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Functional Expression and Characterization of *Aedes aegypti* Dopachrome Conversion Enzyme

Jianmin Fang,* Qian Han,* Jody K. Johnson,† Bruce M. Christensen,† and Jianyong Li*,[1]
**Department of Pathobiology, College of Veterinary Medicine, University of Illinois at Urbana–Champaign, 2001 South Lincoln Avenue, Urbana, Illinois 61802; and †Department of Animal Health and Biomedical Sciences, University of Wisconsin–Madison, 1656 Linden Drive, Madison, Wisconsin 53706*

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A full-length mosquito dopachrome conversion enzyme (DCE) and its truncated form lacking the last 54 carboxyl-terminal amino acid residues are expressed using a baculovirus/insect cell expression system. The full-length recombinant DCE displayed multiple bands during native PAGE with substrate staining, but only one active band was detected when the truncated recombinant DCE was analyzed under identical analysis conditions. Our data suggest that the last 50 some carboxyl-terminal residues are involved in the polymerization of the DCE molecules and that the proposed DCE isozymes likely reflect the presence of multimers of the same DCE molecules. The significance of the recombinant DCE in accelerating the melanization pathway is demonstrated by a rapid production of melanin in a dopa and tyrosinase reaction mixture in the presence of recombinant DCE. The DCE sequence data obtained in our previous study, together with results of functional expression and biochemical characterization achieved in this study, provide a necessary reference for the study of other insect DCEs.

Key Words: Aedes aegypti; **dopachrome conversion enzyme; insect cell; baculovirus; melanization; L-dopachrome.**

Conversion of dopachrome (DC) to 5,6-dihydroxyindole (DI) is a key step in the eumelanin pathway (Fig. 1). For many years this DC to DI step had been considered a nonenzymatic process. However, the rate of nonenzymatic DC conversion to DI seemed too low to be physiologically adequate for some living organisms, especially for insects. In our study dealing with the melanization pathway in *Aedes aegypti* mosquitoes, we determined that an enzyme catalyzing a decarboxylative structural rearrangement of DC to DI is present and that the enzyme significantly accelerates the melanization pathway. Based on its biochemical function, the enzyme was termed dopachrome conversion enzyme (DCE) (1, 2). The same enzyme activity has been detected in a few other insect species (3–6).

DCE is critical for the melanization pathway in insects, but biochemical studies have been hampered by difficulties in the isolation of the enzyme due to limitations in the amount of protein available. It has been equally problematic to clone insect DCEs using molecular approaches because no information was available regarding primary sequences. In mammals, there is an enzyme termed DC tautomerase (DCT) that catalyzes the conversion of DC to 5,6-dihydroxyindole-2-carboxylic acid (DICA) (7, 8). Mammalian DCT is commonly called tyrosinase-related protein 2 based on its sequence homology with tyrosinase (9). Because both mammalian DCT and insect DCE use the same substrate (though their products are different) and both are involved in the melanization pathway (Fig. 1), it was speculated that the two enzymes might share considerable sequence similarity. However, all attempts by us and other researchers to clone insect DCE based on mammalian DCT sequences were unsuccessful.

Recently we purified the mosquito enzyme, obtained internal amino acid sequences from the purified protein, and isolated a cDNA clone based on its partial amino acid sequence (1). However, when the deduced amino acid sequence of the mosquito DCE clone was compared to that of mammalian DCT, there was no sequence homology between the two enzymes. In addition, the isolated mosquito clone shares no sequence homology with mosquito phenoloxidases (PO), whereas human DCT has high sequence homology with human tyrosinase (9). More surprisingly, blast search of the mosquito DCE provided no significant match with other proteins or hypothetical gene products in GenBank protein databases. The only exception was a 30–40% homology with yellow or yellow-related proteins from *Drosophila melanogaster* and the honeybee.

[1] To whom correspondence and reprint requests should be addressed. Fax: +1 217 244 7421. E-mail: jli2@uiuc.edu.

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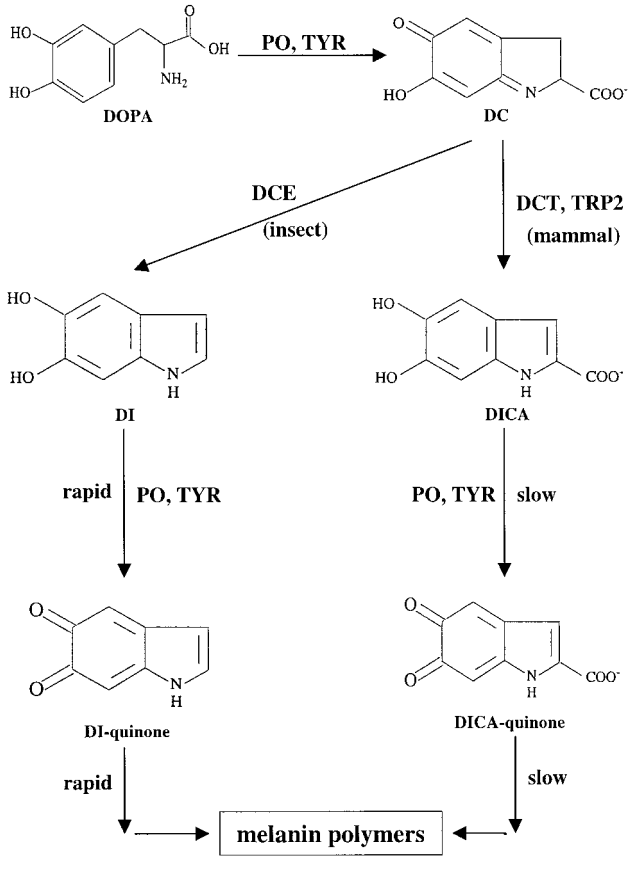

FIG. 1. Melanization pathway. Abbreviations used: DC, dopachrome; DCE, dopachrome conversion enzyme; DCT, dopachrome tautomerase; DI, 5,6-dihydroxyindole; DICA, 5,6-dihydroxyindole-2-carboxylic acid; PO, phenoloxidase; TRP2, tyrosinase-related protein 2; TYR, tyrosinase.

The absence of a similar protein from all other species (except for insects) raises fundamental questions, i.e., why such a unique enzyme evolved only in insects and what is its catalytic mechanism. The primary sequence of a protein dictates its three-dimensional structure, which in turn dictates its biochemical function(s). To understand the structure/function relationship for mosquito DCE, it is necessary to have a relatively large amount of enzyme available for detailed biochemical and structural analysis. It obviously is not practical to directly purify DCE from insects for such studies due to limitations in the amount of protein available; therefore, using a baculovirus/insect cell expression system, we achieved the expression of recombinant *A. aegypti* DCE that is highly active on converting DC to DI. In this report we present data that describe the functional expression of mosquito DCE and the characterization of the recombinant protein in comparison with that expressed in mosquito larvae and pupae. The cloning of *A. aegypti* DCE in our previous study (1), together with the functional expression and biochemical characterization of the enzyme in this study, provides the necessary resources for studying this enzyme in other insect species and also the basis for a more complete understanding of its physiological function(s) and catalytic mechanism. In addition, the characteristics of this enzyme in relation to mammalian dopachrome tautomerase, or tyrosinase-related protein 2 are highly significant and should be of interest to people working on melanogenesis in vertebrates.

MATERIALS AND METHODS

Chemicals and reagents. 3,4-Dihydroxyphenylalanine (dopa), 3,4-dihydroxyphenylethylamine (dopamine), dopa methyl ester, α-methyl dopa, β-mercaptoethanol (β-ME), phenylmethylsulfonyl fluoride (PMSF), sodium periodate ($NaIO_4$), sodium phosphate, trifluroacetic acid, and tyrosinase were obtained from Sigma (St. Louis, MO). The insect cell/baculovirus expression system (MaxBac 2.0) was purchased from Invitrogen Corporation (Carlsbad, CA).

Mosquito rearing and maintenance. Aedes aegypti mosquitoes used in this study were reared according to described methods (1). All mosquitoes were maintained at 25 ± 0.5°C, 60% RH and on a 16-h light:8-h dark cycle with a 90-min crepuscular period at the beginning and end of each light cycle.

Construction of recombinant baculovirus transfer vectors. A forward primer (5′-GGCCTGCAG ATGCTGCGCCTCGTGCTC-3′) and a reverse primer (5′-CCAAGCTTACGGTCTGCGGCCT GTCGG-3′) containing a *Pst*I and a *Hin*dIII restriction site (underlined nucleotides), respectively, were designed based on the *A. aegypti* DCE cDNA sequence (GenBank Accession No. AF288384) and used to amplify the full-length DCE coding sequence from a larval cDNA pool. An additional reverse primer (5′-ACAAGCTTAGTCACAGACGGTGCC-3′) containing the same *Hin*dIII restriction site also was synthesized for amplification of a truncated DCE cDNA lacking 54 carboxyl-terminal amino acid residues. The PCR products were gel-purified and cloned into a PCR2.1 TOPO TA cloning vector (Invitrogen) and then subcloned into baculovirus transfer vector pBlueBac4.5 between the *Pst*I and *Hin*dIII restriction sites. Sequence analysis of recombinant vectors, using the polyhedrin forward and reverse sequencing primers, confirmed that the cloned *A. aegypti* DCE inserts were downstream of the polyhedrin promoter and that the two inserts were in frame.

Isolation of recombinant baculovirus. Spodoptera frugiperda (Sf9) cells were grown in monolayer at 27°C in 25-cm^2 tissue culture flasks using complete TNM-FH medium supplemented with 10% fetal bovine serum (Bio-Whittaker, Walkersville, MD) and 10 μg/ml gentamycin. Recombinant pBlueBac4.5 transfer vectors were cotransfected with triple-cut linearized Bac-N-Blue viral DNA (AcMNPV, *Autographa californica* multiple nuclear polyhedrosis virus), in the presence of InsectinPlus liposomes, into Sf9 cells. Recombinant viruses were purified by plaque assays. Individual putative blue recombinant plaques were transferred to a 12-well microtiter plate and amplified in Sf9 cells. Purity of the recombinant viral clones was determined by PCR amplification of the isolated viral DNA. High-titer viral stocks (HTS) of the identified recombinant viral clones were generated by amplification in suspension culture of Sf9 cells and stored at 4°C.

DCE expression in insect cells and sample preparation. High Five (*Tricoplusia ni*) cells were used for protein expression. The cells were cultured at 27°C in Ultimate Insect serum-free medium supplemented with 10 units of heparin (Sigma, St. Louis, MO) per milliliter of culture in spinner flasks with constant stirring at 80 rpm. When the cell density reached 2.5×10^6 cells/ml, they were inoculated with HTS of recombinant baculovirus at an MOI of 6 and cultured at 27°C. Production of recombinant DCE (rDCE) by the infected cells was based on detection of DCE activity in culture medium supernatant

and/or in soluble cell protein. Briefly, at each 24-h interval after viral inoculation, 2 ml of cell culture was withdrawn and centrifuged to separate supernatant from cells (2000*g* for 10 min at 4°C). The cell pellet was washed with PBS, resuspended in 10 vol of 10 mM phosphate buffer (pH 6.5), and sonicated to lyse the cells. Soluble cell protein was obtained by centrifugation of the cell lysate (20,000*g* for 10 min at 4°C). Both culture medium supernatants and soluble cell protein were assayed for DCE activity. Samples from uninfected cells or cells infected with wild-type baculovirus served as controls.

DCE activity assay. DCE activity was based on a decrease of the 475-nm DC absorbance peak in the reaction mixture (in a cuvette with $d = 1$ cm) using a Hitachi 2001 spectrophotometer. Briefly, DC was generated by mixing 2 mM L-dopa prepared in H_2O with an equal volume of 4 mM $NaIO_4$ prepared in 10 mM phosphate buffer, pH 7.0. The reaction was initiated by mixing 400 μl of DC solution with 100 μl of either supernatant from the culture medium or soluble protein from cell lysates.

Native PAGE with substrate staining. Native PAGE with substrate staining was used to identify bands with DCE activity after electrophoresis. During gel preparation, horseradish peroxidase (HRP) was mixed into a 5% acrylamide solution at a concentration of 0.05 mg/ml prior to polymerization. Soluble cell protein for native PAGE was prepared by mixing collected cell pellets in 2 vol of loading buffer (50 mM Tris, pH 6.8, containing 10% glycerol and a minimum amount of bromphenol blue for visualization), followed by sonication and centrifugation to obtain soluble cell protein. Culture medium supernatants used for native PAGE analysis were prepared by mixing 30 μl of the supernatants with 10 μl of concentrated loading buffer (200 mM Tris, pH 6.8, containing 30% glycerol and minimum amount of bromphenol blue for visualization). Both the culture medium supernatants and the soluble cell protein (20 μl in each sample) were subjected to native PAGE. Electrophoresis was carried out at a constant voltage of 150 V at 4°C using a Hoefer SE 260 Mighty Small II electrophoresis apparatus. After electrophoresis, the gel with the separated proteins was incubated for 10–15 min in a solution containing 2 mM L-dopa and 2 mM H_2O_2 prepared in 20 mM phosphate buffer (pH 6.5). Identification of rDCE was based on formation of black band(s) on the gel.

To compare rDCE with DCE from *A. aegypti*, 40–60% $(NH_4)_2SO_4$ precipitated larval or pupal protein, which contains the majority of DCE activity, was isolated from 3-day-old larvae and 12 hr pupae by a previously described method (1). Briefly, 3-day-old larvae or 12-h-old pupae were collected and homogenized in 20 mM phosphate buffer (pH 7.0), containing 40% saturation of $(NH_4)_2SO_4$, 10 mM β-ME, 1 mM PTU (phenoloxidase inhibitor) and 1 mM PMSF (proteinase inhibitor). Supernatant, obtained by centrifugation, was brought up to 60% saturation of $(NH_4)_2SO_4$. The $(NH_4)_2SO_4$ precipitated protein was collected by centrifugation (18,000*g* for 10 min at 4°C) and dialyzed in 4 liters of 15 mM phosphate buffer (pH 7.0) containing 20 mM β-ME, 1 mM PTU and 1 mM PMSF. The dialysis solution was deoxygenized using helium prior to dialysis. After dialysis, protein samples were analyzed by native PAGE with substrate staining as described for rDCE, and results were compared to those obtained from analysis of the full-length recombinant DCE (f-rDCE) and the truncated recombinant DCE (t-rDCE) in culture medium supernatant or soluble cell protein.

rDCE purification. Both f-rDCE and t-rDCE were purified from culture media supernatants by various chromatographic techniques, including DEAE–Sepharose, phenyl-Sepharose, hydroxyapatite, and UNO-Q column chromatographies. Briefly, the High Five cell culture, inoculated with recombinant virus, was harvested at 4 days postinoculation and centrifuged at 1500*g* for 20 min at 4°C to obtain supernatant. The supernatant was diluted with 2 vol of 5 mM phosphate buffer (pH 7.0) containing 10 mM β-ME and 1 mM PMSF and applied to a DEAE–Sepharose column (5 × 5 cm) and protein was eluted using a linear NaCl gradient (0–300 mM) in 600 ml of 15 mM phosphate buffer containing 10 mM β-ME and 1 mM PMSF. Active DCE fractions were collected and sequentially separated by phenyl-Sepharose, hydroxyapatite, and UNO-Q column chromatographies as described in our previous report (1). The purified f-rDCE and t-rDCE were analyzed by both SDS–PAGE, with Coomassie blue staining, and native PAGE, with substrate staining, respectively.

The effect of rDCE on accelerating melanization reactions. The effect of the purified DCE in accelerating the melanization pathway was assessed by the rate of melanin formation in a reaction mixture containing tyrosinase (Sigma) and L-dopa or dopamine in the presence or absence of DCE samples. The relative amount of melanin polymers produced in the reaction mixtures was based on the relative dimension of the broad absorbance peak with a λ_{max} around 550 nm. During spectral analysis, the solution in the cuvette was stirred constantly using a mini magnetic stir bar.

RESULTS

Production of Recombinant Baculoviruses

Cotransfection of the recombinant pBlueBac4.5 containing DCE inserts with the linearized AcMNPV viral DNA to Sf9 cells led to the successful production of recombinant baculoviruses that were purified by plaque assays. PCR analysis determined the presence of the full-length or truncated DCE insert in the isolated viral DNA. Using the purified DCE recombinant viruses, two viral stocks (HTS), one for the full-length DCE recombinant virus, with a titer of 1×10^9 pfu/ml, and the other for truncated DCE recombinant virus, with a titer of 5×10^8 pfu/ml, were generated and used to infect High Five cells in spinner flasks for large-scale protein expression.

DCE Activity Assay

The expression of DCE in insect cells was based on detection of DCE activity in culture medium supernatants and cell lysates. There was no detectable DCE activity in the supernatant from uninfected cells (Fig. 2, S0). DCE activity was low, with no noticeable change in the levels of DCE activity, from cells collected at 1–5 days after infection with full-length DCE recombinant virus (Fig. 2, C3). In contrast, an almost linear increase in DCE activity was observed in culture media supernatants at 1, 3, and 5 days after viral inoculation (Fig. 2, S1, S3, and S5). Similar results were obtained when culture media from cells infected with truncated DCE recombinant virus were analyzed under identical conditions (not shown). The low DCE activity in soluble cell protein and the high DCE activity in supernatant of culture medium suggest that DCE is secreted into the medium soon after its synthesis. This finding is in agreement with the TargetP program analysis (http://www.cbs.dtu.dk) showing a typical secretion signal peptide consisting of the first 20 amino acid residues at the NH_2-terminus with a potential cleavage site between Gly-20 and Gln-21.

Native PAGE with Substrate Staining of rDCE and Native DCE from Larvae and Pupae

Soluble cell protein from cells infected with full-length DCE recombinant virus had low DCE activity (see Fig. 2, C_3), but when more concentrated soluble cell protein, obtained from cell cultures at 3 day after inoculation, was analyzed by native PAGE with substrate staining, two positive DCE activity bands were detected (Fig. 3A, lane C_3f). The second band, that migrated more slowly on the gel, is presumed to be newly synthesized f-rDCE that has retained the NH_2-terminal signal peptide. Analysis of cell culture supernatants, collected at 1 day after infection, revealed one DCE positive band (band I) that migrated to the same position as the first DCE active band from soluble cell protein (Fig. 3A, lane S_1f). However, a second DCE active band (band II) in supernatant of cultural media became visible at 2 days following infection, and then a third DCE active band (band III) appeared at 4 days after infection (Fig. 3A, lanes S_2f–S_6f). Treatment of the samples with β-ME prior to electrophoresis had no effect on the number of active bands detected, nor did this treatment influence the migration of bands with DCE activity (not shown). Multiple DCE bands also were detected from the 40–60% $(NH_4)_2SO_4$ precipitated larval and pupal protein (Fig. 3A, lanes L and P).

Two DCE active bands also were detected in concentrated soluble cell protein from cells infected with the truncated DCE recombinant virus, but unlike f-rDCE in soluble cell protein, the two t-rDCE bands (Fig. 3B, lane C_3t) migrated much more rapidly on the gel than the f-rDCE bands I and II (Fig. 3B, lane S_6f). However, regardless of the post infection times, only one positive band was detected in supernatants from cell cultures inoculated with the truncated DCE recombinant virus (Fig. 3B, lanes S_3t and S_6t).

The 3 f-rDCE active bands, seen in supernatant from 6-day-old cell cultures, remained well separated after the sample was electrophoresed for 14 h at 4°C on a gradient (5–20%) native polyacrylamide gel (Fig. 3C, lane S_6f). Because the DCE active bands no longer migrated on the gradient gel after 12 h under the applied electrophoresis conditions, the relative position of the DCE active bands on the gradient gel is considered to depend essentially on the relative molecular masses of the individual DCE active bands. The t-rDCE that migrated behind the f-rDCE band III on a 5% native polyacrylamide gel (Fig. 3B, lanes S_6t and S_6f), migrated to the front of the f-rDCE band III (see Fig. 3C, lanes S_6f and S_6t). Based on comparisons with molecular mass standards that were run on the same gel, the t-rDCE active band and the f-rDCE band III represent, at the least, a dimer.

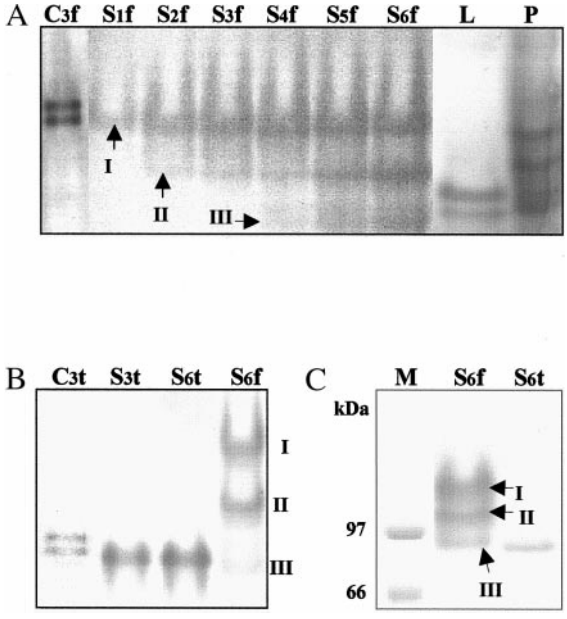

FIG. 3. Native PAGE with substrate staining. A and B were run on 5% native polyacrylamide gel, and C was run on a 5–20% native polyacrylamide gel. Lanes in A represent soluble cell protein from cells collected at 3 days after inoculation with full-length DCE recombinant virus (C_3f), supernatants collected from cell culture at 1 day (S_1f), 2 days (S_2f), 3 days (S_3f), 4 days (S_4f), 5 days (S_5f), and 6 days (S_6f) after inoculation with full-length DCE recombinant virus, and 40–60% $(NH_4)_2SO_4$ precipitated larval (L) and pupal protein (P), respectively. Lanes in B represent soluble cell protein from cells collected at 3 days after inoculation with truncated DCE recombinant virus (C_3t), supernatants collected from cell culture at 3 (S_3t) and 6 (S_6t) days after inoculation with truncated DCE recombinant virus, and supernatants collected from cell culture at 6 days (S_6f) after inoculation with full-length DCE recombinant virus as control. Lanes in C represent supernatants from cells collected at 6 days after inoculation with full-length DCE recombinant virus (S_6f) and truncated DCE recombinant virus (S_6t), respectively, and molecular marker stained with Coomassie blue (M).

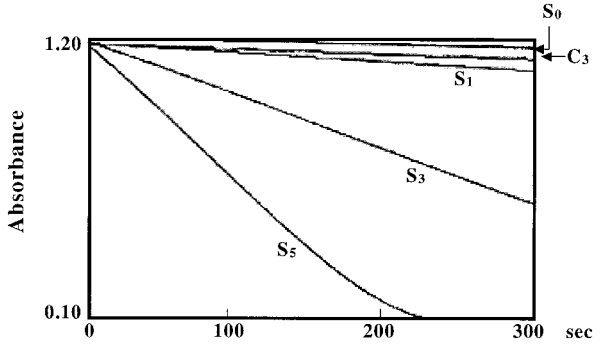

FIG. 2. Recombinant DCE activity assay. Supernatant of the cultural medium or soluble cell protein (100 μl), collected at different time periods following inoculation with full-length DCE recombinant virus, was mixed with DC solution (400 μl), and change of the DC absorbance at 475 nm was continuously monitored for 5 min. Lines represent the absorbance decrease in the reaction mixture containing soluble cell protein from cells at 3 days after viral inoculation (C_3), supernatant of culture medium from uninfected cells (S_0), and supernatants of culture media from cells at 1 (S_1), 3 (S_3), and 5 days (S_5) after viral inoculation, respectively.

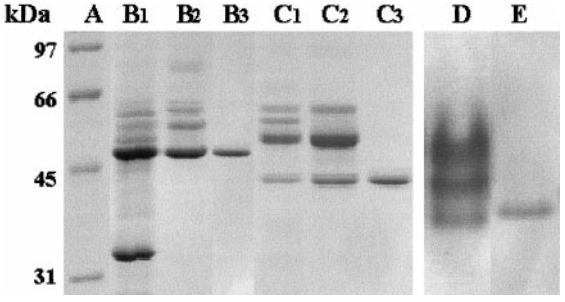

FIG. 4. Purification of recombinant DCE. About 2 liters of supernatant from cell culture inoculated with full-length DCE recombinant virus or truncated DCE recombinant virus were collected at 4 days after inoculation and used for rDCE purification. Lanes in A are protein molecular standard (A), protein profiles of supernatant containing f-rDCE after DEAE–Sepharose (B1), phenyl-Sepharose (B2), and UNO-Q column chromatography (B3), and protein profiles of supernatant containing t-rDCE after DEAE (C1), phenyl-Sepharose (C2), and UNO-Q column chromatography (C3). Lanes in B illustrate the results of native PAGE with substrate staining of the purified f-rDCE (D) and t-rDCE (E).

rDCE Purification

Both the f-rDCE and the t-rDCE were purified from their corresponding culture media supernatants. Figure 4 illustrates protein profiles after separation of f-rDCE by DEAE–Sepharose (Fig. 4, lane B1), phenyl-Sepharose (Fig. 4, lane B2), and hydroxyapatite and UNO-Q column chromatographies (Fig. 4, lane B3) and the protein profiles after separation of t-rDCE by the same chromatographic procedures (Fig. 4, lanes C1–C3). The relative molecular masses of the purified protein bands on SDS–PAGE gel (Fig. 4, lanes B3 and C3) reflected the calculated molecular mass for the f-rDCE (49,000) and t-rDCE (43,000), respectively.

The purified f-rDCE was essentially a single band on SDS–PAGE; however, when the same protein was analyzed by 5% native PAGE with subsequent substrate staining, three DCE positive bands were still observed (Fig. 4, lane D). In contrast, only one DCE active band was observed for the purified t-rDCE (Fig. 4, lane E).

Effect of rDCE in Melanin Formation

Oxidation of L-dopa by tyrosinase resulted in the accumulation of DC to high concentrations (Fig. 5A). Under the applied conditions, essentially all the dopa molecules were oxidized to DC during a 5-min incubation period (oxidation of dopa by tyrosinase or PO could be quite complete because the dopaquinone, the direct enzymatic product, does not accumulate in the reaction mixture and enzymatic dopa oxidation proceeds without being affected by product accumulation), with only a slight decrease in DC concentration observed during the next 20 min of incubation (Fig. 5A). In contrast, addition of f-rDCE at 5 min after dopa oxidation by tyrosinase resulted in the rapid depletion of DC with subsequent accumulation of melanin polymers, which was evidenced by the detection of a broad absorbance peak with a λ_{max} around 550 nm (Fig. 5B). However, when the dopa/tyrosinase reaction mixture was al-

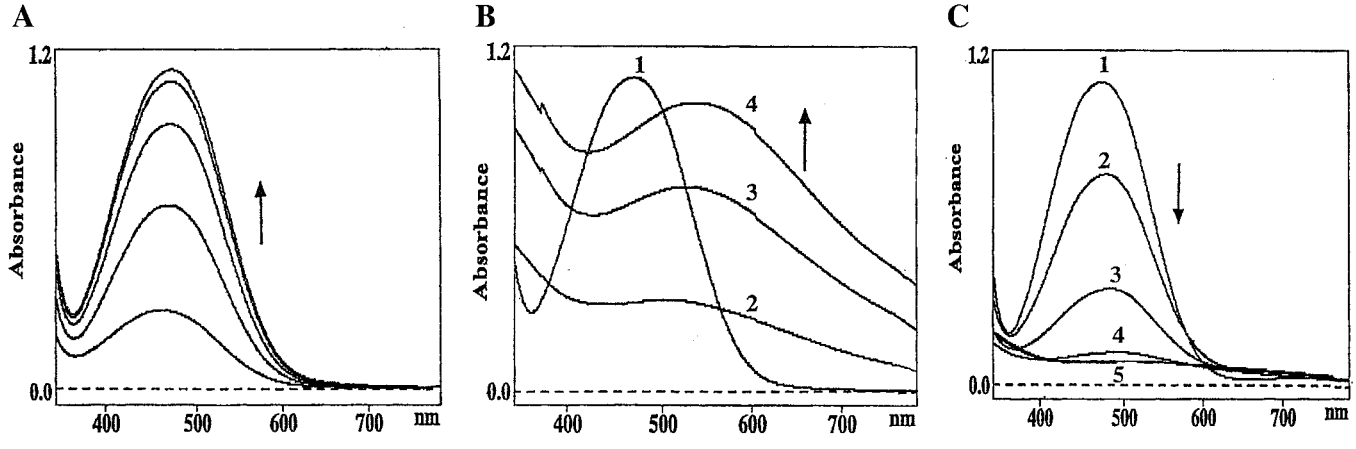

FIG. 5. Effect of recombinant DCE on melanization. L-Dopa (1 mM) and tyrosinase (0.4 mg/ml) were prepared in 10 mM phosphate buffer (pH 7.0) and oxidation of dopa by tyrosinase was initiated by mixing 0.2 ml of tyrosinase solution into 1.8 ml of L-dopa solution. Spectral changes in A illustrate the rapid accumulation of DC in the reaction mixture. Spectral change from 360 to 780 nm was monitored at each 75-s interval, and the highest curve in A represents the 475-nm peak at 5 min after oxidation and the peak slightly lower than the highest one is the absorbance peak at 20 min after oxidation. Spectral changes in B show the 475 nm peak (line 1) in the dopa and tyrosinase reaction mixture at 5 min after oxidation, a rapid decrease of the 475-nm DC peak at 3 min (line 2), and the increase of a broad absorbance peak with a λ_{max} around 550 nm at 6 min (line 3) and 9 min (line 4) after addition of 0.4 μg of purified f-rDCE in 4 μl 50 mM phosphate buffer (pH 7.0). Spectral changes in C illustrate the 475-nm DC absorbance peak in DC solution before the addition of f-rDCE (line 1) and the decrease of the 475-nm peak at 1 min (line 2), 2 min (line 3), 3 min (line 4) and 30 min (line 5) after addition of 0.4 μg of purified f-rDCE. The DC solution in C was generated by tyrosinase as in B, except that the reaction mixture was filtered through a membrane with molecular weight cutoff of 30,000 to separate tyrosinase prior to addition of f-rDCE.

lowed to react for 5 min and centrifuged through a membrane filter (Cetricon YM-30, M_r cutoff 30,000) to separate tyrosinase from DC before the addition of f-rDCE to the DC solution, a rapid decrease in DC concentration was observed without the accumulation of melanin polymers (Fig. 5C).

DISCUSSION

Melanization plays important physiological roles in insects (2–5). It is involved in cuticular tanning, eggshell hardening, defense reactions against pathogens and wound healing. The nature of these biological events requires that the melanization reactions proceed in a timely manner. For example, in mosquitoes the newly formed pupal cuticle is pale, but it turns black within half an hour. The rapid melanin formation in the reaction mixture containing dopa and tyrosinase in the presence of rDCE compared to the rate of melanization in the same reaction mixture in the absence of DCE indicate that DCE is the enzyme responsible for the rapid melanization reactions required for cuticular hardening, defense reactions and wound healing.

Melanization also is an important physiological event in mammals (10–12). Because both mammalian DCT and insect DCE use the same substrate and both are involved in the melanization pathway, one can question why two functionally related enzymes share no sequence homology. The unequivocal functional identification of mosquito DCE provides us the basis to contrast and compare mammalian DCT and insect DCE at both biochemical and molecular levels. In mammals, melanization occurs in melanosomes and proceeds progressively, which is quite different from melanin biosynthesis in mosquitoes. Undoubtedly, mammalian DCT is an important enzyme involved in melanization, but it can be questioned whether this enzyme actually functions in accelerating the melanization pathway. This is because DICA, the product of a DCT-catalyzed reactions, is not oxidized readily by either tyrosinase or PO. In contrast, DI, once formed, is oxidized very rapidly by PO or tyrosinase to form DI-quinone that polymerizes to form melanin polymers (see Fig. 1). The requirement for rapid melanin synthesis may explain why a specific DCE is present in mosquitoes. The same argument for the presence of a specific DCE should be applicable to other insects as well.

Native PAGE with substrate staining has been used to detect the presence of a DCE-like enzyme from *Manduca sexta,* and detection of different bands with DCE activity in developing eggs, larvae, pupae and adults was suggested to result from differential expression and/or processing of the genes coding for the enzyme(s) (13, 14). We previously believed DCE isozymes existed in *A. aegypti* and consequently tried to purify different DCEs without success. The detection of three bands with DCE activity during analysis of the f-rDCE by native PAGE with substrate staining suggests that the multiple DCE bands seen in crude larval or pupal proteins likely results from the formation of multimers from the DCE molecules. Because multiple DCE bands for t-rDCE are not observed, it seems clear that the last 54 or so DCE carboxyl residues are involved in the formation of polymeric DCE.

In summary, expression of the *A. aegypti* DCE using an insect/baculovirus expression system results in the production of biochemically active protein, which provides conclusive evidence for the DCE identity of our previously isolated mosquito cDNA clone. The results of native PAGE analysis of f-rDCE and t-rDCE indicate that the carboxyl terminal residues are involved in polymerization of DCE molecules, which leads to the detection of multiple bands with DCE activity on native gels. The sequence data reported in our previous study (1), together with the functional expression and characterization of *A. aegypti* DCE achieved in this study, should provide valuable references and tools for critical studies of DCE in other insect species.

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