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# A novel quinone: quinone methide isomerase generates quinone methides in insect cuticle

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Insoluble cuticle-bound enzyme(s) of *Manduca sexta* pharate pupae, which is known to convert *N*-acetyldopamine to *N*-acetylnorepinephrine through the intermediate formation of quinone methide, also converted exogenously supplied *N*-acetyldopamine quinone to *N*-acetylnorepinephrine. The presence of a quinone trap such as *N*-acetylcysteine in the reaction mixture containing *N*-acetyldopamine and cuticle prevented the formation of *N*-acetylnorepinephrine but readily yielded *N*-acetylcysteine-*N*-acetyldopamine quinone adduct as a dead-end product. These results indicate the oxidation of *N*-acetyldopamine to its quinone and its enzyme-catalyzed isomerization to quinone methide before yielding *N*-acetylnorepinephrine as the stable product. The role of this newly discovered isomerase in sclerotization of insect cuticle is discussed.

Quinone formation; Quinone methide generation; Phenoloxidase; Quinone methide isomerase; Cuticular tanning; (*Manduca sexta*)

## 1. INTRODUCTION

Sclerotization of insect cuticle is a vital process essential for the survival of most insects [1]. It is responsible for hardening of freshly made, soft larval as well as adult cuticle, egg cases (ootheca) and pupal and/or puparial cases of most insects. It is generally agreed that during this process enzymetically generated oxidation products of *N*-acyldopamine derivatives form covalent cross-links and adducts with cuticular proteins and chitin to form protein-protein as well as protein-chitin cross-links necessary for strengthening of cuticle [2-10]. Based on the reactive intermediates formed, two types of tanning modes have been identified, viz. quinone tanning and $\beta$-sclerotization [4-10]. We discovered quinone methides as additional sclerotizing agents [11] and argued that $\beta$-sclerotization is indeed caused by quinone methides and renamed this process quinone methide sclerotization [9,12]. Although available data indicated the direct formation of quinone methides from 4-alkylcatechols (route C, fig.1), we have now carried out additional experiments that reveal the generation of quinone methides by the combined action of *o*-diphenoloxidase and a novel quinone methide isomerase in insect cuticle (route A-B, fig.1). Here, we present evidence for the operation of this pathway for the generation of *N*-acetyldopamine (NADA) quinone methide in the cuticle of *Manduca sexta*.

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*Abbreviations:* NAcCys, *N*-acetylcysteine; NADA, *N*-acetyldopamine; NANE, *N*-acetylnorepinephrine

## 2. MATERIALS AND METHODS

### 2.1. *Cuticular enzyme preparation*

Eggs of *M. sexta* were donated by Dr J.S. Buckner (Metabolism and Radiation Laboratory, Agricultural Research Service, US Department of Agriculture, Fargo, ND). Larvae were reared on a synthetic medium [13] kept at 25°C during a 16 h light-8 h dark photoperiod. Cuticles from fifth instar larvae which were undergoing pupation but before the onset of browning (pharate pupae) were collected and suspended in ice-cold 0.05 M sodium phosphate buffer, pH 7.5, containing 1 mM phenylthiourea. After removing adhering tissue, cuticle was homogenized in a Waring blender for 1-2 min and passed

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through cheesecloth. The recovered cuticle was washed with water and suspended in 2% sodium tetraborate-1% SDS buffer for several hours to remove soluble proteins. The cuticle was then filtered, washed with 500 ml of water several times followed by ice-cold acetone, dried in air and used for all experiments.

2.2. *HPLC analysis*

HPLC analysis of reaction mixture was performed using a Beckman (Berkley, CA) 332 liquid chromatography system equipped with two model 110 B pumps, a model 420 controller, a model 160 absorbance detector (280 nm) and a model 427 integrator. All separations were carried out on Beckman $C_{18}$-IP ultrasphere reversed-phase columns (5 $\mu$m, 4.6 × 250 mm). Elution of samples was achieved by the use of an isocratic solvent system consisting of 0.05 M acetic acid containing 0.2 mM sodium 1-octanesulfonate in 20% methanol at a flow rate of 0.6 ml/min.

2.3. *Other procedures*

Mushroom tyrosinase (spec. act. 4000 U/mg) was obtained from Sigma (St. Louis, MO). Visible spectral studies were carried out using a Gilford model 2600 spectrophotometer. An authentic sample of NAcCys-NADA-quinone adduct was kindly provided by Dr Hemalatha Dali.

## 3. RESULTS AND DISCUSSION

As shown in fig.1, quinone methides can be directly formed by the oxidation of 4-alkylcatechols (route C) or indirectly formed by the combined action of *o*-diphenoloxidase and an as yet uncharacterized quinone methide isomerase (route A–B). To check the latter possibility NADA-quinone was synthesized by the well-known action

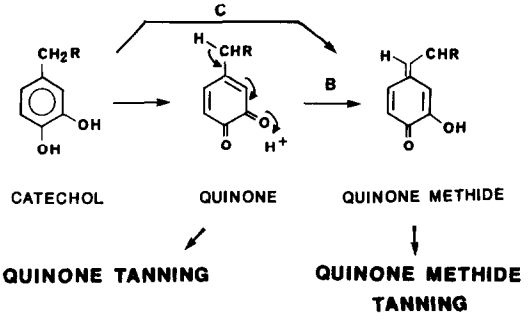


Fig.1. Two different mechanisms for tanning of insect cuticle. 4-Alkylcatechols such as *N*-acetyldopamine (R = $CH_2NHCOCH_3$) or *N*-$\beta$-alanyldopamine (R = $CH_2NHCOCH_2CH_2NH_2$) are oxidized to their corresponding quinone derivatives (route A) which take part in quinone tanning. A similar oxidation at the 1,6-position can produce quinone methides (route C) which participate in quinone methide sclerotization. Enzymatically generated quinones can be also tautomerized to quinone methide (route B) either enzymatically or nonenzymatically.

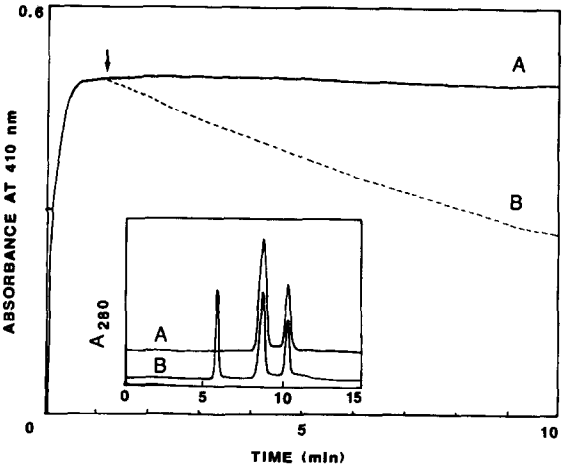


Fig.2. Enzyme-catalyzed conversion of *N*-acetyldopamine quinone to *N*-acetyldopamine quinone methide. A reaction mixture containing 1 mM NADA, 30 $\mu$g mushroom tyrosinase in 50 mM sodium phosphate buffer, pH 6.0, was incubated at room temperature and formation of NADA-quinone was continuously monitored at 450 nm (curve A). At the indicated times, 10 mg *M. sexta* cuticle was added and the fate of preformed NADA-quinone was followed (curve B). (Inset) HPLC analysis of the reaction mixtures A and B. HPLC analysis of (trace A) reactions A, (trace B) reaction B. The peaks at 5.7, 8.5 and 10.1 min are due to NANE, NADA-quinone and NADA, respectively.

of mushroom tyrosinase on NADA and the fate of synthetic NADA-quinone was studied in the presence and absence of pharate *M. sexta* pupal cuticle. Fig.2. shows the effect of cuticle on the fate of NADA-quinone. As is clear, NADA-quinone formed by the action of mushroom tyrosinase on NADA is quite stable and accumulated in the reaction mixture in the absence of cuticle (curve A). However, if the cuticle is added at the indicated time interval, rapid decomposition of NADA-quinone is observed (curve B). Such a phenomenon was not observed with heat-inactivated cuticle thereby indicating the presence of a heat-labile factor in cuticle which causes the decomposition of NADA-quinone (not shown). HPLC studies of the reaction mixture indicated that the disappearance of NADA-quinone is accompanied by the appearance of NANE as the soluble product (fig.2, inset). These results are consistent with the conversion of NADA-quinone to NADA-quinone methide. Since NADA-quinone methide is highly unstable, it undergoes a rapid Michael 1,6-addition reaction with available water molecules to form

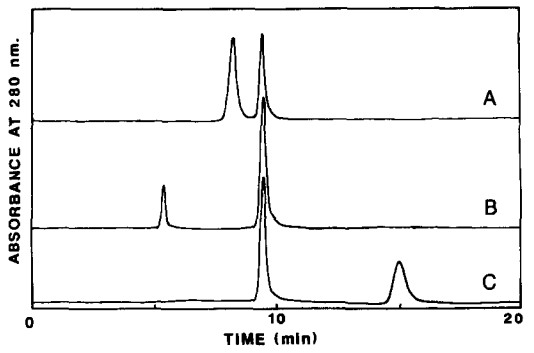


Fig.3. Trapping of transiently formed *N*-acetyldopamine quinone. Standard reaction mixture containing 25 mg cuticle, 1 mM NADA and 50 mM sodium phosphate buffer, pH 6.0, was incubated at room temperature and subjected to HPLC analysis at the indicated time intervals. Curves: A, 5 min reaction in which cuticle was replaced with mushroom tyrosinase (30 $\mu$g); B, 60 min standard reaction; C, 60 min standard reaction containing 2 mM NAcCys. The peaks at 5.4, 8.2, 9.4 and 15.0 min are due to NANE, NADA-quinone, NADA and NAcCys-NADA-quinone adduct, respectively.

racemic NANE as the soluble product [14,15]. Thus, the above experiment clearly indicates the presence of an enzyme system in the cuticle of *M. sexta* which converts NADA-quinone to NADA-quinone methide. However, it does not show that NADA is converted into NADA-quinone methide by this route (route A–B, fig.1) or directly (fig.1, route C). In order to clarify this aspect, the following studies were carried out.

During NADA oxidation by cuticular enzyme(s), generation of NADA-quinone was monitored continuously by visible spectroscopy. As reported earlier, such studies fail to attest to the free generation of NADA-quinone in the reaction mixture [9, 11,12]. In addition, HPLC analysis of the reaction mixture also failed to support the accumulation of NADA-quinone, while NADA conversion to NANE was readily observed (fig.3, trace B). For comparison, HPLC analysis of NADA-mushroom tyrosinase reaction is also shown in fig.3A. It is possible that NADA-quinone may be formed as a transient intermediate below the detection levels and was rapidly utilized by the isomerase in cuticular reactions. In this case, one should be able to trap the NADA-quinone and prevent its further conversion to NADA-quinone methide. Of various quinone traps available, *N*-acetylcysteine was chosen, because it has been demonstrated that cysteine could trap even transiently formed, highly reactive quinones such as dopaquinone to form cysteinyldopas – a reaction which is the basis for all pheomelanin biosyntheses [7,16]. Since *N*-acetylation would not affect the reactivity of the thiol group in cysteine but will avoid any undesired side reactions of free amino groups, *N*-acetylcysteine was used in the present study. Fig.3 shows the oxidation of NADA by *Manduca* cuticular phenoloxidase in the presence and absence of *N*-acetylcysteine. As is evident, in the absence of *N*-acetylcysteine, NANE was readily formed, indicating the conversion of NADA to NADA-quinone methide (trace B). However, in its presence, NANE formation was totally inhibited, but the formation of a new compound could be readily witnessed (trace C). This compound exhibited the same retention time as that of an authentic NAcCys-NADA adduct and co-chromatographed the same as a single symmetrical peak. The present result clearly indicates the presence of a two-component enzyme system in the cuticle of *M. sexta* for the conversion of sclerotizing precursors such as NADA to reactive quinone methides. While the first enzyme converts the alkylcatechols to *o*-benzoquinone derivatives, the second enzyme rapidly causes the isomerization of quinones to quinone methides and provides them for quinone methide sclerotization. We also observed these reactions with *Sarcophaga bullata* and *Periplaneta americana*. In addition, all these three cuticular samples converted *N*-$\beta$-alanyldopamine to *N*-$\beta$-alanylnorepinephrine by the same route. Thus, the observed reactions seem to be of general occurrence for the synthesis of quinone methides.

As early as 1959, Witkop and co-workers [17,18] proposed the tautomerization of alkyl-substituted quinones to quinone methide derivatives for the biosynthesis of norepinephrine. Although the biosynthesis of norepinephrine involves direct enzymatic aerobic hydroxylation of dopamine [19], its acetyl derivative, which is widely found in insect cuticle, undergoes oxidation to the *o*-quinone and 1,6-addition of water to yield *N*-acetylnorepinephrine produced by the route involving isomerization. Currently, we are attempting to solubilize the novel quinone methide isomerase discovered in the present study for further characterization.

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