Enzymes with Peptidase and Proteinase Activity from the Digestive Systems of a Freshwater and a Marine Decapod

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Peptidase and proteinase activities from the digestive systems of langostilla (*Pleuroncodes planipes*) and crayfish (*Pacifastacus astacus*) were evaluated. Hepatopancreas extracts hydrolyzed specific substrates for Leu aminopeptidase, carboxypeptidases A and B, cathepsin C, chymotrypsin, and collagenase. The digestive collagenases were serine proteinases, as shown by inhibition with phenylmethanesulfonyl fluoride. Chymotrypsin-like activity from both systems displayed different inhibition with Phe chloromethyl ketone derivatives and a different pH optima than that characteristic of chymotrypsin from mammals. Results show the decapod chymotrypsins possess different catalytic properties which do not include inhibition by tosyl-Phe chloromethyl ketone. Moreover, decapod chymotrypsin activity was demonstrated when using succinyl-$(Ala)_2$-Pro-Phe-*p*-nitroanilide as substrate and inhibited by carbobenzoxy- Phe chloromethyl ketone.

Keywords: *Chymotrypsin, activity, collagenase, crayfish, decapod, endopeptidase, exopeptidase, langostilla, peptidases, protease, proteinases, shrimp*

INTRODUCTION

Decapod digestive extracts have very high proteolytic enzyme activity (García-Carreño, 1992a). Proteinase, also called endopeptidase (Gibson and Barker, 1979; Osnes, 1985; Honjo *et al.*, 1990; Sun Pan *et al.*, 1991; García-Carreño, 1992a), as well as peptidase, also called exopeptidase (Osnes, 1985; Doke and Ninjoor, 1987; Sun Pan *et al.*, 1991), activities have been reported. The present work is based on the idea that enzymes from decapods possess unique catalytic properties that may be advantageous for their use as food- and feed-processing aids. We are particularly interested in the use of shellfish hepatopancreas as a source of enzymes for acceleration of cheddar cheese ripening. The properties of digestive proteinases from langostilla and crayfish were recently reported (García-Carreño, 1992a; García-Carreño *et al.*, 1993; García-Carreño and Haard, 1993). Most of the digestive enzymes from decapods were serine proteinases, with some metalloproteinases. Recently, a method to separate the peptidase and proteinase activities from the two decapod extracts was reported (García-Carreño and Haard, 1994). The results encouraged us to further characterize individual enzymes from the peptidase and proteinase extracts. Whereas the characterization of the enzyme activities was accomplished, we realized the criteria for identifying an enzyme are often based on the knowledge of the most studied enzymes, i.e., vertebrate source. From this standpoint, the presence or absence of chymotrypsin in decapods has been a subject of debate. Several authors reported the absence of chymotrypsin activity in decapod digestive gland extracts (Gates and Travis, 1969; Galgani *et al.*, 1984; Galgani and Nagayama, 1988; Osnes, 1985; García-Carreño and Haard, 1993). On the other hand, some investigators have identified chymotrypsin activity in decapod digestive systems (Tsai *et al.*, 1986; Jiang *et al.*, 1991, Hernández-Cortés, 1993).

In the present study, activities and properties of peptidases (aminopeptidase, carboxypeptidases A and B, and cathepsin C) and proteinases (chymotrypsin and collagenase) are demonstrated. Additionally, the controversy about the presence of chymotrypsin activity in decapod digestive system is resolved by using the techniques from which the controversy arose, including the specific substrate succinyl-$(Ala)_2$-Pro-Phe-*p*-nitroanilide and the inhibitor carbobenzoxy-Phe chloromethyl ketone.

MATERIALS AND METHODS

Reagents. Substrates succinyl-$(Ala)_2$-Pro-Phe-*p*-nitroanilide (SAPNA), azocasein, benzoyl-Arg-*p*-nitroanilide (BAPNA), and collagen type I, from bovine Achilles tendon, were from Sigma Chemical Co. (St. Louis, MO), and casein Hammarsten was from ICN Pharmaceuticals Inc. (Bucks, England). Inhibitors phenylmethanesulfonyl fluoride (PMSF), tosyl-Lys chloromethyl ketone (TLCK), tosyl-Phe chloromethyl ketone (TPCK), carbobenzoxy-Phe chloromethyl ketone (ZPCK), ethylenediaminetetraacetic acid (EDTA), and 1,10-phenanthroline (PHE), enzymes (1 mg/mL) such as leucine amino peptidase (LAP) (L-5006), carboxypeptidase A (CPA) (C-9762), carboxypeptidase B (CPB) (C-7261), cathepsin C (CC) (C-0385), bovine α-chymotrypsin, porcine trypsin, and collagenase (from *Clostridium histolyticum*), used as internal control of the assays, buffers tris(hydroxymethyl)aminomethane (Tris) and tris(hydroxymethyl)-methyl-2-aminomethane (TES), and molecular weight markers (MWM), trichloracetic acid (TCA), ninhydrin, ethanol, 2-propanol, hydrochloric acid, dimethyl sulfoxide (DMSO), and bovine albumin were from Sigma. Bradford reagent and electrophoresis reagents were from Bio-Rad (Richmond, CA).

Enzyme Preparations. Samples of langostilla were obtained during experimental catch by the "B/O El Puma" vessel in Vizcaino Bay, Baja California Sur, México, 28°40′61″ N, 114°35′54″, in September 1991, at a 125-m depth. The crabs were processed to obtain a proteinase extract as described by García-Carreño (1992a). The extract was frozen at −10 °C until arrival at La Paz Harbor (BCS, México). The sample was transported in ice to the laboratory. A sample of 200 mL was thawed, and lipids were eliminated by centrifugation at 2500*g* for 15 min. The supernatant (4P) was frozen and stored at −15 °C. No change in total proteolytic activity was found during storage for several months.

Crayfish were obtained from California Crayfish Marketing Association and transported live in a cooled ice chest to the

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laboratory. Enzyme preparation was done according to García-Carreño and Haard (1993) as follows. Hepatopancreas was removed from decapitated animals, frozen, and stored at −70 °C prior to use. The thawed tissue was homogenized at 10 °C. Due to the high water content of the tissue, addition of water or any other solution during homogenization was not necessary. To eliminate lipids and tissue debris, the homogenate was centrifuged at 2500*g* for 15 min. The aqueous supernatant (CRF) was frozen and stored at −70 °C.

Enzyme Assay Methods. Leucine aminopeptidase activity was assayed with 1.6 mM L-Leu-*p*-nitroanilide (LeuNA) in 60 mM phosphate buffer, pH 7.0, containing 0.025 M $MgCl_2$. Substrate (1.9 mL) was mixed with 0.1 mL of diluted sample. The increase of the absorbance at 405 nm due to the hydrolysis of the substrate was continuously recorded at 25 °C. One activity unit was expressed as 1 μmol of nitroanilide (NA) released/min at 25 °C (E_{405} = 8800 M^{-1} cm^{-1}).

Carboxypeptidase A activity was evaluated using 1 mM hippuryl-L-Phe (HPA) in 50 mM Tris buffer, pH 7.5, containing 500 mM NaCl. Substrate (2.9 mL) was mixed with 0.1 mL of the enzyme preparation in 10% LiCl. The increase of the absorbance at 254 nm due to the hydrolysis of the substrate was continuously recorded at 25 °C. One activity unit was obtained from the relation U/mg = A_{254} × min^{-1}/0.36 × mg of enzyme × mL of reaction $mixture^{-1}$ at 25 °C (0.36 = molar absorbancy index of hippuric acid, which is formed stoichiometrically).

Carboxypeptidase B activity was evaluated using 1 mM hippuryl-L-Arg (HA) in 50 mM Tris buffer, pH 7.5, containing 100 mM NaCl. Substrate solution (2.9 mL) was mixed with 0.1 mL of the enzyme preparation. The increase of the absorbance at 254 nm due to the hydrolysis of the substrate was continuously recorded at 25 °C. One activity unit was obtained from the relation U/mg = A_{254} × min^{-1}/0.36 × mg of enzyme × mL of reaction $mixture^{-1}$ at 25 °C (0.36 = molar absorbancy index of hippuric acid, which is formed stoichiometrically).

Cathepsin C transferase (CCT) activity was evaluated using 16 mM Gly-Phe-NH_2 in 133 mM hydroxylamine hydrochloride buffer, pH 7.0, containing 8 mM 2-mercaptoethylamine as substrate solution at 25 °C. This solution was prepared as follows: 0.1 mL of 2 M hydroxylamine (fresh from 4 M hydroxylamine hydrochloride adjusted to pH 7.0 with 4 M NaOH), 0.1 mL of 0.125 M 2-mercaptoethylamine hydrochloride (fresh and adjusted to pH 7.0 with 0.1 M NaOH), 0.1 mL of 0.25 M Gly-Phe-NH_2 (adjusted to pH 7.0 with 0.1 M NaOH), and 0.1 mL of water. The reaction was initiated by adding 0.1 mL of the enzyme preparation. The reaction was stopped at appropriate time intervals by addition of 0.5 mL of 20% TCA and 0.5 mL of 5% ferric chloride in 0.1 M HCl. The mixture was adjusted to 2 mL with water and centrifuged. The absorbance at 510 nm was recorded. A calibration curve of 0–10 μmol of Phe-Ala hydroxamate was prepared and used to calculate activity units. One activity unit was expressed as 1 μmol of hydroxamic acid released/min at 25 °C from a standard curve using hydroxamate.

Cathepsin C hydrolase (CCH) activity was evaluated using 40 mM Gly-Phe-naphthylamine in 100 mM phosphate, pH 6.0, containing 20 mM 2-mercaptoethylamine as substrate solution. The mixture was prepared with 1.88 mL of 0.1 M sodium phosphate buffer, pH 6.0, containing 20 mM mercaptoethylamine hydrochloride and 0.1 mL of enzyme preparation. The reaction was initiated by adding 0.02 mL of the substrate solution. The increase of the absorbance at 340 nm due to the hydrolysis of the substrate was continuously recorded at 25 °C. One activity unit was expressed as 1 μmol of naphthylamine released/min at 25 °C (with a molar extinction coefficient of naphthylamine of 1780).

Trypsin amidase activity was assayed using benzoyl-Arg-*p*-nitroanilide as substrate, according to Erlanger *et al.* (1961) and García-Carreño and Haard (1993). BAPNA (1 mM) was dissolved in 1 mL of DMSO and then made to 100 mL with 50 mM Tris buffer, pH 7.5, containing 20 mM $CaCl_2$. The substrate solution and the reaction mixture were maintained at 37 °C during the enzyme assay period. To 1.25 mL of fresh substrate solution was added 25 μL of the enzyme preparation. After 10 min, 0.25 mL of 30% acetic acid was added and then the absorbance at 410 nm was recorded against a water blank. BAPNA units were evaluated according to Dimes *et al.* (1994). BAPNA activity was obtained from the following formula: (A_{410}/min/mg of protein × 1000 × 4) × 8800, where 8800 is the extinction coefficient of *p*-nitroanilide. One activity unit was expressed as 1 μmol of nitroanilide released/min.

Chymotrypsin activity was assayed using succinyl-$(Ala)_2$-Pro-Phe-*p*-nitroanilide as substrate. The assays were conducted at 25 °C. Hydrolysis of SAPNA was continuously recorded as the increase in absorbance at 410 nm. Ten microliters of enzyme preparation was added to 0.490 mL of 0.02 mM SAPNA solution in 0.1 M Tris, pH 7.8, containing 0.01 M $CaCl_2$. The absorbance was recorded for 5 min. One unit of chymotrypsin activity was defined as the absorbance change at 410 nm/min, according to Tsai *et al.* (1986).

Kinetic parameters were obtained using hydrolysis of SAPNA as substrate in a concentration from 0.01 to 0.1 mM. SAPNA hydrolysis was done as previously. The values of SAPNA activity were taken as initial velocity and substituted in a Lineweaver–Burk plot. The kinetic values were obtained using a PC program according to Page (1987), and catalytic efficiency was calculated as the relation between V_{max}/K_m (Whitaker, 1972).

Proteinase or endopeptidase activity was assessed using 2% azocasein in 50 mM Tris, pH 7.5, according to García-Carreño and Haard (1993). In brief, 10 μL of the enzyme preparation was mixed with 0.24 mL of 0.50 M Tris-HCl, pH 7.5, at 25 °C. Reaction was initiated by the addition of 0.50 mL of 2% azocasein and stopped 10 min later by adding 0.50 mL of 20% TCA. The reaction mixture was centrifuged at 6500*g* for 5 min and the absorbance recorded at 440 nm. For the controls, TCA was added before the substrate.

Collagen hydrolysis activity was assessed according to Kim (1991). In brief, 6 mg of bovine tendon collagen was suspended in 1.25 mL of 0.1 M TES buffer, pH 7.5, containing 0.35 mM $CaCl_2$ at 37 °C. Reaction was initiated by adding 25 μL of the enzyme preparation. Five hours later, the reaction mixture was centrifuged at 4000*g* for 5 min. A 0.2-mL sample of supernatant was transferred to test tubes with 0.5 mL of 1.5% ninhydrin. The mixture was incubated at 100 °C for 15 min and then cooled to room temperature, and 2.5 mL of 50% ethanol was added with mixing. Then, the absorbance was recorded at 600 nm. Appropriate blanks were prepared by separately incubating the substrate and the enzyme preparation for 5 h and then mixing them, just before the addition of the ninhydrin solution. Collagenolytic activity was expressed in micromoles of leucine released.

Enzyme Inhibition. The effect of inhibitors (TLCK, TPCK, PMSF, and ZPCK) on SAPNA hydrolysis was evaluated according to García-Carreño (1992b). Five microliters of the inhibitor solution was added to 50 μL of the enzyme preparations. The mixture was incubated for 1 h prior to assay for chymotrypsin activity. The activity was reported as the percentage of the control without inhibitor. Final inhibitor concentrations were 10 mM TLCK in 0.1 M HCl, pH 3, 5 mM TPCK in MeOH, 20 mM PMSF in 2-propanol, and 0.5 mM ZPCK in 50 mM phosphate buffer, pH 7.8.

The effect of chymotrypsin inhibitors on azocasein hydrolysis was studied according to García-Carreño (1992b). Ten microliters of enzyme preparation was incubated with 5 μL of a solution of the inhibitor and 235 μL of buffer for 60 min. Final concentrations of the inhibitors were 0.5 mM TPCK and 0.1 mM ZPCK.

The class of proteinases having collagenolytic activity was assessed by incubating the enzyme preparations with class-specific inhibitors following the method described by García-Carreño (1992b) and measuring the remaining activity according to Kim (1991). Final inhibitor concentrations in the reaction mixture were 2 mM PMSF in 2-propanol, 20 mM EDTA, and 4 mM PHE in methanol.

pH Optima and Thermal Stability. The effect of pH on chymotrypsin and collagenase activities was also determined. Activity was assayed with SAPNA- or collagen-buffered solutions at pH from 4–10 in universal buffer (Stauffer, 1989). Thermostability of chymotrypsin and collagenase was assessed by incubating the enzyme preparation in a water bath at 20, 30, 40, 50, and 70 °C for up to 60 min. Samples were taken every 15 min, and the residual activity was measured. Protein concentration in enzyme preparations was assessed according to Bradford (1976), using bovine albumin as standard.

SDS–Polyacrylamide Gel Electrophoresis. Electrophoresis separation of the proteins in the enzyme preparations was done according to Laemmli (1970), using 12% acrylamide.

Table 1. Peptidase Activity in Decapod Preparations

enzyme	leucine aminopeptidase	carboxypeptidases A	carboxypeptidases B
crayfish	0.0230[a]	31	45
langostilla	0.0066	124	177
LAP[b]	18.5900		
CPA[b]		144	
CPB			3568

[a] Activity units are expressed as follows: leucine aminopeptidase as μmol of nitroanilide released from LeuNA/min and carboxypeptidases A and B as μmol of hippuric acid released/min from HPA and HA, respectively. See text for details. [b] Commercial preparations used as internal control of the assay.

Table 2. Cathepsin C Activity in Decapod Preparations

activity	transferase	hydrolase
crayfish	0.093[a]	0.000
langostilla	0.163	0.013
cathepsin C[b]	11.250	0.033

[a] Cathepsin hydrolysis is expressed as μmol of Phe–Ala hydroxamate acid released/min for transferase activity and μmol of naphthylamine/min for hydrolase activity. See text for details. [b] Commercial preparation from mammal source.

Enzyme preparations were diluted 1:4 in sample buffer, and 5 μL was loaded in a vertical electrophoresis device (Hoffer, San Francisco, CA). Molecular weight standards were included on each plate. Zymograms of endopeptidase activities of fractions separated by electrophoresis were done according to García-Carreño *et al.* (1993).

Enzyme preparations were mixed with TPCK or ZPCK, 5 and 0.5 mM, respectively, and incubated for 1 h. Controls incubated without the inhibitor were prepared with buffer. They were diluted 1:4 in sample buffer, and 5 mL was loaded on SDS–PAGE plates. Electrophoresis and zymograms were done as previously.

All the assays were done in triplicate, and the average was reported. No variation from preparation to preparation was found; neither was the activity changed by storage within the study.

RESULTS AND DISCUSSION

Lactobacilli peptidases such as aminopeptidases and carboxypeptidases play a key role in cheddar cheese ripening (Simard, 1992). We characterized peptidase activities in decapod extracts to know if the enzymes of decapods might be like those of the lactobacilli involved in cheese ripening. Also, the characterization of proteinases in the decapod enzyme extracts was achieved.

Peptidases. The presence of Leu aminopeptidase, carboxypeptidases A and B, and cathepsin C was demonstrated in both langostilla and crayfish enzyme extracts (Tables 1 and 2). Crayfish hepatopancreas had more leucine aminopeptidase and less carboxypeptidase activity than langostilla extracts (Table 1).

Cathepsin C activity was estimated by the two characteristic reactions catalyzed by this enzyme: transferase (CCT) and hydrolase (CCH) activities. Table 2 shows the CCH and CCT activities. The results indicate that both enzyme preparations exhibited transferase activity but only the langostilla extract had hydrolytic activity characteristic of that of mammalian cathepsin C.

Trypsin and Chymotrypsin. Trypsins are recognized by their capability to hydrolyze peptide bonds at the carboxyl side of the positive charged amino acids Arg or Lys. Chymotrypsins hydrolyze the carboxyl side of the hydrophobic amino acids Tyr, Trp, Phe, or Leu. Trypsin activity in decapods has been reported by numerous authors. However, some reports indicate chymotrypsin is absent from decapod digestive systems (Gates and Travis, 1969; Galgani *et al.*, 1984; Galgani and Nagayama, 1988; Osnes, 1985; García-Carreño and Haard, 1993). SAPNA is reported to be a specific substrate for mammalian chymotrypsins, such as a bovine enzyme (DelMar, 1979). Moreover, SAPNA was the substrate used by investigators who reported chymotryptic activity in decapods (Tsai *et al.*, 1986; Jiang *et al.*, 1991; Hernández-Cortés, 1993). Table 3 shows the activities for trypsin and chymotrypsin in the decapod preparations and enzyme standards. The results show that the digestive systems of decapods possess both trypsin and chymotrypsin activities. To further substantiate the presence of decapod chymotrypsin, we used inhibition assays for mammalian trypsins (TLCK) and chymotrypsins (TPCK, ZPCK). Table 4 shows the chymotrypsin-like activity of decapod preparations using SAPNA and inhibitors. Porcine trypsin had almost nonexistent activity with SAPNA, *i.e.*, less than 1% of bovine chymotrypsin (Table 3), which agrees with the results of DelMar (1979). SAPNA hydrolysis by the decapod preparations was not very sensitive to the trypsin inhibitor TLCK nor to the chymotrypsin inhibitor TPCK. On the other hand, the chymotrypsin inhibitor ZPCK was effective in preventing SAPNA hydrolysis by the decapod preparations. In contrast, bovine chymotrypsin was inhibited by both TPCK and ZPCK (Table 4).

Table 3. Hydrolysis of SAPNA and BAPNA by Decapod Preparations

sample	SAPNA[a]	percent[b]	BAPNA	percent (%)
bovine chymotrypsin	1.009	100.00		
porcine trypsin	0.009	0.89	881.20	100.00
langostilla	0.255	25.00	9.46	1.07
crayfish	0.022	2.18	0.65	0.07

[a] SAPNA and BAPNA activity are expressed as mol and μmol of nitroanilide released/min, respectively (see Materials and Methods for further details). [b] Percentage based on chymotrypsin or trypsin activity.

Table 4. Effect of Inhibitors on Hydrolysis of SAPNA and Azocasein

	inhibition (%)[a]					
	SAPNA				azocasein	
sample	PMSF	TLCK	TPCK	ZPCK	TPCK	ZPCK
bovine chymotrypsin	100	1	84	96	90	86
langostilla	100	5	13	93	12	15
crayfish	100	0	0	72	0	3

[a] The percentage of inhibition was calculated using the activity without inhibitors as 100%.

The chymotrypsin-like activity contributing to the hydrolysis of azocasein was examined. TPCK and ZPCK (Table 4) caused some inhibition of azocasein hydrolysis by langostilla enzymes but were not effective with the crayfish enzymes. When the decapod enzyme extracts were assayed, the activity was reduced by an amount which was proportional to the amount of chymotrypsin in the preparation (García-Carreño, 1992a). Kinetic properties of chymotrypsin from different sources using SAPNA as substrate are provided in Table 5. Bovine chymotrypsin had a much lower K_m and, as a consequence, a higher catalytic efficiency (V_{max}/K_m) than the decapod enzymes. Similar results were obtained when the kinetic properties of bovine and cod trypsins were compared (Simpson and Haard, 1984; Simpson *et al.*, 1989).

Collagenase. Enzymes which degrade the helical regions of native collagen are classified according to their mechanism of catalysis in the EC 3.4.24 group of the metalloproteinases (Dixon and Webb, 1979). However, serine collagenases have been identified in the digestive systems of aquatic animals (Sakharov and Litvin, 1990;

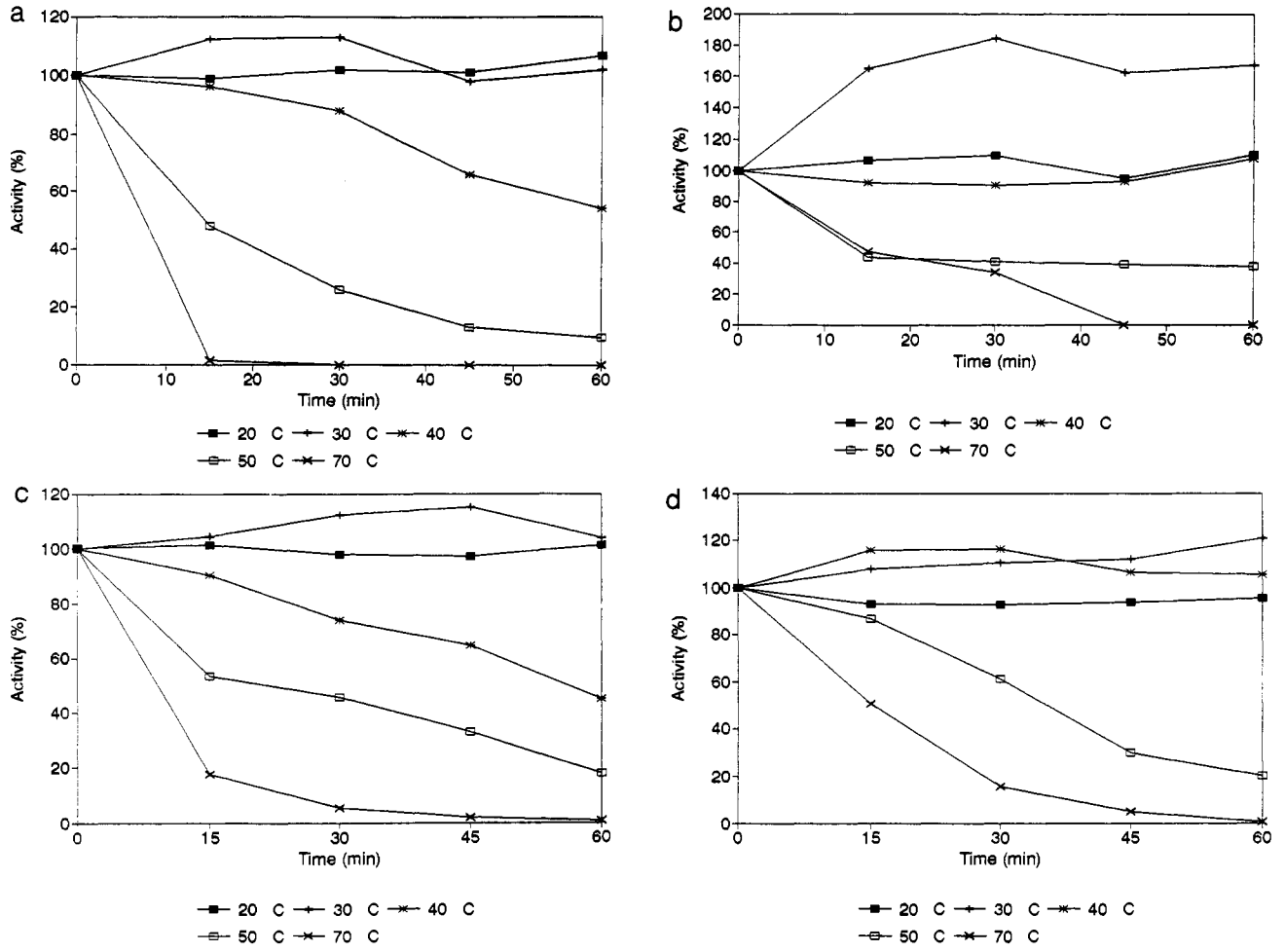

Figure 1. (a) Thermostability of langostilla hepatopancreas extract on SAPNA hydrolysis. Assay was conducted by incubating the enzyme preparation at different temperatures for 60 min. Samples were taken every 10 min and assayed for SAPNA hydrolysis. (b) Thermostability of crayfish hepatopancreas extract on SAPNA hydrolysis. Assay was conducted as in (a). (c) Thermostability of langostilla hepatopancreas extract on collagen hydrolysis. Assay was conducted by incubating the enzyme preparation at different temperatures for 60 min. Samples were taken every 10 min and assayed for collagen hydrolysis. (d) Thermostability of crayfish hepatopancreas extract on collagen hydrolysis. Assay was conducted as in c.

Table 5. Kinetic Constants, Using SAPNA as Substrate

sample	K_m[a]	V_{max}	catalytic efficiency
bovine chymotrypsin	0.002	5.970	2985.00
langostilla	0.283	9.720	34.35
crayfish	0.226	0.271	1.20

[a] K_m (mM). V_{max} (mM/min). Catalytic efficiency (V_{max}/K_m).

Table 6. Collagenolytic Activity of Decapod Preparations

		inhibition (%)		
sample	activity[a]	PMSF	1,10-phenanthroline	EDTA
C. histolyticum	2.453	1	93	55
porcine trypsin	0.101			
langostilla	0.985	95	2	2
crayfish	1.819	94	10	7

[a] Collagenolytic activity is expressed as μmol of leucine released in 5 h.

Klimova *et al.*, 1990; Bracho and Haard, 1991). The collagenolytic activity of porcine trypsin, bacterial collagenase, and decapod extracts is summarized in Table 6.

The hydrolysis rate of collagen by trypsin was less than 2% of that by bacterial collagenase. The bacterial collagenase was inhibited by the metalloproteinase inhibitors EDTA and PHE and was not sensitive to the serine proteinase inhibitor PMSF. It has been suggested that trypsin and chymotrypsin are responsible for the degradation of the collagen in decapod food (Chen *et al.*, 1991). However, trypsins and chymotrypsins are not normally active on tendon collagen, as illustrated with porcine trypsin (Table 6).

Earlier (García-Carreño and Haard, 1993), we showed that serine proteases were responsible for most of the proteolytic activity of langostilla and crayfish digestive enzymes. These results are consistent with the present finding of trypsin, chymotrypsin, and a serine collagenase in these enzyme extracts.

Thermostability of Decapod Digestive Enzymes. Thermostability of enzymes catalyzing SAPNA and collagen hydrolysis was determined for up to 60 min at temperatures ranging from 20 to 70 °C. After 15 min of incubation, chymotrypsin-like activity by crayfish enzymes was inactivated 80% at 30 °C and completely inactivated at 50 and 70 °C (Figure 1a,b). In contrast, langostilla extract was stable up to 60 min between 20 and 30 °C but was rapidly inactivated at 70 °C. Figure 1c,d shows the response of collagenase activity to heat treatment. Crayfish collagen hydrolysis activity was slightly activated between 30 and 40 °C, stable up to 60 min at 20 °C, and completely abolished in 60 min at 70 °C. Langostilla collagenase activity was like that of crayfish since it was stable up to 60 min at 20 °C, completely abolished in 60 min at 70 °C, and slightly activated at 30 °C.

pH Optima of Decapod Digestive Enzymes. The effect of pH on activity of the chmyotrypsin-like and collagenolytic enzymes is shown in Figure 2. For both langostilla and crayfish extracts, chymotrypsin-like activity

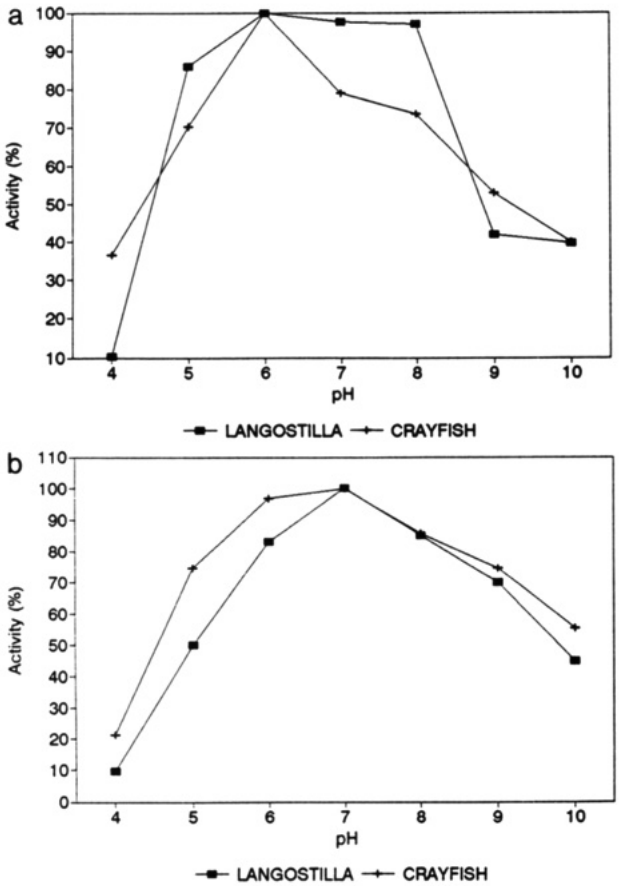

Figure 2. (a) Effect of pH on SAPNA hydrolysis by decapod extracts. Assay was conducted by incubating the reaction mixture of substrate and enzyme preparation at different pHs using universal buffer. (b) Effect of pH on collagen hydrolysis by decapod extracts. Assay was conducted as in (a).

was optimal at pH 6; however, langostilla extract showed high activity over a broad pH from 5 to 8. The pH optimum of mammalian chymotrypsin with amide substrates is about 8.0 (Hess, 1971). Collagenase activity in both langostilla and crayfish was optimal at pH 7 (Figure 2b). Digestive collagenase from fiddler crab has a pH optimum of 8.0 (Eisen *et al.*, 1973) and that from freshwater prawn is 7.5 (Nip *et al.*, 1985).

SDS–PAGE. Zymograms of proteinase activity were done to determine the composition and molecular weight of the enzymes in the decapod extracts. Crayfish extract displayed several active zones having caseinolytic activity. The molecular weight of the active zones ranged from 14 to 100 kDa. One of the active zones had the same migration as bovine chymotrypsin (Figure 3), but no zones had the same migration as bovine trypsin. Langostilla extract displayed several active zones. However, the molecular weight range of langostilla proteases was narrower than that for crayfish. Active zones with similar molecular weights as bovine chymotrypsin and trypsin were found in langostilla extract.

The molecular weights of previously characterized digestive collagenases are similar to that of chymotrypsin, ranging from 25 to 27 kDa (Sakharov *et al.*, 1988; Eisen *et al.*, 1973). All of the active zones in the molecular weight range of 20–30 kDA were partially inhibited by the serine protease inhibitor PMSF (García-Carreño and Haard, 1993). In that study, a higher sensitivity for the serine proteinase PMSF was found for langostilla than for crayfish. On the contrary, crayfish proteinases showed higher sensitivity for the trypsin inhibitor TLCK.

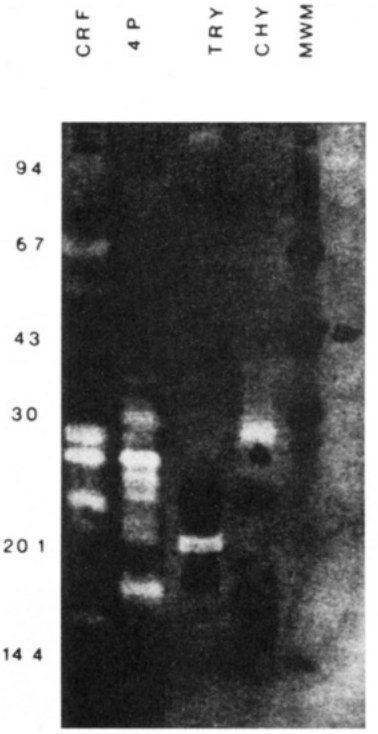

Figure 3. Substrate SDS–PAGE of langostilla and crayfish enzyme preparations. CRF, crayfish, 4P, langostilla; TRY, porcine trypsin, CHY, bovine chymotrypsin; MWM, molecular weight markers. After electrophoresis, the gel was soaked in a casein solution and then stained for protein using Coomassie blue. The band on a shadowy background is where a proteinase was located and the casein in the area hydrolyzed. Black bands are proteins in the preparations with no proteinase activity.

CONCLUSIONS

Langostilla and crayfish hepatopancreas enzymes include amino- and carboxypeptidases, chymotrypsin, trypsin, and serine collagenase. Leucine aminopeptidase activity was higher in crayfish extract than in langostilla, but carboxypeptidases A and B and cathepsin C activities were higher in langostilla extract.

The decapod chymotrypsin activity was insensitive to the chymotrypsin inhibitor TPCK. This helps explain why there have been conflicting reports on the presence/absence of chymotrypsin in decapod digestive systems. The usefulness of SAPNA to evaluate chymotrypsin activity in decapod enzyme preparations was confirmed. Another different property of decapod chymotrypsin activity is the relatively low pH optima for SAPNA hydrolysis.

Chymotrypsin activity in langostilla seems to have higher sensitivity to the chymotrypsin inhibitors TPCK and ZPCK than those from crayfish. The sensitivity of decapod enzymes for ZPCK was higher than that for TPCK which is usually used in the characterization of chymotrypsin activity. On the other hand, ZPCK seems to be a general inhibitor for enzymes having chymotrypsin activity (after we found mammal and decapod enzymes were highly inhibited (Table 4) when using SAPNA as substrate). Moreover, the hydrolysis of SAPNA by mammal and decapod preparations showed higher sensitivity for ZPCK than the hydrolysis of azocasein. Further studies of chymotrypsin from decapod extracts need to be done to obtain kinetic properties, such as K_i for ZPCK and K_m and catalytic efficiency for SAPNA, as well as molecular properties, such as molecular weight and primary and tertiary structure, on a pure preparation.

Collagenase activity in decapod enzyme extracts was classified as a serine proteinase with optimal activity at

neutral pH. The pH optima was somewhat lower than that of decapod digestive collagenases previously identified.

The identity of the enzyme responsible for the collagen hydrolysis needs to be elucidated in order to show if trypsin, chymotrypsin, a concerted activity of them, or another (true collagenase) enzyme is involved in the degradation of the food collagen.

SDS–PAGE zymograms showed the wide variety of decapod proteases and their different molecular weights when compared with mammal references, thus confirming their unique nature.

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