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Further studies on the roles of proteases in the activation of phenolase from Norway lobster (*Nephrops norvegicus*)

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Phenolase activation in Norway lobster (*Nephrops norvegicus*) was found to be a result of proteolysis. Endogenous protease activity is shown to activate the phenolase activity directly. The three protease activities identified in Norway lobster all affected the phenolase-activation process. Protease III, a metal-dependent serine protease, was involved in the activation process, but proteases I and II, two thiol proteases, also affected the activation process by apparently degrading the phenolase present. The phenolase activation in Norway lobster appears to be a result of the balance of the specific proteolytic action of protease III and the presumably less discriminative degradation of protease I and II.

INTRODUCTION

Phenolase, 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). 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 4·7 and 667 000, respectively, of the original natural form of the phenolase (Yan *et al.*, 1990). The activation of phenolase was thus suggested to be associated with proteolysis, since trypsin and broad bacterial protease could accelerate the process. It was also found that the activation required another factor, or factors, which had a low molecular weight (less than 5000), and protease alone did not activate the phenolase activity in Norway lobster. A multiple-component process was therefore suggested (Yan & Taylor, 1991).

Three proteases from Norway lobster have been separated and partially purified and designated as proteases I, II, and III. Proteases I and II were identified as thiol proteases and protease III as a metal-dependent serine protease (Wang *et al.*, 1992).

It was found that the activation of phenolase in Norway lobster needed some specific protease activity. Inhibition of protease III resulted in a proportional reduction of phenolase activation, whereas the inhibition of proteases I and II had little effect on the process. These results indicated that only protease III was involved in the phenolase-activation process in Norway lobster. This conclusion was also supported when the different patterns of phenolase activation at different pHs were studied. A higher activity of protease III led to a higher initial rate of the phenolase activation. However, the sequence of the phenolase-activation process and the exact role of protease activity in the process are not clear. This paper reports further investigations on the roles of these proteases in the activation of phenolase from Norway lobster.

MATERIALS AND METHODS

Materials

Norway lobster (*Nephrops norvegicus*) used in the study were supplied by either Young's English Seafoods (Grimsby, UK) or MAFF (North Shields, UK). 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 phenolase (Yan et al., *1989)*

Frozen Norway lobster heads (100 g) were homogenized with 150 ml of 0·1M phosphate buffer (pH 6·4) with an Ystral homogenizer for 2 min. The homo-

genate was centrifuged at 50 000 *g* (MSE Europa M24) for 20 min at 4°C. The supernatant was used as the crude-phenolase preparation.

Preparation of acetone-precipitated phenolase (Yan et al., 1990)

Crude phenolase (100 ml) was precipitated with 0·5-volume 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 supernatant 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 of phosphate buffer before use.

Assay of phenolase activity

Phenolase activity was measured by using 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 phenolase preparation (Ohshima & Nagayama, 1980). The absorbance at 530 nm was monitored at 25°C by using a Pye Unicam PU8800 UV/Visible spectrophotometer. The increase in absorbance at 530 nm in the first 5 min was determined, and the phenolase activity was expressed as the increase in absorbance (*A*) per min per ml of phenolase.

Assay of protease activity

The reaction mixture contained 2·5 ml of 0·5% casein in either phosphate buffer (0·1M, pH 6·4) or Tris-HCl (0·1M, pH 8·2) and 0·1 ml of phenolase solution. It was incubated in a water bath at 45°C for 30 min. Then 5%(v/w) trichloroacetic acid (TCA) (2·8 ml) was added to stop the reaction and precipitate protein. The mixture was allowed to stand for an hour 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 had been added. The protease activity was expressed as *A*/30 min ml at 280 nm under assay conditions.

Phenolase-free protease preparation

Frozen Norway lobster heads were homogenized with 0·1M phosphate buffer (pH 6·4) in a ratio of 2 : 3 with an Ystral homogenizer for 2 min, and the homogenate was then centrifuged at 50 000 *g* (MSE Europa M24) for 30 min at 4°C. The supernatant collected was treated with acetone, and the precipitate between 1 : 0·75 (enzyme : acetone) and 1 : 1·25 was collected as partially purified protease (Wang *et al.*, 1992). The protease was resuspended in one-quarter of the original volume of 0·1M phosphate buffer (pH 6·4). The partially purified protease was then allowed to pass through an Amicon ultrafiltration cell, model 8200 with membrane MX300 (which allows compounds with a molecular weight of 60 000 or less to pass through and retains larger molecules) to separate phenolase activity from the preparation. The filtrate collected was used as phenolase-free protease preparation.

RESULTS AND DISCUSSION

Studies of trypsin concentration on phenolase activation

Trypsin was observed to accelerate the process of phenolase activation in Norway lobster. When the crude-phenolase preparation was treated with trypsin (2 mg/ml crude phenolase), the activity was observed to increase very rapidly and reach a maximum (between two and three times the original activity) within minutes, whereas, without trypsin, it normally needed hours to reach this maximum (Yan *et al.*, 1990). However, the trypsin added to the phenolase preparation was in excess, and the minimum amount of trypsin needed to reach this maximum was not investigated. When crude-phenolase preparation was mixed with trypsin solution in different concentrations, it was found that phenolase activities reached different maxima (Fig. 1). The higher the final concentration of added trypsin (0–0·5 mg/ml), the higher was the maximum phenolase activity reached, whereas little increase in the activity was observed over 5 h when the phenolase was mixed only with buffer. However, when trypsin was added in a final concentration higher than 0·5 mg/ml, the phenolase did not reach a higher maximum. This indicated that this trypsin activity was sufficient to activate the phenolase present in the preparation and that further addition would not lead to any higher activity because the limitation was the amount of phenolase present.

Effect of storage of acetone-precipitated preparation on activation by trypsin

An increase in activity in acetone-precipitated phenolase still occurred after the partially purified phenolase was resuspended in phosphate buffer (pH 6·4), and trypsin

Table 1. Effect of trypsin on increase in phenolase activity in 30 min

	Phenolase activity (*A*/30 min ml)	
	0 (min)	30 (min)
Normal acetone-precipitated phenolase preparation	0·168 (0·0025)	0·175 (0·002)
Acetone-precipitated phenolase treated with trypsin	0·175 (0·002)	0·267 (0·005)

Solid trypsin was added to acetone-precipitated phenolase (2 mg trypsin/ml enzyme) immediately before being subjected to the zero-time phenolase-activity assay. Data were means of triplicate determinations and typical of five separate experiments. Figures in parentheses are standard deviations of the means.

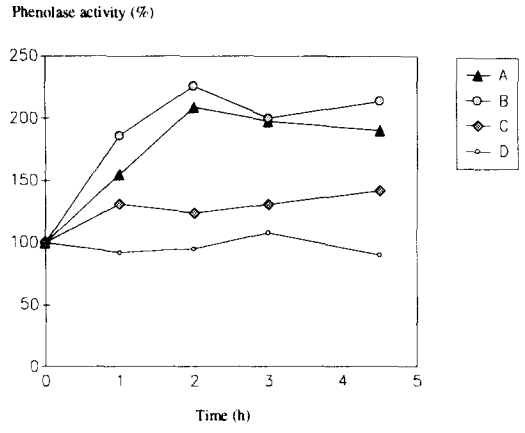

Fig. 1. Effect of trypsin on activation of crude-phenolase preparations. A: 5 ml crude phenolase and 5 ml trypsin (2 mg/ml). B: 5 ml crude phenolase and 5 ml trypsin (1 mg/ml). C: 5 ml crude phenolase and 5 ml trypsin (0·5 mg/ml). D: 5 ml crude phenolase and 5 ml the buffer. Data were means of triplicate determinations.

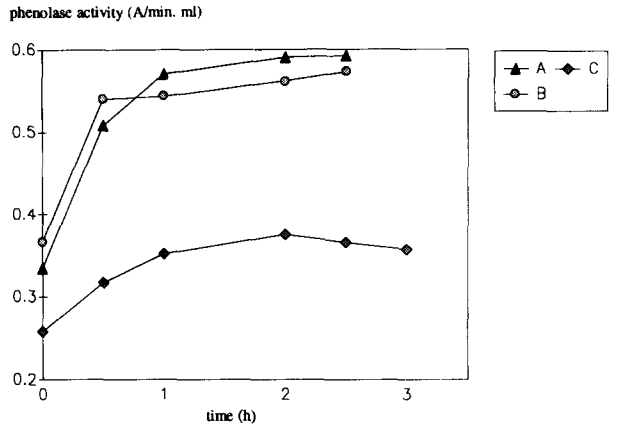

Fig. 2. Effect of trypsin on phenolase activation of acetone-precipitated preparations. Acetone-precipitated phenolases were stored at −15°C for A: 0 day (freshly prepared); B: one day; C: four days before resuspension. Trypsin was added in all samples (2 mg trypsin/ml enzyme preparation) before being subjected to phenolase-activity assay. Data are means of triplicate determinations.

was also observed to be able to accelerate the process. When trypsin was added to the partially purified phenolase (2 mg trypsin/ml enzyme), it was found that the phenolase treated with trypsin reached a much higher activity than the normal preparation within 30 min (Table 1). Thus trypsin can significantly influence the activity increase of an acetone-precipitated-phenolase preparation. This result obviously conflicts with the earlier finding on the phenolase activation from the same species (Yan & Taylor, 1991). It was observed then that trypsin had little significant influence on the activation of the partially purified phenolase. Investigation of the effect of frozen storage of acetone-precipitated phenolase on its activation provided an examination of this apparent discrepancy. It was found that trypsin could still accelerate the process when the acetone-precipitated phenolase was stored at −15°C for between one and four days before it was resuspended in buffer but that the longer the phenolase was stored, the less was the activation by trypsin (Fig. 2). Thus the length of storage of the acetone precipitate at −15°C prior to resuspension may explain the discrepancy.

Activation of acetone-precipitated phenolase by endogenous proteases

The phenolase and three proteases in Norway lobster have been studied and partially purified (Yan *et al.*, 1990; Wang *et al.*, 1992), and these studies provide the possibility of investigation of the relationship between these proteases and the phenolase activation. Because the phenolase molecule is much larger (Form I MW = 660 000, Form II MW = 141 000: Yan *et al.*, 1990), than the proteases (protease I 21 000 and protease III 27 000: Wang *et al.*, 1992; Wang, 1993) in Norway lobster, it is possible to separate phenolase activity from protease activity in partially purified protease preparation by using an ultrafiltration unit with a membrane MX300, which has an exclusion limit of a molecular weight of 60 000. When the acetone-precipitated phenolase was resuspended in the phenolase-free protease preparation, it was found that the phenolase activity increased much more rapidly and reached a much higher maximum than when it was resuspended in buffer (Fig. 3). This result provided direct evidence that the endogenous protease(s) is (or are) involved in the process of phenolase activation in Norway lobster. However, it was also found that, after reaching the maximum activity, the phenolase preparation treated with phenolase-free protease lost its activity more quickly than the normal phenolase preparation.

Protease activities in various phenolase preparations

It was found that the pattern of phenolase activation varied in different phenolase preparations. Figure 4 shows the activations in crude and acetone-precipitated preparations. The activity of the acetone-precipitated-

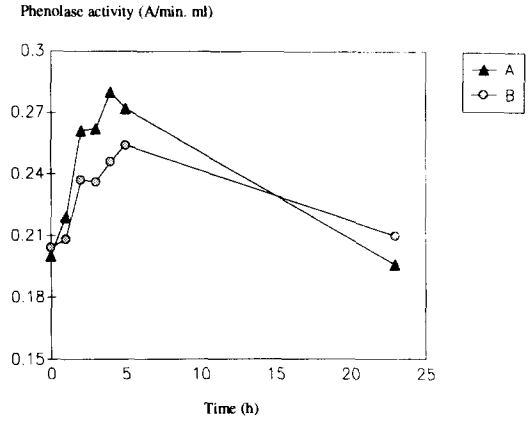

Fig. 3. Effect of endogenous proteases on phenolase activation. A: Acetone-precipitated phenolase resuspended in phenolase-free protease preparation. B: Acetone-precipitated phenolase resuspended in 0·1M phosphate buffer (pH 6·4). Data are means of triplicate determinations and typical of three separate experiments.

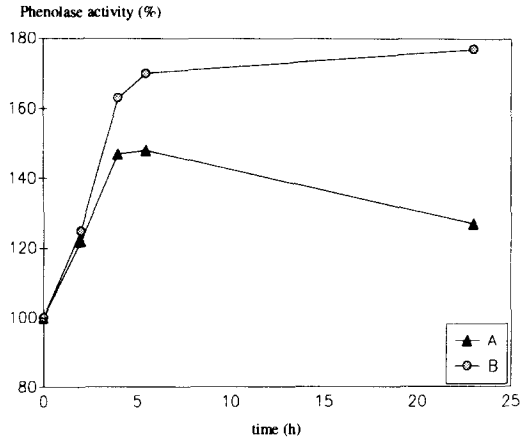

Fig. 4. Difference between activations of phenolase in crude and acetone-precipitated preparations. A: Crude-phenolase preparation. B: Acetone-precipitated-phenolase preparation. Phenolase activity was measured immediately after preparations. Data are means of triplicate determinations.

phenolase preparation always increased more and maintained the maximum for a longer period of time than the crude-phenolase preparation within each batch of enzyme preparation. Since the process of phenolase activation is influenced by protease and other activators, the difference between the patterns of phenolase activation in these two phenolase preparations could be the result of difference in these factors.

Both crude-phenolase preparation and acetone-precipitated-phenolase preparations were assayed for protease activity at pH 6·4 and pH 8·2, and the results are shown in Table 2. It was found that about one-half of the original protease activity at pH 8·2, but only about one-third at pH 6·4, remained in the acetone-precipitated-phenolase preparation. Total protease activity at pH 6·4 was contributed mainly by proteases I and II (Wang *et al.*, 1992), so these two proteases lost much of their activity during the acetone-precipitation procedure used. In contrast, most of the activity of protease III remained in the preparation.

Table 2. Protease activities in different phenolase preparations

	Protease activity (*A*/30 min ml)	
	pH 6·4	pH 8·2
Crude-phenolase preparation	8·79 (0·22)	6·38 (0·17)
Acetone-precipitated phenolase preparation	6·6 (0·34)	5·93 (0·37)

Protease activity was measured by using the standard assay except that 0·1 ml enzyme was added. Data are means of triplicate determinations and figures in parentheses are standard deviations of the means. Acetone-precipitated phenolase was resuspended in half of the original volume.

Role of protease activity in the phenolase activation

Figure 3 showed that endogenous protease activity could significantly increase the rate of phenolase activation in Norway lobster. This result, together with the early findings on phenolase in the animal that the initial form had a higher molecular weight than the more active one, strongly indicated that the activation of phenolase in Norway lobster was due to a proteolytic action, changing the phenolase from a large but less active molecule into a smaller, more active, one.

There are at least three proteases in Norway lobster, namely, proteases I, II, and III, and specific protease activity (i.e. only protease III) was involved in the phenolase-activation process (Wang *et al.*, 1992). Figure 1 shows that a higher protease activity (0–0·5 mg/ml) led to a higher phenolase activation, whereas inhibition of protease III resulted in a proportional reduction in phenolase activation (Wang *et al.*, 1992). These results indicate that the level of phenolase activation may be controlled by the protease activity, which is responsible for the process.

However, the proteolytic action in Norway lobster may result not only in an increase in the phenolase activity but also in the degradation of the enzyme. This was implied in the result given in Fig. 3. Higher endogenous protease activity increased the initial rate of phenolase activation and led to a higher maximum activity but also accelerated the decline of the phenolase activity after it was activated. The phenolase activation at different pHs showed that the decline of the phenolase activity after reaching its maximum activity was more rapid at pH 6·4 than at pH 8·2 (Wang *et al.*, 1992), which suggested that that proteases I and II were involved in the degradation of the phenolase, since these two enzymes had a pH optimum of 6·4. The phenolase activation in Norway lobster can therefore, be considered to be a result of the balance of these three endogenous proteolytic actions. A higher protease III activity can result in a higher rate of the activation and a higher maximum activity of the phenolase, whereas higher thiol-protease activities (protease I and II) may cause a greater degradation of the phenolase. This hypothesis could explain why a greater activation was achieved and why the maximum activity was maintained for a longer time in the acetone-precipitated-phenolase preparations than in crude-phenolase preparations (Fig. 4), since this collates with the different activities of the enzymes in the preparations (Table 2).

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REFERENCES

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

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

Ohshima, T. & Nagayama, F. (1980). Purification and properties of catechol oxidase from the Antarctic krill. *Bull. Jap. Soc. Sci. Fisheries*, **48**, 1036–42.

Wang, Z. (1993). Protease and phenolase activities in Norway lobster (*Nephrops norvegicus*). PhD thesis, University of Humberside, Grimsby, UK.

Wang, Z., Taylor, K. D. A. & Yan, X. (1992). Studies on the protease activities in Norway lobster (*Nephrops norvegicus*) and their role in the phenolase activation process. *Food Chem.*, **45**, 111–16.

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.