed for DCE activity, the yellow mutant showed relatively low levels of DCE activity compared with the wild-type strain, which suggests that the *yellow*-y gene itself is not a DCE, but may exert some influence that impacts the expression of yellow-f, yellow-f2 or other enzymes involved in melanization.

Melanization is an important physiological event in insects and is involved in cuticular tanning, wound healing, foreign organism encapsulation in defence reactions and egg chorion hardening [27,28]. All these biochemical processes must proceed rapidly in a timely manner. Tyrosine is the initial precursor of melanin biosynthesis and the overall process involves hydroxylation of tyrosine to dopa, oxidation of L-dopa to dopaquinone and then to DC, conversion of DC into DHI, oxidation of DHI to DHI-quinone, and polymerization of DHI-quinone to form melanin polymers. Tyrosinase, or phenol oxidase, is capable of catalysing the hydroxylation of tyrosine, oxidation of dopa, and oxidation of DHI to DHI-quinone that polymerizes to produce melanin polymers. The conversion of DC into DHI is a necessary step for melanin production. Although this reaction proceeds non-enzymically under physiological conditions, it is too slow to be physiologically adequate in melanization reactions of insects. The requirement for rapid melanization reactions in insects may explain why specific DCEs have evolved in insects.

In summary, this study demonstrates that *Drosophila* yellow-f and yellow-f2 function as DCEs that likely play an important role during melanin biosynthesis in *Drosophila* larvae, pupae and adults. This represents a first step towards understanding the overall physiological functions of the *Drosophila yellow* gene family and also serves as a reference for studies of other insect DCEs.

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