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Sclerotization of the periostracum of the marine bivalve *Perna viridis* (Linnaeus)

M S BHARATHI and K RAMALINGAM
Department of Zoology, University of Madras, Guindy Campus, Madras 600 025, India

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Abstract. The enzyme phenoloxidase from the periostracum of the bivalve *Perna viridis* (Linnaeus 1758) was extracted and the substrate specificity was studied spectrophotometrically. The enzyme is solubilised by sodium dodecyl sulphate and is activated by trypsin. It shows high activity with the phenolic substrates pyrocatechol, dopamine (3–4 dihydroxyphenylethylamine) and dopa (3–4 dihydroxyphenylalanine). As the enzyme shows catalytic activity with many substrates, it may exist in a multiple form. A qualitative analysis of the phenols occurring in the mantle reveals the presence of dopa and dopamine which may play a role in the tanning of the periostracum. The nature and properties of the enzyme phenoloxidase from the periostracum of *Perna viridis* differ from that occurring in its byssal gland.

Keywords. *Perna viridis*; periostracum; sclerotization; phenoloxidase; mantle; phenols.

1. Introduction

In view of their ecological and economic importance, mussels as a group continue to receive attention in diverse areas of research. One such area is the stabilization of structural proteins. In bivalves scleroproteins are reported to occur as constituents of the periostracum—a thin fibrous layer covering the outer surface of the molluscan shell (Trueman 1950; Brown 1952). The secretion and extrusion of new periostracum from the periostracal groove of the mantle margin is accompanied by the rapid insolubilization, hardening and darkening of the extra-cellular material and this is effected by employing quinone to form quinone-tanned protein (Wilbur 1972). This process of sclerotization presumably gives the structure durability and chemical resistance (Wilbur 1964, 1972; Saleuddin and Petit 1983). Further the quinone tanning of the periostracum has been postulated to be an essential prerequisite for an orderly deposition of calcium carbonate crystals (Beedham and Trueman 1968; Taylor and Kennedy 1969; Petit *et al* 1980).

Among the precursors that go to form the periostracum, the enzyme phenoloxidase occupies a central position as in insects. It is of interest to note that the phenoloxidase from the different regions of an insect, namely blood, cuticle and oothecal wall differs markedly in its nature and substrate preference (Cottrell 1964; Hackman 1971; Brunet 1980). The occurrence of a phenoloxidase in the byssal gland of the bivalve *Perna viridis* (Linnaeus 1758) besides the periostracal phenoloxidase, prompted this study to assess the difference, if any, between these two enzymes (Bharathi 1982; Bharathi and Ramalingam 1983). The nature of the enzyme phenoloxidase (EC 1·14·18·1) from the periostracum of *P. viridis* and its behaviour to substrates have been studied. Further the phenols from the mantle have been extracted and identified to determine whether a correlation exists between the nature of phenols occurring *in situ* and the behaviour of the enzyme to substrates.

2. Materials and methods

Green mussel *P. viridis* was collected from the shore opposite to the University Buildings, Madras. The periostracum was removed by running a sharp scalpel parallel to the shell margin. Approximately 3 g weight of the periostracum was collected from 30 animals.

2.1 *Enzyme preparation and assay*

The enzyme was prepared and assayed following the method of Waite and Wilbur (1976). The enzyme activity was measured spectrophotometrically in a Unicam Sp 800 spectrophotometer at 30°C. The increase in the absorbance was recorded every minute immediately after the addition of the enzyme preparation to a mixture of 1 ml of Tris HCl buffer pH 7·4, and 2 ml of 0·01 M substrate in Tris HCl buffer taken in the sample cuvette. The protein content of the sample was estimated following the procedure of Lowry *et al* (1951). The results are expressed as Δ absorbance mg protein^{-1} min^{-1}.

2.2 *Substrate specificity*

The following chemicals in Tris HCl buffer were used as substrates: 0·01 M L-tyrosine, tyramine, dopa, dopamine, pyrocatechol, protocatechuic acid and hydroquinone (Sigma Chemicals Co., USA). The enzyme-catalysed oxidation of the above catecholic substrates was studied by monitoring the formation of quinones at their wave length of maximal absorption (Waite and Wilbur 1976; Waite 1976). The molar extinction coefficients of the quinones are listed in table 1. Using the molar extinction coefficients the values of Δ absorbance mg protein^{-1} min^{-1} were converted into μmol of substrate oxidized per min (Waite and Wilbur 1976).

2.3 *Extraction of phenols*

The phenolic compounds were extracted from the mantle of *P. viridis* following the method outlined by Andersen (1980). The extracted phenols were characterised by thin layer chromatography and identified (Andersen 1980; Sekeris and Herrlich 1966).

3. Results

The steps in solubilization and activation of enzyme phenoloxidase from the periostracum are given in table 2. Homogenization of the periostracum in aqueous buffer 0·1 M Tris HCl, pH 7·4 liberates some measurable activity into the supernatant. Treatment of the pellet with sodium dodecyl sulphate (SDS) completely solubilises the periostracal phenoloxidase which is still further activated by the addition of trypsin. Keeping the μmol of dopa oxidized by the enzyme/mg protein/min as 1, the relative velocities of the enzyme activity with other substrates have been calculated (table 3). The substrate specificity experiments reveal that the enzyme shows maximum activity with the substrate pyrocatechol and secondly with

Table 1. Extinction coefficients of the oxidation products of the phenolic substrates used.

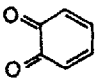
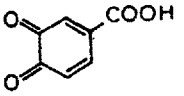
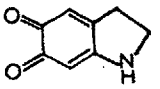
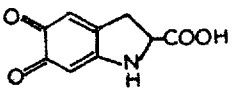
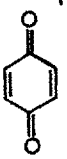
Substrates	Quinone produced		λ_{max} (nm)	E M^{-1} cm^{-1}
Pyrocatechol		*o*-Benzoquinone	390	1417
Protocatechuic acid		1-carboxy, 3,4-benzoquinone	390	1300
Dopamine		5,6-indolequinone	465	2455
Dopa		Dopachrome	480	3388
Hydroquinone		Quinhydrone	440	890

Table 2. Activity of the periostracal phenoloxidase in the supernatant and the pellet (3 4 dihydroxyphenylethylamine used as substrate).

	Activity of periostracal phenoloxidase Δ absorbance mg protein^{-1} min^{-1}
Supernatant of homogenate in 0·2 M Tris HCl, buffer pH 7·4	0·012 ± 0·0013
Pellet treated with 0·5% (w/v) SDS and the resulting supernatant used as enzyme source	0·053 ± 0·0024
The above supernatant treated with 25 μg trypsin/mg protein	0·075 ± 0·0021

Table 3. Activity of phenoloxidase from the periostracum of *P. viridis* on various substrates.

Substrates	μmol of substrate oxidized/mg protein/min	Relative velocity
Pyrocatechol	36·00	2·50
Dopamine	30·50	2·10
Dopa	14·50	1·00
Protocatechuic acid	8·46	0·59
Hydroquinone	5·60	0·39
Tyrosine	0·00	0·00
Tyramine	0·00	0·00

3–4 dihydroxyphenylethylamine. The enzyme does not react with monophenols like tyramine and tyrosine.

The hydrolysate of the mantle contains the following phenolic compounds, identified by their R_f values and the colour formed with the spraying reagents: dopa (R_f 0·27), dopamine (R_f 0·59) and a diphenol (R_f 0·89).

4. Discussion

In insects, the enzyme phenoloxidase has been reported to occur both in soluble and insoluble forms. In cases where the enzyme is bound to the membrane and is insoluble, it has been solubilized by the use of detergents (Hackman and Goldberg 1967; Hughes and Price 1974) or by digestion with proteolytic enzymes (Yamazaki 1969; Andersen 1978). In the periostracum of *P. viridis*, though some phenoloxidase activity is released in the supernatant, the enzyme is completely solubilized by the addition of SDS thus indicating that it may be bound to membrane. This enzyme is further activated by trypsin resembling the periostracal phenoloxidase from *Modiolus* (Waite and Wilbur 1976). In contrast, completely soluble phenoloxidases have been reported from the byssal gland of *P. viridis* (Bharathi 1982) and from the ink of cephalopods (Prota *et al* 1981).

It is seen that the periostracal phenoloxidase from *P. viridis* catalyses *o*-diphenolic substrates like pyrocatechol, dopamine, dopa and protocatechuic acid as well as *p*-phenol hydroquinone, thus exhibiting a wide substrate specificity. Due to its catalytic activity with different substrates, the enzyme could exist in a multiple form resembling the phenoloxidase from the byssal gland of *P. viridis* (Bharathi 1982; Bharathi and Ramalingam 1983). Multiple forms of phenoloxidase have also been reported from insects (Hughes and Price 1975; Pau and Kelly 1975). As the enzyme does not oxidize monophenols, there seems to be no monophenolase activity. Similarly the phenoloxidase from *Modiolus* and *Biomphalaria glabrata* shows no activity with monophenols (Waite and Wilbur 1976; Aragao and Bacila 1976). It is of interest to note that while the periostracal phenoloxidase from *P. viridis* shows a high activity with pyrocatechol, the byssal phenoloxidase shows maximum activity with *p*-phenylenediamine and hydroquinone respectively. Such a diversity is also reported in the insect *Drosophila virilis* in which the cuticular and haemolymphal phenoloxidases differ in their substrate preference (Ohnishi 1954; Yamazaki 1969). Thus the present study elucidates the fact that in *P. viridis* the periostracal phenoloxidase differs in its nature and properties from the byssal phenoloxidase. The *o*-quinones involved in the phenolic tanning of the periostracum of molluscs may be derived from the oxidation of tyrosine or dopa (Bubel 1980).

Histochemical studies have revealed the presence of tyrosine and dopa in the periostracum-secreting cells of the mantle (Hillman 1961; Bubel 1976). Similarly, histochemical localization of the phenols from the byssal gland of *Mytilus edulis* revealed the precursor of the tanning phenol to be dopa, which may get degraded to dopamine and catechol before being oxidized to quinone (Ravindranath and Ramalingam 1972). In the present study the occurrence of dopa and dopamine in the mantle of *P. viridis* suggests a similar pathway. Catechol may be the tanning phenol in the mantle for the enzyme shows higher activity to catechol in *in vitro* studies. Thus, the existence of the periostracal phenoloxidase in multiple form and its relation to tanning phenols deserve to be studied further.

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