Food Chemistry 45 (1992) 111–116

Studies on the protease activities in Norway lobster (*Nephrops norvegicus*) and their role in the phenolase activation process

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(Received 2 August 1991; revised version received and accepted 31 October 1991)

Proteolytic activity in Norway lobster (*Nephrops norvegicus*) was studied. Three proteases were separated and partially purified from Norway lobster heads by a combination of acetone precipitation and DEAE-Sepharose CL-6B column chromatography and designated as enzymes I, II and III. Enzyme III had a pH optimum at around 8·2 towards casein as substrate. Enzymes I and II were found to be very similar in most aspects; they showed multiple pH optima towards casein. Studies on sensitivity to different inhibitors suggested that enzymes I and II were likely to be thiol proteases and enzyme III a metal-dependent serine protease. It was found that only enzyme III was involved in the phenolase activation process in Norway lobster.

INTRODUCTION

Crustaceans are a very important fishery resource in many parts of the world. However, they are a delicate and highly perishable resource, very susceptible to spoilage during post-mortem processing and storage because of their unique biological and biochemical characteristics. 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).

Blackspot development, also known as melanosis and enzymic browning, is usually an initial spoilage of most species of crustaceans and is a result of enzymatically controlled oxidation of the amino acid, tyrosine, to the dark pigment (Yan *et al.*, 1989). The process requires phenolase which is normally an inert enzyme in intact crustaceans. Therefore, blackspot development is very likely to be initiated by phenolase activation (Yan *et al.*, 1990). Proteases are reported to be one of the main factors influencing phenolase activation (Yan & Taylor, 1991). However, it is not clear whether the activation process requires any particular protease specificity and what the exact role of protease in the process is.

Proteases have been purified from several species of crustaceans and their properties described (Kimoto *et al.*, 1981; Nip *et al.*, 1985; Chen & Zall, 1986). Some attempts have been made to identify the active protease activity responsible for mushiness development (Lindner *et al.*, 1988). However, there is no report yet on the study of proteolytic enzymes in Norway lobster, although their role in phenolase activation has been implicated (Yan, 1989). Studies on the proteases in Norway lobster could provide a better understanding of not only the characteristics of the enzymes but also their role in phenolase activation.

This paper reports the separation, partial purification and characterization of proteases in Norway lobster and the identification of active protease activity responsible for the phenolase activation process.

METHODS AND MATERIALS

Materials

Norway lobster (*Nephrops norvegicus*) used in the study were either supplied by Young's English Seafoods (Grimsby, UK) or by MAFF (North Shields, UK).

Food Chemistry 0308-8146/92/$05.00

They were delivered (held in ice) to the school of Food, Fisheries, and Environmental Studies and frozen and stored at −15°C for the experiments.

Methods

Preparation of crude protease

Fifteen grams 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.

Preparation of crude phenolase

One-hundred grams 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.

Assay of phenolase activity

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, 2·2 ml of 0·1 M phosphate buffer (pH 6·4) and 0·2 ml of the crude phenolase preparation (Ohshima & Nagayama, 1980). The absorbance at 530 nm was monitored at 25°C using a Pye Unicam PU8800 UV/Visible spectrophotometer. The increase of absorbance at 530 nm in the first 5 min was taken and the phenolase activity was expressed as the increase in absorbance (A) per minute per millilitre of crude phenolase.

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 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 9·4–10·0, 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 overnight at 4°C, the enzyme preparation was centrifuged at 12 100*g* (MSE Europa M24) for 15 min to remove inactive residues.

DEAE—Sepharose CL-6B column chromatography

Acetone-precipitated protease (35 ml) was applied to the DEAE–Sepharose CL-6B column (2·5 × 35 cm), equilibrated with 10 mM phosphate buffer (pH 7·0). The column was washed with 120 ml of starting buffer to remove unbound materials. Proteases were eluted with a linear gradient of 0–0·5 M KCl in 400 ml produced by a Gradient Mixer GM-1 (Pharmacia Fine Chemicals) and 100 ml of 0·5 M KCl in the same buffer. A flow rate of 60 ml/h was used 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 280 nm using a LKB 2138 Uvicord 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 pre-incubating the enzyme 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, trypsin inhibitor and $MgCl_2$ were dissolved in phosphate buffer (pH 7·0); $CaCl_2$, $CoCl_2$ and $MnCl_2$ were dissolved in Tris–HCl buffer (pH 8·2) and (*p*-chloromercuribenzoic acid (*p*CMB) and phenylmethylsulphonyl fluoride (PMSF) in dimethylsulphoxide (DMSO). A control was run by pre-incubating the enzyme preparation with the appropriate solvent used to dissolve the inhibitors.

Determination of molecular weight

The molecular weight of enzyme III was estimated by gel filtration with a Sepharose 6B column (1 × 90 cm)

employing carbonic anhydrase (mol. wt 29 000), albumin (mol. wt 66 000), alcohol dehydrogenase (mol. wt 150 000), apoferritin (mol. wt 443 000) and thyroglobulin (mol. wt 669 000) as standard proteins. Blue dextran was employed to measure the void volume. The flow rate was 8 ml/h and the temperature 4°C. The enzyme sample was concentrated (by 5 times) using an Amicon Ultrafiltrator (membrane YM10) before application to the column. The eluant volume was monitored for both absorbance at 280 nm and the enzyme activity.

RESULTS AND DISCUSSION

pH-activity profile of crude protease preparation

Protease activity in various species of crustaceans has been studied and many different types of protease activities have been reported, including alkaline (Doke & Ninjoor, 1987), acid (Kimoto *et al.*, 1981) and neutral (Noguchi *et al.*, 1976) types. Thus, the pH-activity of crude protease preparation in Norway lobster was initially examined. Figure 1 shows the relationship between the pH of assay buffer and protease activity of the crude protease preparation from Norway lobster heads, using casein as substrate. Because of the lower solubility of casein in low pH buffers, a lower concentration (0·1%) than that in the standard assay (0·5%) was used in this experiment in order to try to minimize any possible fluctuation caused by the difference in solubility of casein. Three maxima (each with a similar level of activity) were observed at pH 2·4, 6·4 and 8·2. However, it is difficult to interpret the acidic pH optima, since it may be caused by a decrease in activity observed near casein's isoelectric point (pH 4·6) due to its low solubility. Another substrate, bovine albumin (Sigma Chemicals) was also investigated and only one peak was found, with an optimum at about pH 3·0 (Fig. 1). These results suggest that there are probably several proteases present in the crude protease preparation from Norway lobster heads.

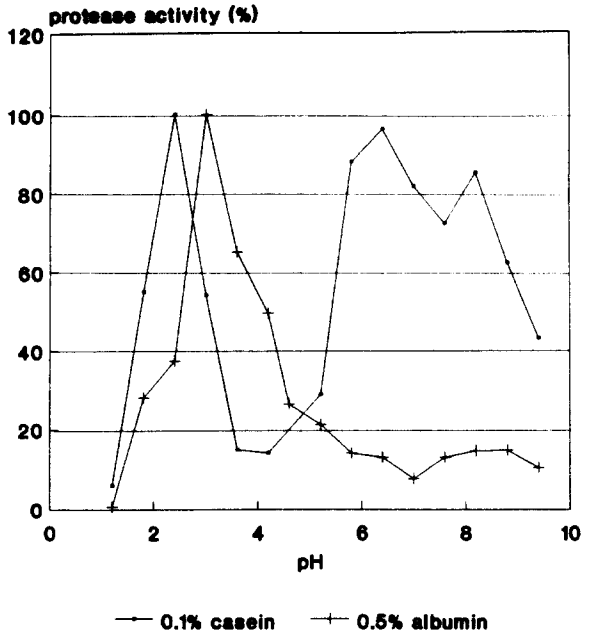

Fig. 1. pH curve of crude protease preparation for Norway lobster. (Data are means of triplicate determinations, and protease activity was measured using 0·1% casein and 0·5% albumin as substrate under the same conditions as in the standard assay.)

Separation and partial purification of proteases

Three proteases, named as enzymes I, II and III, were separated and partially purified from the crude protease preparation using a combination of acetone precipitation and DEAE-Sepharose CL-6B column chromatography (Fig. 2). Table 1 gives a summary of the purification scheme. Reasonable overall degrees of purification were achieved with activity at pH 8·2 in each peak, i.e. 57-fold, 24-fold and 22-fold for enzymes I, II and III, respectively, and high recovery for the

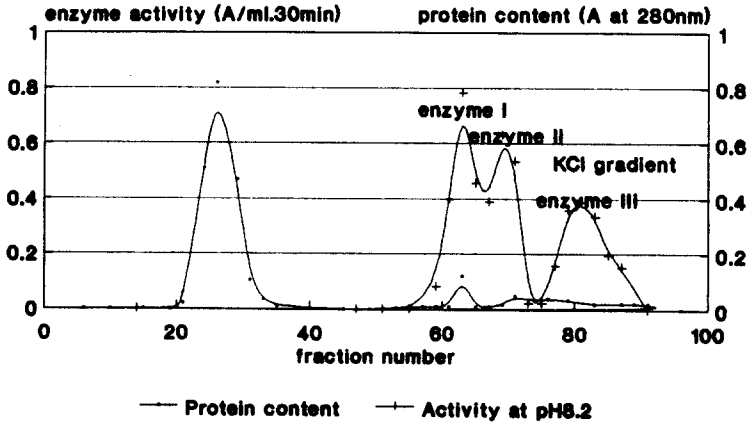

Fig. 2. Separation of protease activities using DEAE-Sepharose column chromatography (2·5 × 35 cm column, 60 ml/h flow rate, 6-ml fractions).

Table 1. Purification scheme for proteases from Norway lobster

	Activity (A/ml per 30 min)	Protein (mg/ml)	Specific activity (A/mg per 30 min)	Ratio of activities	Recovery (%)
Crude					
pH 8·2	1·167		0·187	1	100
6·4	1·640	6·25	0·262	1	100
2·4	1·707		0·273	1·5	100
Acetone-precipitated protease					
pH 8·2	1·527		1·339	1	65·4
6·4	1·823	1·14	1·600	1·19	55·6
2·4	1·343		1·180	0·88	39·3
DEAE-Sepharose CL-6B					
Enzyme I					
pH 8·2	0·433		10·77	1	15·9
6·4	0·373	0·0402	9·28	0·86	9·7
2·4	0·193		4·80	0·44	4·8
Enzyme II					
pH 8·2	0·367		4·503	1	13·4
6·4	0·280	0·0815	3·435	0·76	7·3
2·4	0·177		2·171	0·48	4·4
Enzyme III					
pH 8·2	0·337		4·202	1	12·4
6·4	0·113	0·0802	1·409	0·33	2·9
2·4	0		0	0	0

activity at pH 8·2 was also obtained. As the crude protease contains the sum of activities of the three enzymes, the overall purification for each enzyme is actually higher than those calculated as above. However, much of the activities at both pH 6·4 and 2·4, particularly the latter, were lost during the procedure. The

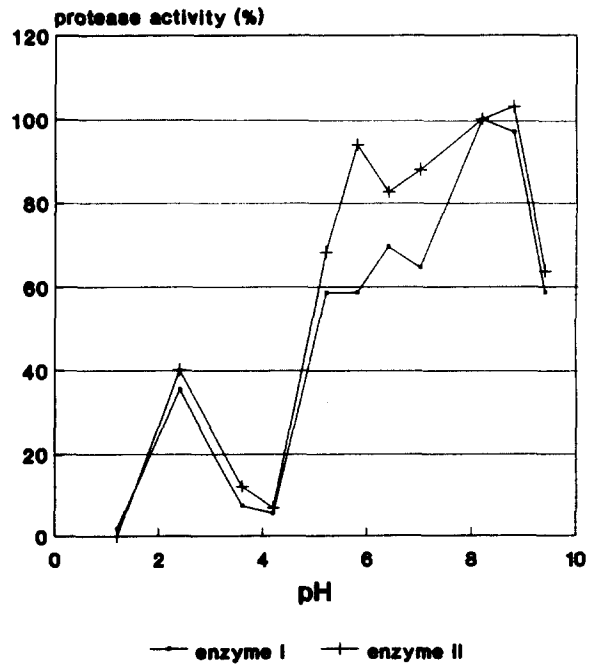

Fig. 3. The effect of pH on enzymes I and II. (Data are means of triplicate determinations, and protease was measured using the standard assay.)

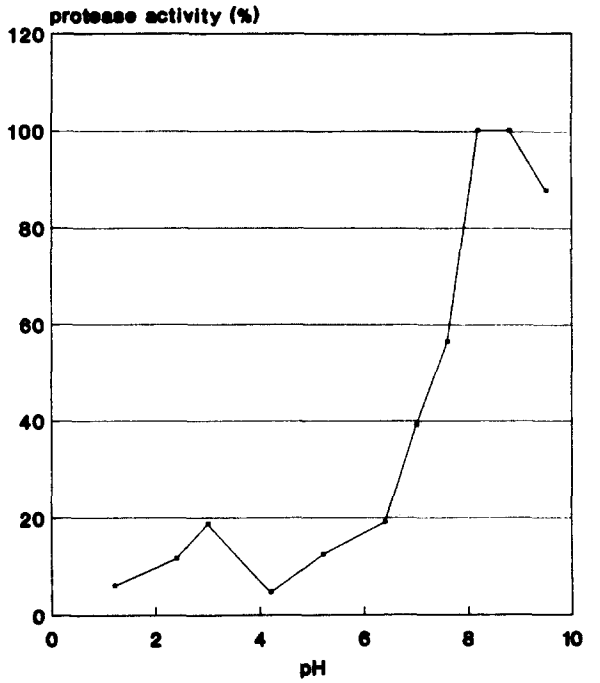

Fig. 4. The effect of pH on enzyme III. (Data are means of triplicate determinations, and protease activity was measured using the standard assay.)

reason for this is not known, but it may be that the enzyme activities at these pH values may need certain activators in order to have full strength of activity. The column chromatography may separate them; therefore, the activities at pH 6·4 and 2·4 may have been considerably lost during the procedure.

Characterization of the partially purified proteases

pH optima

pH optima of enzymes I and II are shown in Fig. 3. It was found that these two enzymes showed multiple pH optima with casein as substrate. Optimum activity of enzyme II occurred at around pH 2·4, 5·8 and 8·2.

Table 2. Effects of inhibitors on protease activity of each enzyme

Compound added	Final concentration (mM)	Activity retained (%)		
		I	II	III
Control	—	100	100	100
Iodoacetamide	2	66	60	94
Trypsin inhibitor	1 (mg/ml)	92	93	68
*p*CMB	2	107	267	142
PMSF	2	90	150	94
$CaCl_2$	2	104	111	111
$CoCl_2$	2	87	88	0
$MnCl_2$	2	87	101	29
$MgCl_2$	2	82	89	100
EDTA	5	121	127	93
8-Hydroxyquinoline	2	108	119	125

N.B. Residual protease activity was measured using the standard assay at pH 8·2 and results are means of triplicate determinations.

Enzyme I had two pH optima, at around pH 2·4 and 8·2 and a very small shoulder at pH 6·4. The effect of pH on the activity of enzymes III is shown in Fig. 4. The pH profile of enzyme III was very different from enzymes I and II, having most activity at around pH 8·2 with only a small shoulder at pH 3·0, which could be an artifact resulting from the reduced solubility of casein near its isoelectric point.

Effect of protease inhibitors

The effects of several protease inhibitors and metal ions on the protease activity of each enzyme fraction are shown in Table 2. Enzymes I and II are very similar in many aspects. Both of then were inhibited by iodoacetamide and stimulated by ethylene diamine tetraacetic acid (EDTA), suggesting that they are likely to be thiol proteases (Doke & Ninjoor, 1987). Trypsin inhibitor caused some inhibition of the activity of enzyme III, and 8-hydroxyquinoline stimulated the activity. Cations such as Co^{2+} and Mn^{2+} inhibited the activity. These properties of enzyme III are very similar to those of an alkaline proteases from shrimp (*Penaeus indicus*) muscle, which was suggested to be a metal-dependent serine protease (Doke & Ninjoor, 1987).

Both *p*CMB and PMSF were dissolved in DMSO, which is commonly used in the study of protease inhibitors as a solvent for those inhibitors that are not water soluble (Chen & Zall, 1986). However, results were compared with controls containing DMSO, which itself had a strong inhibitory effect (greater than 50%) on enzymes I and II. This additional inhibition could possibly mask any effect of *p*CMB and PMSF on enzymes I and II.

Molecular weight

Molecular exclusion chromatography on Sepharose 6B indicated that the molecular weight of enzyme III is 27 000.

Identification of the active protease responsible for phenolase activation

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 not clear whether the activation process needs any particular protease specificity. Therefore, it was of interest to study the influence of three proteases on the activation process by adding different inhibitors. It was found that enzyme III inhibitors (Co^{2+} and Mn^{2+}) inhibited phenolase activation but that inhibitor of enzymes I and II (iodoacetamide) had little effect on the activation process (Fig. 5). This suggests that only enzyme III, which appears to be an alkaline metal-dependent serine protease, is involved in the phenolase activation process in Norway lobster.

Crude phenolase preparations were made using phosphate buffer (pH 6·4) and Tris buffer (pH 8·2) and phenolase activity monitored. The initial rate of phenolase activation in pH 8·2 buffer was much higher than that in pH 6·4, but phenolase activity reached similar maxima after 6–8 h (Fig. 6). This supports the role of enzyme III in the activation process, as this enzyme had a much lower activity at pH 6·4 than at pH 8·2. The initial form of the Norway lobster phenolase (Form I) is converted to Form II (the more active

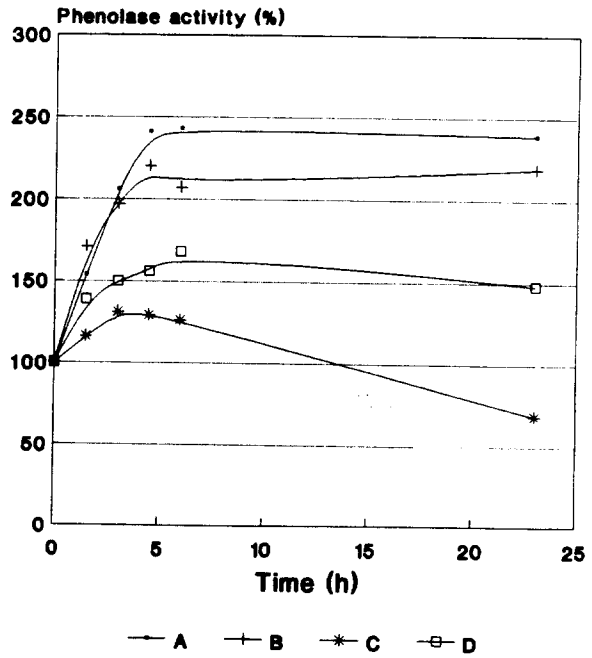

Fig. 5. The effect of protease inhibitors on phenolase activation: blank (A), with 2 mM iodoacetamide (B), with 2 mM $CoCl_2$ (C), with 2 mM $MnCl_2$ (D).

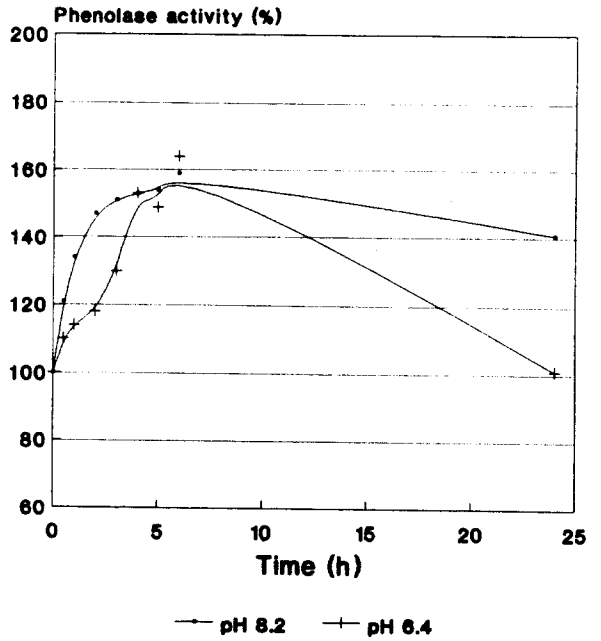

Fig. 6. Phenolase activation in different pH buffers: crude phenolase prepared in Tris buffer (pH 8·2) and phosphate buffer (pH 6·4).

form) by protease activity (Yan & Taylor, 1991). Thus, it would be expected that the maximum phenolase activity would be limited by the initial availability of Form I. However, after 24 h, a lower phenolase activity level was observed at pH 6·4 than at pH 8·2, and this could be due to either more degradation or less activation in the period.

ACKNOWLEDGEMENT

The authors are grateful to Mr T. Thurston for his assistance in acquiring fresh *Nephrops norvegicus* samples.

REFERENCES

Chen, H. C. & Zall, R. R. (1986). Partial purification and characterisation of cathepsin D-like and B-like acid proteases from Surf clam viscera. *J. Food Sci.*, **51** 71–8.

Doke, S. N. & Ninjoor, V. (1987). Characterisation of alkaline proteinase and exopeptidaes from shrimp (*Penaeus indicus*) muscle. *J. Food Sci.*, **52**, 1203–11.

Ferrer, O. J., Koburger, J. A., Otwell, W. S., Gleeson, R. A., Simpson, B. K. & Marshall, M. R. (1989). Phenoloxidase from the cuticle of Florida spiny lobster (*Panulirus argus*): mode of activation and characterisation. *J. Food Sci.*, **54**, 63–7, 176.

Kimoto, K., Thanh, V. V. & Murakami, K. (1981). Acid proteinases from Antarctic krill, *Euphausia superba*: partial purification and some properties. *J. Food Sci.*, **46**, 1881–4.

Lindner, P., Angel, S., Weinberg, Z. G. & Granit, R. (1988). Factors inducing mushiness in stored prawns. *Food Chem.*, **29**, 119–32.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, K. J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, **193**, 265–75.

Nip, W. K., Lan, C. Y. & Moy, J. H. (1985). Partial characterisation of a collagenolytical enzyme fraction from the hepatopancreas of the freshwater prawn, *Macrobrachium rosenbergii*. *J. Food Sci.*, **50**, 1187–8.

Nishimura, K., Kawamura, Y., Matoba, T. & Yonezawa, D. (1983). Classification of proteases in Antarctic krill. *Agric. Biol. and Chem.*, **47**, 2577–83.

Noguchi, A., Yanagimoto, S., Umeda, K. & Kimura, S. (1976). Purification and some properties of proteases of *Euphausia superba*. *J. Agric. Chem. Soc. Japan*, **50**, 415.

Ohshima, T. & Nagayama, F. (1980). Purification and properties of catechol oxidase from the Antarctic krill. *Bull. Jap. Soc. Sci. Fisheries*, **48**, 1035–42.

Yan. X. (1989). Blackspot development and phenolase in Norway lobster (*Nephrops norvegicus*). PhD thesis, CNAA, School of Food, Fisheries and Environmental Studies, Humberside Polytechnic, Grimsby, UK.

Yan, X. & Taylor, K. D. A. (1991). Studies on the mechanism of phenolase activation in Norway lobster (*Nephrops norvegicus*). *Food Chem.*, **41**, 11–12.

Yan, X., Taylor, K. D. A. & Hanson, W. S. (1989). Studies on the mechanism of blackspot development in Norway lobster (*Nephrops norvegicus*). *Food Chem.*, **34**, 273–83.

Yan, X., Taylor, K. D. A. & Hanson, W. S. (1990). Phenolase in Norway lobster (*Nephrops norvegicus*): activation and purification. *Food Chem.*, **36**, 19–30.