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Studies of the Mechanism of Phenolase Activation in Norway Lobster (*Nephrops norvegicus*)

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ABSTRACT

The mechanism of phenolase activation in Norway lobster was studied. It was found that phenolase activation in Norway lobster requires a protease. Trypsin and a bacterial protease from Streptomyces griseus, *accelerate the rate of increase of phenolase activity in crude phenolase preparations from Norway lobster, but have little effect on the enzyme after being partially purified by acetone precipitation. It was also found that the process requires another factor(s), which has low molecular weight and is probably located in a subcellular fraction mainly containing the microsomes. Therefore the increase of phenolase activity appears to be a multiple component process and protease alone does not activate the phenolase activity in Norway lobster.*

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 a cuticular sclerotizing agent (Cobb, 1977). The enzyme in Norway lobster has been shown to be capable of catalysing the oxidation of tyrosine, or its derivatives, resulting in darkening of Norway lobster slurry and presumably in blackspot development (Yan, 1989). The phenolase was shown to be tyrosinase (EC 1.14.18.1), which had both monophenolase and diphenolase activities (Yan *et al.*, 1989).

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Phenolase in insects is very frequently reported to be a mixture of inert proenzymes that require activation (Brunet, 1980). However, the mode of activation is still not fully understood, although most evidence indicates that the process is the result of proteolysis (Brunet, 1980), as many researchers have reported that activators were proteins and that the activation process was paralleled by trypsin, chymotrypsin, and alphachymotrypsin (Ashida & Ohnishi, 1967; Ohnishi *et al.*, 1970; Preston & Taylor, 1970). Sodium dodecyl sulphate (Funatsu & Inaba, 1962), fatty acids (Heyneman & Vercauteren, 1968), acetone and alcohols (Preston & Taylor, 1970) and microbial products (Pye, 1974; Ratclife *et al.*, 1984) have also been observed to activate prophenolases. The mode of activation is not yet clarified.

Little research regarding this activation phenomenon can be found in the seafood area (Ferrer *et al.*, 1989; Yan, Taylor & Hanson, 1990). Phenolases from crustaceans have been found to become active during refrigerated storage and the process was associated with the appearance of a new band of phenolase on an electrophoresis chromatogram (Ferrer *et al.*, 1989; Yan *et al.*, 1990). Natural and activated forms of phenolase have been separated and partially purified (Yan, 1989). Protease was also demonstrated to be involved in the process (Savagaon & Sreenivasan, 1978; Marshall *et al.*, 1984; Ferrer *et al.*, 1989).

Although a majority of the studies have suggested a simple mode of proteolysis for phenolase activation, there have been reports illustrating a more complicated process (Brunet, 1980). The work done by Seybold *et al.* (1975) indicated that the activation process in *Drosophila* phenolase involved at least three steps. Soderhall and Hall (1984) also found that lipopolysaccharides from *Escherichia coli* and *Salmonella abortus* activated a protease, which then caused the activation of phenolase. Further research is needed to study this complex aspect of the process.

A better understanding of the mechanism of phenolase activation may also help to develop alternative methods to sulphites to control post mortem blackspot development, since the original level of phenolase in crustaceans is quite low and melanosis is influenced by the process of phenolase activation (Yan *et al.*, 1990).

The present investigation further examines the possible mechanism of phenolase activation in Norway lobster.

MATERIALS AND METHODS

Preparation of crude enzyme

A hundred grams of frozen Norway lobster heads was homogenized with 150 ml 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 enzyme preparation (Ohshima & Nagayama, 1980).

Assay of phenolase

Spectrophotometric assay
In the proline-catechol spectrophotometric assay, the reaction mixture contained 0·2 ml of 0·5M catechol, 0·2 ml of 0·5M L-proline, 2·2 ml of 0·1M phosphate buffer (pH 6·4), and 0·2 ml of crude enzyme preparation (Ohshima & Nagayama, 1980). The absorbance at 530 nm was monitored at 25°C using a Pye Unicam PU880 UV/Visible spectrophotometer. The increase of absorbance at 530 nm in the first 5 min was taken and the enzyme activity was expressed as the increase in absorbance/min/ml of crude enzyme.

Oxygen uptake assay
In the oxygen uptake assay, the reaction mixture contained 0·2 ml of 0·5M catechol (or other substrates at an appropriate concentration), 2·4 ml of 0·1M phosphate buffer (pH 6·4) and 0·2 ml of the crude enzyme preparation. The oxygen uptake was monitored at ambient temperature (20 ± 2°C) with an oxygen electrode. The activity was expressed in nmol O_2/min/ml of crude enzyme (Ohshima & Nagayama, 1980).

Acetone precipitation

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

Effect of protease

The influence of protease on phenolase activity was determined according to the method of Savagaon and Sreenivasan (1978) by comparing the activity of the phenolase with and without protease. Crude or partially purified enzyme preparations (1 ml) were incubated either with or without protease in an ice bath for 30 min prior to assay (unless stated otherwise) for the

phenolase activity using the standard spectrophotometric assay. The protease used was either 2 mg trypsin (beef pancreas, Fisons, 0·5 Anson unit per gram) or 2 mg bacterial protease (an unusually non-specific protease from *Streptomyces griseus*, Sigma product, 5·8 units per gram).

Effect of protease inhibitors

Effect of protease inhibitor was studied in a similar method to that used by Ferrer *et al.* (1989).

Crude phenolase was prepared as in the standard crude enzyme preparation method except that it was centrifuged at 50 000*g* for 30 min rather than 20 min. The crude enzyme was divided into several aliquots of equal volumes and aliquots were treated with either solid aprotinin or solid trypsin inhibitor to give a final concentration of 0·2%. The mixture was kept in an ice bath for specified periods of time prior to phenolase assay. The phenolase activity was measured by the standard spectrophotometric assay as described above.

Sequential precipitation

Norway lobster head was homogenized and centrifuged according to the scheme in Table 1 to investigate the separation and subcellular location of factors involved in the activation of phenolase.

TABLE 1
Sequential Precipitation Scheme

40 g Norway lobster head and 100 ml 0·1M phosphate buffer, pH 6·4	
Yastral homogenizer, 2 min	
homogenate (slurry)	
centrifuging at 700 g for 10 min, at 4°C MSE Europa M24	
supernatant (1)	precipitate (1)
3 300 g for 10 min, at 4°C	
supernatant (2)	precipitate (2)
16 000 g for 20 min, at 4°C	
supernatant (3)	precipitate (3)
50 000 g for 30 min, at 4°C	
supernatant (4)	precipitate (4)

Effect of concentration

Crude enzyme was concentrated using an Amicon ultrafiltration cell, model 8200 (with membrane YM2, which allows compounds with a molecular weight 5 000 or less to pass through) immediately after preparation, and kept in an ice bath for periods as specified. The effect of concentration was illustrated by comparing the rate of activity increase of preparations with different dilutions. Enzyme activity was estimated using the standard spectrophotometric assay.

Protease assay

The reaction mixture contained 2·5 ml 0·5% casein in 0·1 M phosphate buffer, pH 6·4, and 0·5 ml supernatant or slurry prepared using the standard crude enzyme preparation method. The reaction mixture was incubated in a water bath at 40°C for 30 min. Three millilitres of 5% (w/v) trichloroacetic acid (TCA) was then added to the reaction mixture. It was allowed to stand for 50 min at room temperature. The resultant precipitate was removed by filtration through Whatman No. 1 filter paper. The filtrate was diluted four times before its absorbance was measured at 280 nm in a 1 cm silica cell. A blank was run by adding 3 ml TCA (5%) before incubation. The enzyme activity was expressed as absorbance at 280 nm.

RESULTS AND DISCUSSION

Effect of protease on crude enzyme activation

Activation of phenolase from Norway lobster was observed to take place immediately after making the crude enzyme preparation (Yan *et al.*, 1990). The maximum activity was normally reached 8–12 h after the preparation. The original and active forms of the phenolase have been previously separated and partially purified. The properties of the two forms were studied (Yan *et al.*, 1990). However, the activation mechanism was not fully understood.

When trypsin was added to the crude enzyme preparation, in a ratio of 2 mg trypsin to 1 ml crude enzyme, the activity was observed to increase very rapidly and reach a maximum within minutes, while without trypsin the process normally needed hours (Fig. 1). The activation by protease can also be achieved by a very broad protease (Sigma product, from *Streptomyces griseus*). When this protease was added to the crude enzyme preparation, rapid increase of rate of activation was observed (Fig. 1), and maximum activity was also reached within minutes.

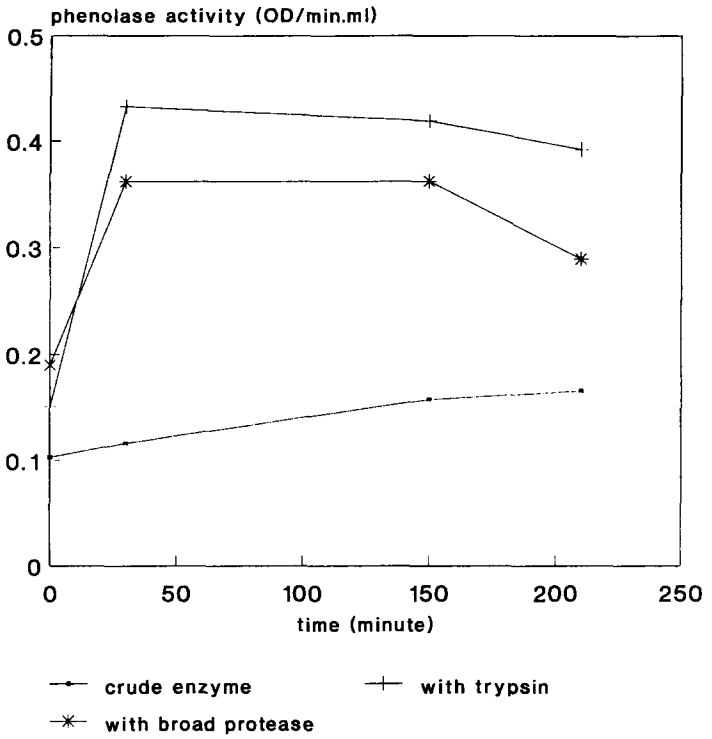

Fig. 1. Effect of added protease on the activation of phenolase. (Phenolase activity was measured using the standard spectrophotometric assay. Data were means of triplicate determinations and a similar trend was observed in a second experiment. Phenolase activity was measured immediately after adding protease.)

These results indicate that a protease may be involved in the increase of activity. This, as observed in studies on activation of phenolase in insects (Brunet, 1980) and more recently in crustaceans (Ferrer *et al.*, 1989), may lead to a hypothesis of limited proteolysis as the mode of activation. However, trypsin did not accelerate the rate of activation of the phenolase which had been partially purified by acetone precipitation, although the partially purified phenolase itself still showed very significant activation (Yan *et al.*, 1990). There is general agreement about the involvement of protease in the activation of phenolase in crustaceans and the activation mechanism of crustacean phenolase seems to be different from that of plant phenolase, which normally is the result of either release of bound phenolase into the soluble fraction or dissociation of phenolase and natural inhibitor/substrate (Brunet, 1980). However, the function of protease in the process is not fully understood and particularly it is not clear whether any other factors are also involved in the process. Phenolase in Norway lobster is a soluble enzyme and activation involves a conversion of a large form into a smaller, more active,

TABLE 2
Influence of Protease Inhibitors on Phenolase Activation

	Phenolase activity (OD/ml.min)		
	0 h	*4 h*	*24 h*
Control (crude enzyme)	0·088	0·147	0·212
	(0·005)	(0·002)	(0·003)
With 0·2% trypsin inhibitor	0·081	0·109	0·104
	(0·005)	(0·004)	(0·002)
With 0·2% aprotinin	0·097	0·130	0·107
	(0·010)	(0·005)	(0·004)

NB: Results were means of triplicate determinations and figures in parentheses are standard deviation of the means.

form (Yan *et al.*, 1990). However, the difference of effect of protease on the activation of crude and partially purified Norway lobster phenolase indicates that this phenolase activation may require other factor(s) and that, without the presence of these factor(s), protease cannot accelerate the process. A multiple stage process has been observed in a study on *Drosophila* phenolase (Seybold *et al.*, 1975). However, most studies on phenolase in insects as well as in crustaceans have reported simple proenzyme and activator interactions. The proposal of Brunet (1980) is that, in these situations, the activator is already 'mature', having itself already undergone activation when it is presented to the proenzyme, which has been described as the initial natural form (Yan *et al.*, 1990).

Effect of protease inhibitors on activation

Crude enzyme was divided into several aliquots of equal volume and each aliquot was treated with either solid trypsin inhibitor or aprotinin at a concentration of 0·2% (w/v). Both inhibitors decreased the activation, but the inhibition was a rather slow process (Table 2). Protease inhibitors have also been observed to inhibit activation of phenolase in other crustaceans (Ferrer *et al.*, 1989). This may be explained as further evidence for the participation of protease in the activation.

Effect of concentration on activation

When crude enzyme was diluted immediately after preparation, the phenolase activity was also reduced in the same proportion as the dilution. However, rate of activity increase was changed dramatically. After 4 h, the original crude enzyme had over 60% increase in activity, while the activity of

samples which had been diluted twice increased only about 20% (Fig. 2). A similar effect of concentration has also been observed in a study of insect larvae (Pye, 1974).

The concentration effect was further investigated by concentrating the crude enzyme using an ultrafiltration cell model 8200 (membrane YM2). The membrane allows compounds with a molecular weight of 5000 or less to pass through, but retains larger molecules. When crude enzyme was concentrated to half its original volume, the initial enzyme activity per millilitre doubled. However, the pattern of percentage activation remained similar to unconcentrated samples (Fig. 2). When the concentrated sample was diluted to its original volume, the initial phenolase activity per millilitre was the same as that of the original crude enzyme. However, the rate of activation was less than half that of the crude enzyme.

This experiment further indicates the participation of another factor(s) in the phenolase activation and illustrates that the other factor(s) has a low molecular weight, and is present in concentrations low enough to be one of

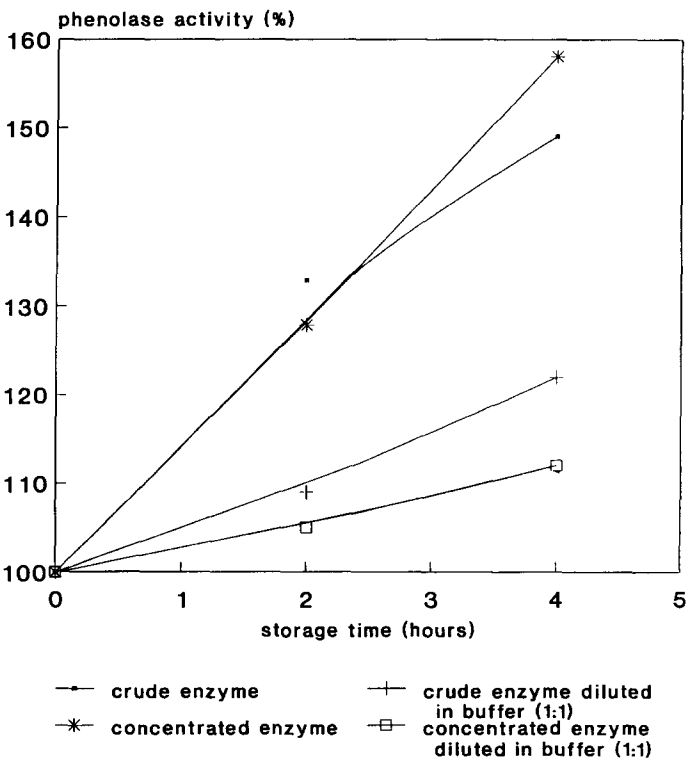

Fig. 2. Influence of concentration on activation. (Enzyme activity, measured using the standard spectrophotometric assay, is expressed as percentage of the activity at time zero. Data were means of triplicate determinations and a similar trend was observed in a second experiment.)

the factors affecting the rate of activation. Ultrafiltration allows it to pass through the membrane, and therefore its concentration remains the same in concentrated crude enzyme preparations. Consequently, the rate of activation was not changed.

Effect of sequential precipitation (subcellular organelles) on activation

When the enzyme was kept with the rest of the slurry, i.e. the whole homogenate without being centrifuged, a higher activity could be reached and the maximum could be maintained for a longer time (Yan *et al.*, 1990). It is thus of interest to investigate which subcellular organelle(s) is responsible for the difference and what produces the difference.

The homogenate was centrifuged at different forces in order to remove, step by step, various subcellular organelles. In order to achieve better separation of subcellular organelles, a higher ratio of phosphate buffer to lobster was used (100 ml buffer to 40 g Norway lobster head, compared to the normal preparation 60 ml buffer to 40 g). Figure 3 illustrates that the

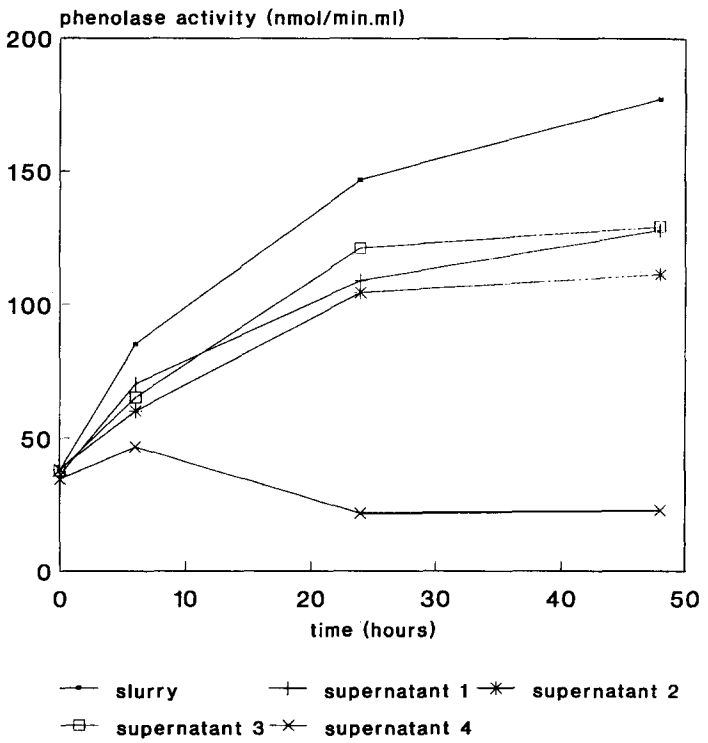

Fig. 3. Influence of sequential precipitation (subcellular organelles) on activation. (Phenolase activity was measured using the standard oxygen uptake assay. Data were means of triplicate determinations and the trend was typical of three separate experiments.)

TABLE 3
Comparison of Protease Activity

Supernatant prepared on day 0		*Supernatant separated from slurry on day 1*		*Sample size*	*Significance of difference*
Mean	*SD*	*Mean*	*SD*	*n*	
0·242	0·037	0·291	0·059	6	not significant

NB: Level of significance was determined by analysis of variance.

activation pattern of the supernatant remains almost the same as that of slurry until the fourth centrifugation. The force of 12 400*g* for 20 min used in the third centrifuging is considered to be able to remove all organelles other than microsomes and 50 000*g* for 30 min in the fourth centrifuging can remove microsomes. No experiment was carried out to check the purity of each fraction, but it is probable that the difference was due to microsomes.

A further investigation was carried out to determine which component was responsible for the difference in activation. One presumption might be that a constant release of protease from microsomes maintained the higher rate of activation. However, Table 3 indicates that there was no significant difference between protease activity in the initial supernatant and that separated from the slurry after 1 day storage at 4°C, which supports the hypothesis of a multiple component process.

CONCLUSION

It was demonstrated in this investigation that the process of phenolase activiation in Norway lobster involves a protease and another factor(s) of low molecular weight, which is possibly located in microsomes. The initial natural form of the phenolase has a molecular weight of 667 000 and that of the active form is 141 000 (Yan *et al.*, 1990), so it is likely that protease may directly modify the phenolase but the activation is very likely a multiple component process. The identify and role of other factor(s) need further investigation.

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REFERENCES

Ashida, M. & Ohnishi, E. (1967). Activation of pre-phenoloxidase in haemolymph of the silkworm, *Bombyx mori*, *Archives of Biochemistry and Biophysics*, **122**, 411–16.

Brunet, P. C. (1980). The metabolism of the aromatic amino acids concerned in the cross-linking of insect cuticle, *Insect Biochemistry*, **10**, 467–500.

Cobb, B. F. (1977). Biochemistry and physiology of shrimp: Effect on use as food. In *Proceedings of the Conference on the Handling, Processing, and Marketing of Tropical Fish*, London, Tropical Products Institute, pp. 405–11.

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 characterization, *Journal of Food Science*, **54**(1), 63–7, 176.

Funatsu, M. & Inaba, T. (1962). Studies on tyrosinase in housefly, Part 1, protyrosinase in the pupae of the housefly and its activation, *Agriculture Biology and Chemistry*, **26**, 535–40.

Heyneman, R. A. & Vercauteren, R. E. (1968). Evidence for a lipid activator of prophenolase in *Tenebrio molitor*, *Journal of Insect Physiology*, **14**, 409–15.

Marshall, M. R., Otwell, W. S. & Walker, B. (1984). Preliminary study on the isolation and activation of polyphenoloxidase from deep sea crab (*Geryon* spp.), *Proceedings of the Ninth Annual Tropical and Subtropical Fisheries Conference of the Americans*, **9**, 118.

Ohnishi, E., Dohke, K. & Ashida, M. (1970). Activation of prophenoloxidase, 2, activation by β-chymotrypsin, *Archives of Biochemistry and Biophysiology*, **139**, 143–8.

Ohshima, T. & Nagayama, F. (1980). Purification and properties of catechol oxidase from the Antarctic krill, *Bulletin of the Japanese Society of Scientific Fisheries*, **46**(8), 1035–42.

Preston, J. W. & Taylor, R. L. (1970). Observations on the phenoloxidase system in the haemolymph of the cockroach *Leucopea muderae*, *Journal of Insect Physiology*, **16**, 1729–44.

Pye, A. (1974). Microbial activation of prophenoloxidase from immune insect larvae, *Nature (London)*, **251**, 610–13.

Ratclife, N. A., Leonard, C. & Rowley, A. F. (1984). Prophenoloxidase activation in insect immunity, *Science*, **226**, 557–9.

Savagaon, K. A. & Sreenivasan, A. (1978). Activation mechanism of pre-phenoloxidase in lobster and shrimp, *Fishery Technology*, **15**, 49–55.

Seybold, W. D., Meltzer, P. S. & Mitchell, H. K. (1975). Phenol oxidase activation in *Drosophila:* A cascade of reactions, *Biochemical Genetics*, **13**, 85–108.

Soderhall, K. & Hall, L. (1984). Lipopolysaccharide induced activation of prophenoloxidase activating systems in crayfish haemocyte lysate. *Biochemica et Biophysica Acta*, **791**, 99–104.

Yan, X. (1989). Blackspot development and phenolase in Norway lobster, PhD Thesis, CNAA, School of Food and Fisheries Studies, Humberside College of Higher Education.

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

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