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Studies on the roles and identification of the small molecule factors on phenolase activation in Norway lobster (*Nephrops norvegicus*)

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The direct effect of small molecule factor(s) on the activation of phenolase in the suggested multiple component process of blackspot development in Norway lobster (*Nephrops norvegicus*) was studied.

When acetone-precipitated phenolase was treated with the small molecule filtrate, the phenolase activation profile was lower compared to the control, particularly at pH 6.7. However, when purified form I phenolase was treated with small molecule filtrate no activation was observed, the assayed phenolase activity being constant but lower than the control. A similar effect was observed when form I phenolase was treated with 1.5 mM dihydroxyphenylalanine (DOPA) (approximately the concentration found in the small molecule filtrate), indicating that this lower pattern resulted from the competition of the phenolase substrates (catechol, DOPA, and possibly tyrosine) in the small molecule filtrate. When the activity of the three proteases in the acetone-precipitated phenolase was studied, it was found that there was a high recovery of proteases I and II in the precipitate but little protease III. This also confirms that the effect of the small molecule filtrate on phenolase activity in acetone-precipitated phenolase was due to its indirect effect on the proteases present.

The modification of the compounds that absorb at 270 nm was studied by a high-performance liquid chromatographic (HPLC) separation method and the quantitative analysis of tyrosine and DOPA in the small molecule filtrate was achieved. Some of the small molecules were identified, by an HPLC method, as tyrosine, DOPA, oxidised DOPA, vitamin C in oxidised form or breakdown product of vitamin C. Oxidised DOPA, DOPA, tyrosine and *N*-acetyldopamine inhibit the proteases to varying degrees. Vitamin C also inhibits all three proteases at pH 6.4; however, at pH 8.2 it markedly activates protease III and slightly activates the two thiol proteases (I and II). © 1997 Published by Elsevier Science Ltd

INTRODUCTION

Phenolase in Norway lobster, which has been shown to be capable of catalysing the oxidation of tyrosine or its derivatives resulting in blackspot development in Norway lobster, is normally an inert enzyme that requires activation, as in most crustacean species (Brunet, 1980; Ferrer *et al.*, 1989; Yan *et al.*, 1990; Simpson *et al.*, 1987). The initial natural form and the more active form of the phenolase have been separated and partially purified. The more active form of the phenolase had a molecular mass of 141 kDa and the original natural form a molecular mass of 667 kDa (Yan *et al.*, 1990).

It was found that only protease II (thiol protease) could initially accelerate phenolase activation at pH 6.7 (which may be expected to approximate the pH of live Norway lobster), whilst protease I (possibly Zn-thiol protease) could activate phenolase when the incubation proceeds (above 9 h). It was also found that protease III (Zn-serine protease) and the two thiol proteases can activate phenolase at pH 8.2 (Zotos & Taylor, 1996).

It was further found that a small molecule factor(s), which has a molecular mass of less than 10 kDa, directly affects the proteases and its effect is influenced by the condition of the samples (Zotos & Taylor, 1997).

However, the compounds in the small molecule filtrate which could affect the proteases were not identified, nor was it proved that they did not directly activate phenolase.

MATERIALS AND METHODS

Materials

Norway lobsters (*Nephrops norvegicus*) used in the study were delivered (frozen) to the School of Applied Science and Technology and stored at −18°C for the experiments.

An Ystral homogeniser and an MSE Europa M24 centrifuge were used for homogenising and centrifuging the samples.

Methods

Preparation of crude phenolase

Frozen Norway lobster heads (100 g) were homogenised with 150 ml of 0.1 M phosphate buffer (pH 6.4) or 0.1 M Tris–HCl buffer (pH 8.2) for 2 min. The homogenate was centrifuged at 50 000*g* for 30 min at 4°C. The supernatant was used as the crude phenolase preparation (Ohshima & Nagayama, 1980).

Acetone precipitation of phenolase

A 60 ml portion of the crude phenolase preparation was precipitated with 30 ml of acetone (precooled to about 15°C) and stirred for 10 min. The supernatant was collected by centrifuging at 12 100*g* for 10 min at 4°C. Precooled acetone was added to the supernatant until the ratio between acetone and enzyme solution was 1:1. The mixture was stirred for another 10 min before it was centrifuged at 12 100*g* for 10 min. The precipitate thus collected was dissolved in 15 ml of 0.1 M phosphate buffer, pH 6.4 or pH 8.2, or Tris–HCl buffer, pH 8.2.

Preparation of form I phenolase

Acetone-precipitated phenolase was prepared as above. The precipitate, in the centrifuge tubes, was stored at 18°C overnight. The following day it was dissolved in 15 ml of 0.1 M phosphate buffer pH 7.0, and a 6 ml aliquot was applied to the Sephacryl S-200 gel filtration column (Zotos & Taylor, 1996) which has a fractionation range for proteins of 5–250 kDa. The void volume of the column was estimated with blue dextran (2000 kDa). The eluant was 0.05 M phosphate buffer (pH 7.0) containing 0.1 M KCl and 0.02% NaN_3, the flow rate 40 ml h^{-1} (8 ml cm^{-2} h^{-1}) and the temperature 4°C. The fractions that were eluting near the void volume (i.e. at a similar time to the blue dextran) with the highest phenolase activity were collected as the fractions containing the form I phenolase (660 kDa).

Preparation of small molecule factor(s)

The small molecule factor(s) was prepared as previously described (Zotos & Taylor, 1997).

Effect of small molecule factor(s) on phenolase activation

The influence of small molecule factor(s) on phenolase activation was studied by comparing the rate of activity increase of acetone-precipitated phenolase or form I phenolase treated with small molecule filtrate and that treated with buffer.

Newly prepared crude phenolase in 0.1 M phosphate buffer (pH 6.4) was partially purified by acetone precipitation. The acetone-precipitated phenolase was then diluted in one-half of the original volume in 0.1 M phosphate buffer (pH 6.4) or 0.1 M Tris–HCl buffer (pH 8.2). The above phenolase fractions were resuspended in a 1:4 ratio with analogous buffer (control) and small molecule filtrate. The form I phenolase preparation was adjusted for pH (6.7 or 8.2) and resuspended in a 1:2 ratio with analogous buffer (control) and small molecule filtrate. The phenolase activity was then measured using 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, 2.0 ml of 0.1 M phosphate buffer (pH 6.4) and 0.2 ml of phenolase preparation.

Preparation of crude protease

Frozen Norway lobster heads (15 g) were homogenised with 150 ml of 0.1 M phosphate buffer (pH 7.0) for 2 min. The homogenate was centrifuged at 12 100*g* for 30 min at 4°C. The supernatant was used as the crude protease preparation.

Assay of protease activity

The reaction mixture contained 2.5 ml of 0.5% casein in Tris–HCl buffer solution pH 8.2 and 0.3 ml of protease solution. It was incubated in a water-bath at 45°C for 30 min. Then, 2.8 ml of 5% (w/v) trichloroacetic acid (TCA) was added to stop the reaction and to precipitate protein. The mixture was allowed to stand for 1 h at room temperature. The precipitate was removed by filtration through Whatman No. 1 filter paper. The absorbance of the supernatant was measured at 280 nm in a 1 cm silica cell. A blank was run by adding the enzyme solution after addition of TCA.

Acetone-precipitated proteases

The acetone-precipitated protease was prepared as previously described (Zotos & Taylor, 1996).

DEAE-Sepharose CL-6B column chromatography

The proteases were separated using the method of Zotos & Taylor (1997).

Electric charge of small molecule factor(s)

The electric charge of the small molecule(s) was determined using the method of Zotos & Taylor (1997).

HPLC quantitative analysis of tyrosine and DOPA and investigation of the compounds detected in the small molecule filtrate

Tyrosine and DOPA (Sigma) were dissolved in 50 mM HCl at a concentration of 10 mM. Standards from 0.25 to 5 mM were then prepared. The untreated, anion (Cl^-)-exchanged and cation (H^+)-exchanged small

molecule filtrates were prepared as described above. Standards and samples were then injected into the HPLC system.

The HPLC system used for the analysis consisted of a Perkin Elmer chromatograph (Model 604), with a UV detector (Pye Unicam PU 4020) and a Trio integrator (Trivector), and a loop of 5 µl. Chromatographic separation was achieved at ambient temperature on a C18 column with 16% carbon, 7.5% phenyl and silica (Alltima C18 4.6 mm×25 mm). The mobile phase consisted of 0.05 M phosphoric acid with 0.05 M potassium hydroxide at pH 2.55 and a flow rate of 1 ml min $^{-1}$. The UV detector was set at a wavelength of 270 nm.

Effect of compounds on proteases

Each protease was mixed in a 1:1 ratio with the solution of the compound under investigation. As a control the protease was mixed 1:1 with either 0.1 M phosphate buffer, pH 6.4, or 0.1 M Tris–HCl buffer, pH 8.2, as appropriate. The mixtures were preincubated for 30 min at room temperature (20°C) prior to assay.

Aliquots of 0.6 ml of the mixtures were then assayed for protease activity with 2.4 ml of 0.5% casein in 0.1 M Tris–HCl (pH 8.2). Then, 3.0 ml of 5% (w/v) TCA was added to stop the reaction and to precipitate protein. The assay control used 0.3 ml of protease with 0.3 ml of the appropriate buffer.

Solutions of the compounds to be investigated were prepared as follows:

3,4-Dihydroxphenylalanine (DOPA) (Sigma) was dissolved in water at concentrations of 4 and 0.2 mM and the solutions were adjusted to pH 6.7 and pH 8.2, respectively, with 0.05 M NaOH.

DOPA was dissolved in water at a concentration of 4 mM and mixed with 0.8 mM ferric sulphate ($Fe_2(SO_4)_3$) in a 1:1 ratio. The mixture was adjusted to pH 8.2 with 0.5 M NaOH and the Fe^{3+} precipitated as Fe_2O_3. The precipitated Fe_2O_3 was removed by filtration through a Whatman No. 1 filter and the filtrate used as oxidised DOPA. Distilled water was also mixed with 0.5 mM $Fe_2(SO_4)_3$ in the same ratio, adjusted to pH 8.2 and filtered through a Whatman No. 1 filter and used as control.

L-Ascorbic acid (Sigma) was dissolved in 0.1 M phosphate buffer (pH 6.4) and in 0.1 M Tris–HCl buffer (pH 8.2) at a concentration of 18 mM.

L-Ascorbic acid (2 mM) was dissolved in water and adjusted to pH 8.2.

Cysteine and cystine (Sigma) were dissolved in 0.1 M Tris–HCl buffer (pH 8.2) and 0.2 M HCl (which had already been adjusted to pH 8.2 with 0.1 M NaOH), respectively, both at a concentration of 10 mM.

Tyrosine (Sigma) was dissolved in 0.2 M HCl (which had already been adjusted to pH 8.2 with 0.1 M NaOH) to give a concentration of 10 mM.

N-Acetyldopamine (Sigma) was dissolved in 0.1 M Tris–HCl buffer (pH 8.2) to give a concentration of 1 mM.

RESULTS AND DISCUSSION

Effect of small molecule factor(s) on acetone-precipitated phenolase

It has been demonstrated that endogenous proteases can activate phenolase in Norway lobster. The proteolysis is limited by the availability of the form I phenolase (less active form) which is converted by the responsible proteases to the more active form II. After incubation at pH 8.2, at which all proteases (I, II and III) can activate phenolase, the maximum activity was higher and was reached faster than at pH 6.7, at which only two proteases (II and I) can activate phenolase (Zotos & Taylor, 1996).

However, the participation of a small molecule factor(s) (molecular mass less than 10 kDa) has been indicated in that it inhibited proteases I, II and III at both pH 6.7 and 8.2, except when it was freshly prepared and then it activated only protease III at pH 8.2. It was also found that the small molecule factor(s) is primarily water-soluble and heat-stable, with heating resulting in a similar change as storage in its effect on proteases. Treatment of the small molecule filtrate with a cation-exchanger increased the inhibition effect on proteases. However, whilst treatment with an anion-exchanger at pH 8.2 reduced inhibition, treatment at pH 4.0 restored activation of protease III. These results imply that the small molecule factor(s) is modified on storage or exists in two forms (Zotos & Taylor, 1997).

Therefore it is necessary to investigate the direct effect of this factor(s) in the phenolase activation. It was demonstrated that the small molecule filtrate did not have any effect on phenolase activation (pH 6.4) (Wang, 1993). However, when acetone-precipitated phenolase was treated with the small molecule filtrate, it was found that the phenolase activation was lower than that of the control (pH 6.7) (Fig. 1). Lower activation of phenolase was also observed at pH 8.2 (Fig. 2) but to a lesser extent. These results indicate that the small molecule factor(s) might directly influence the activation of phenolase.

However, when the recovery of proteases in the acetone-precipitated phenolase was studied, it was found that up to 30% of proteases was recovered in the phenolase acetone precipitation (1:0.5 to 1:1). The recovery of proteases I and II was similar to the recovery achieved with the normal protease acetone-precipitation method (1:0.75 to 1:1.25), but with little activity of protease III.

Thus, the observed effect of the small molecule factor(s) might be via the proteases (Zotos & Taylor, 1997) rather than directly on phenolase. Some lowering of the measured phenolase activity also probably resulted from the competition between catechol (the added substrate for the assay) and the other phenolase substrates (e.g. DOPA and tyrosine) which possibly exist in the small molecule filtrate.

Effect of small molecule factor(s) and DOPA on form I phenolase

To further investigate the effect of the small molecule filtrate, purified form I phenolase preparations were adjusted to pH 6.7 and to 8.2 and treated in a 1:1 ratio with buffer (control), small molecule filtrate and 1.5 mM DOPA (which is a similar concentration to that found in the small molecule filtrate at analogous pH; Table 4).

It was found that the presence of small molecule filtrate and DOPA at both pHs gave lower phenolase activities than the controls and with no indication of any activation process being initiated by the small molecule factor(s) (Figs 3 and 4). This suggests that the lower measured activities could be due to the competition of the other phenolase substrates (e.g. DOPA and possibly tyrosine in the small molecule filtrate) with catechol. This would be expected, as Yan (1989) reported that the ratio of phenolase activity to tyrosine, DOPA and catechol was 1:10:100, respectively.

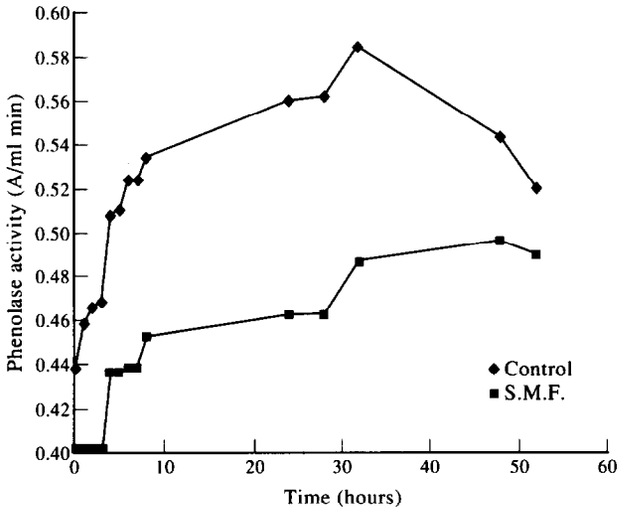

Fig. 1. Effect of small molecule factor(s) on phenolase activity at pH 6.7. Control: acetone-precipitated phenolase resuspended in 0.1 M phosphate buffer, pH 6.4, at a ratio 1:4, respectively. S.M.F.: acetone-precipitated phenolase resuspended in small molecule filtrate obtained using 0.1 M phosphate buffer, pH 6.4, at a ratio 1:4, respectively.

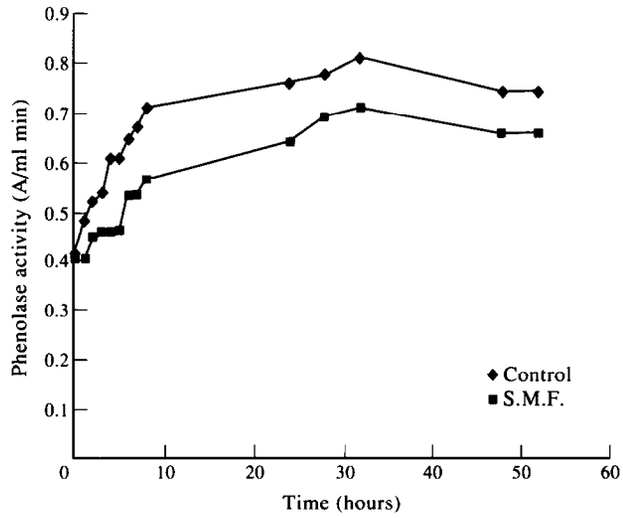

Fig. 2. Effect of small molecule factor(s) on phenolase activity at pH 8.2. Control: acetone-precipitated phenolase resuspended in 0.1 M Tris–HCl buffer, pH 8.2, at a ratio 1:4, respectively. S.M.F.: acetone-precipitated phenolase resuspended in small molecule filtrate obtained using 0.1 M Tris–HCl, pH 8.2, at a ratio 1:4, respectively.

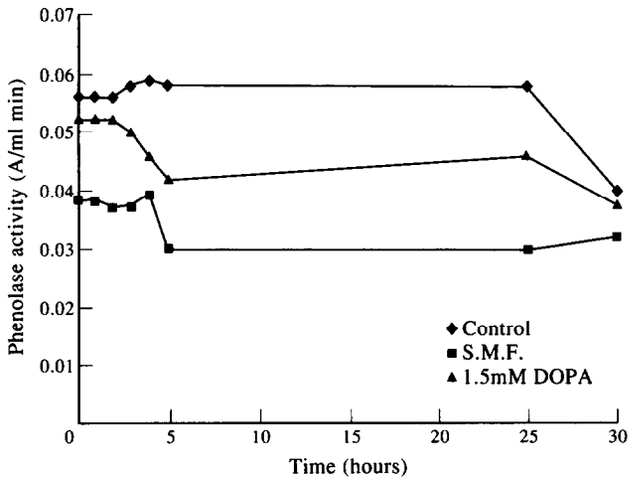

Fig. 3. Effect of small molecule filtrate and DOPA on form I phenolase at pH 6.7. Control: form I phenolase was resuspended in 0.1 M phosphate buffer, pH 6.7, in a 1:2 ratio, respectively. S.M.F: form I phenolase was resuspended in small molecule filtrate, pH 6.7, in a 1:2 ratio, respectively. DOPA: form I phenolase was resuspended in 1.5 mM DOPA, pH 6.7, in a 1:2 ratio, respectively.

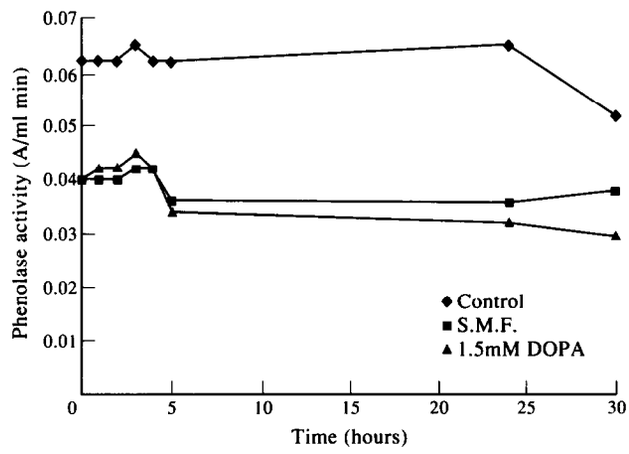

Fig. 4. Effect of small molecule filtrate and DOPA on form I phenolase at pH 8.2. Control: form I phenolase was resuspended in 0.1 M phosphate buffer, pH 8.2, in a 1:2 ratio, respectively. S.M.F: form I phenolase was resuspended in small molecule filtrate, pH 8.2, in a 1:2 ratio, respectively. DOPA: form I phenolase was resuspended in 1.5 mM DOPA, pH 8.2, in a 1:2 ratio, respectively.

Thus, these results confirm that the small molecule factor(s) is an indirect factor(s) in the phenolase activation process. The lower activities observed when acetone-precipitated phenolase was treated with small molecule filtrate resulted from a combination of the competition of the phenolase substrates and the possible inhibition of the small molecule filtrate factor(s) on the proteases.

Quantitative analysis of tyrosine and DOPA, and some studies on the modification of the compounds detected at 270 nm in the small molecule filtrate

It was reported that the small molecule factor(s) is modified on storage or exists in two forms (Zotos & Taylor, 1997). Thus, it was decided to investigate compounds in the small molecule filtrate (i.e. from the Norway lobster flesh). These may be initial substrates or intermediates of the blackspot development sequence or of the sclerotisation process, and might affect the mechanism of blackspot development apart from being phenolase substrates.

An HPLC method was developed for determination of compounds in the initial blackspot development sequence (also phenolase substrates) in the small molecule filtrate.

The small molecule filtrate was extracted on the day of arrival of a batch of samples. Tyrosine was observed only in the small molecule filtrate prepared in 0.1 M Tris–HCl buffer (pH 8.2) and its concentration was very low (0.2 mM). Although the solubility of tyrosine in neutral solutions is low, the extraction should not have been limited by its solubility, which is about 1 mM at 0°C and 2.5 mM at 25°C. The concentration of DOPA in the small molecule filtrate was much higher (1.15 mM and 1.35 mM at pH 6.7 and pH 8.2, respectively). However, apart from tyrosine and DOPA, other compounds were also detected at 270 nm (Fig. 5 and Tables 1 and 2) which might have an effect on proteases and therefore on the sequence of blackspot development.

The levels of these compounds at pH 8.2 and of the three major peaks at pH 6.7 (compounds III, VI and DOPA) were quite constant for the initial 5 days whilst, after the fifth day, a rapid decrease occurred. At pH 6.7 there were more rapid decreases in some minor peaks and the appearance later of new compounds.

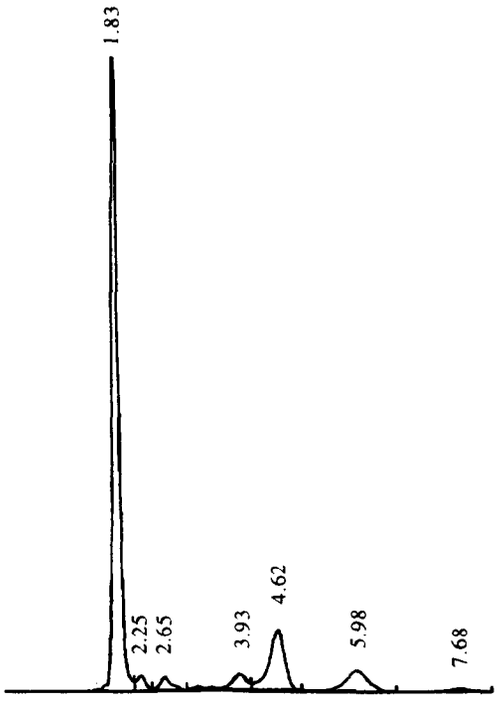

Fig. 5. A typical HPLC profile of the components in the small molecular filtrate (detection 270 nm).

These results confirm that a range of phenols and quinones are present in the small molecule filtrate and that their levels change on storage. This change on storage indicated that phenols and quinones (apart from being phenolase substrates) may participate in the effect of the small molecule factor on proteases, and that this aspect was worthy of further investigation.

Modification of the small molecules treated with the anion (Cl^-)- and cation (H^+)-exchangers

It was found that phenols and quinones are present in the small molecule filtrate.

Table 1. Modification on storage of the compounds detected at 270 nm in a small molecule filtrate prepared in 0.1 M phosphate buffer, pH 6.4

Type of compound	Retention time (min)	Peak area					
		Day 0	Day 1	Day 2	Day 5	Day 6	Day 7
I (unknown)	1.35	—	—	—	—	—	—
II (unknown)	1.62	—	—	—	—	0.6 (0.028)	0.5 (0.001)
III (unknown)	1.85	62.6 (0.12)	63.1 (0.08)	59.4 (0.64)	62.4 (0.3)	48.7 (0.32)	—
IV (unknown)	2.25	0.9 (0.008)	0.5 (0.002)	—	—	—	—
V (unknown)	2.65	0.8 (0.012)	—	—	—	—	—
VI (unknown)	3.95	4.7 (0.016)	4.7 (0.015)	4.5 (0.19)	4.9 (0.07)	0.7 (0.015)	—
VII (DOPA)	4.6	14.6 (0.05)	14.4 (0.09)	13.4 (0.14)	16.9 (0.09)	17.8 (0.17)	—
VIII (unknown)	5.1	—	—	—	1.8 (0.025)	10.2 (0.05)	—
IX (unknown)	5.95	4.6 (0.03)	4.5 (0.04)	2.5 (0.023)	1.4 (0.006)	—	—
X (tyrosine)	7.5	—	—	—	—	—	—

Data are peak areas and means of triplicate determinations. Standard deviations are shown in parentheses.

It was observed that the effects of the small molecule filtrate were strongly modified by treatment with the ion-exchangers (Zotos & Taylor, 1997). Thus, the amphoteric/cationic phenols and quinones could possibly account for the effects of the small molecule filtrate on the proteases. The small molecule filtrate after treatment with the anion-exchanger mainly consisted of the unidentified compound III (1.85 min) and a small amount of the highly polar compound I (1.35 min), whilst the small molecule filtrate after treatment with the cation-exchanger also largely consisted of the compound III (1.85 min), but with a small amount of DOPA (0.15 and 0.16 mM for pH 4.0 and 8.2, respectively) (Table 3). The effect of those mixtures on the proteases was exactly opposite. The anion-exchanged small molecule filtrate increased the inhibition on the proteases whilst the cation-exchanged one restored the activity of the three proteases.

The above results indicate that either compound I (1.35 min) is a great inhibitor of proteases and DOPA is an activator or the combination of those compounds has a reverse effect on the proteases. They also clearly indicate that many of the small molecule(s) are (as removed by both cation- and anion-exchangers) amphoteric compounds. Thus, it is important to try to identify these compounds and to study their effects on proteases.

Identification of the compounds detected in the small molecule filtrate

Identification of the compounds detected in the small molecule filtrate was attempted. The following reference compounds that are readily available were selected because they are either substrates for blackspot development or for sclerosis, or that they can be used to

Table 2. Modification on storage of the compounds detected at 270 nm in a small molecule filtrate prepared in 0.1 M Tris–HCl buffer, pH 8.2

Type of compound	Retention time (min)	Peak area					
		Day 0	Day 1	Day 2	Day 5	Day 6	Day 7
I (unknown)	1.35	—	—	—	—	—	—
II (unknown)	1.6	—	—	—	—	0.7 (0.018)	—
III (unknown)	1.85	75.7 (0.32)	76.1 (0.48)	75.1 (0.28)	74.2 (0.31)	44.9 (0.21)	1.24 (0.009)
IV (unknown)	2.25	2.5 (0.08)	2.1 (0.10)	2.2 (0.07)	2.3 (0.08)	0.8 (0.012)	—
V (unknown)	2.65	2.8 (0.06)	2.8 (0.04)	2.7 (0.05)	2.7 (0.10)	1.0 (0.015)	—
VI (unknown)	3.95	3.3 (0.04)	2.9 (0.10)	3.6 (0.08)	3.5 (0.10)	1.1 (0.026)	—
VII (DOPA)	4.6	17.0 (0.12)	16.6 (0.26)	17.5 (0.12)	15.6 (0.15)	1.6 (0.036)	—
VIII (unknown)	5.1	—	—	—	—	—	—
IX (unknown)	5.95	8.0 (0.13)	8.1 (0.09)	8.1 (0.09)	6.2 (0.12)	—	—
X (tyrosine)	7.5	1.7 (0.04)	1.6 (0.03)	1.6 (0.02)	0.10 (0.009)	—	—

Data are peak areas and means of triplicate determinations. Standard deviations are shown in parentheses.

Table 3. Effect of electrically charged small molecule filtrates (SMF) on the proteases and analysis of the compounds in those filtrates

	Peak area				
	SMF pH 8.2	SMF (Cl^-) pH 8.2	SMF (Cl^-) pH 4.0	SMF ($H+$) pH 8.2	SMF (H^+) pH 4.0
Protease					
I	81[i]	100[i]	100[i]	25[i]	13[i]
II	34[i]	91[i]	100[i]	9[i]	7[i]
III	65[i]	100[i]	100[i]	20[i]	125[a]
Type of compound					
I	—	1.5 (0.012)	1.5 (0.008)	—	—
II	1.1 (0.009)	—	—	—	—
III	75.7 (0.32)	25.2 (0.36)	27.3 (0.23)	17.4 (0.24)	15.9 (0.42)
IV	2.5 (0.08)	—	—	—	—
V	2.8 (0.06)	—	—	—	—
VI	3.3 (0.04)	—	—	—	—
VII (DOPA)	17.0 (0.12) 1.3 mM	—	—	2.2 (0.021) 0.16 mM	2.1 (0.013) 0.15 mM
VIII	—	—	—	—	—
IX	8.0 (0.13)	—	—	—	—
X (tyrosine)	1.7 (0.04)	—	—	—	—

Data are means of triplicate determinations. Standard deviations are shown in parentheses.
i, inhibition; a, activation.

assist in possible identification of other compounds: tyrosine, DOPA, dopamine, *N*-acetyldopamine, catechol, *p*-benzoquinone, L-ascorbic acid and indole.

Some equilibrium reactions were also conducted between the above compounds to obtain as many standards as possible. Thus, DOPA, dopamine, *N*-acetyldopamine and catechol were mixed in a 1:1 ratio with *p*-benzoquinone to identify the retention times of dopaquinone, dopamine-quinone, *N*-acetyldopamine-quinone and *o*-benzoquinone (as well as the resultant reduced hydroquinone), which were expected to be produced in the equilibrium mixtures.

The standard reference compounds and equilibrium mixtures were subjected to HPLC and their retention times identified (Table 4). However, *N*-acetyldopamine and indole (as well as *N*-acetyldopamine-quinone, dopaquinone and *o*-benzoquinone) were not detected under the conditions used in this investigation.

From Tables 1, 2 and 4 the only peak (apart from DOPA and tyrosine) which corresponded with any standards was that of compound IV, which was detected at the same time as L-ascorbic acid. This indicates that compound IV in the small molecule filtrate may possibly be vitamin C. Furthermore, when L-ascorbic acid was stored for some time (4–5 h) and subjected to HPLC or when its pH was changed to 8.2, an additional small peak was observed with a retention time of 2.65 min, corresponding to that of compound V. To further investigate this, the small molecule filtrate was analysed by HPLC at 245 nm (maximum absorbance of vitamin C) and 230 nm. Higher absorption was recorded at the retention times of 2.25 and 2.65 min than at 270 nm, indicating that these components are not phenolic, but could be vitamin C and an oxidised form (or breakdown product) of vitamin C. This was confirmed as higher peaks were recorded at these retention times after addition of L-ascorbic acid to the small molecule filtrate.

When DOPA was oxidised with Fe^{3+} and analysed by HPLC, apart from DOPA (retention time 4.6 min), compound I (with retention time 1.35 min) was also detected. The same peak was also detected when catechol and *N*-acetyldopamine were oxidised and also in the small molecule filtrate treated with the anion-exchanger. It had never been detected in the untreated or stored small molecule filtrate. This highly polar compound might possibly be the result of a further hydroxylation on the benzene ring of the above compounds due to treatment with Fe^{3+} and with the anion-exchanger.

Compound III (retention time 1.85 min) is also a benzene ring compound (phenol/quinone) since its absorbance was decreased six-fold when it was detected at 245 nm or 230 nm instead of at 270 nm. These studies gave no further indication as to the structures of compounds VI, VIII and IX as they showed a similar absorbance at all three wavelengths measured (230, 245 and 270 nm).

Table 4. Reference compounds from HPLC

Compound	Retention time (min)
L-Ascorbic acid	2.25
Dopamine	3.2
DOPA	4.6
Hydroquinone	6.4
Tyrosine	7.65
Dopamine-quinone	12.4
Catechol	16.7
p-Benzoquinone	18.8
N-Acetyldopamine	Not detected
Indole	Not detected
Dopaquinone	Not detected
N-Acetyldopamine-quinone	Not detected
o-Quinone	Not detected

Effect of compounds on proteases

DOPA

When the proteases were treated with DOPA at a final concentration of 2 mM (which is similar to that found in the small molecule filtrate), it was found to inhibit all proteases to a great extent with complete inhibition of the proteases I and III at pH 8.2 (Table 5).

However, when the three proteases were treated with DOPA in a final concentration of 0.1 mM (which is similar to that of 0.08 mM in the small molecule filtrate after treatment with the cation-exchanger), it was found that, at this level of concentration, only the DOPA slightly inhibited the proteases (Table 6). These results indicate that, in the small molecule filtrate treated with

Table 5. Effect of 2 mM DOPA on proteases

	Protease activity at pH 8.2 (A_{280} ml^{-1} per 30 min)		Inhibition (%)
	Control	DOPA 2 mM	
pH 6.7			
Protease I	0.330 (0.003)	0.066 (0.005)	80
Protease II	0.955 (0.009)	0.602 (0.003)	37
Protease III	0.136 (0.004)	0.049 (0.006)	64
pH 8.2			
Protease I	0.370 (0.002)	0	100
Protease II	0.980 (0.016)	0.552 (0.004)	43
Protease III	0.188 (0.004)	0	100

Data are means of triplicate determinations. Standard deviations are shown in parentheses.

Table 6. Effect of 0.1 mM DOPA on proteases

	Protease activity at pH 8.2 (A_{280} ml^{-1} per 30 min)		Inhibition (%)
	Control	DOPA 2 mM	
pH 8.2			
Protease I	0.392 (0.013)	0.292 (0.012)	25
Protease II	0.991 (0.014)	0.968 (0.002)	2
Protease III	0.233 (0.010)	0.201 (0.001)	14

Data are means of triplicate determinations. Standard deviations are shown in parentheses.

Table 7. Effect of compound I and DOPA on proteases

	Protease activity at pH 8.2 (A_{280} ml^{-1} per 30 min)		Inhibition (%)
	Control	Oxidised DOPA	
Protease I	0.284 (0.007)	0	100
Protease II	1.005 (0.012)	0.188 (0.008)	81
Protease III	0.143 (0.006)	0	100

Final concentrations were 0.9 mM for DOPA and 0.4 mM for compound I.
Data are means of triplicate determinations. Standard deviations are shown in parentheses.

the cation (H^+)-exchanger, the recovery of protease activity observed could be a result of the low concentration of DOPA.

It can be concluded that the unknown compound III either does not have any effect on the proteases or may possibly activate protease III, whilst DOPA, apart from being a phenolase substrate, inhibits all proteases and the inhibition depends upon its concentration. Finally, the compound I (retention time 1.35 min) strongly inhibits all proteases even in a very low concentration. Thus, at least some of the small molecule factors which influence the mechanism of blackspot development are amphoteric compounds in addition to cationic ones (Zotos & Taylor, 1997).

Compound I with DOPA

DOPA (4 mM) was oxidised with 0.8 mM $Fe_2(SO_4)_3$ in a 1:1 ratio; distilled water was also mixed with 0.8 mM $Fe_2(SO_4)_3$ in the same ratio (control) and both adjusted to pH 8.2.

Each protease (adjusted to pH 8.2) was resuspended in a 1:1 ratio of control and of oxidised DOPA. It was found that the inhibition of all proteases was very high (Table 7), similar to the inhibition observed when the small molecule filtrate was treated with the anion-exchanger (Zotos & Taylor, 1997) where compound I (1.35 min) was also detected. The concentration of DOPA in the mixture, before being resuspended in the proteases, was 1.8 mM (22.7 peak area), whilst the concentration of the compound I (calculated using the DOPA calibration curve) was estimated as 0.8 mM. Thus, the final concentration of DOPA was 0.9 mM and that of the compound I only 0.4 mM. Those results confirm that the compound obtained from oxidised DOPA (which corresponds to the peak indicated as compound I which was also present when the small molecule filtrate was treated with the anion-exchanger) is the greater inhibitor of the proteases.

Tyrosine

It was found that tyrosine inhibits all the proteases at pH 8.2 (Table 8). This inhibitory effect of tyrosine is less than that of DOPA (Table 5) and so its importance in the *in vivo* system is likely to be less significant, as the tyrosine concentration in the small molecule filtrate is also lower than that of DOPA (Tables 1 and 2).

Table 8. Effect of tyrosine on the proteases

	Protease activity at pH 8.2 (A_{280} ml^{-1} per 30 min)		Inhibition (%)
	Control	Tyrosine	
Protease I	0.076 (0.004)	0.041 (0.004)	46
Protease II	0.409 (0.001)	0.196 (0.004)	52
Protease III	0.099 (0.004)	0.058 (0.002)	41

Control for tyrosine: 0.2 M HCl adjusted to pH 8.2 with 0.2 M NaOH. The final concentration of tyrosine was 5 mM. Tyrosine was dissolved in 0.2 M HCl and adjusted to pH 8.2 with 0.2 M NaOH just before being treated with the proteases.
Data are means of triplicate determinations. Standard deviations are shown in parentheses.

N-*Acetyldopamine*

The role of *N*-acetyldopamine was also considered quite important as it is one of the possible initial compounds in blackspot development. It was found that all proteases were inhibited (Table 9) at a concentration of 0.5 mM.

Comparison of the effect of *N*-acetyldopamine with that of DOPA (Tables 5 and 6) suggests that these compounds have a similar inhibitory effect on the proteases.

L-Ascorbic acid

As an HPLC peak corresponding to ascorbic acid (also with similar ratios of absorbance at 270, 245 and 230 nm) was detected (mainly in the small molecule filtrate prepared in 0.1 M Tris–HCl buffer pH 8.2), and an oxidised form (or breakdown product) was also indicated. It was considered essential to investigate the effect of ascorbic acid on the proteases. It was found that, when the proteases were treated with 18 mM L-ascorbic acid dissolved in 0.1 M phosphate buffer (pH 6.4), all proteases were inhibited by the L-ascorbic acid, although the effect on protease II was least, at 29% (Table 10). This effect was similar to that of the small molecule filtrate.

Furthermore, it was demonstrated that the L-ascorbic acid (dissolved in the buffer) activated the protease III whilst it inhibited the two thiol proteases (Table 11).

Table 9. Effect of 0.5 mM *N*-acetyldopamine on proteases

	Protease activity at pH 8.2 (A_{280} ml^{-1} per 30 min)		Inhibition (%)
	Control	*N*-Acetyldopamine	
Protease I	0.392 (0.013)	0.253 (0.013)	35
Protease II	0.991 (0.014)	0.957 (0.003)	3
Protease III	0.233 (0.010)	0.184 (0.015)	21

The *N*-acetyldopamine was dissolved in distilled water and adjusted to pH 8.2 before treatment.
Data are means of triplicate determinations. Standard deviations are shown in parentheses.

Table 10. Effect of L-ascorbic acid in 0.1 M phosphate buffer (pH 6.4) and mixed with L-ascorbic acid on the proteases

	Protease activity at pH 8.2 (A_{280} ml^{-1} per 30 min)		Inhibition (%)
	Control	L-Ascorbic acid (9 mM)	
Protease I	1.016 (0.004)	0.300 (0.003)	70
Protease II	0.807 (0.001)	0.570 (0.002)	29
Protease III	0.407 (0.003)	0.073 (0.003)	82

Data are means of triplicate determinations. Standard deviations are shown in parentheses.

These results were very similar to the results found when the three proteases were treated with the freshly prepared small molecule filtrates (Zotos & Taylor, 1997).

Thus, the three proteases were treated with L-ascorbic acid at a lower concentration (final 1 mM) to observe its effect on the proteases at a concentration similar to that found in the small molecule filtrate. An activation of all proteases was found (Table 12), particularly of protease III (60%).

However, this would probably be enough to overcome the inhibition by the other compounds (DOPA, oxidised DOPA, etc.). This further indicates that the main activator for protease III in the small molecule filtrate is probably ascorbic acid and possibly the compound V (probably an oxidised form or breakdown product of ascorbic acid).

Effect of cysteine and cystine

It was demonstrated that cysteine increased the colour development in the Norway lobster slurry (Yan, 1989). Further to the observation in the last section that treatment of the small molecule filtrate with L-ascorbic acid restored some of the activity on the proteases at pH 8.2 it was decided to investigate the effect of other compounds (which would not have been detected at 270 nm) that can be involved in reduction/oxidation.

When the proteases were treated with cysteine and cystine, it was found that cysteine activates the two thiol proteases to a great extent (Table 13). This might be expected due to requirement for the thiol group. Protease III was also activated by cysteine. However, cystine had no influence on any of the three proteases.

These results also suggest the possibility that protease III might be activated by reducing agents, as it was activated by cysteine and vitamin C at pH 8.2. At this alkaline pH, protease III can activate phenolase (Zotos & Taylor, 1996).

CONCLUSION

The sequence of the mechanism of blackspot development can now be postulated as follows. Immediately after the lobster is caught (pH about 6.7), protease II in the flesh starts to activate phenolase, converting form I (less active form) to form II (more active one), since the inhibition by the small molecules (DOPA, tyrosine, etc.) on protease II is not very high. Protease I, which can also activate phenolase at pH 6.7 after several hours (Zotos & Taylor, 1996), is inhibited by the small molecules and mainly located in the viscera and so it is unlikely to participate in the mechanism. However, no visible blackspot was detected whilst the pH of the

Table 11. Effect of L-ascorbic acid in 0.1 M Tris–HCl buffer (pH 8.2) on the proteases

	Protease activity at pH 8.2 (A_{280} ml^{-1} per 30 min)		Inhibition (%)	Activation (%)
	Control	L-Ascorbic acid (9 mM)		
Protease I	0.273 (0.006)	0.162 (0.005)	40	
Protease II	0.924 (0.005)	0.680 (0.003)	26	
Protease III	0.164 (0.005)	0.241 (0.004)		47

Data are means of triplicate determinations. Standard deviations are shown in parentheses.

Table 12. Effect of L-ascorbic acid on the proteases at pH 8.2 (final concentration 1 mM)

	Protease activity at pH 8.2 (A_{280} ml^{-1} per 30 min)		Activation (%)
	Control	L-Ascorbic acid	
Protease I	0.392 (0.013)	0.457 (0.006)	16
Protease II	0.991 (0.014)	1.050 (0.001)	6
Protease III	0.233 (0.010)	0.374 (0.005)	60

Data are means of triplicate determinations. Standard deviations are shown in parentheses.

Table 13. Effect of cysteine and cystine on the proteases

	Protease activity at pH 8.2 (A_{280} ml^{-1} per 30 min)		Activation (%)
	Control	Cysteine	
Protease I	0.346 (0.010)	0.730 (0.013)	111
Protease II	0.691 (0.004)	1.489 (0.003)	117
Protease III	0.119 (0.008)	0.169 (0.006)	42
	Control	Cystine	
Protease I	0.236 (0.004)	0.234 (0.003)	0
Protease II	0.409 (0.001)	0.435 (0.003)	6
Protease III	0.099 (0.004)	0.098 (0.002)	0

Control for cysteine: Tris–HCl buffer pH 8.2. Control for cystine: 0.2 M HCl adjusted to pH 8.2 with 0.2 M NaOH. The final concentration of both cysteine and cystine was 5 mM. Cysteine was dissolved in Tris–HCl buffer (pH 8.2); cystine was dissolved in 0.2 M HCl and adjusted to pH 8.2 with 0.2 M NaOH just before being treated with the proteases. Data are means of triplicate determinations. Standard deviations are shown in parentheses.

heads was about 7.0. The blackspot developed in the Norway lobster heads after at least 24 h storage at $3 \pm 1°C$ (or in only 2 h at the ambient temperature) when the pH had increased (pH above 8.0).

Thus, the pH of the stored Norway lobster rapidly changes towards the alkaline area (pH above 8.0) where all proteases can activate phenolase, despite the inhibition from the small molecules. In particular, protease III, which is strongly activated by the ascorbic acid which forms at this pH, is likely to be involved. The activation of protease III by added cysteine, implies that a more reducing environment, as would be expected post mortem, might be a significant factor. The contribution of protease II is also quite considerable since it is the least sensitive protease to almost all inhibitors. Protease I, which is very sensitive, presumably hardly participates in the mechanism.

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