Comparative analysis of Anopheles gambiae L-tyrosine decarboxylase and L-DOPA decarboxylase

by

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(ABSTRACT)

A major pathway of tyramine and dopamine synthesis in insects is through the decarboxylation of tyrosine and DOPA, respectively. Although tyrosine decarboxylase (TDC) has been mentioned in some reports, it has never been critically analyzed. The high sequence identity shared by tyrosine decarboxylase and DOPA decarboxylase in insects, and the similar structures of the substrates, tyrosine and DOPA, raise the possibility that both tyrosine decarboxylase and DOPA decarboxylase (DDC) have activities to tyrosine and DOPA. In this study, after tyrosine decarboxylase and DOPA decarboxylase enzymes of Anopheles gambiae were expressed, their substrate specificities and biochemical properties were critically analyzed. My results provide clear biochemical evidence establishing that the mosquito tyrosine decarboxylase functions primarily on the production of tyramine with low activity to DOPA. In contrast, mosquito DOPA decarboxylase is highly specific to DOPA with essentially no activity to tyrosine.
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CHAPTER 1

INTRODUCTION

Tyramine plays several critical roles in insect physiology. Tyramine is the only nonpeptide neurotransmitter and/or neuromodulator other than octopamine that is limited to invertebrates. Immunohistochemical studies have identified a group of larval neurons that contain only tyramine [1,2]. Tyramine has been described as an inhibitor for the cockroach fat body glycogenolysis. It has also been shown that tyramine is synthesized in non-neuronal cells and functions as a paracrine signal in the renal system of the fruit fly [2], increasing chloride concentration and stimulating the production of higher amounts of urine. Tyramine also appears to play a role in the regulation of olfactory responses, as it has a significant influence on olfaction. In addition to its function as neurotransmitter, tyramine is the direct precursor in octopamine synthesis[3,4,5]. Octopamine has been shown to affect many processes in insects [2,6]. Because octopamine must be produced from tyramine, the protein involved in tyramine production is undoubtedly essential to insect survival.

In insects, tyramine and dopamine can be formed from tyrosine and DOPA through the decarboxylation of their α-carboxyl group by decarboxylase-mediated reactions. The enzymes involved in the decarboxylation of tyrosine and DOPA are commonly named tyrosine decarboxylase (TDC) and DOPA decarboxylase (DDC), respectively (Figure 3)[2,10,11]. While many species have either DDC or TDC, insects are exceptional in that they have both DDC and TDC. For example, two sequences in Drosophila melanogaster have been assigned as TDCs, based primarily on the negative effect of their mutants on tyramine production[12,13,14,15]. However, the biochemical characteristics of these
proposed TDC proteins have not been clearly established[10]. For example, it is not
known if insect TDC can also use L-DOPA or other aromatic amino acids as substrates;
or how distinctive the substrate profile and kinetic properties of insect TDC may be
compared with those of insect DDC.

The predicted TDC in *Drosophila melanogaster* shares high sequence identity
(~50%) with its DDC. The TDC present in plants seems to be active to both tyrosine and
DOPA (Figure 4) [16,17]. Based on high sequence identity of the proposed *Drosophila*
TDC with DDC, and the similarity of the structures of the substrates, DOPA and tyrosine
(Figure 1), we speculate that insect TDC may also be able to use DOPA as a substrate or
have DDC activity. Recently, a number of insect genome sequences have become
available, including *Anopheles gambiae, Aedes aegypti, Tribolium castaneum, and Apis*
mellifera. BLAST search of these available insect genomes revealed that each insect
species contains a number of DDC-like sequences that are commonly named aromatic
amino acid decarboxylases (AAADs). These insect AAADs share more than 40% 
sequence identity within a species. For example, *Anopheles gambiae* contains 6
individual AAADs with the accession numbers of XP_308519, XP_308521, XP_319838,
XP_319840, XP_319841 and XP_319749, respectively. These predicted AAADs share
42-98% sequence identity among themselves. Based on the level of sequence identity of
the *Anopheles gambiae* AAADs with predicted or functionally verified DDC and TDC
sequences, we predict that the *Anopheles gambiae* XP_308521 and XP_319840
sequences correspond to insect TDC and DDC (Figure 2). To understand their similarities
and differences, I expressed the *An. gambiae* DDC and putative TDC and analyzed their
substrate specificity and biochemical properties. The following sections provide details
regarding our functional expression and biochemical characterization of *An. gambiae* DDC and TDC.

Figure 1: The similarity of Tyrosine and DOPA chemical structure. The main difference between Tyrosine and DOPA is the extra hydroxyl group in the aromatic ring of DOPA.
Figure 2: Sequence alignment of *Anopheles gambiae* TDC and DDC. Letters with a pink background represent the identical amino acid residues between *Anopheles gambiae* TDC and DDC sequences. Letters with a blue background represent amino acid residues that have similar chemical properties.
CHAPTER 2

LITERATURE REVIEW

2.1 The Role of Aromatic Decarboxylase Enzymes in Animals and Plants.

Aromatic amino acids, such as tyrosine and DOPA, can fill a number of diverse roles, not only functioning as the building blocks for protein synthesis, but also as primary and secondary messengers in cellular signaling cascades. Frequently, these amino acids have been found to be decarboxylated in vertebrates, invertebrates and plants by the aromatic amino acid decarboxylase enzymes (AADCs) [18,19]. AADCs, in most organisms, are pyridoxal-5'-phosphate (PLP)-dependent enzymes of ancient evolutionary origin. In mammals, insects and plants, AADCs are responsible for catalyzing the decarboxylation of aromatic L-amino acids to aromatic amines that serve as important neurotransmitters or precursors of secondary metabolites [20].

Plant and animal AADCs have significant similarities in structural subunit, molecular mass, and kinetic properties, but they also display remarkable differences in substrate specificity. For example, plant TDC can be specific to either L-amino acids with an indole ring or L-amino acids with a phenol ring, but to not both. In contrast, DDCs in mammals and insects accept a wide range of aromatic L-amino acids [20].

In insects, a variety of functions have been reported for the decarboxylase enzymes. For instance, decarboxylase enzymes catalyze the synthesis of neurotransmitters or neuro-modulators such as dopamine, serotonin (5HT) and tyramine in the nervous system, indicating that these enzymes play a crucial role in important physiological processes, such as learning and memory [21].
In addition to their role as regulators for several secondary metabolic pathways, plant AADCs have other interesting properties corresponding to animal AADCs, such as the physicochemical basis for their unique substrate specificities, biochemical properties as PLP-dependent enzymes, possible degradation via an ubiquitin mediated pathway, and an evolutionary relationship with DDC in animals [20]. The metabolites that are synthesized via AADCs are classified as essential compounds for growth and they are regularly engaged as an interface between plants and their biotic and abiotic environments.
2.2 Physiological Functions of Plant and Animal Tyrosine Decarboxylase (TDC)

In the nervous system of vertebrates and insects, tyrosine metabolic pathways have been classified under two main categories according to tyrosine transformation to dopamine and octopamine (Figure 3)[11]. Tyrosine is decarboxylated by tyrosine decarboxylase (TDC) to tyramine. Subsequently, tyramine is converted to octopamine by tyramine-β-hydroxylase (TβH), which adds a hydroxyl group in the β-position of tyramine [2,11]. Therefore, the synthesis pathway of octopamine cannot take place without TDC enzyme action [2]. Accordingly, TDC and TβH enzymes exist in all nervous system cells or any type of cell that synthesizes tyramine and octopamine. They are rate limiting since both of them are necessary for the tyramine and octopamine pathway [2]. The TDC knockout mutation results in a phenotype lacking both octopaminergic and tyraminergic mediated neurotransmission [2]. This phenotype is seen in the *Drosophila* inactive (*iav*) mutant, which demonstrates a decrease in both tyramine and octopamine concentration [22]. Moreover, the *iav* mutant was used to trace the molecular basis of sensitization toward cocaine and the results showed inability of the *iav* mutant to sensitize, most probably due to a shortage of tyramine [2,23].

In plants, L-Tyrosine/L-DOPA decarboxylase (TYDC) plays an important role in several different metabolic pathways and its enzymatic function appears to be ubiquitous (Figure 4). For instance, the formation of the simple alkaloid phytoalexin, hordinine, in *Hordeum vulgare* and of the hydroxyphenylethanol glycoside, verbascoside, in *Syringa vulgaris* is supported by the TDC enzyme [26,27,20]. In addition, it was reported that TDC was found to be very important as a ubiquitous plant defense response gene that is
induced by pathogens and participates in cell wall-bound hydroxycinnamic acid amide biosynthesis [20,28].

Plant TDC enzyme was found to be involved in the biosynthesis of complex alkaloids as well [26,27]. These complex alkaloids are principally of the benzylisoquinoline type, which includes more than 2500 known compounds found mostly in five plant families. Benzylisoquinoline alkaloids are pharmacologically important, because they comprise a number of widely prescribed pharmaceuticals, such as papaverine (a muscle relaxant); morphine and codeine (analgesic and antitussive drugs) from *Papaver somniferum*; sanguinarine (an antibiotic used in oral hygiene products) from *Sanguinaria canadensis*; colchicine (a microtubule disrupter and gout suppressant) from *Colchicum autumnale*; and (+)-tubocurarine (a non-depolarizing muscle relaxant, used as an adjuvant to anesthesia, that produces temporary paralysis) from *Chondodendron tomentosum*[20,28]. The biosynthesis of all benzylisoquinoline alkaloids starts with the formation of (S) norcoclaurine via the condensation of dopamine and 4-hydroxyphenylacetaldehyde (4-HPAA) [29], which is derived from tyrosine. Dopamine could be also be derived from tyrosine via the hydroxylation of tyramine or the decarboxylation of DOPA. Because there is a possibility that TDC is involved in the formation of both dopamine and 4-HPAA [20], it may be indispensable in the synthesis of benzylisoquinoline alkaloids.
2.3 Physiological Function of Animal DOPA Decarboxylase (DDC)

3,4-dihydroxyphenylalanine decarboxylase (DDC) or L-DOPA decarboxylase is a multi-functional enzyme that catalyzes more than one reaction. It is a pyridoxal-5-phosphate (PLP)-dependent enzyme that decarboxylates 3,4-dihydroxyphenylalanine (DOPA) and 5-hydroxytryptophan (5-HTP) to dopamine and to 5-hydroxytryptamine (serotonin), respectively[10,30,31]. DDC is an aromatic L-amino acid decarboxylase (AAAD); and has the ability to catalyze the decarboxylation of other aromatic L-amino acids as well. It is found in most living species, from the most simple organisms such as bacteria to the most complex species such as humans. In animals, L-DOPA decarboxylase has been the focus of many studies because it is involved in several key biochemical and/or physiological processes, such as the catalysis of a key step in the biosynthesis of the monoamine neurotransmitters serotonin, dopamine, epinephrine, and norepinephrine in the central and peripheral nervous systems of most mammalian species and insects[10,32,33]. It has been suggested that the DOPA/5HTP decarboxylase enzyme that exists in the insect brain might be similar to the 5HTP/DOPA decarboxylase in the vertebrate brain[31].

At least five AAADs or DDC-like sequences were found in Drosophila via a protein BLAST search, while only one sequence was found in mammals [10]. Among the five DDC-like sequences in Drosophila, one was found to be identical to the typical DDC, whereas of the other DDC-like sequences, two have been suggested to be TDC, and the other two have been named α-methyl DOPA resistant proteins.
In addition to the TDC mediated pathway to tyramine, a second route of transformation for tyrosine utilizes the dopamine pathway (Figure 3) [1,2]. Tyrosine in this pathway is hydroxylated to 3,4-dihydroxyphenylalanine (DOPA) by tyrosine hydroxylase. Subsequently, DOPA is decarboxylated to dopamine by DOPA decarboxylase. After this point, a salvage pathway of tyramine might occur [2]. Although the transformation of dopamine to tyramine has never been established in insects, it might be possible for dopamine to be dehydroxylated into tyramine by dopamine dehydroxylase. However, the hydroxylation of tyrosine to DOPA via tyrosinase has been reported in peripheral tissues [34].
Figure 3. Tyrosine metabolic pathways. There are two main tyrosine pathways; the first transforms tyrosine to tyramine by tyrosine decarboxylase, followed by tyramine hydroxylation to octopamine by tyramine β-hydroxylase (TβH); the second transforms tyrosine to DOPA by tyrosine hydroxylase (TH), and then DOPA is decarboxylated to dopamine by DOPA decarboxylase (DDC).
Figure 4. Major biosynthetic functions of L-tyrosine/L-DOPA decarboxylase (TYDC) in plant metabolic pathways that produces benzylisoquinoline alkaloids, monoterpenoid indole alkaloids and cell wall-bound hydroxycinnamic acid amides.
2.4. The Importance of Tyramine in Invertebrates

Tyramine’s function as a nonpeptide neurotransmitter is limited to invertebrates, but its structure is related to adrenaline and noradrenaline and has similar biological functions [2]. It has not been clearly established whether both tyramine and octopamine are used as neurotransmitters in neurons or if tyramine is released only from the neurons that lack the tyramine-β-hydroxylase enzyme (TβH). Immunohistochemical research has identified a group of larval neurons that contain only tyramine [1,2].

The importance of the role of tyramine as an intermediate in the octopamine pathway [3,4,5] can hardly be overstated. Octopamine modulates skeletal muscles such as flight muscles in locusts and visceral muscles such as oviduct muscles in Drosophila and foregut muscles in Manduca sexta [7,48]. It is active at presynaptic and postsynaptic sites [44,45], modulates all sense organs [2], and stimulates the light organ of the firefly [8,53]. Octopamine increases metabolism, affecting the heart, air sacs and corpora cardiaca and mobilizing lipids in the fat body [2,52]. It elicits the immune response of hemocytes, fat body and barrier epithelia [9], as well as increasing the rate of phagocytosis and nodule configuration [59].

It has been reported that octopamine increases cAMP or Ca2+ levels, whereas tyramine has been found to have the opposite effect [39]. This has also been observed at the systemic level [2]. Tyramine has also been described as an inhibitor for the cockroach fat body glycogenolysis [40]. Saraswati et al. confirmed the opposite effects of octopamine and tyramine on the locomotion of Drosophila larvae by using morphometric
methods to examine the activity of mutants, which lack octopamine (inactive \textit{ian}). They determined that a balance between octopamine and tyramine is required for normal behavior [41]. This balance may regulate other processes as well.
2.5. Tyramine roles in insect physiology

The physiological role of tyramine has not been defined clearly in insects [2]. Honoka (a mutant Drosophila line) has a decreased tyramine receptor (TAR) concentration. This mutant did not have many phenotypic differences when compared to the wild type as there were only slight differences in olfactory and TA-induced muscle contractions as well as activity levels. Tyramine is synthesized in non-neuronal cells and it functions as a paracrine signal in the renal system of the fruit fly [2]. Tyrosine or tyramine can be obtained from the diet, so tyramine probably works directly on the stellate cells of the Malpighian tubules. Tyrosine can be taken in by principal cells of the tubules where it is decarboxylated to tyramine and subsequently released to trigger the tyramine receptors on the stellate cells. This activation increases chloride concentration, resulting in an influx of water, which produces a higher volume of urine.

Evidence for the involvement of tyramine in sensory inputs is seen in the defects of olfactory responses in the Drosophila honoka mutant, which has a strongly reduced tyramine receptor expression. [42] It is not known if tyramine is involved in other sensory perceptions.
2.6. Tyramine Roles in Plants.

Tyramine plays a crucial role as an alkaloid precursor [20]. Tyramine has been found as a jasmonic acid conjugate in petunia pollen [57]. Moreover, in tobacco, tyrosine has been considered a limiting factor for sexual reproduction [58]. It has also been implicated in xylem as a component of the constitutive cell wall [59] and as a wound-induced periderm cell wall constituent [60]. In the cell wall, tyramine has also been considered both as a free amine and as a hydroxycinnamic acid-conjugated amide deposited in response to viral infection [61].

In addition to its role in the general physiology of plants, tyramine in some species, such as parsley and Arabidopsis that do not synthesize benzylisoquinoline alkaloids, plays a role as an integral component of the plant defense response. This fact was suggested via some studies on the isolation of TDC genes from parsley and Arabidopsis[62,63].

In the cell wall, the deposition of hydroxycinnamic acid amides, and other phenolics generates a barrier against pathogens by decreasing cell wall digestibility and/or by directly inhibiting the growth of fungal hyphae [20]. Amides are synthesized by the transfer of hydroxycinnamic acids from hydroxycinnamoyl-CoA esters to hydroxyphenethylamines, such as tyramine.

As one of several structurally related substituted phenethylamines, such as octopamine, adrenaline, norepinephrine, N- methyltyramine and dopamine, tyramine exhibits extreme toxicity toward wild-type callus tissue cultures of many species, including corn, sunflower, soybean and tobacco [64].
In contrast, the genetically modified crown gall tissues, generated by modified strains of *Agrobacterium tumefaciens*, have the ability to resist the toxicity of substituted phenethylamines, including tyramine. This resistance was formed via the expression of isopentyl transferase, a key enzyme in cytokinin biosynthesis[64]. In crown gall cultures (cytokinin-treated), tyramine is rapidly incorporated into cell walls. The integration of tyramine into the cell wall as insoluble amides appears to be a mechanism to control their toxicity as soluble metabolites in plants [20].
CHAPTER 3

MATERIALS AND METHODS

3,4-dihydroxy-L-phenylalanine (L-DOPA), L-tyrosine and the pH buffers of Tris, HEPES and MES were obtained from Sigma Chemical Co.

3.1. cDNA Synthesis and Amplification

3.1.1. cDNA Synthesis

Total RNAs were extracted from Anopheles gambiae larvae and early pupae using Trizol reagent (Gibco-BRL) according to the manufacturer’s instructions. Subsequently first-strand cDNA was synthesized using the SuperScript™ III First-Strand Synthesis System (Invitrogen™) according to the manufacturer’s instructions (Cat No. 18080-051). The cDNA was used to amplify the putative DDC and TDC sequences of Anopheles gambiae using RT-PCR.

3.1.2. Primer Design

The pairs of oligonucleotide forward and reverse primers (Table 1) were designed, synthesized and used to amplify Anopheles gambiae full-length coding sequences for both the TDC (protein ID: XP_308521) and DDC (protein ID: AAC16249) from the combined larval and pupal cDNA pools.

The underlined nucleotides in the forward and reverse primers of DDC correspond to an NdeI and an EcoRI endonuclease restriction sites, respectively; while the underlined nucleotides in the forward and reverse primers of TDC correspond to an NdeI and an XhoI endonuclease restriction site, respectively. An additional reverse primer
containing an *XhoI* restriction site was also designed and used in combination with the same forward primer to amplify a predicted TDC catalytic domain.

<table>
<thead>
<tr>
<th></th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
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<tbody>
<tr>
<td>1- DDC</td>
<td>ACTCCATATGCAGCGCCAGAGTTCAA</td>
<td>ACTCGAATTCACTTTCTTCTGCTCGGC CA</td>
</tr>
<tr>
<td>2- TDC</td>
<td>ACTCCATATGAAATACGGAAGAGTTTCGCA</td>
<td>ACTCCTCGAGTCAATTGCTGGCCTCCTCC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ACTCCTCGAGTTACGTCGCCGCGCAGTGGCCT</td>
</tr>
</tbody>
</table>

Table 1. The sets of forward and reverse primers used for *Anopheles gambiae* TDC and DDC amplification and cloning.

### 3.1.3. PCR Reaction and Conditions

The PCR reaction mixture consisted of 13.5 µl H2O, 2.0 µl 10x PCR buffer, 0.5 µl 40 µM Primers, 0.8 µl 1.25 µM dNTP, 0.6 µl 50 µM mgcl2, 0.2 µl *Taq* polymerase and 2.0 µl cDNA for a total reaction volume of 20 µl. The PCR conditions were 95 °C 30 sec, 55 °C 30 sec and 72°C for 1.30 min for 36 cycles.
3.2. Protein Expression and Purification

3.2.1. cDNA Cloning and Subcloning

The amplified cDNA products of TDC or DDC were cloned into a pGEM vector (pGEM®-T from Promega) and then subcloned into a pTYB1 plasmid (Impact™-CN from New England Biolabs) according to the manufacturer’s instructions. The expressed fusion proteins of recombinant TDC and DDC contained a chitin-binding domain. Cloning and subcloning were achieved via the restriction sites that were designed in the ends of the forward and reverse primers. The PCR products of DDC or TDC were recovered by ethanol precipitation and digested with NdeI and EcoRI, in the case of DDC, or NdeI and XhoI, in the case of TDC. The digested fragments were gel-purified and then ligated between the NdeI and EcoRI or NdeI and XhoI restriction sites in the TA cloning vector or protein expression vector. The recombinant plasmids were propagated in Escherichia coli DE3 for protein expression.

3.2.2. Plasmid Transformation

Recombinant TDC and DDC were prepared using recombinant Impact™-CN plasmids. The recombinant TDC or DDC plasmids were transformed into E.coli cells using the heat shock method. Briefly, 10 µl of recombinant reaction was mixed with 50µl of the component cells. The mixture was incubated in ice for 30 min, exposed to heat shock at 42°C for 45 sec, and subsequently incubated in ice for 5 min. The cells were cultured in 100 µl SOC medium for 60 min at 37 °C then plated on an agar-LB plate containing 50 µg/ml ampicillin and incubated overnight. A single colony was selected
from the agar-LB plate and inoculated into 10 ml of LB broth containing 50 µg/ml ampicillin. Transformed bacterial cells were cultured for 10 h at 37°C, transferred to a large volume of LB broth (1000 ml) containing 50 µg/ml ampicillin and incubated at 37°C. The culture then was induced with 0.2 mM isopropyl-1-thio-b-D-galactopyranoside (IPTG), and cultured at 15°C for 24 hrs. The cells were centrifuged at (3,000g for 20 min at 4°C) and subsequently the TDC and DDC recombinant proteins were extracted.

### 3.2.3. Protein Extraction

The transformed *E. coli* cells were disrupted by sonication. Briefly, the cells were pelleted by centrifugation at 5000 g for 10 min at 4°C and then resuspended in 50 ml ice-cold lysis buffer. The mixture was sonicated using a (BRANSON SONIFIER 450). The supernatant containing the protein was obtained by centrifugation at 20,000 g for 30 min at 4°C.

### 3.2.4. Protein Purification

The soluble fusion proteins of recombinant TDC and DDC were separated by column chromatography using packed chitin beads according to the Impact™-CN’s instruction manual. They were subsequently hydrolyzed under reducing conditions. The affinity purification resulted in the isolation of recombinant TDC or DDC proteins at about 75% purity. Further purification of the recombinant proteins was achieved by Mono-Q, hydroxylapatite (DEAE-Sepharose) and gel-filtration chromatographies. Purity of the recombinant proteins were evaluated by SDS-PAGE and the concentrations of the purified recombinant proteins were determined via a Bio-Rad protein assay kit (Hercules, CA) using bovine serum albumin as a standard.
3.3 Protein Activity Assay

The individual recombinant proteins were examined for TDC and DDC activities. Briefly, reaction mixtures of 0.1 ml, containing 10 µg of *Anopheles gambiae* recombinant DDC or TDC and 5 mM of L-tyrosine or L-DOPA was prepared in 0.2 M phosphate buffer (pH 7.0) containing 0.1 mM pyridoxal-5’-phosphate (PLP). The reaction was incubated for 5 min at 25 °C then stopped by adding an equal volume of 0.8 M formic acid. The acidified reaction mixtures were centrifuged at (15,000 g for 10 min at 4 °C). Supernatants (4 µl) were injected into an HPLC-ED for analysis. L-tyrosine and tyramine or L-DOPA and dopamine were detected electrochemically by oxidation.
CHAPTER 4

RESULTS

4.1. cDNA Synthesis and Amplification

cDNA of both Anopheles gambiae DDC and TDC were synthesized successfully from the extracted mRNA using RT-PCR technique. Using combined larval and pupal cDNA pools, the cDNAs of An. gambiae TDC and DDC were amplified using a set of forward and reverse primers that were designed to be highly specific to TDC and DDC coding sequences (Figure 5). The results suggest that both TDC and DDC are expressed in Anopheles gambiae larvae and pupae.

Figure 5. Agarose gel analysis of amplified mosquito TDC and DDC. (A) amplified DDC PCR products and (B) amplified DDC PCR products. Both DDC and TDC cDNA were amplified by a highly specific set of forward and reverse primers. The results suggest that both TDC and DDC proteins are expressed in Anopheles gambiae larvae and pupae.
4.2. Protein Expression and Purification:

Both *Anopheles gambiae* DDC and TDC were expressed using a bacterial protein expression system. Their recombinant proteins in supernatants of bacterial extracts were purified first by chitin-bead chromatography, resulting in the purification of their recombinant proteins to approximately 75%. The isolated DDC and TDC fractions were then further purified by mono-Q and gel filtration chromatography. TDC and DDC recombinant proteins were present as a single band on SDS polyacrylamide gel after Mono-Q, hydroxylapatite and gel filtration chromatographies (Figure 6).

![SDS-PAGE analysis of purified Anopheles gambiae TDC and DDC](image)

Figure 6. SDS-PAGE analysis of purified *Anopheles gambiae* TDC and DDC. Purified TDC and DDC on SDS polyacrylamide are present as a single band at size ~ 50 KDa.
Analysis of the protein TDC fractions from SDS polyacrylamide revealed that TDC behaved as a protein with a relative molecular weight of ~ 50 KD. Using the retention time of TDC in comparison with protein molecular weight standards during gel filtration chromatography, TDC was determined to have a relative molecular weight of 110,000, indicating that TDC is a dimer in physiological buffer (Figure 7). Under physiological pH conditions (pH = 7.0), DDC displayed a large absorbance peak with a $\lambda_{\text{max}}$ around 335 nm and a smaller visible peak with a $\lambda_{\text{max}}$ around 425 nm (Figure 8A), which is similar to those of mammalian DDC [10]. Under identical conditions, TDC showed a similar absorbance spectrum, but its 425 nm peak was greater than its 335 nm peak (Figure 8B). It is generally considered that the peaks with $\lambda_{\text{max}}$ around 335 and 425 nm correspond to the protonated and unprotonated internal aldimine, respectively [10], but no noticeable dynamic changes of the 335 nm peak and 425 nm peak in DDC were observed by changing the pH of the buffer within a range of 5.0 – 9.5 (not shown). Similarly, no apparent dynamic changes of the two peaks were observed when TDC was prepared in buffers ranging from pH 5.0 – 9.5 (not shown).
Figure 7. Analysis of the protein TDC fractions from gel filtration chromatography. (A) retention time of TDC at 32 min. (B) protein molecular weight standards. TDC behaved as a protein with a relative molecular weight of 110,000, indicating that TDC is a dimer in physiological buffer.
Figure 8. Analysis of purified mosquito TDC and DDC by spectrometry. (A) Purified DDC in 50 mM phosphate buffer (pH 7.0). (B) Purified TDC in 50 mM phosphate buffer (pH 7.0). The presence of absorption peaks with a $\lambda_{\text{max}}$ at 335 and 410 nm for DDC or 335 and 420 nm for TDC indicates the association of the PLP cofactor with *Anopheles gambiae* DDC and TDC.
4.3. Substrate Specificity

4.3.1. *Anopheles gambiae* TDC and DDC Activity Assays with L-DOPA

Reaction mixtures of 0.1 ml, containing 10 µg of *Anopheles gambiae* recombinant DDC or TDC and 5 mM L-dopa, were incubated at 25°C. The reaction was stopped by adding an equal volume of 0.8 M formic acid at 5 min after incubation. The acidified reaction mixtures were centrifuged for 10 min (15,000 g for 10 min at 4°C). Supernatants (4 µl) were immediately injected for HPLC-ED analysis. In figure 9, chromatograms A and B illustrate the accumulation of dopamine in the reaction mixtures containing recombinant DDC and TDC, respectively.

4.3.2. *Anopheles gambiae* TDC and DDC Activity Assays with L-tyrosine

Reaction mixtures of 0.1 mL, containing 10 µg of *Anopheles gambiae* recombinant DDC or TDC and 5 mM L-tyrosine, were incubated at 25 °C. The reaction was stopped by adding an equal volume of 0.8 M formic acid at 5 min after incubation. The acidified reaction mixtures were centrifuged for 10 min (15,000 g for 10 min at 4°C). Supernatants (4 µl) were immediately injected for HPLC-ED analysis. In figure 10, chromatogram A illustrates the inability of DDC to catalyze the decarboxylation of tyrosine, and chromatogram B shows the accumulation of a high concentration of tyramine in the reaction mixture.
Figure 9. Substrate specificity of mosquito DDC and TDC with L-dopa. Reaction mixtures of 0.1 ml, containing 10 µg of *Anopheles gambiae* recombinant DDC or TDC and 5 mM L-dopa, were incubated at 25°C. The reaction was stopped by adding an equal volume of 0.8 M formic acid at 5 min after incubation. The acidified reaction mixtures were centrifuged (15,000 g for 10 min at 4°C) and then analyzed by HPLC with electrochemical detection. Chromatograms A and B illustrate the accumulation of dopamine in the reaction mixtures containing recombinant DDC and TDC, respectively.
Figure 10. Substrate specificity of mosquito TDC and DDC with L-tyrosine. Reaction mixtures of 0.1 ml, containing 10 µg of *Anopheles gambiae* recombinant DDC or TDC and 5 mM L-tyrosine, were incubated at 25°C. The reaction was stopped by adding an equal volume of 0.8 M formic acid at 5 min after incubation. The acidified reaction mixtures were centrifuged (15,000 g for 10 min at 4°C) and then analyzed by HPLC with electrochemical detection. Chromatogram A illustrates inability of DDC to catalyze the decarboxylation of tyrosine and chromatogram B shows the accumulation of a high concentration of tyramine in the reaction mixture.
CHAPTER 5

DISCUSSION

The physiological importance of tyramine has attracted considerable attention to the proteins that are responsible for tyramine production across a number of organisms ranging from vertebrates to plants and bacteria. However, in insects, the biochemical characteristics of tyrosine decarboxylases have not been clearly established [10]. A protein BLAST search using pig DDC sequence listed five AAADs or DDC-like sequences from the Drosophila genome. Among them, one is the typical DDC that has been extensively characterized, two are predicted TDCs and the other two are named α-methyl DOPA-resistant proteins [10]. The two presumed TDC sequences were based on the negative effect of their mutants on tyramine production [12,13,14,15]. In addition, knockout studies (conducted in Drosophila) of the two proposed TDCs determined that both of these DDC-like sequences are essential for tyramine production [10]. The insect DDC-like sequences share a high sequence identity (about 40%) with one another and their level of identity increases (to 50%) when only their putative catalytic domains are compared.

Although there has been speculation regarding the functional activities of DDC and TDC (with regard to the production of dopamine and tyramine), mentioned sporadically within the literature, this concept has never been critically analyzed or clearly discussed, especially with regard to their role in insects. There are several considerations that might lead one to hypothesize that both DDC and TDC have overlapping activities for producing tyramine from tyrosine or dopamine from DOPA.
First, tyrosine and DOPA have similar chemical structures (Figure 1) and therefore likely fit in the active site of both DDC and TDC; secondly, DDC and TDC share high sequence identity with their predicted substrate interacting residues high conserved; and thirdly plant TDC, the counterpart of insect TDC, is capable of catalyzing the decarboxylation of both tyrosine and DOPA (Figure 4) [2].

This study provides data that demonstrate that TDC is a dimer under physiological pH conditions. Moreover, the results showed that the true mosquito DDC is highly specific for DOPA with essentially no activity to tyrosine. However, although the mosquito TDC primarily catalyzes the decarboxylation of tyrosine, it can also catalyze the decarboxylation of DOPA. These data indicate that TDC is a multi functional enzyme which catalyzes more than one reaction.

The high substrate specificity of DDC with DOPA and the capacity of TDC to decarboxylate both tyrosine and DOPA may be crucial to insects. Perhaps the specific activities of TDC and DDC are conserved due to evolutionarily significant physiological requirements during development. In insects, tyramine is involved in many biochemical processes and events, impacting a wide swath of tissues and systems. For example, tyramine serves as a precursor for the production of octopamine. Studies of the action of octopamine in insects have revealed a biologically diverse role for octopamine, affecting the function and development of nearly every major tissue and physiological system [2]. For example, octopamine regulates foregut activity, the fat body, the sensory organs, the development of skeletal muscles, as well as all of the elements in an insects’ rudimentary immune system.
Dopamine is also involved in many biochemical processes/events, some of which do not seem to occur outside of insects [65]. For instance, dopamine can be conjugated with β-alanine to form N-β-alanyldopamine (NBAD). As NBAD has been found to be essential for the process of cuticle sclerotization, dopamine is also important for this biological process. During larval development, insects need to periodically produce a new cuticle when the old one is shed. Once the new cuticle is formed, it must be hardened rapidly by sclerotization and melanization to protect the insect’s body from physical and UV-mediated damage. Based on this function alone, dopamine would be critical for both the growth and the survival of insects. But it has other roles as well. Therefore, the availability of tyramine and dopamine is an apparent prerequisite in insect survival. These physiological requirements may explain why a highly specialized TDC has evolved in mosquitoes [65].

Although tyramine might be produced from dopamine through dopamine dehydroxylation, as mentioned previously, the transformation of dopamine to tyramine has not yet been established in insects [43]. Based on our results, the major pathway of tyramine production is from tyrosine via TDC (because insect DDC has no any TDC activity). Conversely, the major pathway for dopamine production from DOPA is catalyzed via both DDC as well as TDC.
REFERENCES


