Quinone methide formation from 4-alkylcatechols: a novel reaction catalyzed by cuticular polyphenol oxidase

M. Sugumaran and H. Lipke

Department of Biology, University of Massachusetts at Boston, Dorchester, MA 02125, USA

Received 5 March 1983

Oxidation of 4-alkylcatechols by cuticular polyphenol oxidase gives quinone methides as initial products as opposed to the conventional quinones. This new reaction accounts for several conflicting observations on the catecholamine chemistry in insect cuticle.

Oxidation of catechols *Quinone formation* *Tanning mechanism* *Quinone methide generation* *Arylation* *Cuticular polyphenol oxidase*

1. INTRODUCTION

The key role played by cuticular polyphenol oxidase and the catecholamine derivatives during sclerotization of arthropod cuticle is well established [1]. Sclerotization is generally visualized as a process involving covalent coupling of enzymatically generated quinones with structural proteins [2,3]. Cuticular polyphenol oxidase is believed to catalyze the conversion of catechols to quinones for this process [3,4]. This communication, on the other hand, reports that the products of the reactions catalyzed by cuticular polyphenol oxidase are quinone methides, tautomers of 4-alkyl substituted quinones. This new finding reconciles several conflicting observations on the fate of catecholamines in insect cuticle.

2. MATERIALS AND METHODS

Sarcophaga bullata larvae were reared on dog food. Cuticle was harvested during the wandering stage. Larvae were homogenized in a Virtis Omnimixer at 2°C, in water at half-maximum speed and twice at full speed. Cuticle sheaths were recovered by sieving through cheese cloth, washed with water and suspended in buffer A consisting of 2% sodium tetraborate–1% SDS–0.01% phenylthiourea (pH 9.0) for 30 min at room temperature to remove soluble structural proteins. Following filtration, the tissue was washed 3 times with water and 0.05 M sodium phosphate buffer (pH 7.0) and the residue again washed with water followed by acetone at 0°C and dried in air.

Larval cuticle (500 mg) was suspended in 75 ml 0.05 M sodium phosphate buffer (pH 6.0) with 100 μmol specified catechol at 37°C for 1 h. The reaction was arrested by the addition of 0.5 ml conc. HCl and filtered. Ether-soluble phenols were extracted from the filtrate. Aqueous layers were lyophilized, taken up in methanol, filtered and used for HPLC analysis. Residual cuticle was washed successively with 200 ml water, 100 ml buffer A, 500 ml water, 50 ml 1 M HCl, 200 ml water and 50 ml acetone and dried in air.

Hydrolysis of *o*-diphenol treated cuticle was performed at 108°C for 24 h with 1 M HCl. The catechols were identified by nitrite/molybdate complex formation directly on the unhydrolyzed tissue or on methanol extracts of evaporated hydrolyzates [5]. Spectral changes associated with the oxidation of catechols were monitored using a Gilford Spectrophotometer model 250. HPLC analysis of catechols was performed as in [6]. Thin-layer chromatographic analyses of catechols

Abbreviations: NADA, *N*-acetyldopamine; NANE, *N*-acetylnorepinephrine; SDS, sodium dodecyl sulfate

Published by Elsevier Science Publishers
00145793/83/$3.00

were done as in [7]. Catechols were identified by spraying with nitrite/molybdate reagent followed by 1 M NaOH.

Catechols were supplied by Aldrich Chemicals (Milwaukee WI). *N*-Acetyldopamine was prepared from dopamine by acetylation [7]. Crystalline mushroom tyrosinase was purchased from Sigma Chemicals (St Louis MO).

3. RESULTS AND DISCUSSION

The chitin bound tyrosinase from *Sarcophaga bullata* readily oxidized a number of *o*-diphenols such as catechol, 3-methylcatechol, 4-methylcatechol, 3,4-dihydroxybenzoic acid, 3,4-dihydroxyphenylacetic acid, 3,4-dihydroxyphenylglycol, 3,4-dihydroxyphenethyl alcohol, caffeic acid, 3,4-dihydroxyphenylpropionic acid, chlorogenic acid, dopa, dopamine, NADA, epinephrine and norepinephrine. This broad specificity is in accord with polyphenol oxidases recovered from other insect cuticles [8–10].

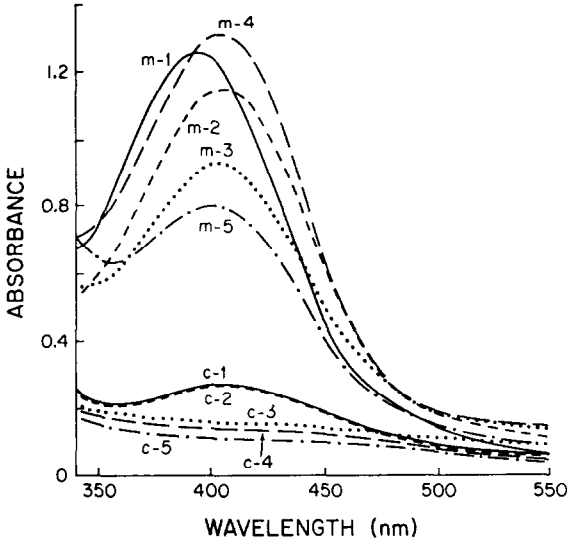

Fig.1. Spectral changes occurring during the oxidation of catechols with mushroom tyrosinase (m-series) and cuticular polyphenol oxidase (c-series). Substrates used: (1) catechol; (2) 4-methylcatechol; (3) *N*-acetyldopamine; (4) 3,4-dihydroxyphenethyl alcohol; (5) 3,4-dihydroxyphenylacetic acid. Assay conditions: 1 mM substrate in 1 ml 0.05 M sodium phosphate buffer (pH 6.0) and crystalline mushroom tyrosinase (18 μg) or cuticle powder (25 mg).

Although specificity was similar to mushroom tyrosinase, the visible spectral changes associated with the initial stages of oxidation differed markedly for the two enzyme systems (fig.1). During the oxidation of 5 representative catechols by the fungal enzyme, characteristic quinone spectra were generated with absorption maximum at around 400 nm. Cuticle enzyme, however, failed to produce such spectra for catechols with alkyl side chain with the exception of 4-methylcatechol. Even prolonged incubations failed to elicit the quinone spectra for these compounds suggesting that products other than quinones are formed. Thin-layer chromatographic examination of the soluble products accumulating in the reaction mixture supported this contention.

Table 1 gives the products formed by incubation of various catecholic compounds with cuticular polyphenol oxidase. All 4-alkylcatechols yielded side-chain hydroxylated products during the incubation. These results were also confirmed by HPLC studies. Fig.2 for instance, presents the HPLC analysis of the 4-methylcatechol reaction mixture. In addition to the unknown non-catecholic metabolite, formation of both 3,4-dihydroxybenzyl alcohol and 3,4-dihydroxybenzaldehyde could be noted. The major product arises by the rapid oxidation of the alcohol under the experimental conditions employed.

The above results can be explained by the formation of quinone methides rather than quinones as

Table 1

Catecholic products formed by the action of cuticular polyphenol oxidase on various catechols

No.	Substrate used	Catecholic products formed
1.	Catechol	Nil
2.	3,4-Dihydroxybenzoic acid	Nil
3.	4-Methylcatechol	3,4-Dihydroxybenzyl alcohol + 3,4-dihydroxybenzaldehyde
4.	3,4-Dihydroxyphenethyl alcohol	3,4-Dihydroxyphenylglycol
5.	3,4-Dihydroxyphenylacetic acid	3,4-Dihydroxymandelic acid
6.	*N*-Acetyldopamine	*N*-Acetylnorepinephrine

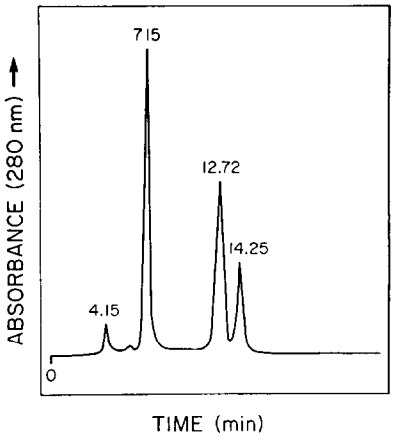

Fig.2. HPLC of products of 4-methylcatechol side chain oxidation by cuticular polyphenol oxidase. Elution times: 3,4-dihydroxybenzyl alcohol (4.15 min); 3,4-dihydroxybenzaldehyde (7.15 min); unknown non-catecholic metabolite (12.72 min); 4-methylcatechol (14.25 min).

the inition products of catechol oxidation. Quinone methides are analogues of quinones with one oxygen atom of the quinone replaced by a methylene group [11]. In the present study, they happened to be the tautomers of 4-alkylquinones and hence their formation by a 2-electron oxidation of 4-alkylcatechols is quite possible (fig.3). In aqueous systems, hydration of quinone methide is rapid, spontaneously affording side-chain hydroxylated products [11].

Quinone methides also rapidly react with nucleophiles [11]. Since cuticle is abundant with histidyl and lysyl residues, quinone methides generated would be expected to react with cuticle and get covalently bound to them. Hence, after conducting the reaction, cuticle was reisolated and checked for the presence of bound catechols. Preliminary experiments with nitrite/molybdate reagent applied directly to the tissue confirmed this contention. Further, hydrolysis of the treated cuticle released free catechols confirming the covalent binding. Only those catechols with alkyl side chain bound covalently to the cuticle with the retention of the *o*-diphenol group. Catechol and 3,4-dihydroxybenzoic acid did not show this reaction.

Side-chain hydroxylation could also be a consequence of dopamine-β-hydroxylase activity. However, the operation of this route is unlikely in the cuticle since:

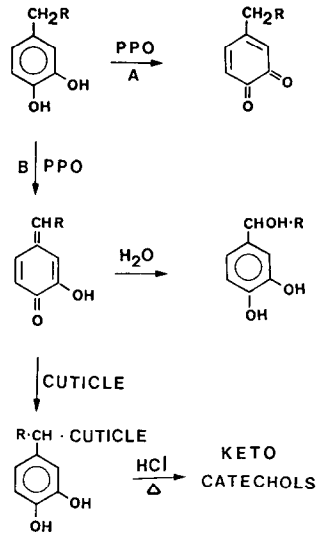

Fig.3. Formation and reactions of quinone methides. Catecholase activity of polyphenol oxidase (PPO) produces quinones from catechols (pathway A). Cuticular polyphenol oxidase catalyzes the conversion of 4-alkylcatechols to quinone methides (pathway B) which react either with water to give side-chain hydroxylated products or, with cuticle, to become immobilized to them. Hydrolysis of catechol-bound cuticle releases the ketocatechols.

(i) All the above reactions occur in the absence of ascorbic acid reductant [12];

(ii) NANE formed from NADA by the action of cuticular enzyme is known to be optically inactive [13] discounting a dopamine-β-hydroxylase type enzyme and favouring the non-stereo-specific water addition to quinone methide derivative [11].

(iii) Immobilization of *o*-phenols to cuticle rules out any possible involvement of dopamine-β-hydroxylase activity.

Quinone methide formation and its subsequent reactions account for a number of conflicting observations on the catecholamine metabolism in insect cuticle:

(i) It accounts for the presence of colourless cuticle in some species;

(ii) It explains the release of ketocatechols by a number of tanned cuticle upon acid hydrolysis [14];

(iii) It aids the unique release of tritium from the β-positions of tritiated *N*-acetyldopamine

upon incubation with cuticular enzymes which could not be accounted for in [4,15,16].

The preference of such enzymes for 4-alkyl-substituted catechols and the lactase activity of the cuticle are in harmony with quinone methide forming enzyme system [8–10].

This is the first report on the formation and utilization of quinone methides in biological processes. Quinone methides have been implicated as one of the reaction intermediates during the biosynthesis of the second most abundant biopolymer, lignin [17] and bioreductive alkylations observed during the action of drugs with an alkyl substituted quinone structure [18,19]. However, evidence for the proposal is still lacking. Our studies establish the unique formation of quinone methides and their biological significance during sclerotization of insect cuticle. Further studies are in progress to understand the biochemical events following the quinone methide formation.

ACKNOWLEDGEMENTS

We thank G. DeMatteo, D. Godbey and M. Burke for their skilled assistance. This work was supported by a grant from the National Institute of Health 2R01-AI 14753-04.

REFERENCES

[1] Andersen, S.O. (1979) Annu. Rev. Entomol. 24, 29–61.
[2] Sugumaran, M. and Lipke, H. (1982) Proc. Natl. Acad. Sci. USA 79, 2480–2484.
[3] Brunet, P.C.J. (1980) Insect Biochem. 10, 467–500.
[4] Andersen, S.O. (1979) Insect Biochem. 9, 233–239.
[5] Arnow, L.E. (1937) J. Biol. Chem. 118, 531–537.
[6] Sugumaran, M. and Lipke, H. (1982) Anal. Biochem. 121, 251–256.
[7] Andersen, S.O. (1980) in: Cuticle Techniques in Arthropods (Miller, T.A. ed) pp.185–215, Springer-Verlag, New York.
[8] Yamazaki, H.I. (1972) Insect Biochem. 2, 431–444.
[9] Andersen, S.O. (1978) Insect Biochem. 8, 143–148.
[10] Barrett, F.M. and Andersen, S.O. (1981) Insect Biochem. 11, 17–23.
[11] Wagner, H.U. and Grompper, R. (1974) in: The Chemistry of the Quinonoid Compounds (Patai, S. ed) pp.1145–1178, Wiley, London, New York.
[12] Taylor, K.B. (1974) J. Biol. Chem. 249, 454–458.
[13] Peter, M.G. (1980) Insect Biochem. 10, 221–227.
[14] Andersen, S.O. and Roepstorff, P. (1978) Insect Biochem. 8, 99–104.
[15] Andersen, S.O. (1976) in: The Insect Integument (Hepburn, H.R. ed) pp.121–144, Elsevier, Amsterdam, New York.
[16] Andersen, S.O. (1977) Symp. Zool. Soc. Lond. 39, 7–32.
[17] Zeikus, J.G. (1981) Adv. Microbiol. Ecol. 5, 211–243.
[18] Moore, H.W. (1977) Science 197, 527–532.
[19] Walsh, C. (1982) Tetrahedron 38, 871–909.