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Partial purification and characterization of proteases from Norway lobster (*Nephrops norvegicus*) and their role in the phenolase activation process

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Proteolytic activity in Norway lobster (*Nephrops norvegicus*) was studied. An improved separation and partial purification of the three proteases (designated as proteases I, II and III) was achieved from Norway lobster heads by a combination of acetone precipitation and DEAE-Sepharose CL-6B column chromatography.

The purification achieved was 63-, 25- and 217-fold at pH 8.2, and 40-, 25- and 160-fold at pH 6.4 for protease I, II and III, respectively.

With casein as substrate, protease III was most active at pH 8.2, whilst proteases I and II showed activity over a wide range of pH.

Protease III was characterized as an alkaline Zn-serine protease as it was strongly inhibited by PMSF, soybean trypsin inhibitor, Co^{2+}, Mn^{2+} and 1–10 phenanthroline. Protease I was strongly inhibited by *p*-benzoquinone, iodoacetamide, heavy metals (Ag^{+}, Cu^{2+}) and 1–10 phenanthroline and was thus characterized as a Zn-thiol protease. Protease II was also inhibited by the same inhibitors as protease I (but to a lesser extent) and was characterized as a thiol protease.

The molecular weights were determined to be 22.5, 45 and 42.5 kDa (with activity at 18 kDa) for proteases I, II and III, respectively.

It was found that protease III activates phenolase at pH 8.2, whilst proteases II and I can activate phenolase at both pH 6.7 and 8.2. Copyright © 1996 Published by Elsevier Science Ltd.

INTRODUCTION

Tissue proteases have been implicated as adversely affecting the quality of stored muscle foods due to the sustained action of endopeptidases and exopeptidases that are involved in the complete breakdown of tissue proteins (Goll *et al.*, 1983).

Proteases are reported to be responsible for many of the spoilages, such as autolysis (Nishimura *et al.*, 1983) and mushiness (Lindner *et al.*, 1988). Proteases are also reported to be involved in blackspot development in crustaceans (Ferrer *et al.*, 1989; Yan *et al.*, 1990; Yan & Taylor, 1991; Wang *et al.*, 1991, 1993).

Proteases have been purified from several species of crustaceans and their properties have been described (Kimoto *et al.*, 1981; Nip *et al.*, 1985*a*). Some attempts have been made to identify the protease activity responsible for mushiness development (Lindner *et al.*, 1988). A recent study has investigated the proteases in Norway lobster and some interesting results have been reported (Wang *et al.*, 1993). However, further studies on the proteases in Norway lobster could provide a better understanding of the sequence and relationship of molecules involved in blackspot development in Norway lobster.

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METHODS AND MATERIALS

Materials

Norway lobsters (*Nephrops norvegicus*) used in the study were supplied by MAFF (North Shields, UK). They were delivered (frozen) to the School of Applied Science and Technology and stored at −18°C for the experiments.

Methods

Preparation of crude protease

Fifteen grammes of frozen Norway lobster heads were homogenized with 150 ml of 0.1 M phosphate buffer (pH 7.0) with an Ystral homogenizer for 2 min. The homogenate was centrifuged at 12 100 *g* (MSE Europa M24) 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 (except where otherwise stated) in buffer solution 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.

HCl–KCl, citrate–phosphate, phosphate, Tris–HCl and tetraborate–NaOH buffers were used in pH ranges of 1.2–1.8, 2.4–5.2, 5.8–7.6, 8.2–8.8 and 9.2–10.2, respectively. The 30 min incubation time was selected by running a time course of the assay; within this time interval, the reaction was zero order. The enzyme activity was expressed as A/ml per 30 min at 280 nm under assay conditions.

Acetone precipitation

Acetone, which was precooled to about −15°C, was slowly added to the crude protease preparation until the ratio between enzyme solution and acetone was 1:0.75, and the mixture was stirred for 10 min. The precipitate was separated from the supernatant after centrifuging at 12 100*g* (MSE Europa M24) for 10 min at 4°C.

Precooled acetone was added to the supernatant until the ratio between enzyme solution and acetone was 1:1.25. 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 about 2 h at 4°C, the protease preparation was centrifuged at 12 100 *g* (MSE Europa M24) for 15 min to remove inactive residues.

Preparation of crude phenolase

One hundred grammes of frozen Norway lobster heads were homogenized with 150 ml of 0.1 M 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 phenolase preparation.

Acetone precipitation of phenolase

Forty millilitres of crude phenolase was precipitated with 20 ml of acetone precooled in about −15°C and stirred for 10 min. The supernatant was collected by centrifuging at 12 100*g* (MSE Europa M24) 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 12 100*g* (MSE Europa M24) for 10 min. The precipitate thus collected was dissolved in 20 ml of 0.1 M phosphate buffer, pH 6.4 (Yan *et al.*, 1990).

DEAE-Sepharose CL-6B column chromatography

Acetone-precipitated protease (35–38 ml) was applied to the DEAESepharose 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 (except where otherwise stated) produced by a Gradient Mixer GM-I (Pharmacia Fine Chemicals) and a further 100 ml of 0.5 M KCl, when 0–0.5 gradient was used, in the same buffer. A flow rate of 8 ml/cm^2/h (40 ml/h) and 12 ml/cm^2/h (60 ml/h) was used, and 4 and 6 ml fractions were collected using a LKB 2212 HeliRac fraction collector. The column was run at 4°C. Protein content of the eluate was estimated by monitoring the absorbance at 280nm using a LKB 2138 Unicord S UV monitor, and protease activity was monitored by measuring the activity of each fraction using the standard protease assay.

Protein determination

The protein concentration was determined using the method of Lowry *et al.* (1951).

Inhibition of proteases

The effect of inhibitors on protease activity was determined by preincubating the protease preparation with the inhibitor at room temperature for 30 min before assay and the residual activity was estimated by the standard protease assay at pH 8.2. Iodoacetamide, 1–10 phenanthroline, *p*-benzoquinone, *N*-diazoacetyl-DL-norleucine + Cu^{2+} (N-DANL + Cu^{2+}), phenylmethylsulphonyl fluoride (PMSF) and soybean trypsin inhibitor were dissolved in phosphate buffer (pH 7.0); $CoCl_2$ and $MnCl_2$ were dissolved in Tris–HCl buffer (pH 8.2) and $AgNO_3$ and $CuCl_2$ in distilled water. A control was run by pre-incubating the protease preparation with the appropriate solvent used to dissolve the inhibitors.

Determination of molecular weight

The molecular weights of proteases (separated by DEAE Sepharose column chromatography) were estimated by gel filtration with Sephacryl S-200 column (2.5×60 cm) employing cytochrome C (mol. wt 12 400), carbonic anhydrase (mol. wt 29 000), albumin (66 000), alcohol dehydrogenase (mol. wt 150 000) and β-amylase (mol. wt 200 000) as standard proteins. Blue dextran was employed to measure the void volume. The eluant was 0.05 M phosphate buffer (pH 7.0) containing 0.1 M KCl and 0.02% NaN_3, the flow rate was 8 ml/cm^2/h (40 ml/h) and the temperature 4°C. The protease samples were concentrated (by 10 times) using an Amicon

Ultrafiltrator (membrane YM5) before application to the column. The eluant volume was monitored for both absorbance at 280 nm and the protease activity.

Effect of endogenous proteases on phenolase activation
The influence of the endogenous proteases on phenolase activation was determined by comparing the activity increase of acetone-precipitated phenolase with and without added protease activity.

The separated proteases (I, II and III) were mixed with acetone precipitated phenolase in a ratio (1:3), respectively, adjusted at pH 6.7 and 8.2, and incubated on refrigeration.

Phenolase activity was 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 phosphate buffer (pH 6.4) and 0.8 ml of acetone-precipitated phenolase–protease mixture. 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 phenolase activity was expressed as the increase in absorbance (A) per min per ml of acetone-precipitated phenolase. The control comprised 0.35 M KCl in 10 mM phosphate buffer (pH 7.0) mixed with acetone-precipitated phenolase in the same ratio (3:1, respectively), adjusted to the above pH and incubated on refrigeration.

RESULTS AND DISCUSSION

Acetone precipitation

Higher yields of active Norway lobster proteases were obtained using acetone precipitation than using ammonium sulphate precipitation (Wang, 1993). Consequently, different ratios of crude extract volume:acetone volume, 1:0.5, 1: 1, 1:0.75, 1.25, 1.1 and 1.5, were investigated. However, the second procedure was found to be the most effective, giving both highest recoveries and specific activities. Thus, acetone precipitation, at the ratio 1:0.75 and then 1:1.25 (proteases volume:acetone volume), was used as the first step to partially purify proteases in Norway lobster and the acetone-precipitated protease was further used for purification and separation.

Separation and partial purification of proteases

When an eluant of potassium chloride solution, 0–0.5 M, was used as stated by Wang *et al.* (1991), three different protease peaks were obtained (Fig. 1). These results were similar to those were obtained by Wang *et al.* (1991) except that clearer separation of the initial two peaks was achieved in this work. This may be a result of the longer column used (40 cm instead of 35 cm used by Wang *et al.* (1991)) and from the slower flow rate (8 ml/cm^2/h, 40 ml/h instead of 12 ml/cm^2/h, 60 ml/h).

However, the three proteases were still eluted very close to each other and emerged almost at the end of the separation process. Consequently the initial ion strength of potassium chloride was increased to 0.3 M, i.e. a gradient from 0.3 to 0.5 M was used with the same flow rate (8 ml/cm^2/h, 40 ml/h). Although the peaks emerged much earlier there was no clear separation between protease II and III.

A further increase of the initial strength of potassium chloride to 0.35 M, together with an increase in flow rate from 8 ml/cm^2/h (40 ml/h) to 12 ml/cm^2/h (60 ml/h), resulted in a very clear separation of the three proteases which also emerged faster from the column (Fig. 2). This figure also shows that the majority of proteins are eluted before the proteases. The most active fractions of each protease peak were collected for further investigation.

A combination of acetone precipitation and DEAE-Sepharose CL-6B column chromatography was used to separate and purify the proteases in Norway lobster, the purity and yield of each step throughout the whole purification process is shown in Table 2. The improvement achieved in the separation process in this work

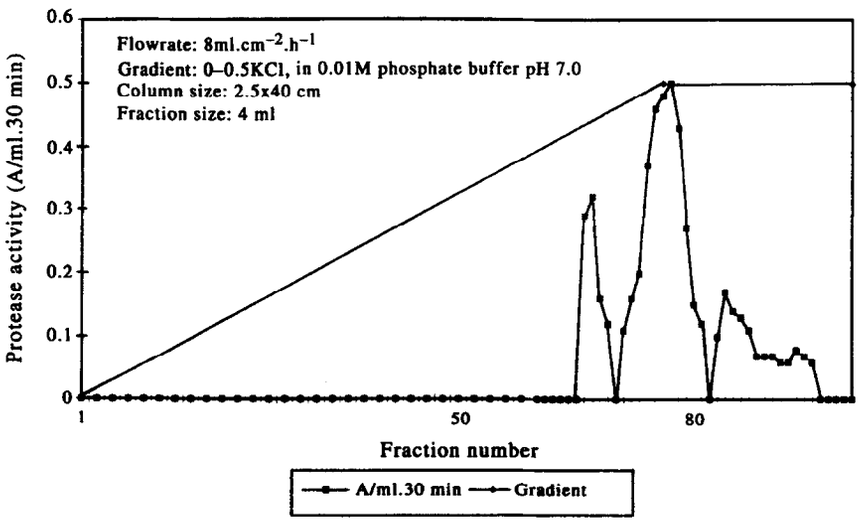

Fig. 1. Ion-exchange separation of acetone-precipitated protease activities. Flow rate, 8 ml/cm^2/h. Gradient, 0–0.5 M KCl in 0.01 M phosphate buffer, pH 7.0. Column size, 2.5×40 cm. Fraction size, 4 ml.

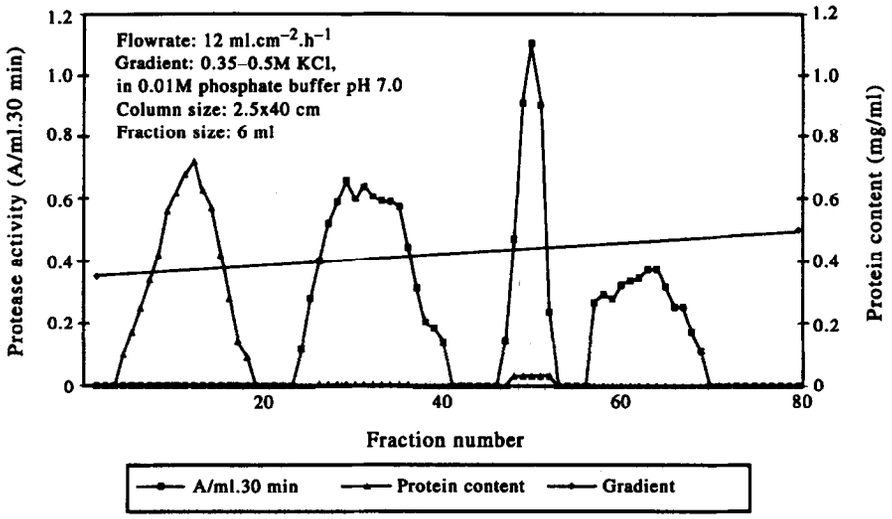

Fig. 2. Ion-exchange separation of acetone-precipitated protease activities. Flow rate, 12 ml/cm^2/h. Gradient, 0.35–0.5 M KCl in 0.01 M phosphate buffer, pH 7.0. Column size, 2.5×40 cm. Fraction size, 6 ml.

gave higher degrees of purification than those obtained by Wang *et al.* (1991). The overall purification at pH 8.2 was 63-, 25- and 217-fold, at pH 6.4, 40-, 25- and 160-fold in protease I, protease II and protease III, respectively. As can be observed the highest purification as well as the highest recoveries were obtained for the activity at pH 8.2 (Table 1). Considering that the crude protease contains the sum of the three activities at three peaks, the overall purification for each peak is actually higher than those calculated as above. However, at pH 2.4 most of the activity was lost during the procedure with the only activity found in protease II (a 12-fold purification). Wang *et al.* (1991) also recovered a small amount of activity at this pH in protease I.

Characterization of the partially purified proteases

pH optima

It was found that proteases I and II showed similar multiple pH optima with casein as substrate (Fig. 3). However, the pH profile of protease III was very different to proteases I and II, having most activity at around pH 8.2 with a small shoulder at pH 6.4 (Fig. 4).

Effect of protease inhibitors

The effect of a range of inhibitors on samples was investigated (Table 2). It was found that protease III was strongly inhibited by PMSF, trypsin inhibitor and 1–10 phenanthroline, Mn^{2+} and Co^{2+} (Table 2) indi-

Table 1. Purification scheme for proteases from Norway lobster

	Vol. (ml)	Protease activity (A/ml/30 min)	Proteincontent (mg/ml)	Specific activity (A/ml/30 min)	Recovery %
Crude extract					
pH 2.4		1.110 (0.011)		0.241	100
pH 6.4	120	0.750 (0.011)	4.6	0.163	100
pH 8.2		0.907 (0.010)		0.197	100
Acetone precipitated proteases					
pH 2.4		1.383 (0.003)		1.92	62
pH 6.4	60	1.033 (0.002)	0.72	1.44	69
pH 8.2		1.233 (0.003)		1.71	68
Protease I					
pH 2.4		0		0	0
pH 6.4	66	0.377 (0.003)	0.06	6.6	28
pH 8.2		0.710 (0.009)		12.5	43
Protease II					
pH 2.4		0.513 (0.004)		3.1	12
pH 6.4	30	0.693 (0.002)	0.17	4.1	23
pH 8.2		0.870 (0.004)		5.2	24
Protease III					
pH 2.4		0		0	0
pH 6.4	36	0.273 (0.003)	0.01	26	11
pH 8.2		0.553 (0.006)		52	18

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

cating clearly that it is a Zn-serine protease. The 100% inhibition was achieved by N-DANL + Cu^{2+} was half that obtained with the $CuCl_2$ alone. This alkaline protease has very similar characteristics to the alkaline protease from shrimp (Doke & Ninjoor, 1987). Protease I was inhibited by *p*-benzoquinone, iodoacetamide, heavy metals such as $AgNO_3$, $CuCl_2$ and 1–10 phenanthroline (Table 2) and so was characterized as a Zn-thiol

Table 2. Effect of some inhibitors on proteases from Norway lobster with no visible blackspot development

	Final concentration (mM)	Activity retained (%)		
		Protease I	Protease II	Protease III
Control	—	100	100	100
Iodoacetamide	2	54	79	75
1–10 phenanthroline	2	32	54	38
p-Benzoquinone	2	11	62	13
N-DANL + Cu	2	2	42	0
PMSF	2	92	89	0
Trypsin	0.5 mg/ml	103	111	0
$CoCl_2$	2	127	100	0
$MnCl_2$	2	92	104	0
$AgNO_3$	2	11	41	79
$CuCl_2$	2	8	39	58

N-DANL, *N*-diazoacetyl-DL-norleucine. PMSF, phenylmethylsulphonyl fluoride. Activity was measured at pH 8.2, with casein as substrate. Data are means of triplicate determinations.

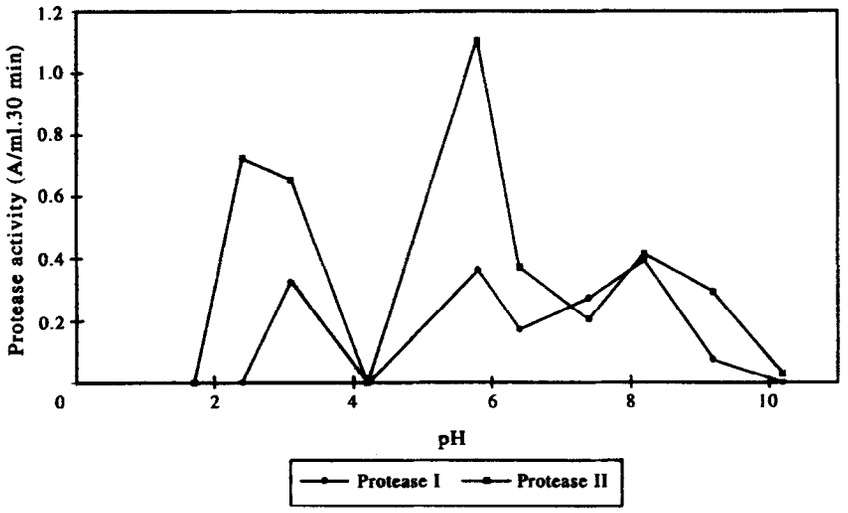

Fig. 3. Effect of pH on protease I and II. Protease I and II was prepared using a DEAE-Sepharose CL-6B, and protease activity was determined under the standard conditions with 0.2% casein as substrate. Data are means of triplicate determinations with a maximum value range of 0.010.

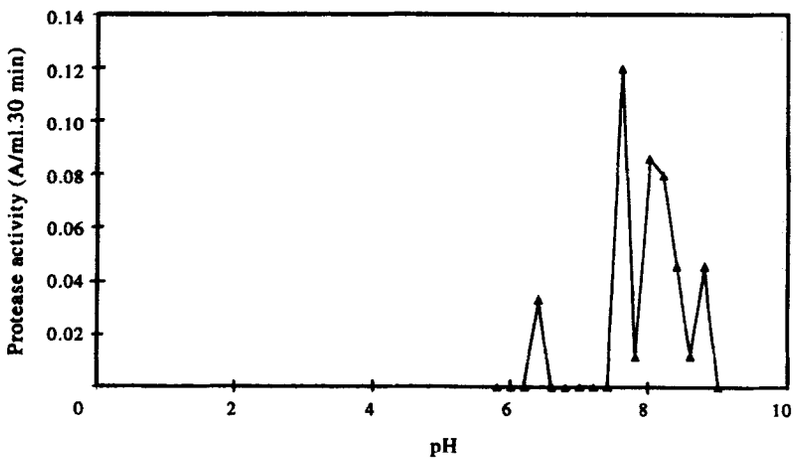

Fig. 4. Effect of pH on protease III. Protease III was prepared using a DEAE-Sepharose CL-6B, and protease activity was determined under the standard conditions with 0.2% casein as substrate. Data are means of triplicate determinations with a maximum value range of 0.005.

Table 3. Effect of some inhibitors on proteases from Norway lobster with visible blackspot development

	Final concentration (mM)	Activity retained %		
		Protease I	Protease II	Protease III
Control	—	100	100	100
Iodoacetamide	2	84	78	62
PMSF	2	114	31	243
Trypsin	0.5mg/ml	122	108	124
$CoCl_2$	2	40	98	40
$MnCl_2$	2	46	81	66
$AgNO_3$	2	42	62	87
1–10 phenanthroline	2	34	52	56
N-DANL + Cu^{2+}	2	16	56	51

N-DANL, *N*-diazoacetyl-DL-norleucine. PMSF, phenylmethylsulphonyl fluoride. Activity was measured at pH 8.2, with casein as substrate. Data are means of triplicate determinations. PMSF = Phenylmethylsulfonyl fluoride.

protease. Protease II was also inhibited by the same inhibitors as protease I but to a lesser extent and so was also characterized as a thiol protease. Protease II has very similar characteristics to the thiol protease found by Kimoto *et al.* (1986).

However, inhibition studies on proteases isolated from samples which had strongly visible blackspot development showed changes in inhibition, particularly with respect to protease III (Table 3). This indicates that the proteases from Norway lobster may change their properties on storage, and this could well be a factor in blackspot development.

Molecular weight

Protease I, the Zn-thiol protease, was estimated to have a molecular weight of 22.5 kDa (Fig. 5). Protease II, the second thiol protease, which was quite similar to the thiol protease, was found by Kimoto *et al.* (1986) in krill, had a molecular weight of 45 kDa, lower than the molecular weight reported by the above author. Two molecular weights were observed in the protease III fraction, namely 42.5 and 18 kDa. This could be due either to the dissociation of the alkaline protease into two subunits or to the existence of two different proteases.

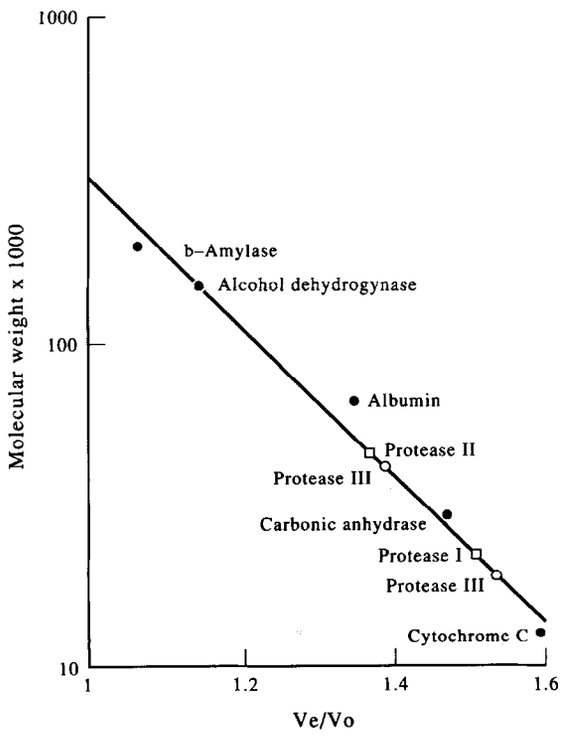

Fig. 5. Estimation of molecular weight of proteases by gel filtration.

Acetone precipitation of phenolase

The phenolase was precipitated with acetone from a ratio of 0:0.5 to a final ratio 1:1 (Yan *et al.*, 1990). The recovery of phenolase using this method was quite high (above 50%). However, it was also found that up to 30% of proteases were recovered in this precipitate.

Effect of the three endogenous proteases on phenolase activation

It was suggested that phenolase activation in Norway lobster is a result of proteolysis by endogenous protease activity, resulting in phenolase changing from its large less active form (mol. wt 667 kDa) into a smaller more active one (mol. wt 141 kDa) (Yan *et al.*, 1990). Although the role of protease activity in the process of phenolase activation was implicated in several studies on crustacean phenolase (Yan & Taylor, 1991) it was considered that protease III was responsible for the process, whilst proteases I and II were involved in the degradation of phenolase (Wang *et al.*, 1993) it was not clear whether the activation process needed any particular protease specificity. Therefore, it was of interest to study the influence of the three separated proteases by mixing them with acetone precipitated phenolase.(Fig. 6)

Acetone-precipitated phenolase was mixed with the separated proteases at a ratio 1:4, and incubated under refrigeration at pH 6.7 and pH 8.2. It was found that only protease II (thiol protease) initially accelerates phenolase activation at pH 6.7, which would be approximately the pH of live Norway lobster, whilst protease I (Zn-thiol protease) participates in phenolase activation when the incubation proceeds (above 9 h)

(Fig. 7). This may indicate that protease II and I (to a lesser extent) are mainly responsible for the initiation of blackspot development in Norway lobster.

It was observed that all proteases can activate phenolase at pH 8.2. Protease I initially seems to degrade phenolase but then, after longer periods of incubation, contributes to phenolase activation (as also observed at pH 6.7) to a greater extent than the other two proteases. This observation for protease I leads to the conclusion that this protease requires certain conditions to participate in the mechanism of blackspot development.

Although, the activity of protease III was more than 8-fold lower than the activity of the other two proteases (II and I), it rapidly activates phenolase at pH 8.2

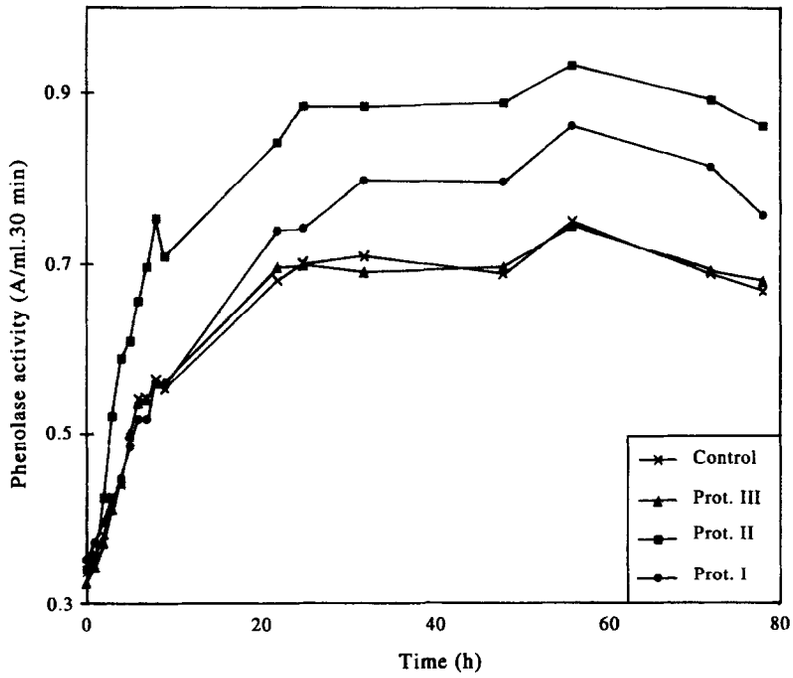

Fig. 6. Effect of endogenous proteases on phenolase activation at pH 6.7. Control, acetone-precipitated phenolase resuspended in 0.35 M KCl in phosphate buffer, pH 7.0, at a ratio of 1:4. Protease I, II and III: acetone-precipitated phenolase resuspended in the separated proteases I, II and III (after ion exchange) at a ratio of 1:4. Incubated at 3°C at pH 6.7.

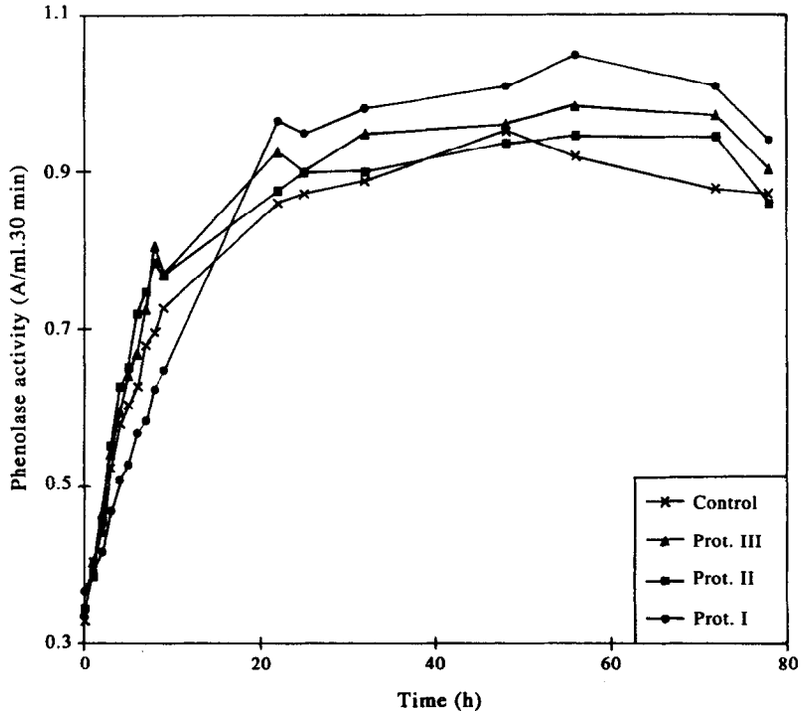

Fig. 7. Effect of endogenous proteases on phenolase activation at pH 8.2. Control: acetone-precipitated phenolase resuspended in 0.35 M KCl in phosphate buffer, pH 7.0, at a ratio of 1:4. Protease I, II and III: acetone-precipitated phenolase resuspended in the separated proteases I, II and III (after ion exchange) at a ratio of 1:4. Re-adjusted to pH 8.2 with 0.1 M NaOH and incubated at 3°C.

slightly more than protease II. This may indicate that the contribution of protease III in the mechanism of blackspot development might be considerable.

Furthermore, when the pH of Norway lobster heads was measured, using samples (with no visible blackspot development) which were almost alive before being frozen down to −20°C, it was found that the pH of the heads was 8.05, and after 5 h at 18°C the pH increased to 8.25. This might indicate that the alkaline pH range (above 8.00) is the critical pH range for blackspot development. At this alkaline pH area, protease III starts activating phenolase, whilst the other two proteases can activate phenolase from pH 6.7.

These results demonstrate that endogenous proteases can activate phenolase in Norway lobster. The proteolysis is limited by the availability of the Form 1 phenolase (less active form) which is converted by the responsible proteases to the more active Form II. After incubation at pH 8.2, where all proteases (III, II and I) can activate phenolase, the maximum activity was higher, was achieved sooner and declined more rapidly than at pH 6.7, where only two proteases (II and I) can activate phenolase.

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