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Characteristics of phenoloxidases in larval cuticle of the coconut pest, *Oryctes rhinoceros*

R JEYARAJ, T THANGARAJ, C A VASUKI and M ARUCHAMI
Department of Zoology, Kongunadu Arts and Science College, Coimbatore 641 029, India

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Abstract. Characteristics of cuticular phenoloxidases of the coconut pest, *Oryctes rhinoceros* were studied biochemically with respect to substrate specificity, pH optima, thermostability and the behaviour of the enzyme in the presence of various chemicals. Biochemical studies indicate that the larval cuticle contains tyrosinase (EC, 1·10·3·1 Ortho diphenol: O_2 oxidoreductase), and laccase type of enzymes (EC, 1·10·3·2. Para diphenol: O_2 oxidoreductase). These enzymes showed similarity to enzyme types A and C with respect to the enzyme activity in the larval cuticle of *Calliphora vicina*. In the electrophoretic study two fractions are discernable in the gel which confirms the occurrence of two enzymes namely tyrosinase and laccase types.

Keywords. Phenoloxidases; *Oryctes rhinoceros*; tyrosinase; laccase.

1. Introduction

The insect phenoloxidases are known to play important roles in cuticular sclerotization and defensive functions (Andersen 1979; Brunet 1980). Subsequent to the report of Dewitz (1902a, b) in the blow fly, various workers have studied the nature and mode of sclerotization in insects. Two enzymes namely tyrosinase and laccase types of enzymes have been reported in insects. The enzyme tyrosinase is reported to occur both in the cuticle and blood, whereas, the latter is found to occur in the cuticle alone.

The enzyme associated with defensive function is said to be soluble whereas, the cuticle sclerotizing enzyme is reported to occur as soluble and insoluble forms. Yamazaki (1969, 1972) has reported that presclerotized pupal cuticle of *Bombyx mori* and *Drosophila* contain an insoluble enzyme and could be solubilised by trypsin digestion. Subsequently, Andersen (1978) has purified the trypsin soluble phenoloxidase in the cuticle of desert locust, *Schistocerca gregaria* and the enzyme was found to oxidise both ortho and paraphenols. Further studies in the above animal have revealed that the cuticle contains an insoluble enzyme which also activate the side chain of N-acetyl dopamine (NADA). However, it is not resolved yet that these two enzymes are one and the same entity (Barrett and Andersen 1981). Soluble phenoloxidases have been separated and purified in the larval cuticle of *Calliphora vicina* by Barett and Andersen (1981) and are reported to be involved in wound healing (tyrosinase type A) and cuticular stabilization (laccase types B and C). Recently, Barrett (1984) has reported that soluble enzyme, similar to that of enzyme type A in *Calliphora vicina* is known to occur in the cuticle of *Calpodes ethlius* and named it as wound healing phenoloxidase. In this paper, we report the characteristics of soluble enzyme(s) from the cuticle of coconut pest, *Oryctes rhinoceros*.

2. Materials and methods

Third instar larvae of *O. rhinoceros* (5 cm-length) were collected from the manure pits in the garden nearer to the college campus and were reared in plastic troughs (capacity 5 liters) by providing cow dung as the source of food. For the preparation of enzyme, larval cuticles were removed carefully and washed several times in distilled water and the adhering tissues were removed. Thoroughly cleaned cuticles were observed under microscope for the adhering tissues and blood cells if any. Cuticles were cut into small pieces and ground well using morter and pestle in ice cold borate buffer 0·003 M (0·1%) pH 9·0. The crude enzyme was separated after centrifugation at 5000 rpm for 20 min. The enzyme was precipitated by ammonium sulphate (50%) and dialysed against the phosphate buffer (pH 7·0) extensively for 48 h at 10°C. The flocculent materials were removed and the clear solution was used as the enzyme source.

Phenoloxidase activity was measured following the method of Andersen (1980), with little modifications. 100 µl of the enzyme was incubated with 100 µl of 0·01 M substrate dissolved in 0·01 M phosphate buffer (pH 7·0) (the pH of the incubation mixture was 7·2). After 15 min at 30°C 1·8 ml of (0·01 M) phosphate buffer (pH 7·0) was added and the intensity of colour developed was measured at 480 nm against the blank using Spectronic 21-spectrophotometer (Bausch–Lomb). Protein content was measured following the method of Lowry *et al* (1951). The enzyme was separated electrophoretically following the method of Jolley and Mason (1965) and were localised by incubating the gels in suitable substrates (0·01 M). (4-methyl catechol, Dopamine, Dopa, Hydroquinone) for a period of 20 h and the bands were visualized.

3. Results and discussion

The enzymes separated from the cuticle of *O. rhinoceros* showed activity towards the following substrates in the order of Dopamine 4-methyl Catechol methylhydroquinone hydroquinone Dopa tyramine (table 1). The enzyme showed activity towards O-

Table 1. Activity of enzyme, phenoloxidase on various substrates.

Substrates	Enzyme activity (absorbance/mg protein/min)
Monophenols	
Tyrosine	0·0
Tyramine	0·02
Diphenols	
4-methyl catechol	0·13
Methyl hydroquinone	0·11
Hydroquinone	0·10
Dopa	0·04
Dopamine	2·0
Protocatechuic acid	0·0
Resorcinol	0·0

Each value represents average of 3 different determinations.

diphenol and paradiphenol as well as monophenols suggesting thereby that both tyrosinase and laccase type of enzymes are present in the cuticle of *O. rhinoceros.* However, the enzyme showed inability to oxidize the protocatechuic acid. Similar observation has also been reported in the case of Calliphora larval cuticle, in which 3 types of enzymes have been separated (enzyme types A, B and C). Enzyme A is designated as tyrosinase and it is suggested that it may perform the function of wound healing whereas, B and C are of laccase types and are involved in cuticular sclerotization.

Since the enzyme obtained from the cuticle after ammonium sulphate precipitation, it is not possible to distinguish at this level whether the cuticle contains more than one enzyme. Recently Barrett (1984) has reported that the cuticle of *Calpodes ethlius* is known to contain only the enzyme A type which showed similar properties to that of the enzyme A type in the *Calliphora erythrocephala.*

The cuticular enzyme in the *O. rhinoceros* showed similarity to the enzyme type A as in the case of *C. vicina* (Barrett and Andersen 1981) and *C. ethlius* (Barrett 1984). All these enzymes are sensitive to phenylthiourea, sodium diethyl dithiocarbamate and are unstable above 60°C. These enzymes showed pH optimum around 6·5–7·2 (tables 2, 3 and 4).

Electrophoretically it has been identified that there are two fractions in 7% gel. One fraction was discernable at the top of the gel, whereas another fraction is just below the first fraction (figure 1).

It has been suggested that the enzyme tyrosinase occurring in the cuticle as proenzyme is performing the function of wound healing. However, a detailed study on this aspect is wanting. The enzyme laccase is reported to be involved in the cuticular sclerotization. Andersen and Roepstorf (1982) have suggested that cuticular sclerotization is mediated by two ways that N-acetyl dopamine is oxidised to quinone by the enzyme phenoloxidase and the same compound can also be oxidized in side chain of NADA giving rise to unsaturated NADA by desaturase which can further be oxidised to unsaturated quinone by the diphenol oxidase. Based on these findings they have suggested that saturated quinone involving pathway is leading to the formation of coloured cuticle whereas, the unsaturated quinone forming pathway

Table 2. Effect of various chemicals in the activity of the enzyme, phenoloxidase.

Chemicals	Concentration (mg/ml)	Activity (absorbance/mg protein/min)	Inhibition (%)
Without treatment	0·0	2·0	0·0
Phenylthiourea	1·0	0·001	96·3
	0·5	0·002	92·6
Thiourea	1·0	0·013	51·86
	0·5	0·013	51·86
Sodium fluoride	1·0	0·019	29·63
	0·5	0·018	33·34
Sodium azide	1·0	0·015	44·45
	0·5	0·016	40·75
Diethyldithio carbamate	1·0	0·003	88·89
	0·5	0·004	85·19

Each value represents average of 3 different determinations.

Table 3. Effect of temperature on the stability (absorbance/mg protein/30 min) and activity of the enzyme, phenoloxidase.

Temperature (0°C)	Activity recovered after treatment with various temperatures	Activity
20	0·060	0·069
30	0·164	0·170
40	0·188	0·178
50	0·220	0·270
60	0·234	0·310
70	0·040	0·050
80	0·040	0·010

Each value represents average of 3 different determinations.

Table 4. Effect of pH on the activity of enzyme, phenoloxidase.

pH	Activity (absorbance/mg protein/30 min)
5·5	0·090
6·0	0·100
6·5	0·155
7·0	0·175
7·5	0·140
8·0	0·100
8·5	0·080

Each value represents average of 3 different determinations.

leads to colourless cuticle. In this context it is of interest to mention that *O. rhinoceros* is having the white cuticle in the larval stage while, it is turned to brownish red during pupal and black colour in the adult stage. It may be suggested that the sclerotization of larval cuticle may be involved by producing the unsaturated quinone involving the phenoloxidase and desaturase in the larval stage.

It has been suggested that the sclerotization of white cuticle is mediated by the formation of quinone methide rather than the formation of unsaturated NADA involving the enzymes phenoloxidase and desaturase (Sugumaran and Lipke 1983). This alternative scheme justifies the formation of catechol derivatives, tritium release from β-position and catechol dimerization by the formation of quinone methides. Since NADA is generally accepted as the unique sclerotising agent, it has been suggested the possibility of the synthesis of tautomeric quinone methide and the quinone from NADA. However, formation of quinone methide from NADA becomes more complicated than the substrates containing methylene group at 4th position (Sugumaran and Lipke 1983). It is relevant to mention that the phenoloxidase oxidases strongly 4 methyl catechol and methyl hydroquinone comparatively equivalent to Dopamine as has been observed in the present study. Similar observation has also

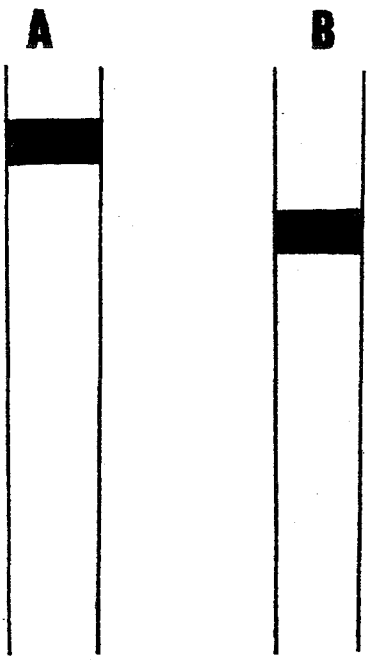

Figure 1. Localization of phenoloxidases in 7% polyacrylamide gel. **A.** Tyrosinase. **B.** Laccase.

been reported in the case of *B. mori* (Yamazaki 1972) in the locust cuticle *S. gregaria* (Andersen 1978).

The electrophoretic study revealed that there are atleast 2 enzymes occurring in the larval cuticle of *O. rhinoceros*. A detailed study is underway to elucidate the various properties of these enzymes.

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References

Andersen S O 1978 Characterization of a Trypsin-Solubilized Phenoloxidase from locust cuticle; *Insect Biochem.* **8** 143–148

Andersen S O 1979 Characterization of the Sclerotization enzymes in locust cuticle; *Insect Biochem.* **9** 233–239

Andersen S O 1980 Cuticular Sclerotization; in *Cuticle techniques in Arthropods* (ed) T A Miller (Springer-Verlag) pp 185–215

Andersen S O and Roepstorf Q 1982 Sclerotization of insect cuticle III. An unsaturated derivative of N-acetyldopamine and its role in sclerotization; *Insect Biochem.* **12** 269–276

Barrett F M 1984 Wound healing phenoloxidase in larval cuticle of *Calpodes ethlius* (Lepidoptera: Hesperiidae); *Can. J. Zool.* **62** 834–838

Barrett F M and Andersen S O 1981 Phenoloxidases in larval cuticle of the blowfly *Calliphora vicina*; *Insect Biochem.* **11** 17–23

Brunet P C J 1980 The metabolism of the aromatic amino acids concerned in the crosslinking of insect cuticle; *Insect Biochem.* **10** 467–500

Dewitz J 1902a Untersuchungen uber die Verwandlungen der Insektenlarven; *Arch. Anat. Physiol. Abt. Physiol.* pp 327–340

Dewitz J 1902b Weitere Milleilunge zu meinen 'Un-tersuchungen uber die Verwandlung der Insektenlarven'; *Arch. Anat. Physiol. Abt. Physiol.* pp 425–442

Jolley R L and Mason H S 1965 The multiple forms of tyrosinase; *J. Biol. Chem.* **240** 1489–1491

Lowry O H, Rosebrough N J, Farr A L and Randall R J 1951 Protein measurement with Folin phenol reagent; *J. Biol. Chem.* **193** 265–275

Sugumaran M and Lipke H 1983 Quinone methide formation from 4-alkyl catechols-a noval reaction catalysed by cuticular polyphenoloxidase; *FEBS Lett.* **195** 65–68

Yamazaki H I 1969 The cuticular phenoloxidase in *Drosophila virilis*; *J. Insect Physiol.* **15** 2203–2211

Yamazaki H I 1972 Cuticular phenoloxidase from the silkworm *Bombyx mori* properties, solubilization and purification; *Insect Biochem.* **2** 431–444