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o-Quinone/quinone methide isomerase: a novel enzyme preventing the destruction of self-matter by phenoloxidase-generated quinones during immune response in insects

Steven J. Saul and Manickam Sugumaran

Department of Biology, University of Massachusetts at Boston, Harbor Campus, Boston, MA 02125, USA

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Melanization and encapsulation of invading foreign organisms observed during the immune response in insects is known to be due to the action of activated phenoloxidase. Phenoloxidase-generated quinones are deposited either directly or after self-polymerization on foreign objects accounting for the observed reactions. Since the reactions of quinones are nonenzymatic, they do not discriminate self from nonself and hence will also destroy self-matter. In this report we present evidence for the presence of a novel quinone/quinone methide isomerase in the hemolymph of *Sarcophaga bullata* which destroys long-lived quinones and hence acts to protect the self-matter. Quinone methides, formed by the action of this enzyme on physiologically important quinones, being unstable undergo rapid hydration to form nontoxic metabolites.

Immunity; Phenoloxidase; Melanization; Quinone tautomerase; Quinone methide; (*Sarcophaga bullata*)

1. INTRODUCTION

The participation of phenoloxidase in melanization and encapsulation of invading foreign organisms [1–5], sclerotization of cuticle [6–8], and wound healing [9,10] in insects is well established. Phenoloxidase catalyzes the conversion of *o*-diphenols to *o*-benzoquinones, which directly participate in melanizing foreign objects in insect hemolymph [1–5]. Dopa quinone and dopamine quinone, formed by the enzymatic oxidation of the *o*-diphenols, dopa and dopamine, undergo rapid intramolecular cyclization and subsequent polymerization to melanin, and hence do not react with external nucleophiles on proteins [8,11,12]. However, quinones of other *o*-diphenols, most notably *N*-acyl derivatives of dopamine and norepinephrine, which are abundant in insect hemolymph [13], do not exhibit internal reactivity but add onto the external nucleophiles of proteins [6–8,11,12]. Hence, these quinones are potentially toxic to the insect because they can react not only with foreign objects but also with self-matter. Here, we report the presence of a new enzyme in the hemolymph of *Sarcophaga bullata* larvae which catalyzes the conversion of phenoloxidase-generated quinones to transient 2-hydroxy-*p*-quinone methides. The unstable quinone methides thus formed undergo rapid hydration to form nontoxic metabolites [6–8] thereby preventing the destruction of self-matter.

Correspondence address: M. Sugumaran, Department of Biology, University of Massachusetts at Boston, Harbor Campus, Boston, MA 02125, USA

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

2. MATERIALS AND METHODS

2.1. *Partial purification of* o-*quinone isomerase*

Larvae of *S. bullata* were raised on a dog food diet. 15-day-old larvae were injected with a decoagulation buffer containing 15 mM NaCl, 100 mM glucose, 10 mM EDTA, 30 mM trisodium citrate and 26 mM citric acid (pH 4.6). Animals were bled immediately and the hemolymph collected was centrifuged at $800 \times g$ for 5 min. The clear supernatant obtained was lyophilized and stored at $-20°C$ until use.

The lyophilized hemolymph was dissolved in ice-cold distilled water and 100 mg protein was chromatographed on a Sepharose

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6B column (2.5 × 84 cm) with 0.05 M phosphate buffer (pH 6.0), containing 0.2 M NaCl at a flow rate of 30 ml/h. Arylphorin which is the major hemolymph protein eluted between 240 and 270 ml, while the isomerase which comprises approx. 1% of the total hemolymph proteins eluted at around 312 ml. It was collected and used as the partially purified enzyme.

2.2. *Zymosan-treated hemolymph*

Lyophilized hemolymph of *S. bullata* prepared as outlined above was desalted on a Sephadex G-25 column (1 × 30 cm) with 10 mM cacodylate buffer (pH 7.0) containing 5 mM $CaCl_2$. The protein fraction was treated with zymosan A (100 μg/ml) overnight at 4°C to activate the prophenoloxidase system and used as zymosan-treated hemolymph.

2.3. *HPLC analysis*

HPLC analysis of reaction mixtures 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 μm, 4.6 × 250 mm). Elution of samples was achieved by the use of an isocratic solvent system consisting of 50 mM acetic acid containing 0.2 mM sodium 1-octanesulfonate in 20% methanol at a flow rate of 0.6 ml/min.

2.4. *Other procedures*

Mushroom tyrosinase (spec. act. 4000 U/mg), dopa, dopamine, 4-methylcatechol, 3,4-dihydroxyphenylglycol, NADA and zymosan A were obtained from Sigma (St. Louis, MO). Visible spectral studies were carried out using a Gilford model 2600 spectrophotometer. The rest of the procedures are outlined in the figure legends.

3. RESULTS AND DISCUSSION

S. bullata larval hemolymph contained phenoloxidase in an inactive proenzyme form which was readily activated by treatment with zymosan, confirming its key role in the immune response [14]. When zymosan-activated hemolymph was assayed for phenoloxidase activity, quinone formation was witnessed only with certain compounds such as dopa, dopamine, catechol and 4-methylcatechol. Physiologically important catecholamine derivatives such as *N*-acetyldopamine (NADA) and *N*-β-alanyldopamine, which constitute the majority of hemolymph phenols [13], were not converted to stable quinone derivatives although oxygen uptake studies confirmed the oxidative nature of the reaction. These observations indicated to us that *N*-acyldopamine quinones are either highly unstable or undergo further enzymatic transformations in the hemolymph.

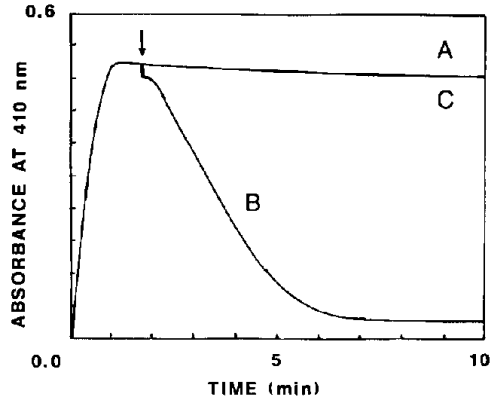

Fig.1. Stability of *N*-acetyldopamine. A reaction mixture containing 1 mM *N*-acetyldopamine (NADA), and mushroom tyrosinase (30 μg) in 50 mM sodium phosphate buffer (pH 6.0) was incubated at room temperature, formation of NADA quinone being continuously monitored at 410 nm (curve A). At the indicated time, 20 μl of *S. bullata* larval hemolymph (15 days old) was added to the reaction mixture and the fate of preformed NADA quinone was followed [curve B, native hemolymph; curve C, heat-treated (60°C, 10 min) hemolymph].

Fig.1 shows the time course of formation and accumulation of NADA quinone in a reaction mixture containing NADA and mushroom tyrosinase. The product, NADA quinone, is quite stable and accumulates in the reaction mixture (trace A). Theoretically, accumulated quinone in an in vivo system would slowly react with nucleophiles on protein molecules [5–8] and destroy not only foreign matter but also self-matter. Yet, very rarely does one observe the destruction of self-matter during the immune response of insects. Therefore, it is logical to expect the presence of a protective mechanism against accumulation of toxic quinones in the hemolymph. To investigate this possibility, a sample of hemolymph from *S. bullata* was added to the reaction mixture in which NADA quinone was formed. As shown in fig.1B, addition of hemolymph caused rapid decomposition of preformed NADA quinone. In control experiments with mushroom tyrosinase, heat-inactivated hemolymph (at 60°C for 10 min), and NADA, the NADA quinone generated was found to be stable (fig.1C). These results are consistent with the presence of a heat-labile factor in the native hemolymph which destroys NADA quinone.

Using its ability to destroy NADA quinone as a marker, this protein was purified by chromatography on a Sepharose 6B column. Purified enzyme,

which was devoid of any *o*-diphenoloxidase activity, readily transformed a variety of quinones. In order to identify the product(s) of the reaction, HPLC studies were carried out. Fig.2 shows the results obtained from HPLC of a reaction mixture containing NADA and mushroom tyrosinase analyzed at zero time (trace A) and 5 min (trace B) after initiating the reaction. When the hemolymph enzyme was added to this reaction (fig.2C), NADA quinone disappeared rapidly with concomitant formation of *N*-acetylnorepinephrine (NANE). Similarly, *N*-β-alanyldopamine quinone and hydroxyethyl-*o*-benzoquinone were converted to *N*-β-alanylnorepinephrine and 3,4-dihydroxyphenyl glycol, respectively. In addition, when the quinones of the side-chain-hydroxylated compounds as NANE quinone, *N*-β-alanylnorepinephrine quinone and 3,4-dihydroxyphenyl glycol quinone were provided to the enzyme, they were converted to *N*-acetylarterenone, *N*-β-alanylarterenone and 2-hydroxy-3′,4′-dihydroxyacetophenone, respectively, albeit at a much slower rate than the methylene-substituted quinones.

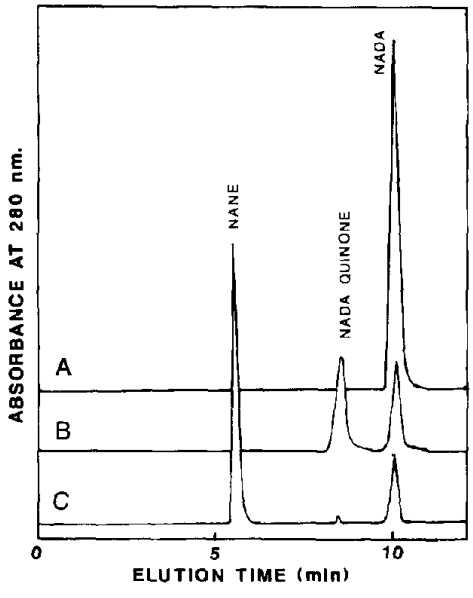

Fig.2. HPLC analysis of the *N*-acetyldopamine reaction. A reaction mixture containing 1 mM *N*-acetyldopamine (NADA) and mushroom tyrosinase (30 μg) in 50 mM sodium phosphate buffer (pH 6.0) was incubated at room temperature. An aliquot of the above mixture (5 μl) was subjected to HPLC analysis as outlined in section 2. (A) 0 time reaction, (B) 5 min reaction, (C) B + 50 μl partially purified isomerase after 10 min incubation.

These conversions can be accounted for by the enzyme-catalyzed isomerization of alkyl-substituted quinones to 2-hydroxy-*p*-quinone methide derivatives and the subsequent nonenzymatic transformations of the quinone methides formed (fig.3). While the quinone methides derived from NADA, *N*-β-alanyldopamine and 3,4-dihydroxyphenethyl alcohol, undergo rapid Michael 1,6-addition reactions with water to yield side-chain-hydroxylated catechols - NANE, *N*-β-alanylnorepinephrine and 3,4-dihydroxyphenyl-glycol, respectively - those derived from the side-chain-hydroxylated compounds undergo rapid tautomerization to yield *N*-acetylarterenone, *N*-β-alanylarterenone and 2-hydroxy-3′,4′-dihydroxyacetophenone, respectively (fig.3).

To determine whether the isomerase can perform these transformations in vivo, zymosan-activated *S. bullata* larval hemolymph was tested for its ability to carry out the above transformations. As shown in fig.4, activated phenoloxidase readily generated NADA quinone from NADA (fig.4A,

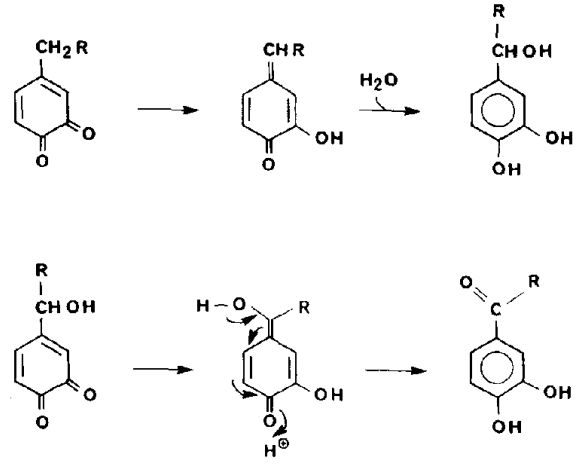

Fig.3. Proposed mechanism for the observed reactions. Quinone-quinone methide isomerase converts NADA quinone (R = $CH_2NHCOCH_3$), *N*-β-alanyldopamine quinone (R = $CH_2NHCOCH_2CH_2NH_2$) and hydroxyethyl-*o*-benzoquinone (R = CH_2OH) to their quinone methide derivatives which are highly unstable and undergo rapid nonenzymatic Michael 1,6-addition reaction with water to yield side-chain-hydroxylated compounds (upper row). The quinones of these compounds, viz. NANE quinone, *N*-β-alanylnorepinephrine quinone and 3,4-dihydroxyphenyl glycol quinone (lower row), upon isomerase action, yield the quinone methides which are enols. Rapid nonenzymatic ketonization of the quinone methide produces *N*-acetylarterenone, *N*-β-alanylarterenone and 2-hydroxy-3′,4′-dihydroxyacetophenone, respectively.

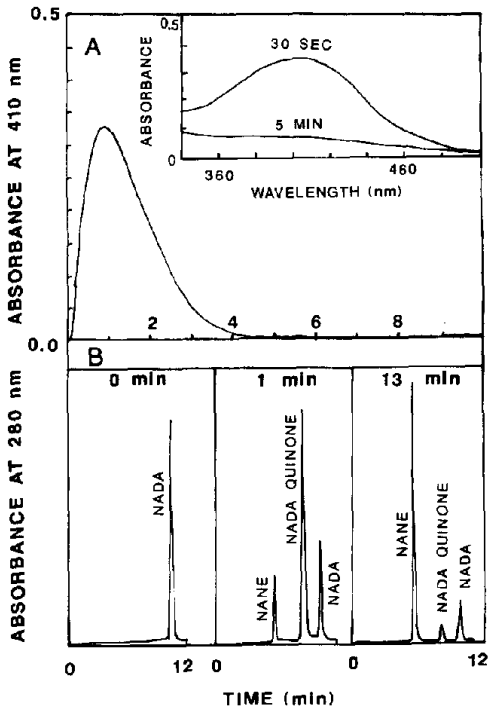

Fig.4. (A) Transient accumulation of NADA quinone in the hemolymph. Zymosan-treated hemolymph was prepared as outlined in the text and an aliquot (20 μl) was incubated with 1 mM NADA in 0.1 M sodium phosphate buffer, pH 6.0 (total volume 1 ml). The formation of NADA quinone in this reaction mixture was monitored at 410 nm. (Inset) Visible spectrum of the reaction mixture at the indicated time intervals. The 30 s spectrum corresponds to NADA quinone. (B) HPLC analysis of the above reaction. Aliquots (5 μl) of the above reaction were subjected to HPLC analysis as outlined in fig.2 at the indicated time intervals.

inset). However, NADA quinone accumulated in the reaction mixture only transiently (fig.4A). HPLC analysis (fig.4B) revealed that the disappearance of NADA quinone is coincident with the appearance of NANE in the reaction mixture. Addition of *N*-acetylcysteine, which is a quinone trap, totally abolished the appearance of NANE while quantitatively trapping NADA quinone as *N*-acetylcysteinyl(*N'*-acetyl)dopamine (not shown). Thus, the above results confirm the occurrence of NADA conversion to NANE via NADA quinone and NADA quinone methide in the larval hemolymph of *S. bullata*. We have detected quinone isomerase activity not only in *S. bullata* larvae but also in other insects, such as *Drosophila melanogaster* and *Tenebrio molitor*.

Quinone methide formation from dopamine and its subsequent hydration was originally suggested as a mechanism for the biosynthesis of norepinephrine by Witkop and co-workers [15,16]. Although a direct enzymatic hydroxylation route was established for norepinephrine biosynthesis [17], we have recently established that its acetyl derivative, viz. NANE, is produced by the route involving isomerization of quinone to quinone methide in the cuticle of *S. bullata*, *Manduca sexta* and *Periplaneta americana* in relation to cuticular sclerotization [18,19]. Thus, this novel enzyme seems to be involved not only in protection of self-matter during invasion by foreign organisms, but also in sclerotization of insect cuticle. Currently, we are engaged in a detailed study of the quinone isomerase which is involved in these two vital processes in insects.

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