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Phenolase in Norway Lobster (*Nephrops norvegicus*): Activation and Purification

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ABSTRACT

Increase in phenolase activity from Norway lobster was observed to take place immediately after making a crude enzyme preparation; the total enzyme activity increased by a factor of 3–4 over a period of 8–12 h. The process was associated with the appearance of a more active form of the phenolase. The initial natural form (Form I) and the more active form (Form II) of the enzyme were separated and partially purified by a combination of acetone precipitation and DEAE cellulose column chromatography. The natural form (Form I) was found to have an optimum temperature of 40°C, an isoelectric point of 4·7, a molecular weight of 667 000 and a K_m *towards catechol of 21·8 mM, whereas the more active form (Form II) was found to have an optimum temperature of 45°C, an isoelectric point of 6·1, a molecular weight of 141 000, and a* K_m *towards catechol of 2·84 mM.*

INTRODUCTION

Phenolase, also known as phenol oxidase, tyrosinase and catechol oxidase, is involved in the enzymic browning of fruits and vegetables. In live crustaceans, phenolase is involved in the production of *N*-acetyldopamine and its derivatives, which were reported to be a cuticular sclerotizing agent (Cobb, 1977). *Post mortem*, the enzyme can catalyze the oxidation of tyrosine or its derivatives to melanin resulting in blackspot development. Until recently,

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the mechanism of blackspot development and the exact role of the enzyme in the process were not fully understood. In previous work (Yan *et al.*, 1989), it was demonstrated that phenolase in Norway lobster played a crucial role in post-mortem blackspot development and it was suggested that the process is an enzymatically-controlled oxidation of tyrosine or its derivatives to melanin. The phenolase was shown to be tyrosinase (EC 1.14.18.1), which had both monophenolase and diphenolase activity. The monophenolase activity measured as tyrosinase was directly related to diphenolase activity measured as catechol oxidase. The phenolases in fruit and vegetables, have been extensively studied, while in crustaceans, the enzyme has only been partially purified from a limited number of species (Savagaon & Sreenivasan, 1978; Ohshima & Nagayama, 1980; Farias, 1982; Simpson *et al.*, 1987) and the properties of the enzyme are yet to be comprehensively studied.

The potential hazards associated with the use of sulphite to control blackspot development stimulate the need to develop alternative methods of control. A better understanding of the behaviour and properties of the phenolase would be useful in screening alternative methods.

Phenolase activity in some species of crustaceans was shown to increase very significantly after making a crude enzyme preparation; this increase could be accelerated by trypsin and an endogenous enzyme with tryptic activity (Savagaon & Sreenivasan, 1978). It was suggested that the process might involve a change from a latent form to an active one. Activation of phenolase in the premoulting stage was also reported in crab phenolase (Summers, 1967). However, the activation process was not observed when shrimp phenolase was studied (Simpson *et al.*, 1987). The process has not yet been further studied on other species of crustaceans.

This paper reports the observation of activation of phenolase in Norway lobster and describes the separation and purification of the natural and more active forms of the enzyme.

MATERIALS AND METHODS

Norway lobsters (*Nephrops norvegicus*) used in this study were supplied in ice by Young's English Seafoods, Grimsby. They were immediately frozen and stored at −15°C for not more than 3 months.

Preparation of crude enzyme

One hundred grams of frozen Norway lobster heads were homogenized with 150 ml of 0·1 M phosphate buffer (pH 6·4) using an Ystral homogenizer for

2 min. The homogenate was centrifuged at 50 000*g* (MSE Europa M24 centrifuge) 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 recorded and the enzyme activity was expressed as the increase in absorbance/min per ml of crude enzyme.

Oxygen uptake enzyme assay

In the oxygen uptake assay, the reaction mixture contained 0·2 ml of 0·5 M catechol (or other substrates at an appropriate concentration), 2·4 ml of 0·1 M 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 per ml of crude enzyme (Ohshima & Nagayama, 1980).

Acetone precipitation

One hundred millilitres of crude enzyme were precipitated with 0·5 vol acetone precooled to about −15°C and stirred for about 10 min. The supernatant was collected by centrifugation at 12 400*g* for 10 min at 4°C. Precooled acetone was added to the supernatant until the ratio between enzyme and acetone was 1:1. The mixture was stirred for another 10 min before it was centrifuged at 12 400*g* for 10 min. The precipitate thus collected was dissolved in 50 ml 10 mM phosphate buffer, pH 6·4. After standing overnight, the solution was centrifuged at 12 400*g* for 20 min to remove inactive residues.

DEAE cellulose column chromatography

Forty millilitres of acetone-treated phenolase was applied to a DEAE cellulose column (2·5 × 48 cm), equilibrated with 10 mM phosphate buffer, pH 6·4. The column was first washed with 150 ml of the same phosphate buffer. Phenolase was eluted with a linear gradient of 0 to 1·3 M KCl in 500 ml of the same buffer. A flowrate of 60 ml/h was used and 4 ml fractions were collected using a fraction collector. The column was run at 4°C.

Determination of protein

Protein concentration was determined by the method of Lowry *et al.* (1951).

Molecular weight determination

The molecular weights of partially purified Form I (natural) and of Form II (activated) phenolase were estimated by gel filtration on Sepharose 6B. The column conditions were: size 10 × 950 mm; flowrate 8 ml/h; temperature 4°C. The column was calibrated with carbonic anhydrase (M_w 29 000), albumin, bovine serum (M_w 66 000), alcohol dehydrogenase, yeast (M_w 150 000), apoferritin (M_w 443 000), and thyroglobulin, bovine (M_w 669 000). The void volume was determined by the eluate volume of blue dextran (M_w 2 000 000).

The enzyme samples were concentrated by 5–8 times using an Amicon Ultrafiltration Cell model 8200 (membrane XM50) before application to the column. The eluant volume was monitored for both absorbance at 280 nm and the enzyme activity.

Isoelectric focusing

Isoelectric focusing was performed on agarose IEF gel with flat bed apparatus FBE-3000 (Pharmacia), according to the standard procedure described by Pharmacia Fine Chemicals (Anon., 1982). The ampholyte 'Pharmalyte' covered a pH range of 3 to 10. pI values were calibrated with Pharmacia broad pI calibration kit (pH 3–10). The protein was stained with 0·2% brilliant Blue R in 35% ethanol and 10% acetic acid. Phenolase activity was detected by staining using 0·25M catechol and 0·25M proline solution in 10 mM phosphate buffer, pH 6·4.

RESULTS AND DISCUSSION

Activation of phenolase from Norway lobster

In this investigation, the phenolase activity in Norway lobster was measured using catechol as a substrate. However, the enzyme activity with catechol has been shown to be directly related to that using tyrosine or DOPA as a substrate: the enzyme was consequently suggested to be tyrosinase (EC 1.14.18.1), which had both monophenolase and diphenolase activity (Yan *et al.*, 1989). Activation of phenolase from Norway lobster was observed to take place immediately after making the crude enzyme preparation. The maximum activity was normally reached 8–12 h after

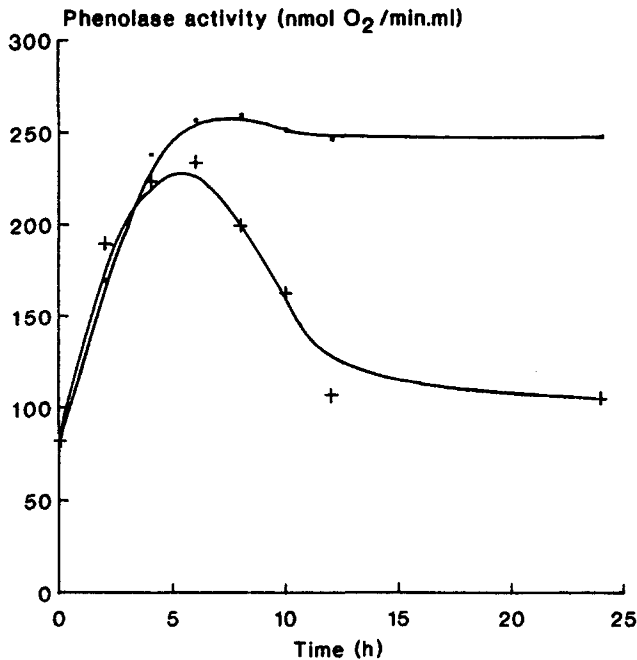

Fig. 1. Activation of phenolase from Norway lobster (1). Slurry, prepared as the standard method for crude enzyme preparation but without the final centrifugal step. Slurry (· · ·); crude enzyme (+).

preparation, although in some preparations a longer time was taken to reach this maximum. Activity then began to decrease (Fig. 1). However, when the enzyme was kept with rest of the slurry, i.e. the whole homogenate without being centrifuged, a higher activity could be reached and the maximum could be maintained for 3 to 5 days (Fig. 2). When these homogenates were centrifuged each day, the enzyme activity remained in the soluble fraction, not in the precipitate.

Triton X-100 influence on activation of the phenolase

As discussed above, the activation process was enhanced when the enzyme was kept together with the rest of the slurry. This might be due to the enzyme being within organelles or a membrane and that a release of phenolase from certain subcellular organelles maintained the 'activation' process. In fruits and vegetables, phenolase has often been reported to be a bound enzyme, which could be released by biological detergents such as Triton X-100 (Mayer & Harel, 1979). However, Triton X-100 had no influence on phenolase activity from Norway lobster, with an average enzyme activity of 65·8 nmol O_2/min ml for three normal preparations and 59·9 nmol

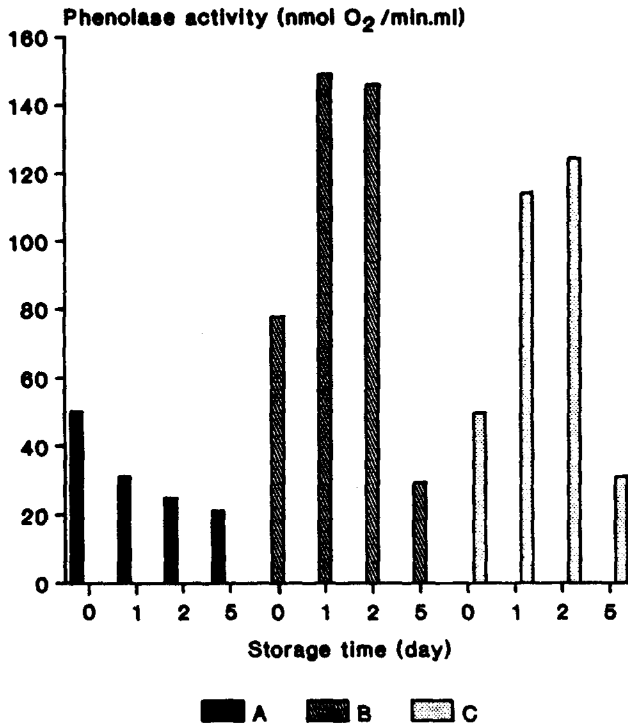

Fig. 2. Activation of phenolase from Norway lobster (2). A: crude enzyme preparation; B: slurry; C: crude enzyme preparation separated from the slurry each day.

O_2/min ml for three preparations using the same extracting buffer except that it contained 1·0% Triton X-100. A *t*-test showed no significant difference ($P > 0{\cdot}2$). This suggested that the enzyme was unlikely to be bound.

Influence of trypsin on phenolase activity

The influence of trypsin was determined by the method of Savagaon & Sreenivasan (1978). Enzyme preparations, partially purified by acetone precipitation, were incubated with trypsin (1 ml enzyme with 1 mg trypsin) in an ice bath for 30 min prior to assay for the activity. The average enzyme activity was 0·153 OD change/min ml for three normal enzyme preparations and 0·146 OD change/min ml for the same preparations after trypsin treatment. Thus, trypsin had little influence on the activity increase of these preparations. On the contrary, it may degrade the enzyme, leading to less activity. In this respect, the activation of phenolase from Norway lobster differed from that of phenolase from lobster and shrimp (Savagaon & Sreenivasan, 1978). This implies that the mechanism of phenolase activation

in Norway lobster might be different from that in lobster and shrimp, which was suggested to involve limited proteolysis by an activating enzyme to form isoenzymes.

IEF studies of the activation and isoelectric point determination

When freshly prepared crude enzyme was subjected to isoelectric focusing on Agarose gel, only one band of enzyme activity was observed, but after storing the preparation at 4°C for 1 day, isoelectric focusing resulted in the appearance of two bands. This indicated that the increase in phenolase

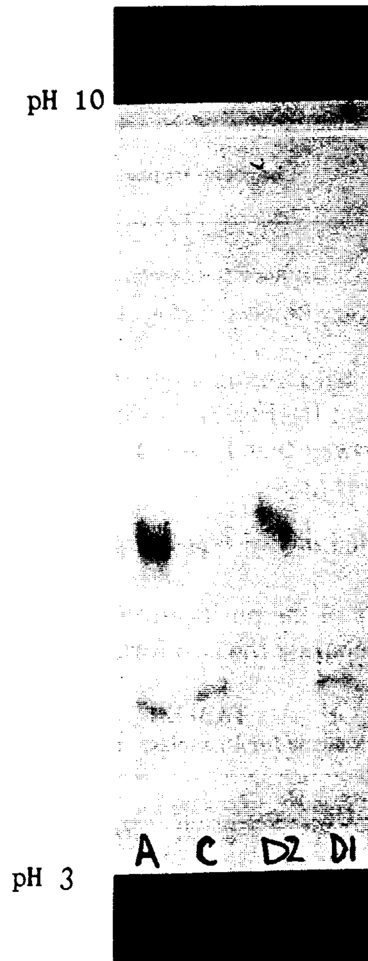

Fig. 3. IEF chromatogram of phenolase on agarose. A: crude enzyme stored at 4°C for one day; C: freshly prepared crude enzyme; D1: Form I of the phenolase; D2: Form II of the phenolase.

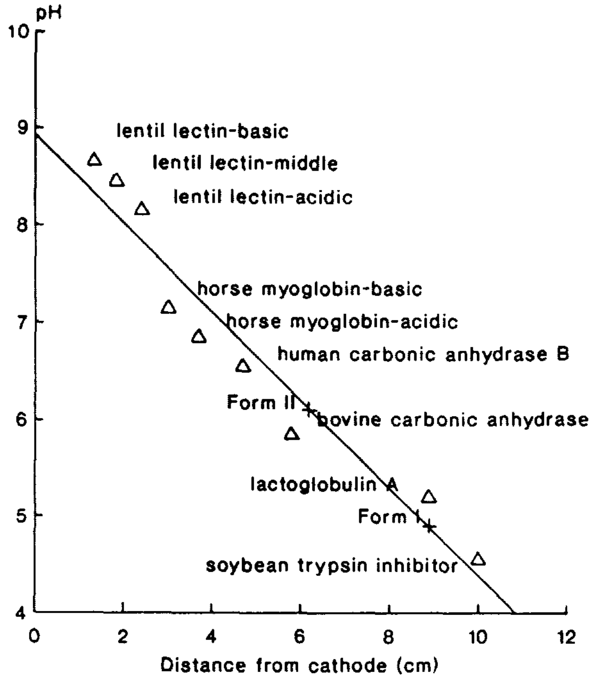

Fig. 4. Estimation of isoelectric point.

activity was associated with the change from one form of the enzyme (Form I) to another (Form II) (Fig. 3). Comparison to IEF chromatograms of standard proteins showed that Forms I and II had isoelectric points of 4·7 and 6·1, respectively (Fig. 4).

Separation and purification of two types of phenolase

Table 1 gives a summary of the purification scheme. This scheme could be used very efficiently to separate the two forms of the phenolase (Fig. 5). An

TABLE 1
Purification Scheme for Phenolase from Norway Lobster

Steps	*Volume (ml)*	*Protein (mg/ml)*	*Specific activity (OD/min/mg protein)*	*Recovery (%)*
Crude	80	23·3	$11·3 \times 10^{-3}$	100
Acetone (ppt)	40	18·7	$21·0 \times 10^{-3}$	76·0
Form one from DEAE cellulose	32	0·67	$270·0 \times 10^{-3}$	28·2
Form two from DEAE cellulose	36	6·00	$19·3 \times 10^{-3}$	21·0

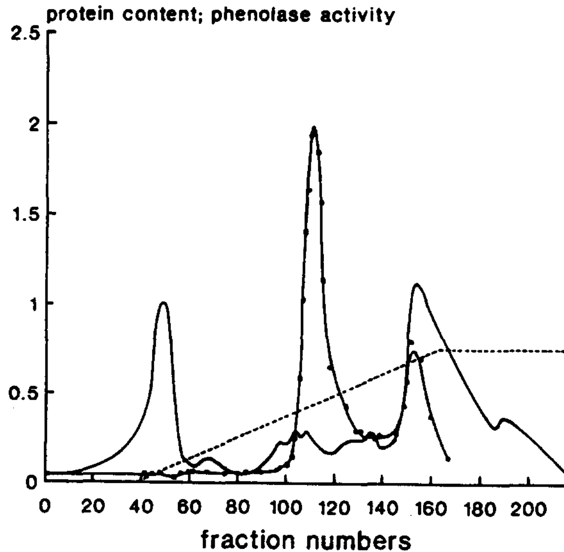

Fig. 5. Column chromatogram of phenolase on DEAE cellulose. Protein content is expressed as absorbance at 280 nm; enzyme activity is expressed as OD at 530 nm/min ml. Protein (—); KCl gradient 0–1·3M (---); enzyme × 10 (-■-).

overall 26-fold purification was achieved with Form II. Since the activity of the crude enzyme was measured 1 day after preparation, i.e. after activation or the increase in activity, the crude enzyme activity would include the sum of activity of the two forms. The actual degree of purification should be higher than values obtained by simply dividing specific activity of purified enzymes by crude ones. This was particularly true with Form I, which was identified to be the initial naturally occurring form, since after activation the majority of the phenolase activity in crude enzyme preparation was contributed from Form II. By comparison with other similar ion-exchange procedures (Savagaon & Sreenivasan, 1978; Ohshima & Nagayama, 1980; Farias, 1982), this procedure was better with respect to the overall degree of purification and recovery. Moreover, this procedure efficiently separated the two forms of the phenolase.

Molecular weight

Molecular weights of forms I and II were determined by Sepharose column chromatography. This method indicated that Form I had a molecular weight of 667 000, while Form II had a molecular weight of 141 000 (Fig. 6). This indicated that the activation process was likely to be a latent natural form dissociating into 4–5 subunits. When activation was observed in lobster (*Panulirus homarus*), three isoenzymes were found to be present in the

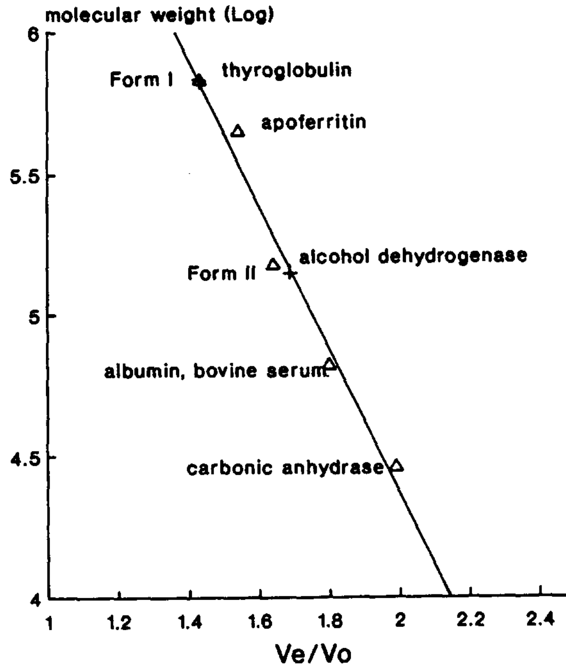

Fig. 6. Estimation of molecular weight. Vo: void volume estimated by the eluate volume of blue dextran; Ve: eluate volume of individual protein.

crude enzyme preparation although the possible relationship between these isoenzymes was not explored (Savagaon & Sreenivasan, 1978), while the enzyme from shrimp (*Penaeus setiferus*) revealed only one band in electrophoresis, and no activation was observed (Simpson *et al.*, 1987).

Effect of temperature on crude and purified phenolase

The effect of temperature on the assay procedure was studied with crude and purified enzymes. Both the crude enzyme and Form II were observed to show a maximum activity at a temperature of about 45°C (Fig. 7), while Form I showed maximum activity at a slightly lower temperature of about 40°C.

Affinity towards catechol

As discussed above, the activation process possibly involved the dissociation of Form I (naturally occurring form) into Form II (activated form). Figure 8 shows that the affinity of the enzyme for the substrate catechol, as indicated by the K_m, changed during the activation process. Since the crude enzyme preparation, after 1 day's storage, mainly consisted of Form II of the phenolase, the K_m was quite similar to Form II (approximately 2·8 mM), while

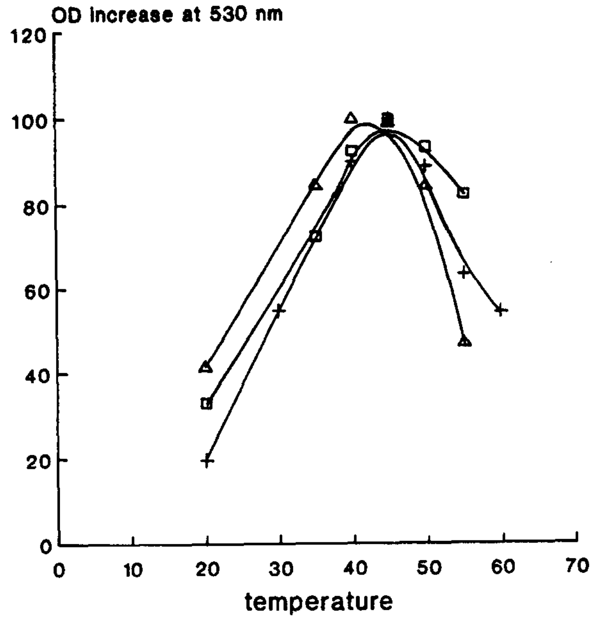

Fig. 7. Effect of temperature on the phenolase activity. The enzyme activity was determined by the spectrophotometric method. Crude enzyme (+); Form I (△); Form II (□).

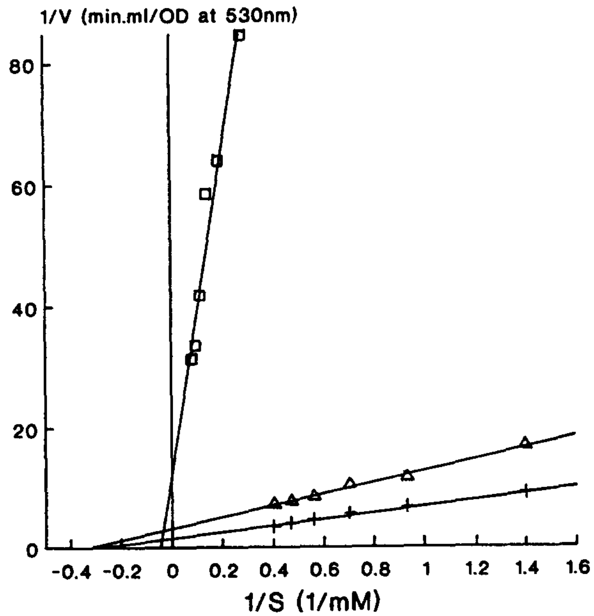

Fig. 8. Lineweaver–Burk plot for the oxidation of catechol. S: catechol concentration; V: rate of reaction. Crude enzyme (+); Form I (□); Form II (△).

Form I, which was the natural form, had the much higher K_m of 21·8 mM (i.e. a lower affinity) towards catechol. This indicated that, during the activation process, not only more enzyme working units were produced but also the enzyme changed its affinity towards substrates. This might have a significant implication in live stage sclerotization and post-mortem blackspot development. Phenolase in crustaceans might exist in a low activity latent form (Form I). By activating phenolase and increasing its affinity towards substrates (i.e. conversion to Form II), crustaceans could control the sclerotization process. Higher activity was reported in crab phenolase during the premoulting stage (Summers, 1967). The phenolase would be gradually activated *post mortem* without any control, which could eventually lead to serious blackspot development.

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