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Studies on the Mechanism of Blackspot Development in Norway Lobster (*Nephrops norvegicus*)

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ABSTRACT

*The mechanism of blackspot development in Norway lobster (*Nephrops norvegicus*) was studied. A linear relationship was observed between phenolase activity and rate of colour development in homogenates. It was found that tyrosine could significantly influence the rate of colour development. Production of dihydroxyphenylalanine (DOPA) during colour development was demonstrated using an HPLC method. The production of DOPA, i.e. tyrosinase activity, and the rate of colour development in homogenates of Norway lobster were found to be directly related to the phenolase activity as measured using catechol oxidase activity. It is proposed that tyrosine is the initial substrate for blackspot development in Norway lobster and that the process is an enzymatically controlled oxidation. It is suggested that phenolase from Norway lobster is tyrosinase (EC.14.18.1) which has both monophenolase and diphenolase activity.*

INTRODUCTION

Crustaceans are a very important fishery resource and in many parts of the world they are considered to be luxury foods. However, because of their unique biological and biochemical characteristics they rapidly deteriorate

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due to various post-mortem spoilage processes. The initial spoilage is usually due to blackspot development, also called melanosis or enzymic browning. Recent research has shown that it does not necessarily affect the eating quality, but obviously it is not appealing to consumers (Cobb, 1977; Ogawa *et al.*, 1983). Melanosis of crustaceans was first reported by Fieger (1951). Initially, it was suggested to be the result of mold growth (Fieger, 1951). Later research dismissed the possibility of microbial action and involvement of phenolase was observed (Kakimoto & Kanazawa, 1956). It was then generally considered that post-mortem blackspot development was a result of enzymatically controlled oxidation of tyrosine or its derivatives to form melanin, although the mechanism was not well understood (Cobb, 1977; Farias, 1982; Finne & Mige, 1985). Many researchers reported tyrosinase activity of phenolase in crustaceans (Faulkner *et al.*, 1954; Kakimoto & Kanazawa, 1956; Naguchi *et al.*, 1979), but most did not use tyrosine as a substrate and few demonstrated the production of dihydroxylphenylalanine (DOPA) (Summers, 1967). More recently researchers have found the phenolases in the species they studied did not show any detectable tyrosinase (EC.14.18.1) activity but only catechol oxidase (EC.1.10.3.1) activity (Nagayama *et al.*, 1979; Ohshima & Nagayama, 1980; Nakagama & Nagayama, 1981). These results led to alternative suggestions for the mechanism of blackspot development; for example, autoxidation of unstable diphenols such as DOPA, or enzymatically controlled oxidation of stable diphenol such as the 3-*O*-sulphate derivative of *N*-acetyldopamine, which is an intermediate in cuticle sclerotization (Cobb, 1977).

Under most circumstances, blackspot can be controlled by treatment with sulphite, but potential hazards associated with the use of sulphite stimulated the need to develop some alternative ways to prevent blackspot development. A better understanding of melanosis is necessary to develop methods to fully control the process.

The present investigation examined the possible role of tyrosine and the function of phenolase in blackspot development, in order to help to clarify the mechanism of blackspot development.

MATERIALS AND METHODS

Norway lobsters (*Nephrops norvegicus*) used in this study were supplied by Young's Seafoods, Grimsby. They were delivered in ice to the School of Food and Fisheries Studies, and were frozen and stored at −15°C for not more than 3 months.

Preparation of crude enzyme

One hundred grams of frozen Norway lobster heads, or different parts of the animal in the study of enzyme distribution, were homogenized with 150 ml 0·1M phosphate buffer (pH 6·4) with an Ystral homogenizer for 2 min. The homogenate was centrifuged at 50 000*g* (MSE Europa M24) for 20 min at 4°C. The supernatant was used as the crude enzyme preparation.

Spectrophotometric enzyme assay

In the proline-catechol spectrophotometric assay, the reaction mixture contained 0·2 ml of 0·5 M catechol, 0·2 ml of 0·5 M L-proline, 2·2 ml of 0·1 M phosphate buffer (pH 6·4) and 0·2 ml of crude enzyme preparation (Ohshima & Nagayama, 1980). The absorbance at 530 nm was monitored at 25°C using a Pye Unicam PU8800 UV/visible spectrophotometer. The increase of absorbance at 530 nm in the first 5 min was taken and the enzyme activity was expressed as the increase in absorbance/min/ml of crude enzyme.

Oxygen uptake enzyme assay

In the oxygen uptake assay, the reaction mixture contained 0·2 ml of 0·5M catechol (or other substrates at an appropriate concentration), 2·4 ml of 0·1M phosphate buffer (pH 6·4) and 0·2 ml of the crude enzyme preparation. The oxygen uptake was monitored at ambient temperature (20 ± 2°C) with an oxygen electrode. The activity was expressed in nmol O_2/min/ml of crude enzyme (Ohshima & Nagayama, 1980).

Assay of tyrosinase

The method was a modification of those used by Summers (1967) and Haavik and Flatmark (1980). The reaction mixture contained 5 ml 4% ascorbic acid neutralized with NaOH, 5 ml enzyme preparation, 50 mg L-tyrosine and 5 ml 0·1M phosphate buffer (pH 6·4). The reaction mixture was stirred at 3°C for 6 h and the reaction was stopped by adding an equal volume of ice-cold ethanol, containing glacial acetic acid, to give a pH of 4·7. The mixture was allowed to settle for 10 min before being centrifuged at 12 400*g* for 10 min at 4°C (MSE Europa M24). Samples of 2 μl were directly injected into the liquid chromatograph for determination of DOPA.

An HPLC (Perkin–Elmer 604) with a fluorescence detector (Merck, Hitachi F1000) and Trio integrator (Trivector) was used for the studies. The chromatographic separation was achieved at ambient temperature on a

sulphonated fluorocarbon polymer coated on a pelicular silica support (Zipax SCX, 2·1 mm × 100 cm). The mobile phase, consisting of 10 mM acetate buffer (pH 3·7) with 1% (v/v) of propanol, was pumped at a flow rate of 1·5 ml/min. The fluorescence detector was set at an excitation wavelength of 281 nm and emission wavelength of 314 nm.

Determination of the rate of colour development

Samples for determination of the rate of colour development were prepared as follows: 60 g of Norway lobster heads were homogenized with 60 ml 0·1M phosphate buffer (pH 6·4) for 2 min. Twenty millilitres of homogenate was mixed with 10 ml of phosphate buffer or 2 mM tyrosine or 2 mM DOPA. The slurry was poured into a petri dish, which was then stored in a refrigerator (at 4°C) overnight (20 ± 2 h).

The upper layer of slurry was taken and placed on a clear glass plate and its L value (which measures darkness and lightness in the Hunter L, a, b system) was measured with an Erichsen 511 colorimeter directly, using a black box as background to avoid influence of external light. The instrument was standardized with a white colour standard. The rate of colour development was expressed as a difference of values before and after refrigerated storage.

RESULTS AND DISCUSSION

Influence of tyrosine on the rate of blackspot development

Oxidation of tyrosine to DOPA was originally considered to be the first step in melanosis. However, several recent studies on phenolases in various species of crustaceans did not show any tyrosinase activity when spectrophotometric and oxygen uptake assays were used (Nagayama *et al.*, 1979; Ohshima & Nagayama, 1980).

In this study tyrosinase activity was not detected in enzyme preparations from Norway lobster when the oxygen uptake in the reaction mixtures was monitored (Fig. 1), but a low level of activity towards DOPA was detected. This differed from the results of Ohshima and Nagayama (1980) on Antarctic krill, which showed that the phenolase only had catechol oxidase activity and did not catalyse the oxidation of DOPA. Since phenolase from crustaceans was found to have more affinity for catechol and DOPA than tyrosine as substrate (Summers, 1967), oxygen uptake assay might not be sensitive enough to detect the presence of any tyrosinase activity, and in some cases even DOPA oxidase activity.

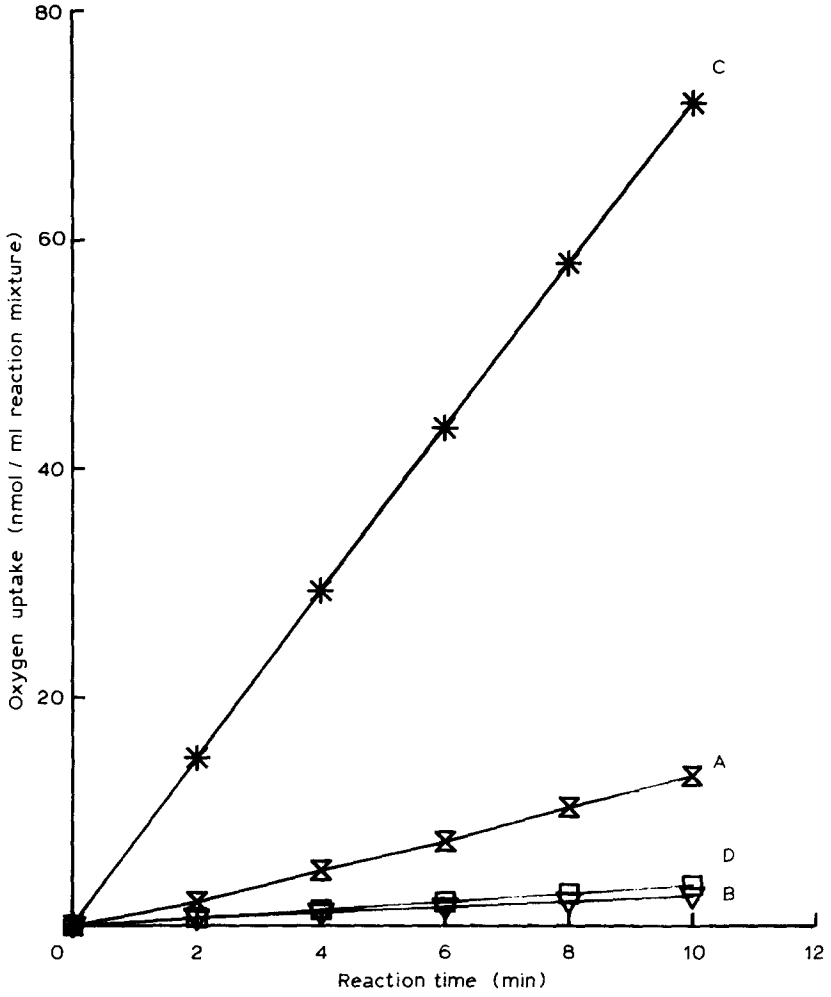

Fig. 1. Oxygen uptake of reaction mixture. Oxygen uptake was measured as in the standard method. Substrates added were: A, 0·2 ml 20 mM DOPA; B, 0·2 ml 2·2 mM tyrosine; C, 0·2 ml 0·5 M catechol; D, blank.

In order to examine the possible role of tyrosine in the blackspot development, and any possible tyrosinase activity, a reflectocolorimetric method was developed. The rate of darkening was measured as the difference in L values of a slurry of Norway lobster after 20 hours' storage at 4°C. Samples with tyrosine added developed more darkness in a similar time period to the samples without added substrate. More darkness was also observed when DOPA or catechol was added, and the initial rate of colour development within 3 h was more rapid by factors of approximately 10 and 100, respectively. The difference in colour development between samples

TABLE 1
Influence of Tyrosine on the Rate of Blackspot Development
(five independent experiments)

Sample	*Rate of blackspot development* ($L_0 - L$)				
Tyrosine	20·0	22·4	23·5	24·3	24·1
Blank	12·9	18·9	19·5	16·0	18·8

Analysis of variance for the difference between tyrosine and blank.
Blank: mean 17·2, SD 2·77. Tyrosine: mean 22·9, SD 1·76.
$F = 15{\cdot}1$, $F_{0{\cdot}01} = 11{\cdot}3$.

with and without tyrosine was highly significant ($P < 0{\cdot}01$) (Table 1). This indicated that tyrosine played a role in blackspot development, even if tyrosinase activity was not detected.

Production of DOPA catalysed by crude enzyme preparation

The fact that tyrosine increased the rate of colour development in the slurry might be due to the result of interaction of tyrosine with oxidation products of other phenols and/or tyrosine being directly oxidized to form melanin. If tyrosine is an initial substrate for an enzymic reaction, the production of DOPA in the reaction mixture should be observed when further oxidation to dopaquinone is blocked. When 4% ascorbic acid was added to the reaction mixture containing crude enzyme and tyrosine, production of DOPA was demonstrated (Fig. 2). Production of DOPA in the reaction mixture not only indicated that tyrosine could be an initial substrate for blackspot development but also suggested the presence of tyrosinase activity.

Relationship between tyrosinase activity and catechol oxidase activity

To further confirm the presence of tyrosinase activity, the relationship between tyrosinase activity (i.e. production of DOPA) and catechol oxidase activity was examined with several different crude enzyme preparations. It was found that the amount of DOPA produced in the reaction mixture was directly related to the catechol oxidase activity present in the system (Fig. 3). When activities towards catechol and DOPA were measured in nmol O_2/min/ml enzyme preparation and tyrosinase activity was measured in nmol DOPA produced/min/ml enzyme preparation, the ratio between activities towards tyrosine, DOPA and catechol were found to be approximately 1:10:100. A similar ratio was found in other studies (Summers, 1967). It was apparent that the oxygen uptake assay could not detect such levels of

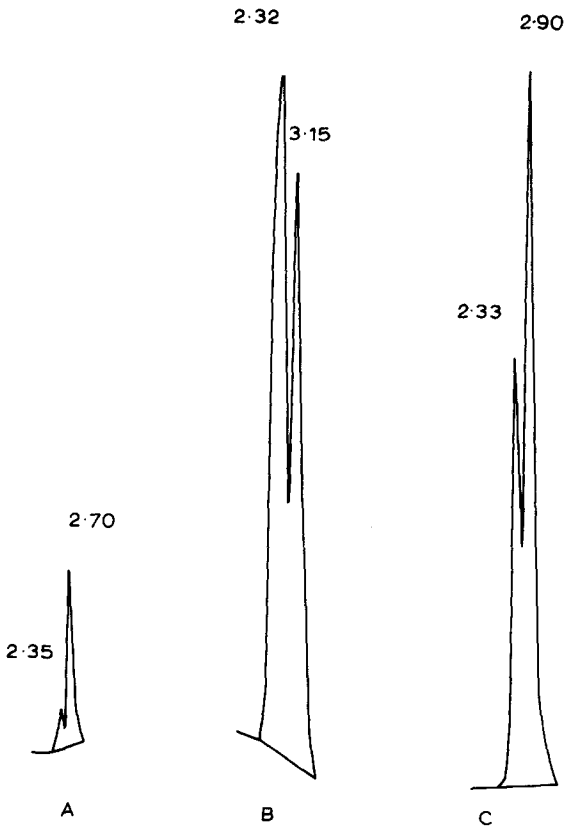

Fig. 2. HPLC chromatograms for estimation of tyrosine and DOPA. A, chromatogram of reaction blank (no tyrosine added); B, chromatogram of 167 pmol DOPA and 334 pmol tyrosine; C, chromatogram of reaction mixture.

tyrosinase activity. An interesting comparison is a study on the fiddler crab, where the Km towards catechol was found to be 0·3 mM, and activities towards tyrosine, DOPA and catechol were detected (Summers, 1967), while in the case of Antarctic krill the Km for catechol as substrate was 0·2M and the phenolase could only apparently catalyse the oxidation of catechol (Ohshima & Nagayama, 1980). In this investigation the Km was 3·5 mM, and activities towards DOPA and catechol were detected. It is suggested that tyrosinase activity might be present in Norway lobster, and possibly in some other crustaceans, in very low levels, and failure to observe any tyrosinase activity in some studies might be due to the spectrophotometric and oxygen uptake assays not being sensitive enough to detect its presence. Therefore it is suggested that phenolase in Norway lobster is tyrosinase (EC.1.14.18.1), which also has diphenolase activity, rather than being catechol oxidase

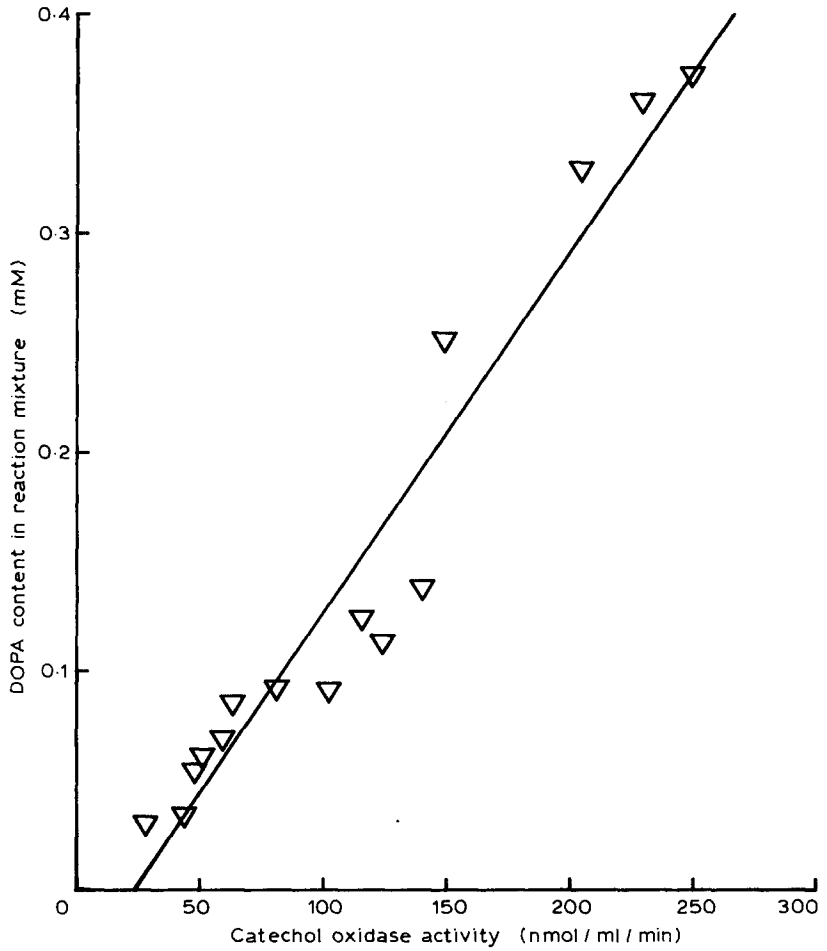

Fig. 3. The relationship between catechol oxidase activity and the rate of DOPA production. Graph was plotted with results from several different crude enzyme preparations.

(EC.1.10.3.1). This may also be true in some other crustaceans. Further studies on those species are suggested.

Relationship between the rate of blackspot development and phenolase activity

It is a common observation that different parts of crustaceans have a different tendency to develop blackspot with the highest rate in the head and very low or negligible rate in the flesh (Cobb, 1977). The levels of phenolase activity and the rate of colour development in slurries from different parts of Norway lobster were examined. A direct relationship was found between the

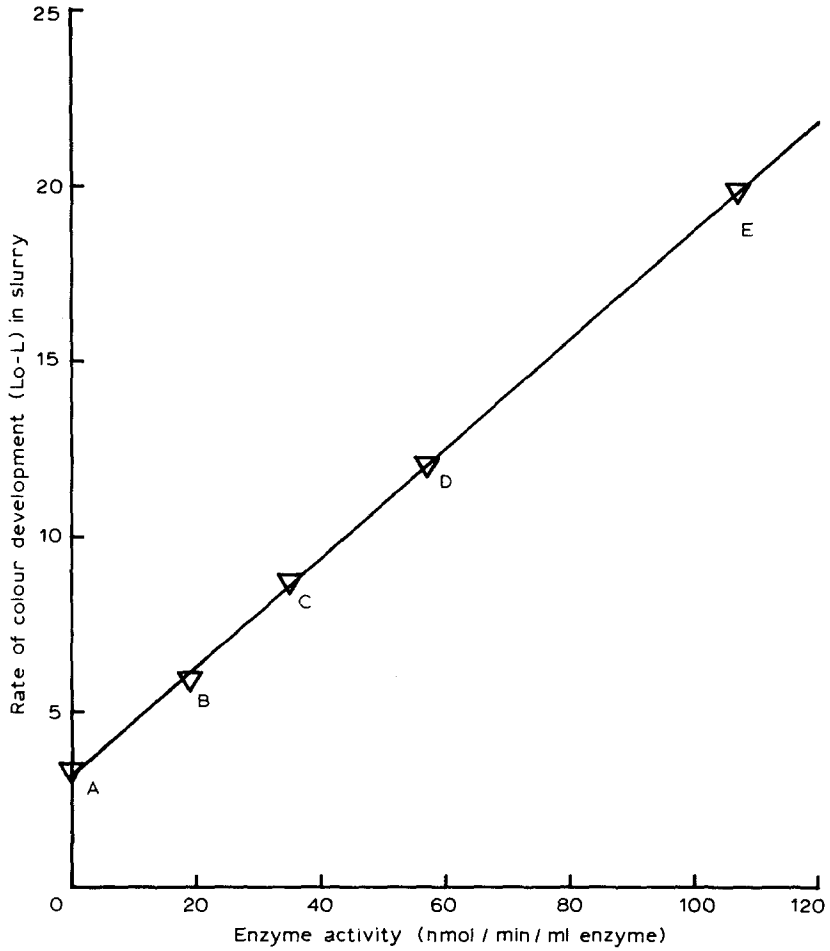

Fig. 4. The relationship between the enzyme activity and rate of blackening. A, flesh; B, tail; C, shell; D, telson; E, head.

phenolase activity and the rate of colour development (Fig. 4). This indicated that blackspot development in Norway lobster is very likely to be an enzymatically controlled process.

CONCLUSION

Tyrosine was found to significantly influence the rate of colour development in slurries prepared from Norway lobster, which suggested a possible role of tyrosine in the process of blackspot development. Tyrosinase activity of phenolase from Norway lobster was detected by an HPLC assay. The

activity towards tyrosine was directly proportional to activities towards DOPA and catechol, but with a lower affinity. It is suggested that the phenolase is tyrosinase (EC.1.14.18.1), which also has diphenolase activity, rather than catechol oxidase (EC.1.10.3.1). The phenolase activity was observed to be directly related to the rate of colour development in homogenates from different parts of Norway lobster. Thus it is suggested that blackspot development in Norway lobster is the result of enzymatically controlled oxidation of tyrosine or its derivatives to form melanin. This might also be the case in some other species of crustaceans where tyrosinase activity was found to be undetectable when spectrophotometric and oxygen uptake assay were used.

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