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Study of the Proteolytic Activity of the Hepatopancreas of the Freshwater Prawn, *Macrobrachium rosenbergii*, and its Role in Inducing Mushiness in Muscle Tissue During Post-Mortem Storage

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ABSTRACT

The proteolytic activities of crude homogenates from the hepatopancreas of the prawn Macrobrachium rosenbergii *were investigated. The caseinolytic and haemoglobin-hydrolysing activities are mainly due to thiol proteases with a minor contribution of trypsin-like activity. The hepatopancreas proteolytic activity is less efficient than trypsin in degrading myofibrillar proteins in intact tissue. The collagenolytic activity in the homogenates differs in substrate specificity from bacterial collagenase. The specificity of these enzymic activities and their relation to mushiness in prawns stored in ice are discussed.*

INTRODUCTION

The shelf life of prawns (*Macrobrachium rosenbergii*) stored on ice is limited to about 1 week (Nip *et al.*, 1985*a*; Angel *et al.*, 1985), because of the mushy texture which appears in the abdominal tail segments on cooking. The involvement of enzymes diffusing into the tail segments from the

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disintegrating hepatopancreas during ice storage, was suggested by several authors. Baranowski *et al.* (1984) and Nip *et al.* (1985*b*) suggested that collagenase was the main enzyme responsible for the mushiness phenomenon. Based on electron microscopic studies of cooked muscle of prawns stored in ice, Rowland *et al.* (1982) suggested that proteolytic enzymes diffusing from the digestive tracts were responsible for the deterioration after cooking.

The aim of this work was to partially characterise the proteolytic activities in hepatopancreas homogenates and to relate them to the mushiness occurring after cooking of prawns previously stored on ice.

MATERIALS AND METHODS

Materials

Adult prawns (*Macrobrachium rosenbergii*) in the intermolt stage were used in all experiments. If not otherwise indicated, enzymes, substrates, inhibitors and insoluble collagen were purchased from Sigma. Bacterial collagenase (*C. histolyticum*) was purchased from Worthington. Synthetic substrate for collagenase was purchased from Boehringer Mannheim.

Preparation of hepatopancreas-homogenates

Freshly prepared homogenates were used in all experiments. The homogenates were prepared by the method described by Baranowski *et al.* (1984).

Enzymatic assays of the homogenates

Caseinolytic and haemoglobin hydrolytic activities were measured at 0°C or 30°C, at pH 7·4 if not indicated otherwise, as previously described (Lindner *et al.*, 1988). The substrates for this assay were 0·6% casein or 1·5% haemoglobin denaturated in 6M urea, prepared according to Wirnt (1971). Release of trichloroacetic acid (TCA) soluble material was measured by the method of Lowry *et al.* (1951), with tyrosine as standard. A unit of these proteolytic activities was defined as the activity releasing 1 nmol tyrosine/min in the TCA supernatant.

For inhibition studies, the enzyme preparations tested were preincubated with the inhibitors for 30 min at room temperature. Stock solutions of 30 mM phenylmethanesulphonylfluoride (PMSF) and iodoacetamide were pre-

pared in DMF. Preincubation of the homogenates with DMF without inhibitors had no effect on the enzymic activities of interest. Since EDTA was found to interfere with the assay by the method of Lowry, when its effect on the proteolytic activity was tested, the activity was measured by the increase in absorbance at 280 nm. Protein was assayed by the method of Lowry *et al.* (1951).

Trypsin-like and chymotrypsin-like activities were measured at room temperature on the substrates benzoyl-*L*-arginyl-*p*-nitroanilide (BAPNA) (Fritz *et al.*, 1971) and glutaryl-*L*-phenylalanine-*p*-nitroanilide (GPNA) (Erlanger *et al.*, 1966), respectively. To 1 ml reaction mixture at pH 8·2, containing 100 μmol triethanolamine (TEA) HCl buffer and 1·2 μmol substrate, 0·015–0·050 ml of homogenate was added. Release of *p*-nitroaniline was followed in a spectrophotometer at 410 nm. The rate of the splitting of the substrate was calculated from the slope using $E_{410} = 8{\cdot}8\,cm^2\,\mu mol^{-1}$. A unit of activity was defined as the activity releasing 1 nmol substrate/min. The results were compared to the activities of freshly prepared solutions of trypsin and chymotrypsin of known proteolytic activities. Collagenolytic activity was measured by two methods:

(a) *Release of hydroxyproline from insoluble collagen.* To 1 ml reaction mixture at pH 7·4, containing 7 mg insoluble collagen, 50 μmol Tris HCl and 5 μmol $CaCl_2$, 0·005–0·05 ml homogenate was added. Samples without homogenate in the reaction mixture were used as controls. After incubation at 30°C the reaction was stopped by adding 7 ml of cold water, the collagen was removed by filtration and the supernatant was passed through a 1·2 μm millipore filter. An aliquot of 6 ml of the filtrate was lyophilised, dissolved in 3 ml of 6M HCl and hydrolysed for 12 h at 108°C. The hydroxyproline in the hydrolysate was measured according to the method of Neuman & Logan (1950). A unit of collagenolytic activity was defined as the activity releasing 1 nmol of hydroxyproline/min.

(b) *Splitting of the synthetic substrate for collagenase.* 4-Phenylazobenzyl-oxycarbonyl-L-Pro-L-Leu-Gly-L-Pro-D-Arg, was measured according to the method of Wunch & Heidrich (1963). A unit of activity was defined as the activity hydrolysing 1 nmol of substrate/min.

Electrophotetic study of the effect of hepatopancreas homogenate on myofibrillar proteins in intact muscle tissue

Incubation of prawn tail segments with hepatopancreas homogenates and trypsin, extraction of myofibrillar proteins from the digested segments and preparation of the samples for electrophoresis were carried out as previously described (Lindner *et al.*, 1988). Electrophoresis of the extracted myofibrillar proteins was carried out by the procedure of Porzio & Pearson (1977).

Gel filtration of hepatopancreas homogenate proteins

Gel filtration was carried out at cold room temperature. Twenty milligrams of protein precipitated from hepatopancreas homogenates by ammonium sulphate at 75% saturation, were loaded on a 750 × 16 mm column of Ultrogel ACA54. The gel was eluted at a rate of 12 ml/h with a solution of 50 mM Tris.HCl, 5 mM $CaCl_2$ and 100 mM NaCl at pH 7·4. Fractions of 3·0 ml were collected and analysed for enzymatic activities.

Haemoglobin-hydrolysing activity was assayed on 1·5% haemoglobin in 6M urea. The reaction mixture containing 1·2 ml haemoglobin and 0·2 ml effluent (or 0·2 ml of elution buffer in controls) was incubated for 45 min at 30°C. The reaction was stopped by addition of 2·6 ml of 15% TCA. After 1 h the samples were centrifuged for 15 min at 1100 *g* and the absorbance of the supernatants was measured at 280 nm. The results are reported as ΔOD_{280} where the absorbance of the controls was used as reference. Collagenolytic and trypsin-like activities were measured as described above.

RESULTS

Hepatopancreas homogenates contained 6–16 mg protein. The pH profile at 30°C of the proteolytic activity of the homogenate on haemoglobin is shown in Fig. 1. The optimal pH is 7·3; about 30% of the activity remains at pH 4·0.

The proteolytic and trypsin-like amidase activities of several preparations of interest are shown in Table 1. Compared on the basis of equal haemoglobin hydrolysing activity, hepatopancreas homogenates contain only a little trypsin-like BAPNA splitting activity (< 10% as compared to trypsin) and a little collagenase activity, when measured on a specific substrate on which bacterial collagenase is active (< 1% compared to a partially purified preparation from *C. histolyticum*). The collagenolytic activity of hepatopancreas homogenates, measured by solubilisation of hydroxyproline from insoluble collagen, was 15-fold more than trypsin but 5- to 10-fold less than the above-mentioned collagenase. Hepatopancreas homogenates did not split GPNA, a synthetic substrate of chymotrypsin, in the pH range 7.3–8·8.

The results of inhibition studies of various activities are shown in Table 2. Inhibition of the caseinolytic and haemoglobin hydrolytic activities was exerted by SH blocking agents like *p*-hydroxymercuribenzoate (PHMB) and iodoacetamide while PMSF, a potent inhibitor of serine proteases, was only slightly effective, even at a concentration of 5 mM. The chloromethyl ketones of *N*-tosyl-*L*-lysine and *N*-tosyl-*L*-phenylalanine (TLCK and TPCK, respectively) both inhibited the haemoglobin-hydrolysing activity while

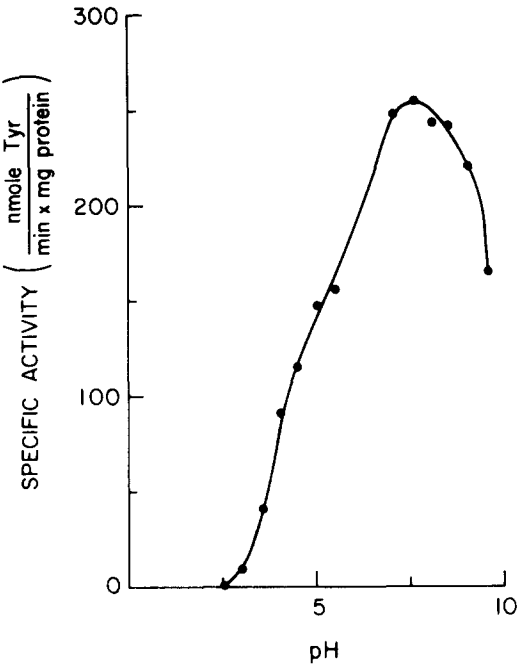

Fig. 1. pH dependence of the proteolytic activity of hepatopancreas homogenate at 30°C, with urea-denatured haemoglobin as substrate.

TABLE 1

Characteristics of Three Preparations with respect to Various Proteolytic Specific Activities

Activity:	*Proteolytic*[a]		*Trypsin-like*[b]	*Collagenase*		*Activity ratios*	
Substrate:	*A Haemoglobin*	*B Casein*	*C BAPNA*	*D Synthetic*[c]	*E Insoluble collagen*[d]	*D/A*	*E/A*
Hp homogenate[e]	180–400	90–200	2–8	0·5–2·0	500–2 000	0·01–0·001	2–6
Trypsin	15 000	7 000	15 500	6	9 600	0·0004	0·14
Collagenase[g]	180	400	—[f]	200	3 800	1·1	21

All specific activities are expressed as: activity units/mg protein at 30°C (see 'Material and Methods').
(a) activity units: nmol tyrosine released/min.
(b) activity units: nmol BAPNA released/min.
(c) activity units: nmol synthetic substrate hydrolysed/min.
(d) activity units: nmol hydroxyproline released/min.
(e) hepatopancreas homogenate.
(f) no measurable activity.
(g) from *C. histolyticum*.

10 mM EDTA was without effect. BAPNA-splitting activity was unaffected by PHMB and iodoacetamide, partially inhibited by PMSF and totally inhibited by soy trypsin inhibitor. Similar results were obtained after partial purification of the homogenate by precipitation with ammonium sulphate at 75% saturation. The collagenolytic activity, measured as solubilisation of

TABLE 2
Effect of Inhibitors on Proteolytic Activities[a] of Hepatopancreas Homogenate

Activity:	*Proteolytic*	*Trypsin-like*	*Collagenolytic*
Substrate:	*Haemoglobin or casein*	*BAPNA*	*Insoluble Collagen*
Inhibitor			
—	100	100	100
1·5 mM PMSF	95[b] 97[c]	60	93
5·0 mM PMSF	85[b]	40	71
1·0 mM PHMB	12[b] 15[c]	100	91
1·5 mM iodoacetamide	12[b]	100	
3·0 mM iodoacetamide	8[c]		
0·5 mg/ml soy trypsin inhibitor	47[b]	0	54
10 mM EDTA	100[b,c]	100	
1·0 mM TLCK	4[c]		
0·2 mM TPCK	4[c]		

[a] % activity relative to control.
[b] 0·28 mg/ml homogenate protein in preincubation mixture, substrate 0·6% casein.
[c] 3·0 mg/ml homogenate protein in preincubation mixture, substrate 1·5% haemoglobin in 6M urea.

hydroxyproline, was not inhibited by 1 mM PHMB or 1·5 mM PMSF but partially inhibited by soy trypsin inhibitor or 5 mM PMSF.

The gel filtration elution pattern of hepatopancreas homogenate proteins is shown in Fig. 2. The haemoglobin hydrolytic and trypsin-like activities appeared to co-elute (molecular weight range of 15 000–30 000) but were not exactly superimposed. The BAPNA splitting activity eluted at the higher molecular weight part of the peak. Two zones with different inhibition patterns could be located under the peak of the haemoglobin hydrolytic

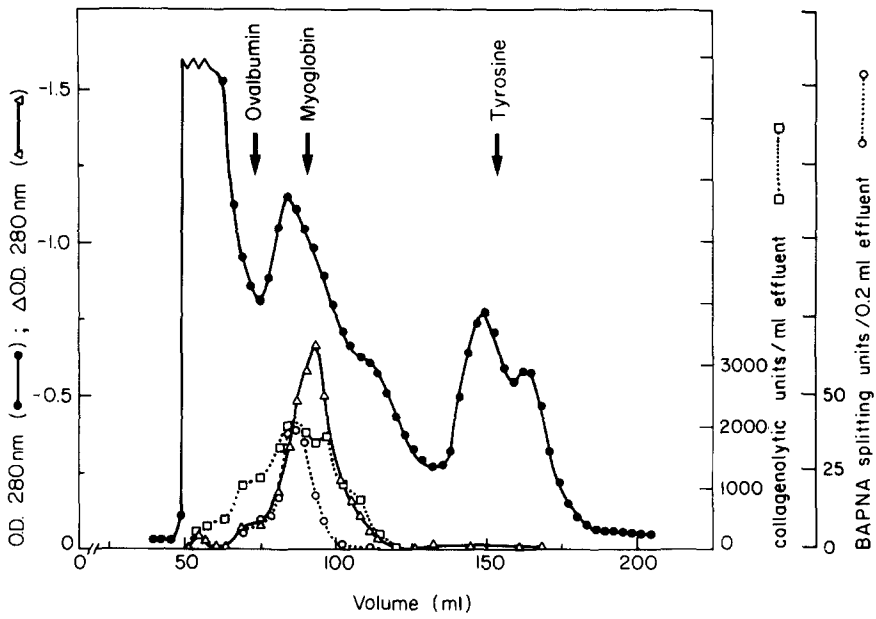

Fig. 2. Gel filtration of proteins from hepatopancreas homogenate on ultrogel ACA54. Column: 750 × 16 mm, V_0: 50 ml, V_t: 152 ml. Flow rate: 12 ml/h, fraction size: 3·0 ml. Elution buffer: 50 mM Tris.HCl and 5 mM $CaCl_2$ in 100 mM NaCl, pH 7·4. Sample: 20 mg protein in 2 ml elution buffer. Haemoglobin hydrolytic activity, ΔOD_{280}, △——△; BAPNA splitting activity, ○-----○; collagenolytic activity, □-----□. Inhibition of haemoglobin hydrolytic activity of the effluent:

Zone	Range	1 mM PMSF	1 mM PHMB
A	79–85 ml	32%	21%
B	96–102 ml	13%	85%

activity. In zone A the haemoglobin hydrolytic activity was partially inhibited by PMSF but only slightly affected by PHMB, while in zone B it was only slightly affected by PMSF and inhibited by PHMB. The collagenolytic activity had a broad molecular weight range covering the range of both trypsin and haemoglobin hydrolytic activities.

The sodium dodecyl sulphate-polyacrilamide gel electrophoresis (SDS-PAGE) patterns of myofibrillar proteins extracted from prawn tail segments incubated with hepatopancreas homogenate and trypsin are shown in Fig. 3. Although the added haemoglobin hydrolytic activity from hepatopancreas homogenate was twice that of trypsin, it was less effective in degrading myofibrillar proteins. Under the prevailing experimental conditions, hepatopancreas homogenate seemed to degrade mainly myosin while trypsin degraded also species of lower molecular weight. The band below

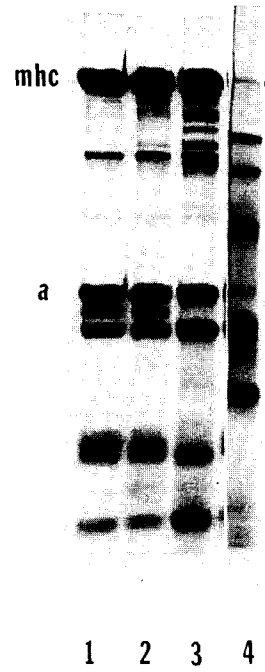

Fig. 3. SDS-PAGE of myofibrillar proteins extracted from prawn tail segments incubated 18 h at 0°C and pH 7·0 with: Lane 1, Buffer only (control); Lane 2, 1·0 mg/ml protein from hepatopancreas homogenate (18 caseinolytic units/ml at 0°C); Lane 3, 0·010 mg/ml trypsin (7 caseinolytic units/ml at 0°C); Lane 4, molecular weight markers: myosin, 205 000 (weak band); β-galactosidase, 116 000; phosphorylase B, 97 000; bovine serum albumin, 68 000; carbonic anhydrase, 29 000. Electrophoresis by the method of Porzio & Pearson (1977), 40 μg protein/lane; mhc, myosin heavy chains; a, actin.

actin (troponin-T or tropomyosin) disappeared after treatment with trypsin but was little affected by hepatopancreas homogenate.

DISCUSSION

The effective inhibition of the haemoglobin hydrolytic and caseinolytic activities in prawn hepatopancreas by the thiol blocking agents PHMB and iodocetamide, suggests that this activity is mainly due to thiol proteases. The inhibitory effect of both TLCK and TPCK, known as potent inhibitors of the serine proteases trypsin and chymotrypsin, respectively, is mainly due to their ability to inhibit certain thiol proteases (Glazer & Smith, 1971). This holds especially for TPCK since the homogenate showed no chymotrypsin-like activity on the synthetic substrate GPNA. Since the BAPNA splitting activity is inhibited by soybean trypsin inhibitor while unaffected by thiol blocking agents like PHMB and iodoacetamide, this activity is assumed to be due to a trypsin-like enzyme. The inhibition studies on the caseinolytic and haemoglobin hydrolytic activities, however, suggest that this trypsin-like activity makes only a small contribution to the total proteolytic activity of the homogenate at pH 7·4. The results of the inhibition studies on the proteolytic fraction isolated by gel filtration (see Fig. 2) also suggest that this

amidase activity is due to a trypsin-like protease. PMSF, which is a potent inhibitor of serine proteases, is also an inhibitor of thiol proteases (Glazer & Smith, 1971). The reason for the low efficiency of PMSF at concentrations normally used in inhibition studies (0·5–1·5 mM) is not clear. The presence of a PMSF scavenger in the crude homogenate is suggested by the increased efficiency of this inhibitor in diluted homogenates. However, even when applied to partially purified homogenates (fractionation by $(NH_4)_2SO_4$ and gel filtration) it was not as effective as soy trypsin inhibitor in inhibiting amidase activity on BAPNA.

The presence of weak chymotrypsin activity in prawn hepatopancreas homogenates was reported by Baranowski *et al.* (1984), and Lee *et al.* (1980). In these studies the various activities were characterised by using synthetic substrates. For an assessment of the relative contributions of the various activities to the total proteolytic activity of a crude preparation, these studies must be combined with inhibition studies on the proteolytic activity of this preparation.

The solubilisation of hydroxyproline from insoluble collagen by trypsin suggests that the substrate used was partially denatured. Unlike bacterial collagenase, collagenase from prawn hepatopancreas is not active on the synthetic substrate 4-phenylazobenzyl-oxycarbonyl-L-Pro-L-Leu-Gly-L-Pro-D-Arg. This confirms that the homogenates were not contaminated with bacterial collagenase. The collagenase activity of the hepatopancreas homogenate is demonstrated by its 15- to 40-fold higher specificity for insoluble collagen as compared to trypsin (see ratio E/A in Table 1). From the results of inhibition studies (see Table 2) it can be concluded that the caseinolytic activity in the homogenate makes only a small contribution to its collagenolytic activity. A trypsin-like collagenase from the hepatopancreas of the fiddler crab, *Uca pugilator*, was characterised by Eisen *et al.* (1973). No sufficient evidence was produced in this work to decisively confirm the activity of a similar enzyme in homogenates prepared from the hepatopancreas of the prawn, *Macrobrachium rosenbergii*. Even if this will be confirmed by future work, the fact that the collagenolytic activity of hepatopancreas homogenates from prawns was only partially inhibited by trypsin inhibitors, suggests that other types of collagenases are also present in these preparations.

In previously published works, it was shown that mushiness is not related to the number of proteolytic bacteria (Angel *et al.*, 1985) and is not caused by an endogenous proteolytic system in the muscle (Lindner *et al.*, 1988). Only a little breakdown of myofibrillar proteins is observed in tail segments which turned mushy on cooking (Lindner *et al.*, 1988). This and the low efficiency of hepatopancreas homogenates in digesting myofibrillar proteins in intact tissue (see Fig. 3) suggest that collagenolytic activity diffusing from the

disintegrating hepatopancreas may be responsible for the onset of tissue deterioration leading to mushiness, in prawns stored on ice. This suggestion is supported by the results of Nip *et al.* (1985*a*) who measured an increase in soluble collagen in muscle of prawns stored in ice.

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