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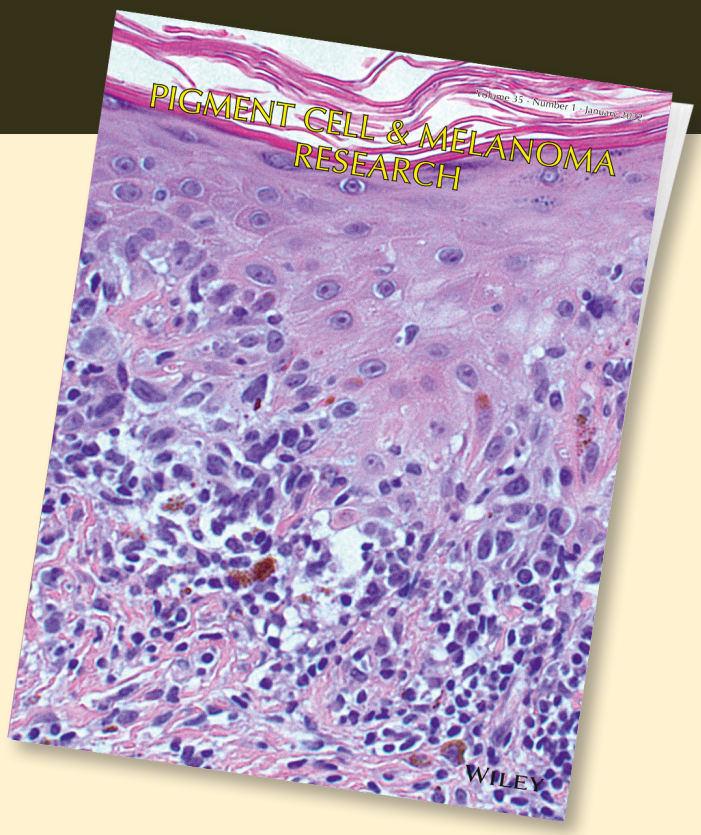

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ORIGINAL ARTICLE

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Drosophila yellow-h encodes dopaminechrome tautomerase: A new enzyme in the eumelanin biosynthetic pathway

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Abstract

Melanin is a widely distributed phenolic pigment that is biosynthesized from tyrosine and its hydroxylated product, dopa, in all animals. However, recent studies reveal a significant deviation from this paradigm, as insects appear to use dopamine rather than dopa as the major precursor of melanin. This observation calls for a reconsideration of the insect melanogenic pathway. While phenoloxidases and laccases can oxidize dopamine for dopaminechrome production, the fate of dopaminechrome remains undetermined. Dopachrome decarboxylase/tautomerase, encoded by *yellow-f/f2* of *Drosophila melanogaster*, can convert dopaminechrome into 5,6-dihydroxyindole, but the same enzyme from other organisms does not act on dopaminechrome, suggesting the existence of a specific dopaminechrome tautomerase (DPT). We now report the identification of this novel enzyme that biosynthesizes 5,6-dihydroxyindole from dopaminechrome in *Drosophila*. Dopaminechrome tautomerase acted on both dopaminechrome and *N*-methyl dopaminechrome but not on dopachrome or other aminochromes tested. Our biochemical and molecular studies reveal that this enzyme is encoded by the *yellow-h* gene, a member of the *yellow* gene family, and advance our understanding of the physiological functions of this gene family. Identification and characterization of DPT clarifies the precursor for melanin biosynthetic pathways and proves the existence of an independent melanogenic pathway in insects that utilizes dopamine as the primary precursor.

KEYWORDS

dopamine, dopaminechrome, dopaminechrome tautomerase, eumelanin biosynthesis, insect melanogenesis

1 | INTRODUCTION

Melanin is the main skin and hair pigment found throughout the animal kingdom. It is responsible for protecting animals from damaging solar radiation and plays a role in all aspects of animal coloration (Hill, 1992). Mammalian pathways of melanin biosynthesis have been extensively studied (d'Ischia et al., 2015; Ito & Wakamatsu, 2008; Sugumaran & Barek, 2016). The two major types of melanin include brown-to-black eumelanin and yellow-to-red pheomelanin, both arising from tyrosine and its hydroxylated product, dopa. Tyrosinase

Hanine Barek and Heya Zhao contributed equally to this work.

converts tyrosine and dopa to dopaquinone. The non-enzymatic cyclization of dopaquinone generates leucochrome, which is rapidly oxidized by dopaquinone to dopachrome (Figure 1). Dopachrome tautomerase (DCT) isomerizes dopachrome to 5,6-dihydroxyindole-2-carboxylic acid (DHICA) (Aroca et al., 1990; Pawelek, 1990). The oxidative polymerization of DHICA results in the brown colored DHICA eumelanin (Boissy et al., 1998; Kobayashi et al., 1994). Dopachrome is believed to be non-enzymatically converted to 5,6-dihydroxyindole (DHI) by decarboxylation and aromatization in mammalian cells, although in many insect species an enzyme catalyzing this reaction, called dopachrome decarboxylase/tautomerase (DCDT), formerly known as dopachrome conversion factor, has been identified (Aso et al., 1989; Cherqui et al., 1998; Han et al., 2002; Sugumaran & Semensi, 1991). Oxidative polymerization of DHI produces black colored DHI eumelanin. On the other hand, pheomelanin is biosynthesized from cysteinyldopa, which is formed by the condensation of tyrosinase-generated dopaquinone with the amino acid cysteine (Figure 1). Oxidative polymerization of cysteinyldopa then produces yellow-to-red pheomelanin (d'Ischia et al., 2015; Ito & Wakamatsu, 2008). Tyrosinase appears to be the sole enzyme responsible for pheomelanin biosynthesis.

Melanin formation is absolutely essential for all insects as they enlist melanogenesis for a variety of physiologically important processes (Eleftherianos & Revenis, 2011; Shamim et al., 2014; Sugumaran, 1996; 2002, 2010; Theopold et al., 2004; True, 2003; Wittkopp & Beldade, 2009). Extensive deposition of insoluble melanin at a wound site prevents uncontrolled bleeding. During invasion by foreign organisms, insects limit the spread and multiplication of the intruders by encapsulating and melanizing them in the body cavity (Nappi & Christensen, 2005; Sugumaran, 2002, 2009). Significant melanogenesis also occurs in the cuticle, providing coloration for camouflage and defense. In addition, cuticular melanin also serves other functions, such as thermal regulation and protection from desiccation. As a result, melanogenesis is indispensable for the survival and reproductive success of all insects (Shamim et al., 2014; True, 2003; Wittkopp & Beldade, 2009).

It is widely accepted that insect melanogenesis utilizes the same pathway as that used by mammals. However, latest studies suggest that certain key aspects of insect melanogenesis are significantly different from mammalian melanin biosynthesis (Barek et al., 2018; Sugumaran & Barek, 2016). Although phenoloxidases were assumed to be the enzymes responsible for the oxidation of catecholamines in the insect cuticle by a number of groups, recent studies point out that laccases and not phenoloxidases perform this reaction (Arakane et al., 2005; Dittmer & Kanost, 2010). More importantly, we have shown decisively that dopamine rather than dopa is the main precursor for both eu- and pheomelanin in the cuticle (Barek et al., 2018; Sugumaran & Barek, 2016). Large amounts of dopamine are needed for the biosynthesis of N-acetyldopamine (NADA) and N-β-alanyldopamine (NBAD), two of the most important catecholamine derivatives that are required for cuticular sclerotization and hardening to protect the soft-bodied insects (Sugumaran, 2010). The insect enzyme dopa decarboxylase (*Ddc*) readily converts dopa present in epidermal cells to dopamine (Arakane et al., 2009; Paskewitz & Andreev, 2008). Dopamine thus formed is acylated and used primarily for cuticle sclerotization and hardening reactions. However, some of the unused NBAD is hydrolyzed back to dopamine and is then diverted for melanin biosynthesis (Figure 1).

Significance

The *yellow* gene family is classified as one of the most enigmatic family of genes discovered in insects, as the functions of most Yellow-like proteins remain unknown. We have uncovered the biological activity of the Yellow-h protein in *Drosophila melanogaster*. The *yellow-h* gene encodes a novel enzyme, dopaminechrome tautomerase (DPT), that catalyzes the conversion of dopaminechrome to 5,6-dihydroxyindole in the eumelanin biosynthetic pathway originating exclusively from dopamine. DPT does not act on the dopa derived dopachrome, which is the standard melanogenic precursor in most animals.

Given the central role of dopamine in insect melanogenesis, we asked whether a specific enzyme catalyzing the conversion of dopaminechrome to DHI exists in insects. It has been shown that dopaminechrome is a poor substrate for several insect dopachrome decarboxylase/tautomerases (DCDT) (Aso et al., 1989; Cherqui et al., 1998; Sugumaran & Semensi, 1991). Therefore, one would expect that similar to DCDT, there should be an enzyme that would more specifically act on dopaminechrome. A previous study (Han et al., 2002) demonstrated that the *Drosophila melanogaster* DCDT encoded by the *yellow-f* and *yellow-f2* genes catalyzes the conversion of both dopachrome and dopaminechrome into DHI, however, a possible existence of a specific enzyme that would convert dopaminechrome to DHI has not been reported. Here, we present an identification of such an enzyme that we call dopaminechrome tautomerase (DPT), characterize its biochemical properties, and show that it is encoded by the *yellow-h* gene in *Drosophila*. In addition to identifying a novel enzyme, this work expands our understanding of the insect Yellow family of proteins and confirms the operation of a unique pathway for eumelanogenesis that originates from dopamine.

2 | MATERIALS AND METHODS

2.1 | Partial purification of DPT

Drosophila adults were raised in population cages at room temperature. Embryos were collected by placing the apple juice Petri dishes with yeast paste into the cages for indicated time windows. Solutions for preparing apple juice plates contained 700 ml of water, 300 ml of

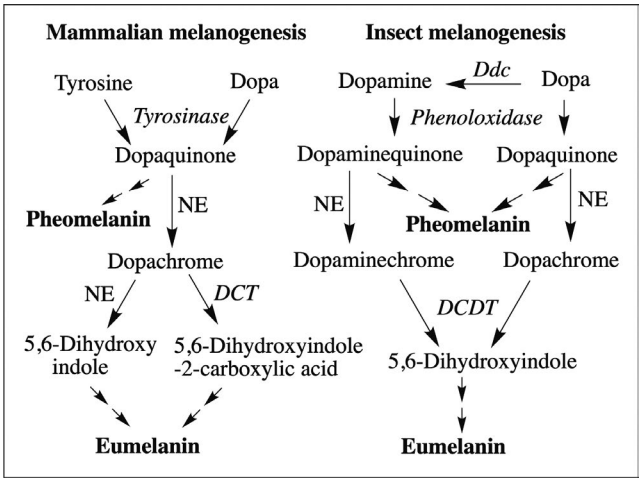

FIGURE 1 Comparative analysis of the eumelanogenesis pathway in mammals and insects. In mammals, tyrosinase converts tyrosine and dopa to dopaquinone which is further converted non-enzymatically (NE) to dopachrome. Dopachrome tautomerase (DCT) catalyzes the production of 5,6-dihydroxyindole-2-carboxuylic acid (DHICA). 5,6-dihydroxyindole (DHI) is believed to be formed by non-enzymatic reactions (NE). Oxidative polymerization of DHI and DHICA produces the eumelanin pigment. In insect cuticle, dopa is primarily decarboxylated to dopamine by the action of dopa decarboxylase (Ddc). Dopamine is first converted to N-β-alanyldopamine (NBAD) and N-acetyldopamine, which are the sclerotizing precursor required for cuticle hardening. Unused NBAD is later hydrolyzed back to dopamine and then used for melanin production (not shown in the figure). It is oxidized by the cuticular laccase to dopamine quinone which is non-enzymatically converted to dopaminechrome. Dopaminechrome can be isomerized to DHI by dopachrome decarboxylase/tautomerase (DCDT)

apple juice, and 0.5 g of methyl-*p*-hydroxybenzoate fortified with 2.0% agar. For the initial characterization of DPT, 15 g of embryos collected between 16 and 19 hr was used. All subsequent operations were carried out at 0–5°C. All centrifugations were carried out for 25 min at 15,000 × g. The embryos were homogenized in a glass homogenizer with 30% ammonium sulfate in 50 mM sodium phosphate buffer pH 7.0. The precipitated proteins and cell debris were discarded, and the supernatant was subjected to 30%–45% ammonium sulfate fractionation. The proteins precipitated were collected after centrifugation and the supernatant was subjected to 45%–50% ammonium sulfate fractionation followed by centrifugation. Both 30%–45% and 45%–50% ammonium sulfate precipitated fractions were either used immediately or stored at −80°C for later use. An estimated 26 mg of 45%–50% ammonium sulfate fraction was subjected to size exclusion chromatography on a Sephacryl S-200 column (42.5 × 2.5 cm) equilibrated with 50 mM sodium phosphate buffer, pH 7.0 at a flow rate of 2.5 ml/min. Fractions of 2 ml were collected and assayed for both protein content and dopaminechrome isomerase activity. Protein fractions containing DPT activity were pooled, concentrated by lyophilization and used for further work.

2.2 | Further purification of DPT

An estimated 68 mg of protein from 45% to 50% ammonium sulfate fraction was subjected to Phenyl Sepharose column chromatography (5 × 2.0 cm) using 50 mM sodium phosphate buffer, pH 7.0 containing 1 M ammonium sulfate. After washing the column with six column volumes of buffer, bound proteins were eluted with a linear gradient of 1 M ammonium sulfate to 0 M ammonium sulfate at a flow rate of 0.7 ml/min. Fractions of 3 ml were collected and assayed for DPT activity. The tubes containing active fractions (2.74 mg) were pooled and concentrated using a Centricon tube with a molecular weight cutoff of 10 kDa. The pH of the concentrated solution was adjusted to 8 by adding a few drops of 1 N NaOH and loaded on a DEAE-column (5 × 2.0 cm) equilibrated with 50 mM sodium phosphate buffer, pH 7.0, containing 50 mM sodium chloride. The column was washed with four column volumes of the same buffer. Bound proteins were eluted with 50 mM sodium phosphate buffer, pH 7.0 containing 1 M sodium chloride. Protein fractions possessing DPT activity (1.02 mg protein) were pooled, lyophilized, and subjected to size exclusion chromatography on a Sephacryl S-200 column (12. × 2.5 cm) equilibrated with 50 mM sodium phosphate buffer, pH 7.0 containing 0.05 M sodium chloride at a flow rate of 1.5 ml/min. Fractions of 3 ml were collected. The active fractions were pooled, dialyzed against water and concentrated by lyophilization. This purified sample was used for all biochemical studies. It was also subjected to polyacrylamide gel electrophoresis. The band containing DPT activity was analyzed by nanoLC-MS/MS at the Taplin Mass Spectrometry Facility at Harvard Medical School.

2.3 | Protein estimation

Protein content in various preparations was determined using Bradford assay as outlined by Bio-Rad protein estimation manual and/or by monitoring the absorbance at 280 nm.

2.4 | Assay of DPT and DCDT

Dopaminechrome and dopachrome needed for the enzyme assay were prepared in situ just before conducting the assay. They were typically made by mixing 100 μl of 0.25 mM of dopamine or dopa and 100 μl of 0.5 mM of sodium periodate and used immediately. The reaction mixture contained 200 μl of aminochrome prepared as described above, 200 μl of water and 100 μl of enzyme solution. No buffer was used in the assays, as metal ions tend to have an adverse effect on the stability of dopaminechrome. The reaction pH was about 7. After quickly mixing, the decrease in absorbance at 475 nm of the reaction mixture was measured spectrophotometrically. Other chromes were prepared by mixing their respective catecholic precursors with two molar equivalents of sodium periodate and used in assays.

2.5 | Polyacrylamide gel electrophoresis and activity staining of DPT

A portion of purified protein (5 μg) was subjected to polyacrylamide gel electrophoresis under native conditions on a Mini-PROTEAN II apparatus as per manufacturer's instructions. The electrophoresis was carried out at 75 V for 8 hr. After the electrophoresis, the gel was collected and used for protein staining with Coomassie blue or activity staining with dopaminechrome and/or dopachrome to detect the activities of DPT and/or DCDT. The chromes were made as outlined above and incubated with the gel. The bands corresponding to DPT and DCDT converted the chromes to 5,6-dihydroxyindole (DHI). DHI being extremely sensitive to aerial oxidation rapidly got oxidized to produce blue colored melanochrome within 10–15 min (Nicklas & Sugumaran, 1995). At the purple to blue stage, the gel was repeatedly washed with distilled water to remove all soluble compounds. Within an hour, the blue band on the chrome stained gel became black due to insoluble DHI melanin deposition and thus revealing the location of the enzymes on the gel. The stained gels can be stored in a refrigerator indefinitely without the loss of color.

2.6 | Cloning of yellow-y, yellow-f, and yellow-h

Yellow-y was cloned from genomic DNA that was extracted from the *iso-1 Drosophila melanogaster* strain. The final construct contained only the coding region without the intervening intron. The open reading frame was assembled from two pieces amplified by PCR and cloned into pIE-4 vector with the following primers and NEBuilder HiFi DNA assembly kit:

1st piece:

Fwd: 5′-AAACTAGTCGCGAGGCCTGCAACATGTTCCAGGACAAAGGGTGGATCC-3′.

Rev: 5′-GCCGGAATCCCATCACGCCAGCGGGGAACAGTGAC-3′.

2nd piece:

Fwd: 5′-CTGGCGTGATGGGATTCCGGCCACTCTGACCTATATAAAC-3′.

Rev: 5′-TTTTGTTTTTTGGATCTATTAACCTTGATGCTGATGATGCCAC-3′.

To establish Yellow-y-SBP expressing stable S2 cell line, Yellow-y was cloned from pIE-4 vector into pMK33-SBP-C vector (Yang & Veraksa, 2017) with the following primers:

Fwd: 5′-CTACCTCGAGCAACATGTTCCAGGACAAAGGGTG-3′.

Rev: 5′-CATCGGTACCACCTTGATGCTGATGATGCC-3′.

Yellow-f was cloned into pMK33-SBP-C vector (Yang & Veraksa, 2017) from a cDNA in the *Drosophila* Gene Collection with the following primers:

Fwd: 5′-CTACCTCGAGCAACATGTTATCGCTGGACGTTC-3′.

Rev: 5′-CATCGGTACCCTCACAAACTGTTCCCCTC-3′.

Yellow-h was cloned from genomic DNA that was extracted from Oregon R *Drosophila melanogaster* strain into pMK33-SBP-C vector (Yang & Veraksa, 2017) with the following primers and overlapping PCR:

1st piece:

Fwd: 5′-GGGATCTCGAGGGATCCGATCAACATGCAGTCTATGACTATCTTTAATATAATTAC-3′.

Rev: 5′-GGTACAAAGTCTCCATTTAAAATAGATTGTTGGCGTTG-3′.

2nd piece:

Fwd: 5′-CTATTTTAAATGGAGACTTTGTACCAAAAAATAATTTAC-3′.

Rev: 5′-GCACCATGAACTCCTTGAAACTTGCCATTGGATGAAAATAAAG-3′.

3rd piece:

Fwd: 5′-GCAAGTTTCAAGGAGTTCATGGTGCCTATGAATATC-3′.

Rev: 5′-GTCCATGCCACTAGTGGTACCAAAACACTGTCCTTCTTCTATCAAC-3′.

All constructs were verified by sequencing.

2.7 | Establishment of stable cell lines

Drosophila S2 cells were transfected with the plasmids using Effectene transfection reagent from Qiagen. Stable cell lines were selected in Schneider M3 medium with 300 μg/ml hygromycin.

2.8 | Purification of Yellow-y-SBP and Yellow-f-SBP proteins

Stable cells were induced overnight with 0.35 mM $CuSO_4$. Cells were centrifuged at 4°C at 500 × *g* for 5 min. Supernatant was incubated with 50 μl of packed streptavidin beads on a rotator for 5 hr at 4°C. After washing five times with 1xPBS, the proteins were eluted with 300 μl of 2 mM biotin in 1xPBS (Yang & Veraksa, 2017).

2.9 | Purification of Yellow-h-SBP protein

Stable cells were induced overnight with 0.35 mM $CuSO_4$. Cells were centrifuged at 4°C at 500 × *g* for 3 min then washed once with 1xPBS and centrifuged for 5 min. Cells were lysed with 0.2% NP-40 (IGEPAL) in 1xPBS containing 2xComplete protease inhibitor (Roche) for 20 min on ice, then centrifuged for 15 min. Yellow-h-SBP protein from the cell lysate was purified and isolated using the same purification protocol outlined above for Yellow-y-SBP protein.

2.10 | HPLC analysis of DPT reaction

A reaction mixture (1 ml) containing 1 mM dopamine and 2 mM sodium periodate and 5 μg of Yellow-h-SBP protein in water was incubated at room temperature. An aliquot of the reaction mixture (10 μl) was subjected to HPLC analysis on Agilent 1100 HPLC series C_{18} cartridge (Agilent Technologies, Santa Clara, CA) using isocratic elution with 20% methanol and 80% MilliQ water at a flow rate of 0.7 ml/min. The absorbance of the peaks was monitored at 280 nm using a diode array spectrophotomer.

2.11 | Mass spectrometry conditions for DPT reactions

An Orbitrap Fusion Lumos mass spectrometer (Thermo Fisher) coupled online to an EASY-nLC 1200 (Thermo Fisher) was used to detect and characterize the products. The mass spectrometer was operated in the small molecule mode. The global settings were as follows: ion source type NSI, positive voltage of 1900 V, and an Ion Transfer Tube Temp of 275°C. Ions for the MS scans were detected in the Orbitrap with a resolution of 30,000. The mass range was normal, and the scan range was set to 100–1000 m/z. The RF lens was set to 30% and the AGC target and maximum injection time were 4.0×10^5 and 50 ms, respectively. The data-dependent MS2 CID scans were run in conjunction with a targeted mass filter in which the targeted masses corresponded to the following protonated species: dopamine (154.086804 m/z), dopamine-leucochrome (152.071154 m/z), DHI (150.055503 m/z), and DHI dimer (297.087533 m/z). An intensity threshold of 2.0×10^3 was set on each mass with a mass tolerance of ±10 ppm. Ions for the ddMS2 CID were isolated in the ion trap with a rapid scan rate and with an isolation window of 2 m/z. Ions were fragmented via CID with a fixed collision energy of 40%. The Q parameter for the CID activation was set to 0.25. The AGC target and maximum injection time were set to 1.0×10^4 and 500 ms. The cycle time for the data-dependent acquisition was set to 3 s. Standard assay conditions were used for conducting the DPT reaction. An aliquot of the reaction (100 μl) was quenched with 900 μl of 1% trifluroacetic acid and the diluted reaction was directly injected into the mass spectrometer.

2.12 | Mass spectrometry conditions for protein identification

The band containing DPT activity was analyzed by nanoLC-MS/MS following trypsin digestion. SEQUEST (ver. 28, rev. 13) was used to search a library of annotated *Drosophila* proteins downloaded from FlyBase (30,620 entries). Mass tolerance for precursor ions was 50 ppm, mass tolerance for fragment ions was 1 Da. Data were filtered based on the peptide FDR of 0.26%. Complete proteomic data are available at ProteomeXchange via the PRIDE partner repository with the dataset identifier PXD023295. A complete list of identified proteins is provided as Table S1.

2.13 | Substrate specificity analysis

A reaction mixture containing chrome prepared as outlined under "Assay of DPT and DCDT" (above), with the exception of different catecholic substrates in the place of dopa/dopamine was incubated with 2 μg of Yellow-h-SBP protein at room temperature, and the disappearance of the chrome was continuously monitored at 475 nm using a spectrophotometer.

3 | RESULTS

3.1 | Purification of drosophila DPT

The *yellow* gene family in insects has been associated with several functions including pigmentation, behavior, and protection from desiccation (Arakane et al., 2010; Drapeau, 2001; Ferguson et al., 2011; Li et al., 2011; Paskewitz & Andreev, 2008; Walter et al., 1991; Wittkopp, True, et al., 2002; Wittkopp, Vaccaro, et al., 2002; Xia et al., 2006). Despite considerable research efforts, the biochemical functions of most *yellow* gene family members remain unknown. In *Drosophila melanogaster*, 14 *yellow* related genes have been identified (Drapeau, 2001). The *yellow-y* gene is the founding member of the family and is required for eumelanin production, as *yellow-y* mutant flies have a yellow-colored cuticle (Drapeau, 2001; Walter et al., 1991; Wittkopp, Vaccaro, et al., 2002). Studies aimed at deciphering the function of the Yellow-y protein resulted in the characterization of *yellow-f/f2* gene products as DCDT (Han et al., 2002). We reasoned that DPT is likely to be encoded by a *yellow* family member. Based on the high-throughput RNA expression data (FlyBase), we chose embryos to screen for specific DPT activity to minimize possible interference from *yellow-f/f2* co-expression (Figure S2).

Proteins extracted from embryos collected at 3-hr intervals were subjected to different ammonium sulfate fractionations (35%–45% and 45%–50%) and tested for tautomerase activity against both dopaminechrome and dopachrome. Both ammonium sulfate extracts possessed significantly more DPT activity than dopachrome tautomerase activity (Figure 2a,b). This result suggested that there is an enzyme that acts more readily on dopaminechrome and that it is likely to be different from DCDT. Analysis of the 30%–45% ammonium sulfate fractions revealed that although the conversion of dopaminechrome at the 20–24 hr embryonic stage was maximal, it also contained significant amount of DCDT activity (Figure 2b). In contrast, the 16–19 hr time point in the 45%–50% ammonium sulfate fraction specifically exhibited DPT activity (Figure 2a). For this reason, embryos aged 16–19 hr and 45%–50% ammonium sulfate fractionation were chosen for further identification and purification of DPT. Using the protein purification scheme outlined in Figure 2c, DPT was partially purified from the crude extracts of 16–19 hr *Drosophila* embryos.

3.2 | DPT is encoded by the yellow-h gene

To identify the gene that codes for DPT, the purified enzyme fraction was separated by SDS-PAGE and subjected to DPT activity staining using the same protocol that was developed for detecting dopachrome tautomerase activity, with the exception of using dopaminechrome as a substrate (Nicklas & Sugumaran, 1995). A single band of DPT activity appeared in the gel. A corresponding protein band stained with Coomassie blue on the duplicate gel was excised and subjected to tryptic digestion followed by nanoLC-MS/MS analysis and protein identification. In our analysis of mass spectrometry

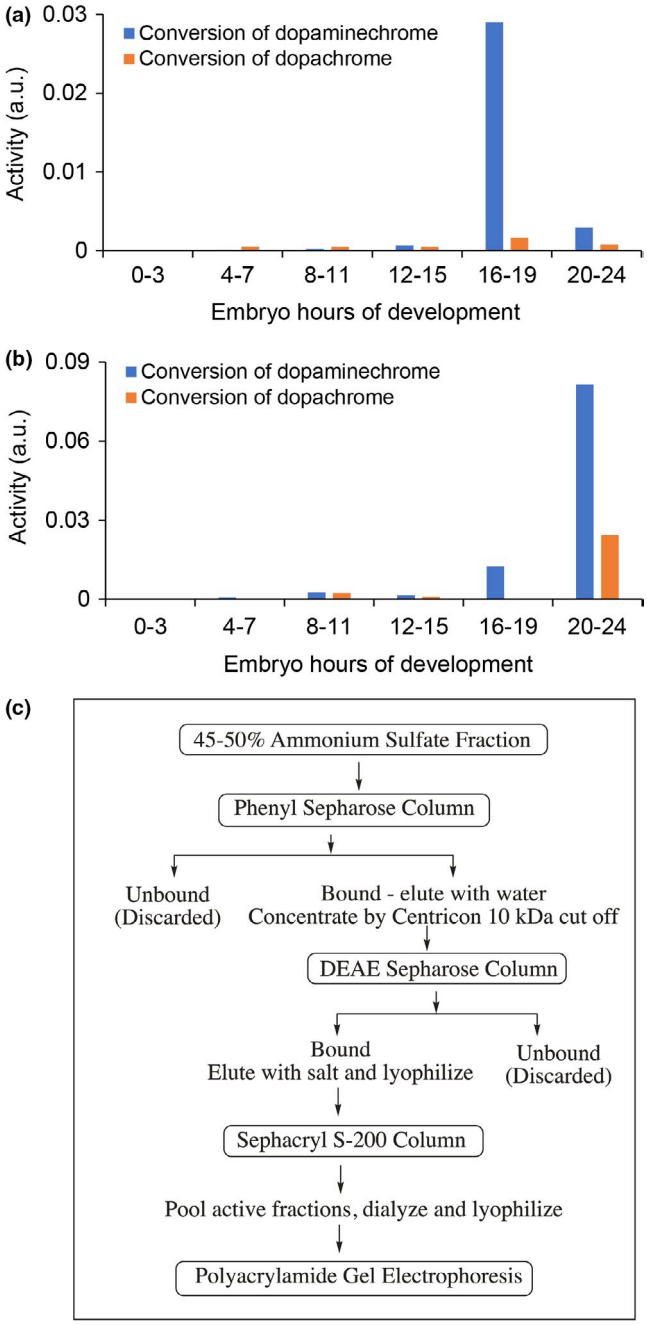

FIGURE 2 Purification of dopaminechrome tautomerase (DPT) from *Drosophila* embryo extracts. (a, b) Time course of appearance of dopaminechrome and dopachrome conversion activities in 45%–50% (a) and 30%–45% (b) ammonium sulfate fractions from embryonic extracts. The results were average of two experiments. (c) Purification scheme for DPT

TABLE 1 Proteins identified by mass spectrometry analysis of the DPT band

	Number of peptides in the embryo sample	
	Total	Unique
CG8193 (PPO2)	36	35
PPO-A1	29	28
Laccase-2	3	3
Yellow-h	10	8

Note: A complete dataset showing peptide numbers for all identified proteins is provided as Table S1, which also includes information on Intensity% and Coverage%.

Abbreviations: DPT, dopaminechrome tautomerase; PPO, prophenoloxidase.

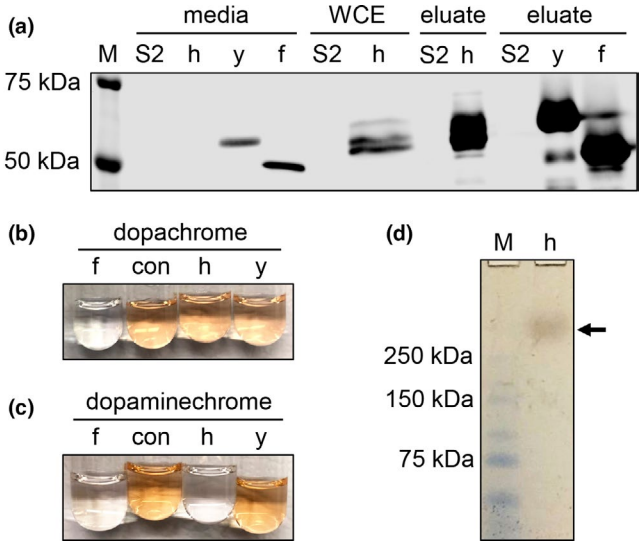

FIGURE 3 Expression and characterization of dopaminechrome tautomerase (DPT). (a) Expression of SBP-tagged Yellow proteins in cultured S2 cells. Western blot with anti-SBP antibody of cell media, whole cell extract (WCE), and eluates after streptavidin purification. M, molecular weight marker, S2, untransfected cells, y, Yellow-y-SBP, h, Yellow-h-SBP, f, Yellow-f-SBP. Yellow-h-SBP was absent from the media and was purified from WCE. (b,c) Solution assays of recombinant protein activity using dopachrome (b) and dopaminechrome (c). con, controls (no protein added), f, Yellow-f-SBP, h, Yellow-h-SBP, y, Yellow-y-SBP. (d) Activity assay of Yellow-h-SBP on a polyacrylamide gel using dopaminechrome as a substrate. M, molecular weight marker, h, Yellow-h-SBP protein (2 μg). Arrow indicates location of staining

data, we focused on the proteins that were identified with multiple peptides and that were representing enzyme families which have been previously implicated in melanogenesis. Phenoloxidases (PPO1 and PPO2) were the major proteins in the excised band (Table 1; complete mass spectrometry data are included in Table S1). To test whether phenoloxidases were responsible for the DPT activity, we utilized a *Drosophila* line that was double null mutant for *PPO1* and *PPO2* (Dudzic et al., 2015). If indeed one or more phenoloxidases could also possess DPT activity, this line would not exhibit the DPT reaction. However, protein extracts obtained from the double mutant line still displayed DPT activity, thereby ruling out phenoloxidase being responsible for the observed DPT activity (Figure S1).

The DPT band also contained several peptides from the Yellow-h protein, a member of the *yellow* gene family (Table 1). To test whether Yellow-h protein acts as DPT, we expressed it in S2 cells as a carboxy-terminally tagged fusion with streptavidin binding peptide (SBP) (Keefe et al., 2001).

As controls, we also expressed Yellow-y-SBP and Yellow-f-SBP. All three proteins were isolated from the culture medium and purified using a streptavidin affinity column. Whole cell extracts, cell culture medium, and the eluates from the streptavidin column were analyzed using Western blotting with anti-SBP antibody. Yellow-h-SBP was not secreted into the medium, unlike Yellow-y-SBP or Yellow-f-SBP that could be detected as secreted proteins in the media and were present in high amounts in streptavidin precipitates (Figure 3a). This result suggests that the Yellow-h protein is either a cytoplasmic protein or a protein that is attached to the plasma membrane with exposure on the extracellular side of the cell. Accordingly, we purified Yellow-h-SBP from whole S2 cell extracts.

3.3 | DPT specifically converts dopaminechrome

All recombinant proteins were then assessed for DCDT and DPT activities. As expected, recombinant Yellow-f-SBP converted both dopaminechrome and dopachrome (orange color) to 5,6-dihydroxyindole (colorless), whereas recombinant Yellow-y-SBP did not exhibit any activity towards either dopaminechrome or dopachrome (Figure 3b,c). Recombinant Yellow-h-SBP acted solely on dopaminechrome and did not exhibit any significant activity towards dopachrome (Figure 3b,c). To further confirm this result, recombinant Yellow-h-SBP was subjected to polyacrylamide gel electrophoresis and assessed for tautomerase activities by the staining protocols developed for DCDT and DPT (Nicklas & Sugumaran, 1995; Xia et al., 2006). Within 10 min, a blue melanochrome band appeared on the gel stained with dopaminechrome which turned into black melanin product after 30 min (Figure 3d), whereas no such band appeared in the gel stained with dopachrome (Figure S3). These results demonstrate that Yellow-h functions as a specific DPT.

Substrate specificity of recombinant Yellow-h-SBP protein was studied using several potential substrates. It readily bleached dopaminechrome followed by N-methyldopaminechrome, but did not convert any other chromes tested, including dopachrome

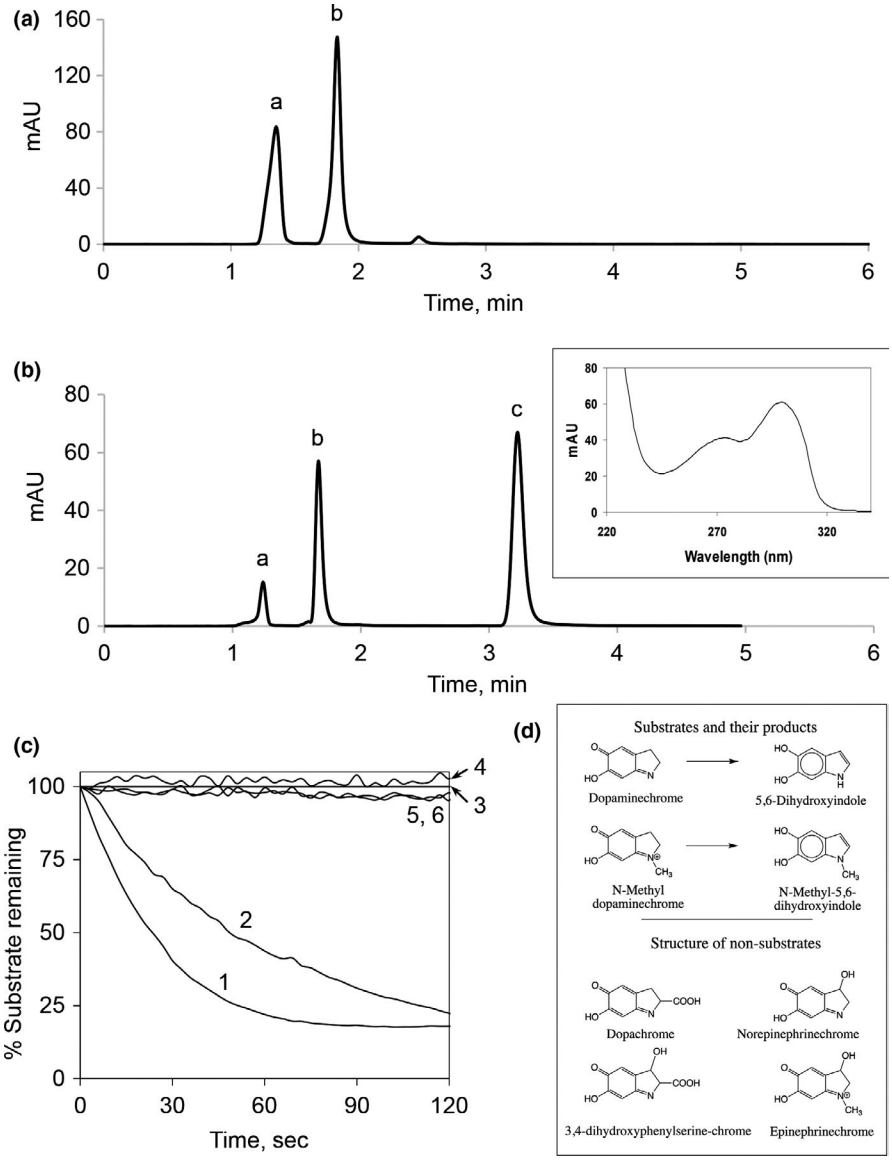

FIGURE 4 Enzyme activity of dopaminechrome tautomerase (DPT). (a, b) HPLC analysis of a reaction of Yellow-h-SBP protein with dopaminechrome. (a) Zero time point. (b) 2 min time point. Peak a, unoxidized dopamine, peak b, dopaminechrome, peak c, DHI. Inset: UV spectrum of DHI formed in the reaction mixture (peak c). (c) Substrate specificity of DPT. Time courses of reactions monitored at 475 nm. Substrates used were: 1, dopaminechrome, 2, *N*-methyl-dopaminechrome, 3, dopachrome, 4, 3,4-dihydroxyphenyl-serine-chrome, 5, norepinephrinechrome, 6, epinephrinechrome. (d) Structures of substrates and non-substrates of DPT, corresponding to the data in (c)

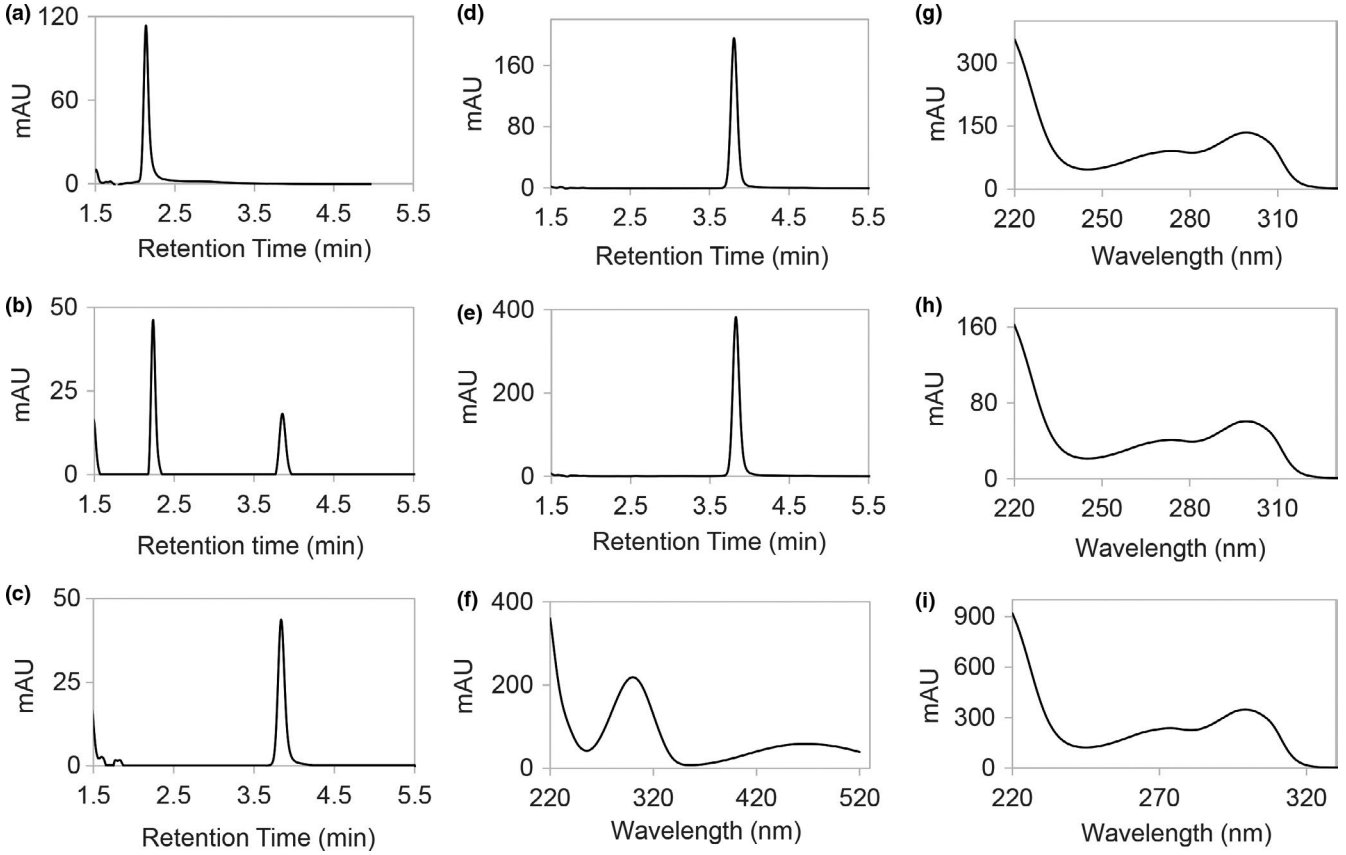

FIGURE 5 HPLC analysis of the dopaminechrome reaction mixture. A reaction mixture containing dopamine with twice the amount of sodium periodate and Yellow-h-SBP protein in water was incubated at room temperature and an aliquot was subjected to HPLC analysis as described in Materials and Methods. (a) Control zero-time reaction before the addition of enzyme. The peak eluting at 2.14 min is due to dopaminechrome. (b) HPLC analysis of the 1 min enzymatic reaction. The peak at 2.14 is due to dopaminechrome, and the peak at 3.8 min is due to DHI. (c) HPLC analysis of the 6 min reaction. Note a complete conversion of dopamine chrome to DHI. (d) HPLC of the DHI standard. The peak eluting at 3.82 min is due to DHI. (e) Co-chromatography of the 6 min reaction peak with the DHI standard. Note the elution of a single symmetrical peak at 3.82 min. (f) UV/visible spectrum of dopaminechrome peak from (a). (g) UV/visible spectrum of the authentic DHI peak from (d). (h) UV/visible spectrum of elution peak at 3.82 min from the 1 min reaction. (i) UV/visible spectrum of the co-chromatography peak from (e)

(Figure 4a–c). These results conclusively prove that the protein encoded by the *yellow-h* gene is DPT, with high specificity towards dopaminechrome, and devoid of any detectable activity on dopachrome. The structures of the compounds that served as the substrate and the products of the reaction are shown in Figure 4d. To identify the product of the reaction, HPLC studies were carried out. The reaction mixture contained 1:2 ratio of dopamine to periodate to ensure the complete conversion of dopamine to dopaminechrome. The conditions for HPLC analysis of various reactions are given in Materials and Methods. The elution profile of dopaminechrome on a reversed phase C_{18} column using isocratic elution with 20% methanol in water is shown in Figure 5a. Dopaminechrome eluted at about 2.14 min. It's UV/visible spectrum is shown in Figure 5f. Even a short incubation (1 min) of dopaminechrome with Yellow-h-SBP protein resulted in a rapid conversion of dopaminechrome to a new compound eluting at 3.82 min (Figure 5b). By 6 min incubation, complete conversion of the 2.14 min peak to the 3.82 min peak was observed (Figure 5c). This peak exhibited the same elution profile as that of the DHI standard (Figure 5d). Moreover, the UV spectrum of the 3.82 min peak (Figure 5h) matched that of authentic DHI shown in Figure 5g. When the 3.82 min peak was collected and subjected to co-chromatography with the DHI standard, both compounds eluted as a single symmetrical peak (Figure 5e) and exhibited the same UV/visible spectrum (Figure 5i) as that of DHI, confirming the identity of the enzymatic product as DHI.

To further confirm the identity of the product, mass spectral studies were carried out. Figure 6a shows the average electrospray mass spectrum of the reaction. Two ions observed at 150 and 172 are due to M + 1 and M + 23, corresponding to the protonated and sodiated forms of DHI. The m/z value of the 150 product (150.0551) is within 3 ppm of the theoretical mass of DHI. Similarly, the sodiated product observed with an m/z value of 172.03689 is within 3 ppm of the theoretical mass of sodiated DHI, thus confirming the identity of the product of dopaminechrome conversion by DPT as DHI. The collision induced decomposition spectrum of the 150 ion is shown in Figure 6b. Essentially, the same fragmentation pattern was observed for the DHI standard. Since DHI is a very simple bicyclic compound, the fragmentation pattern was not very informative.

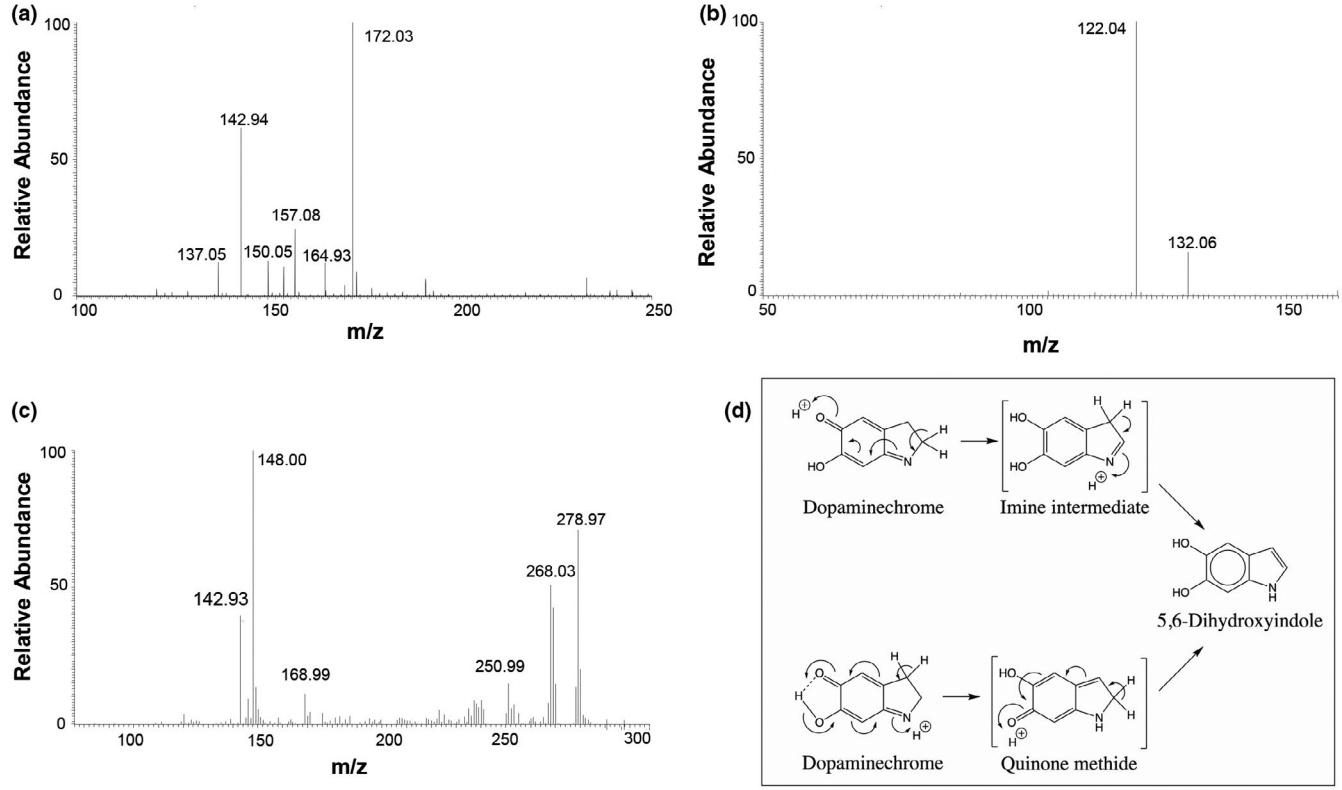

FIGURE 6 Mass spectral analysis of the DPT reaction and a possible mechanism of conversion of dopaminechrome to 5,6-dihydroxyindole (DHI). (a) The average electrospray mass spectrum of the DHI reaction. Note the presence of dopaminechrome isomerase reaction product, DHI, in the protonated form at m/z value of 150.05 and in the sodiated form at 172.03, confirming its production. (b) The collision induced decomposition mass spectrum of the product ion at m/z 150.05. (c) The collision induced decomposition mass spectrum of the dimeric product ion at m/z 297. (d) Two possible routes of DHI generation from dopaminechrome

We have recently reported that DHI is extremely unstable in solution and readily exhibits dimerization and oligomerization reactions (Sugumaran et al., 2020). Accordingly, we could also observe the presence of trace amounts of DHI dimers formed in the reaction mixture at m/z 297. The observed mass of this dimer (297.0880) was within 1.6 ppm of the theoretical mass of the protonated DHI dimer (297.087533). The collision induced decomposition spectrum of this dimeric product (shown in Figure 6c) matched the recently reported mass spectrum of the DHI dimer peak by peak (Sugumaran et al., 2020), confirming the fact that DHI is the product formed in the reaction, and that it also exhibits non-enzymatic dimerization in solution as reported (Sugumaran et al., 2020).

Two possible mechanisms can account for the conversion of dopaminechrome to DHI (Figure 6d). The first route calls for the transient formation of an imine intermediate and its isomerization to DHI. The second route invokes the production of a quinone methide intermediate and its isomerization to DHI. The imine intermediate route invokes the electron donating properties to the C=N bond. In reality however, the C=N is electron withdrawing in the C → N direction and hence, it is easier to protonate the nitrogen. As a consequence, quinone methide will be formed more favorably than the imine intermediate. A similar quinone methide intermediate formation has been established for the non-enzymatic transformation of dopachrome to DHI (Sugumaran et al., 1990) as well as enzymatic transformation of dopachrome to DHI catalyzed by DCDT (Sugumaran & Semensi, 1991). Therefore, the quinone methide route appears to be the more preferred route for DHI formation than the imine intermediate route. At present, it is not clear if any metal ions are associated with the enzymatic transformation of dopaminechrome to DHI. The mammalian DCT has been shown to be a zinc enzyme (Solano et al., 1994), but the presence or absence of a metal ion in insect DCDT remains unresolved. We could not examine the metal ion content of DPT due to the fact that the enzyme is induced in the cells with a large excess of copper. Experiments with metal chelators were inconclusive. Therefore, the role of metal ions in enzymatic catalysis could not be assessed. The metal ion such as zinc may be necessary for coordinating the quinonoid moiety of dopaminechrome and assisting the transition of dopaminechrome to quinone methide. However, the active site topology of the DCTs or DCDTs remains uncertain, given the lack of 3D structures. A detailed discussion on different tautomerases is presented elsewhere (Sugumaran & Barek, 2016).

4 | DISCUSSION

Melanin formation in insects is associated with many physiological processes such as cuticular tanning, innate immune response, and

wound healing (Eleftherianos & Revenis, 2011; Shamim et al., 2014; Sugumaran, 1996, 2002, 2010; Theopold et al., 2004; True, 2003; Wittkopp & Beldade, 2009). Some of these pathways operate on short time frames and therefore require rapid melanin synthesis. Melanin synthesis in insects also occurs in the open circulatory system of insect hemolymph, in contrast to melanin synthesis in mammals, where it is confined to specialized melanocytes. Therefore, melanogenesis in insects must occur significantly faster to avoid the potential toxicity caused by the reactive intermediates. Although dopamine has been known to be one of the precursors for melanin biosynthesis in insects, recent chemical analyses revealed that insect melanins are mostly derived from dopamine and not dopa (Barek et al., 2018; Sugumaran & Barek, 2016). Given this new finding, we hypothesized that insects must possess an independent pathway for conversion of dopamine to melanin. Broad-specificity phenoloxidases can oxidize both dopa and dopamine, and convert them to their quinones, which can rapidly cyclize and form aminochromes. However, further conversion of aminochromes calls for the use of conversion factors such as DCDT. While *Drosophila* DCDT has the potential to convert both dopachrome and dopaminechrome to the same end product, DHI, other insect DCDTs appear to be recalcitrant to dopachrome, necessitating the use of a separate DPT. This protein is now identified in the current work as the *Drosophila yellow-h* gene product.

Since the conversion of aminochrome to 5,6-dihydroxyindole is a crucial step in melanin formation and must occur fast due to the toxicity of these molecules (Galzigna et al., 1999; Matsunaga et al., 1999), insects appear to employ more than one protein to catalyze aminochrome tautomerization. The results presented here reveal that insects such as *Drosophila* produce dopamine-derived melanin *via* a novel enzyme, DPT. Based on this finding, we propose a revised melanin biosynthesis pathway in *Drosophila* (Figure 7), in which we add *yellow-h* to the known set of genes associated with melanization and sclerotization.

According to this model, the amino acid tyrosine is first hydroxylated to dopa by tyrosine hydroxylase (*pale*). Dopa decarboxylase (*Ddc*) then converts dopa to dopamine which is used to make NADA and NBAD. These two molecules are primarily used for cuticular sclerotization reactions (Barek et al., 2017; Sugumaran, 2010). Either during or after sclerotization reactions, part of NBAD is converted back to dopamine by the action of NBAD hydrolase, *tan* (Aust et al., 2010). The dopamine thus regenerated is oxidized by cuticular laccase to its quinone (Arakane et al., 2005). The resultant dopaminequinone is non-enzymatically cyclized and oxidized to dopaminechrome. We propose here that dopaminechrome can serve as a substrate for either DCDT encoded by *yellow-f/f2* or, as shown in this study, for DPT encoded by *yellow-h*. We speculate that the existence of multiple enzymes that catalyze this reaction in *Drosophila* is related to the high toxicity of dopaminechrome. In addition, DCDT and DPT may be expressed at different times during development and/or in different tissues, ensuring the availability of aminochrome converting activity.

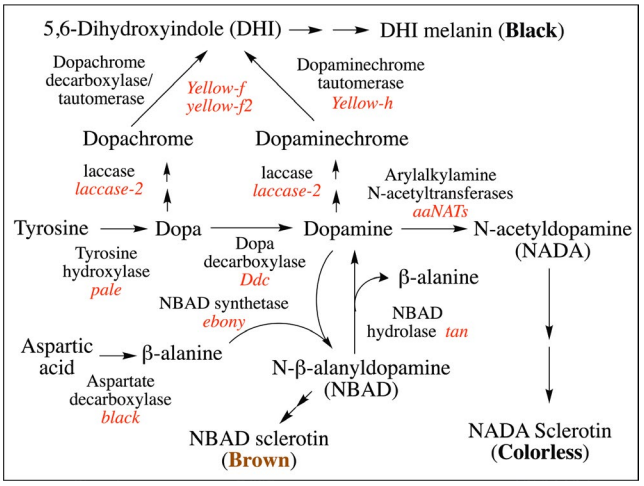

FIGURE 7 Biosynthetic pathways for cuticular melanogenesis in *Drosophila melanogaster*. Cuticular sclerotization and melanization are often coupled in most insects. Both processes start with the conversion of tyrosine to dopa by the action of tyrosine hydroxylase coded by the gene *pale*. Dopa decarboxylase (*Ddc*) generates dopamine from dopa. Dopamine is primarily converted to NBAD and NADA by the action of NBAD synthetase (*ebony*) and *N*-acetyl transferases (*aaNATs*), respectively. NBAD and NADA serve as the precursor for the sclerotization pathway. Excess NBAD that remains after sclerotization is converted back to dopamine by NBAD hydrolase (*tan*). Both dopa and dopamine can be oxidized by cuticular laccase to their corresponding quinones and after their cyclization to chromes are converted to DHI by either dopachrome decarboxylase/tautomerase (*yellow-f/f2*) or as presented in this study by dopaminechrome tautomerase encoded by *yellow-h*. Oxidative polymerization of DHI makes the black colored melanin in cuticle. The genes associated with these enzymes are shown in red

We note that macrophage migration inhibition factor (MIF) which has D-dopachrome tautomerase activity (Matsunaga et al., 1999) has no sequence homology to any of the tautomerases in the melanogenic pathway, which act only on the natural substrate L-dopachrome and related compounds. Its concentration is high in the brain. This coupled with the fact that it also possesses DPT activity along with other activities suggests that it may be associated with detoxifying dopaminechrome in mammals.

Of the 14 different *yellow*-related genes in *Drosophila*, the biochemical function was identified for only three: highly related *yellow-f* and *yellow-f2*, which encode DCDT (Han et al., 2002), and *yellow-h* that encodes DPT (current study). The molecular functions of other *yellow* family genes remain unknown. Yellow-y protein was originally thought to possess dopachrome or dopaminechrome converting activity, however this was shown not to be the case (Han et al., 2002), and we have confirmed this result in this study (Figure 3b,c). How the Yellow-y protein promotes black pigmentation in the *Drosophila* cuticle is still an unresolved puzzle. We note that the *yellow-h* gene exhibits a similar temporal expression pattern as *yellow-y* in both *Tribolium castaneum* and *Drosophila melanogaster* (Figure S2) (Nicklas & Sugumaran, 1995; Thurmond et al., 2019).

In the swallowtail butterfly *Papilio xuthus*, a Yellow-h like protein, Yellow-h3, is expressed in a pattern that is similar to Yellow-y and in places where the black pigment is deposited in the cuticle (Futahashi et al., 2011). These observations suggest that the Yellow-h type proteins may somehow participate in melanin biosynthesis together with Yellow-y. Further studies are needed to elucidate the exact molecular function of the remaining *yellow* family members.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest with the contents of this article.

AUTHOR CONTRIBUTION

H.B., H.Z., A.V., and M.S. designed experiments, H.B., H.Z., and K.H. performed experiments, H.B., A.V., and M.S. wrote the manuscript.

DATA AVAILABILITY STATEMENT

All data presented in the paper are contained in the manuscript. Complete proteomic data are available at ProteomeXchange via the PRIDE partner repository with the dataset identifier PXD023295.

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SUPPORTING INFORMATION

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