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Studies on the influence of small molecule factor(s) on protease activities in Norway lobster (*Nephrops norvegicus*)

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The interactions of small molecule(s) and proteases in the suggested multiple component process of phenolase activation in Norway lobster (*Nephrops norvegicus*) were studied.

It was observed that protease I is mainly a visceral protease, whilst proteases II and III exist in approximately equal amounts in viscera and flesh, respectively.

It was found that the small molecule factor(s) (molecular weight less than 10 000) inhibited proteases I, II and III at pH 6.7 and 8.2, except when it was freshly prepared and then it activated only protease III and only at pH 8.2. This indicates that the small molecule(s) may be modified on storage.

It was found that the small molecule factor(s) is primarily water-soluble and heat-stable, with heating resulting in a similar change to storage in its effect on proteases. Treatment of the small molecule filtrate with a cation-exchanger also 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 also infer that the small molecule factor(s) is modified on storage or exists in two forms.

INTRODUCTION

Phenolase, also known as phenol oxidase, tyrosinase and catechol oxidase, is involved in the production of *N*-acetyldopamine and its derivatives, which were reported to be cuticular sclerotising agents (Cobb, 1977). The enzyme in Norway lobster has been shown to be capable of catalysing the oxidation of tyrosine or its derivatives, resulting in blackspot development, although normally it is an inert enzyme that requires activation (Yan *et al.*, 1990) in most crustacean species (Brunet, 1980; Ferrer *et al.*, 1989). The enzyme was shown to be tyrosinase (EC 1.14.18.1), which had both monophenolase and diphenolase activities (Yan *et al.*, 1990). 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 pI of 6.1 and molecular weight of 141 000 in comparison with values of 4700 and 667 000, respectively, for the original natural form of the phenolase (Yan *et al.*, 1990).

Three endogenous proteases from Norway lobster have been separated and partially purified and designated proteases I, II and III (Zotos & Taylor, 1995). It was found that only protease II (thiol protease) could initially activate phenolase at pH 6.7 (which may be expected to be approximate to the pH of live Norway lobster), whilst protease I (Zn–thiol protease) could activate phenolase during the course of incubation (longer than 9 h). This may indicate that proteases II and I (to a lesser extent) are mainly responsible for the initiation of blackspot development in Norway lobster. It was also found that protease III (Zn–serine protease) and the two thiol proteases can activate phenolase at pH 8.2. Protease I initially appears to degrade phenolase but then, after a longer period of incubation, contributes to phenolase activation (as also observed at pH 6.7) (Zotos & Taylor, 1995).

It was suggested that the activation required another factor, or factors, which had a low molecular weight (less than 5000). A multiple-component process was therefore suggested (Yan & Taylor, 1991). This paper reports the effects of small molecule(s) present, prepared by filtration, on the activities of the separated proteases.

MATERIALS AND METHODS

Materials

The Norway lobsters (*Nephrops norvegicus*) used in the study were supplied by the Ministry of Agriculture, Fisheries and Food (MAFF, North Shields, UK). They

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, respectively.

Methods

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 TCA was added.

Distribution of protease activities in Norway lobster
Frozen Norway lobster heads (15 g), or heads with the intestines removed, 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 supernatants were used as the crude protease preparation.

Acetone precipitation of proteases
Acetone, precooled to about −15 °C, was slowly added to the crude protease preparation until the ratio between acetone and enzyme solution was 0.75:1, and the mixture was stirred for 10 min. The precipitate was separated from the supernatant after 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 1.25:1. The mixture was stirred for another 10 min before being centrifuged at 12 100*g* for 10 min at 4 °C. The precipitate collected was dissolved in half of the original volume of 0.1 M phosphate buffer (pH 7.0). After standing for about 2 h at 4 °C, the protease preparation was centrifuged at 12 100*g* for 15 min to remove inactive residues. After standing for about 2 h at 4 °C, the protease preparation was centrifuged at 12 100*g* for 15 min to remove inactive residues and was then applied to a DEAE–Sepharose CL-6B column for chromatography.

DEAE–Sepharose CL-6B column chromatography
The acetone-precipitated protease solution (35–38 ml) was applied to a DEAE–Sepharose CL-6B column (2.5×40 cm), equilibrated with 10 mM phosphate buffer (pH 7.0). The column was washed with about 250 ml of starting buffer to remove unbound materials. Proteases were eluted with a linear gradient of 0.35–0.5 M KCl (in 450 ml) produced by a gradient mixer GM-1 (Pharmacia Fine Chemicals). A flow rate of 60 ml h^{-1} was used and 6 ml fractions were collected using an LKB 2212 HeliRac fraction collector. The column was run at 4 °C. The protein content of the eluate was estimated by monitoring the absorbance at 280 nm using an LKB 2138 Unicord S UV monitor, and the protease activity was monitored by measuring the activity of each fraction using the standard protease assay.

Preparation of small molecule factor(s)
Frozen Norway lobster heads were homogenised with 0.1 M phosphate buffer (pH 6.4) and with 0.1 M Tris–HCl (pH 8.2) in the ratio 1:4 for 2 min. The homogenate was centrifuged at 50 000*g* for 30 min at 4 °C. The supernatant was then allowed to pass through an Amicon ultrafiltration cell (Model 8200) with membrane YM10 (which allows compounds with a molecular weight of 10 000 or less to pass through and retains all bigger molecules). The filtrate collected was used as a small molecule preparation named 'small molecule filtrate'.

Effect of small molecule factor(s) on proteases
The influence of small molecule factor(s) on protease activity was determined by comparing the activities of separated protease with and without added small molecule filtrate.

A 1:1 ratio of each protease solution and either buffer (pH 6.4 and 8.2) or small molecule filtrate (pH 6.4 and 8.2) was preincubated for 30 min at room temperature (20 °C). Then 0.6 ml of each mixture was assayed for protease activity with 2.4 ml of 0.5% casein in 0.1 M Tris–HCl (pH 8.2). Finally, 3.0 ml of 5% (w/v) TCA was added to stop the reaction and to precipitate protein. The difference in activity observed would result from the influence of the small molecules on the proteases.

Heat stability of small molecule factor(s)
The heat stability of the small molecule factor(s) was determined by studying the influence of heated and unheated small molecule filtrate on the separated proteases.

Each protease solution (adjusted to pH 8.2) was resuspended in a 1:1 ratio of Tris–HCl buffer (pH 8.2) (control), in small molecule filtrate (prepared using Tris–HCl buffer (pH 8.2) without any treatment, and in small molecule filtrate that had been heated at 85–95 °C for 45 min. They were preincubated and then assayed.

Solvent/water solubility of small molecule factor(s)
Small molecule filtrate prepared in Tris–HCl buffer (pH 8.2) was extracted five times with diethyl ether each time

in a 1:1 ratio using a separating funnel. After the fifth extraction, the lower layer was drained and collected as the water-soluble fraction.

Each protease solution (adjusted to pH 8.2) was resuspended in a 1:1 ratio of Tris–HCl buffer (pH 8.2) (control), in small molecule filtrate (prepared using Tris–HCl buffer (pH 8.2)) without any treatment, and in small molecule filtrate which had been treated five times with diethyl ether. They were preincubated and then assayed.

Electric charge of small molecule factor(s)

Small molecule filtrate was prepared in Tris–HCl buffer (pH 8.2 and 4.0). Amberlite IRA 420 (Cl^-) or Amberlite CG-50 (H^+) was mixed with small molecule filtrate in a ratio 2.5:1. The mixture was then stirred for 2 h before the Amberlite was removed by filtration through filter paper (Whatman No. 1).

Each protease was resuspended in a 1:1 ratio of Tris–HCl buffer (pH 8.2 or pH 4.0) (control), in small molecule filtrate (prepared using Tris–HCl buffer (pH 8.2 or pH 4.0)) without any treatment, in small molecule filtrate treated with Amberlite IRA 420 (anion-exchanger), and in small molecule filtrate treated with Amberlite CG-50 (cation-exchanger). They were preincubated and then assayed.

RESULTS AND DISCUSSION

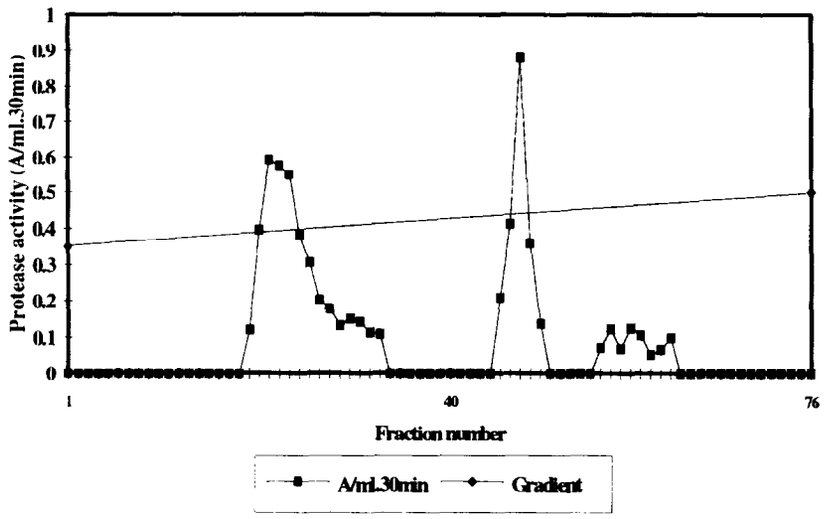
Distribution of protease activities in Norway lobster

It is widely reported that proteases are likely to be responsible for much of the deterioration in Norway lobster and most of them are considered to be visceral enzymes. It has been found that the levels of protease activity in viscera (heads) are about 20 times as high as those in other parts of the animal (Wang, 1993). Diffusion of proteases from viscera of the animal may explain why Norway lobster tails can be kept much longer than whole animals under the same conditions and why they are usually tailed on board ship. Konagaya (1980) reported that autolysis of Antarctic krill could be caused by visceral enzymes which brought about additional digestive breakdown of the organs themselves and of the surrounding tissues when the viscera were physically destroyed during handling. Lindner *et al.* (1988) also reported that the main cause of mushiness following ice storage appeared to be diffusion of proteolytic and collagenolytic enzymes from the hepatopancreas.

When the distribution of the three separated proteases was examined (whole heads and heads from which the viscera were removed), the enzymes were partially purified with acetone and applied to the ion-exchange column, it was observed that protease I is mainly visceral, whilst proteases II and III exist in about equal amounts in both viscera and flesh (Figs 1 and 2). As proteases can participate in the process of blackspot development by activating phenolase (Zotos & Taylor, 1995), and as blackspot development starts immediately after the lobster is caught, this distribution might explain the activation of phenolase.

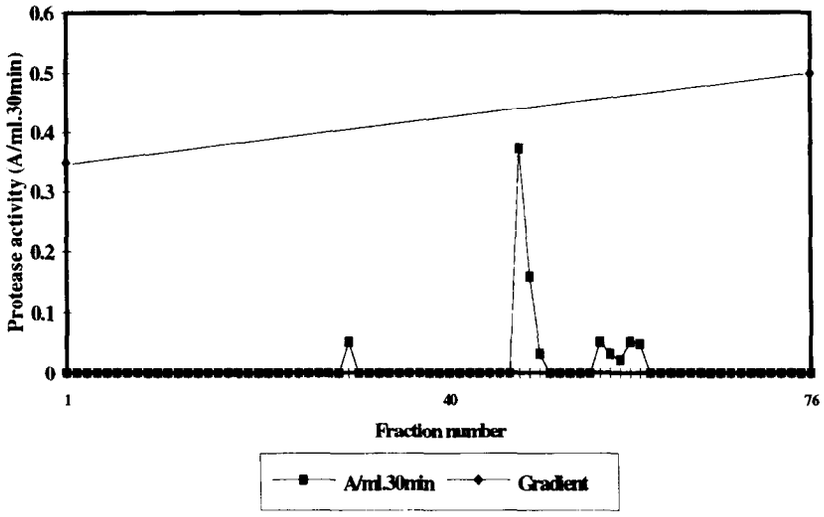
Effect of the small molecule factor(s) on the proteases

It has been demonstrated that endogenous proteases can activate phenolase in Norway lobster. The proteolysis is



Flowrate 60ml.h^{-1}
Gradient 0.35 to 0.5M KCl,
in 0.01M phosphate buffer pH 7.0
Column size 2.5x40cm
Fraction size 6ml

Fig. 1. Ion-exchange separation of acetone-precipitated protease (whole heads).



Flowrate 60ml.h^{-1}
Gradient 0.35 to 0.5M KCl,
in 0.01M phosphate buffer pH 7.0
Column size 2.5x40cm
Fraction size 6ml

Fig. 2. Ion-exchange separation of acetone-precipitated protease (heads without intestines).

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, where only two proteases (II and I) can activate phenolase (Zotos & Taylor, 1995).

However, the participation of low molecular weight factor(s) in the activation of phenolase has been indicated. Thus, a multiple component process was suggested by Yan and Taylor (1991). It was also reported that the small molecule factor(s) works as an indirect factor and activates the proteases rather than the phenolase itself (Wang, 1993).

When the proteases were treated with freshly prepared small molecule filtrate, activation of protease III at pH 8.2 was observed whilst the other two proteases were inhibited (Table 1). After storage at 3 °C, all

Table 1. Effect of small molecule factor(s) on the activity of proteases incubated at pH 8.2

	Protease activity at pH 8.2 (A ml^{-1} per 30 min)		Inhibition (%)	Activation (%)
	Control	SMF-0		
Protease I	0.671(0.004)	0.317(0.001)	53	
Protease II	0.921(0.019)	0.568(0.007)	38	
Protease III	0.264(0.004)	0.405(0.008)		53
	Control	SMF-1		
Protease I	0.671(0.009)	0.125(0.005)	81	
Protease II	0.941(0.001)	0.558(0.009)	40	
Protease III	0.201(0.001)	0.069(0.004)	65	
	Control	SMF-2		
Protease I	0.576(0.012)	0.039(0.002)	93	
Protease II	0.802(0.004)	0.372(0.004)	53	
Protease III	0.198(0.008)	0	100	

SMF-0, small molecule filtrate treated on day of preparation with the proteases (0 day).
SMF-1, small molecule filtrate stored for 1 day at 3 °C.
SMF-2, small molecule filtrate stored for 2 days at 3 °C.
Data are means of triplicate determinations. Standard deviations are shown in parentheses.

Table 2. Effect of small molecule factor(s) on the activity of proteases incubated at pH 6.7

	Protease activity at pH 8.2 (A ml^{-1} per 30 min)		Inhibition (%)
	Control	SMF-0	
Protease I	0.654(0.004)	0.332(0.004)	50
Protease II	0.918(0.019)	0.568(0.027)	34
Protease III	0.248(0.001)	0.133(0.003)	46
	Control	SMF-1	
Protease I	0.718(0.009)	0.292(0.005)	59
Protease II	0.890(0.003)	0.661(0.019)	26
Protease III	0.254(0.002)	0	100
	Control	SMF-2	
Protease I	0.649(0.014)	0.451(0.007)	30
Protease II	0.897(0.005)	0.734(0.016)	18
Protease III	0.249(0.003)	0.030(0.003)	88

SMF-0, small molecule filtrate treated on day of preparation with the proteases (0 day).
SMF-1, small molecule filtrate stored for 1 day at 3 °C.
SMF-2, small molecule filtrate stored for 2 days at 3 °C.
Data are means of triplicate determinations. Standard deviations are shown in parentheses.

proteases were inhibited by the addition of the filtrate. When the proteases were treated at pH 6.7, all proteases were inhibited, even with the freshly prepared small molecule filtrate (Table 2). These results indicate that the small molecule factor(s) is a compound(s) that is either modified on storage or exists in two forms.

Thus, the small molecule factor(s), which has a molecular weight of less than 10 000, directly affects the proteases, but its effect is strongly influenced by the condition of the samples used.

Properties of small molecule(s)

Heat stability

The heat stability of the small molecule factor(s) was determined by studying the influence of heated and unheated small molecule filtrate on the proteases. Thus, the three separated proteases were resuspended in Tris–HCl buffer (pH 8.2), in small molecule filtrate without any treatment (control) and in small molecule filtrate which had been heated at 85–95 °C for 45 min. It was found that heating the small molecule filtrate (Table 3) had an effect similar to that of storage (Table 1), with a resultant higher inhibitory action on all proteases.

Table 3. The effect of prior heating of the small molecule filtrate on the proteases

	Protease activity at pH 8.2 (A ml^{-1} per 30 min)		Inhibition (%)	Activation (%)
	Control	SMF-0		
Protease I	0.359(0.008)	0.061(0.007)	83	
Protease II	0.813(0.003)	0.550(0.016)	32	
Protease III	0.105(0.004)	0.135(0.004)		28
	Control	SMF-h		
Protease I	0.359(0.008)	0	100	
Protease II	0.813(0.003)	0.267(0.008)	67	
Protease III	0.105(0.004)	0	100	

SMF-0, small molecule filtrate without any treatment.
SMF-h, the small molecule filtrate was further diluted at a ratio of 1:1 with Tris–HCl buffer (pH 8.2) and heated at 85–95 °C for 45 min.
Data are means of triplicate determinations. Standard deviations are shown in parentheses.

Table 4. The effect of prior treatment of the small molecule filtrate with diethyl ether on the proteases

	Protease activity at pH 8.2 (A ml^{-1} per 30 min)		Inhibition (%)	Activation (%)
	Control	SMF-0		
Protease I	0.359(0.008)	0.061(0.007)	83	
Protease II	0.813(0.003)	0.550(0.016)	32	
Protease III	0.105(0.004)	0.135(0.004)		28
	Control	SMF-d		
Protease I	0.421(0.015)	0.153(0.007)	63	
Protease II	0.807(0.011)	0.521(0.009)	35	
Protease III	0.109(0.008)	0.107(0.008)	0	0

SMF-0, small molecule filtrate without any treatment.
SMF-d, the small molecule filtrate treated with diethyl ether in a ratio 1:1 (five extractions).
Data are means of triplicate determinations. Standard deviations are shown in parentheses.

Table 5. Effect of Amberlite IRA 420 (anion-exchanger) on the small molecule filtrate at pH 8.2 and pH 4.0

	Protease activity at pH 8.2 (A ml^{-1} per 30 min)		Inhibition (%)
	Control	SMF-0	
pH 8.2			
Protease I	0.671(0.009)	0.125(0.005)	81
Protease II	0.841(0.001)	0.558(0.009)	34
Protease III	0.201(0.001)	0.069(0.004)	65
	Control	SMF-a	
Protease I	0.671(0.009)	0	100
Protease II	0.841(0.001)	0.074(0.004)	91
Protease III	0.201(0.001)	0	100
pH 4.0			
	Control	SMF-0	
Protease I	0.782(0.019)	0.291(0.012)	63
Protease II	0.945(0.003)	0.420(0.009)	56
Protease III	0.144(0.008)	0.047(0.003)	67
	Control	SMF-a	
Protease I	0.782(0.019)	0	100
Protease II	0.945(0.003)	0	100
Protease III	0.144(0.008)	0	100

SMF-0, small molecule filtrate without any treatment.
SMF-a, small molecule filtrate treated with Amberlite IRA 420 (Cl^-).
Data are means of triplicate determinations. Standard deviations are shown in parentheses.

Solvent/water solubility

Small molecule filtrate was extracted five times with diethyl ether, in a ratio 1:1 each time, in a separating funnel. After the fifth extraction, the lower layer was drained and collected as water-soluble fraction. The difference of the inhibitory action between this fraction and the untreated small molecule filtrate was not great and, although the activation of protease III was lost (Table 4), this is a similar level to the storage level. Thus it is probable that the small molecule factor(s) is predominantly water-soluble.

Electric charge

It was reported from studies using carboxymethylcellulose or DEAE-cellulose ion-exchangers, that the small molecule(s) is likely to be slightly negatively charged at pH 6.4 (Wang, 1993).

When the three proteases were resuspended in the small molecule filtrate which had been treated with Amberlite IRA 420 (Cl^-, anion-exchanger), the inhibitory action of the small molecule filtrate on the proteases further increased (Table 5). This indicates that the small molecule(s) is not removed by the anion-exchanger as the effect is similar to that of storage and thus it is likely to be cationic. When the small molecule filtrate was treated with Amberlite CG-50 (H^+, cation-exchanger) (Table 6) the inhibition was reduced, which might imply that the small molecule(s) involved is cationic. However, this could also be regarded as effectively a reversal of the increase in inhibition observed on storage of the filtrate. This view is supported by the observation

Table 6. Effect of Amberlite CG-50 (cation-exchanger) on the small molecule filtrate at pH 8.2 and pH 4.0

	Protease activity at pH 8.2 (A ml^{-1} per 30 min)		Inhibition (%)	Activation (%)
	Control	SMF-0		
pH 8.2				
Protease I	0.671(0.009)	0.125(0.005)	81	
Protease II	0.841(0.001)	0.558(0.009)	34	
Protease III	0.201(0.001)	0.069(0.004)	65	
	Control	SMF-c		
Protease I	0.671(0.009)	0.502(0.002)	25	
Protease II	0.841(0.001)	0.760(0.015)	9	
Protease III	0.201(0.001)	0.160(0.004)	20	
pH 4.0				
	Control	SMF-0		
Protease I	0.782(0.019)	0.291(0.012)	63	
Protease II	0.945(0.003)	0.420(0.009)	56	
Protease III	0.144(0.008)	0.047(0.003)	67	
	Control	SMF-c		
Protease I	0.782(0.019)	0.676(0.012)	13	
Protease II	0.945(0.003)	0.871(0.008)	7	
Protease III	0.144(0.008)	0.325(0.019)		125

SMF-0, small molecule filtrate without any treatment.
SMF-c, small molecule filtrate treated with Amberlite CG-50 (H^+).
Data are means of triplicate determinations. Standard deviations are shown in parentheses.

that, after the small molecule filtrate was mixed with cation-exchanger at pH 4.0, the activation of protease III at pH 8.2 (Table 6) was similar to freshly prepared filtrate (Table 1). This implies that ion-exchangers can modify the small molecule(s) in a similar way to that which occurs on storage or its reversal.

These results indicate that the small molecule factor(s) is either modified on storage or exists in two forms.

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