Purification and characterization of a digestive cysteine proteinase from the American lobster (*Homarus americanus*)

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A new cysteine proteinase was isolated from the digestive juice of the American lobster (*Homarus americanus*). The enzyme was purified by a combination of affinity and ion-exchange chromatography and gel filtration. The cysteine proteinase accounted for 80 % of the proteolytic activity in the lumen of the hepatopancreas. The most potent heavy-metal inhibitors were Hg, Cu, and Ag ions. Inhibition by organic proteinase inhibitors, including E-64 [L-*trans*-epoxysuccinyl-leucylamido-(4-guanidino)butane] and activation of the enzyme by 2-mercaptoethanol and dithiothreitol are characteristic of cysteine proteinases. Several similarities to papain are noted and include the N-terminal sequence, of which 22 of the first 28 amino acids are identical. Some notable differences are the higher M_r of 28 000 compared with 23 350 for papain, and the low isoelectric point (pI 4.5) of the lobster enzyme. The effects of pH and temperature on catalytic activity of the lobster proteinase were studied with benzyloxycarbonylalanine *p*-nitrophenyl ester as the substrate. The k_{cat}/K_m value was effectively temperature-independent between 10 and 60 °C. The pH-activity profile for the lobster enzyme revealed four apparent protonation states, of which only two are active.

INTRODUCTION

Cysteine proteinases have been isolated from a wide range of sources, including higher-plant fruit and latex, animal tissues and bacteria. For papain, the most studied of these enzymes, the structure and mechanism of action is known in considerable detail (Brocklehurst *et al.*, 1987). Cysteine proteinases do not often function in a digestive role. However, Reid & Rauchert (1976) and Chen & Zall (1986) found digestive cathepsin-B type enzymes in clams, and Garcia *et al.* (1978), Houseman (1978) and Houseman & Downe (1982) reported the principal digestive proteinase of some blood-feeding insects to be thiol-dependent. Recently, Kitch & Murdoch (1986) described a thiol-dependent proteinase from the digestive tract of larvae of the bruchid beetle *Callosobruchus masculatus*.

Digestive enzymes in the gastric juice of the American lobster (*Homarus americanus*) were surveyed by Brockerhoff *et al.* (1970), who reported at least seven proteolytic enzymes separable by ion-exchange chromatography, and with M_r values in the range 12 500–50 000.

It was suggested that one of these enzymes was similar to the so-called 'low molecular weight protease' isolated from gastric juice of the freshwater crayfish (*Astacus fluviatilis*) (Pfleiderer *et al.*, 1967). The apparent low M_r of 11 000 for the crayfish enzyme has since been shown to be attributable to its anomalous behaviour on Sephadex columns (Titani *et al.*, 1987).

Our preliminary studies with lobster gastric juice showed that more than 80 % of the proteolytic activity, as assayed using casein as the substrate, did behave in a similar manner to the crayfish proteinase on Sephadex columns, but had the quite distinct catalytic properties characteristic of a cysteine proteinase. In the present paper we describe the purification and characterization of this new cysteine proteinase.

MATERIALS AND METHODS

Gastric juice was obtained from lobsters starved for 1 week and kept in seawater tanks at 5–10 °C. A 10 cm length of poly(vinyl chloride) (PVC) tube (3 mm outer diameter; 1 mm inner diameter) was inserted into the stomach via the mouth, and 6–10 ml of fluid was drawn into a syringe. The accumulated juice was either freeze-dried or used immediately. Thiopropyl-Sepharose 6B was obtained from Pharmacia, and substrates, inhibitors and papain from Sigma and coumaride substrates from IAF BioChem. International, Quebec, PQ, Canada.

Analytical methods

Protein purity was monitored by polyacrylamide-gel electrophoresis (PAGE) in 12 % slab gels in the presence of 0.1 % SDS by the method of Laemmli (1970).

The pI was determined by isoelectric focusing in an LKB apparatus at 2 °C with a stabilizing sucrose concentration gradient and an Ampholine gradient, pH

Abbreviations used: PAGE, polyacrylamide-gel electrophoresis; PVDF, poly(vinylidene difluoride); PTH, phenylthiohydantoin; Cbz-Lys-ONp, benzyloxycarbonyl-lysine *p*-nitrophenyl ester; DTT, dithiothreitol; QAE, quaternary aminoethyl; E-64, L-*trans*-epoxysuccinyl-leucylamido-(4-guanidino)butane; Nbs₂, 5,5'-dithiobis-(2-nitrobenzoic acid) ('DTNB').

range 3–10, at 1000 V for 72 h. The column was fractionated and enzyme activity and pH determined for each fraction.

Protein was determined by the Coomassie Blue method of Bradford (1976).

Amino acid analyses were performed on protein samples hydrolysed for 24, 48 and 72 h with 6 M-HCl in evacuated sealed tubes at 110 °C with a Durrum D500 analyser. Serine and threonine were calculated by linear extrapolation to zero time. Half-cystine was measured as cysteic acid after performic oxidation. Tryptophan was analysed after hydrolysis with methanesulphonic acid as described by Liu & Chang (1971).

Samples for sequence analysis (5 µg, 400 pmol) were loaded on to a mini gel (10 cm × 10 cm, 0.5 mm thick) and electrophoresed through a pre-aged (1 week at 4 °C) 15% acrylamide running gel as described by Laemmli (1970), using a Bio-Rad Mini-Protein II cell (Bio-Rad Laboratories, Richmond, CA, U.S.A.). After electrophoresis the gel was soaked in transfer buffer and electroblotted on to poly(vinylidene difluoride) (PVDF) membrane (Millipore Corp., Bedford, MA, U.S.A.) by the method of Matsudaira (1987). The staining procedure for the electroblotted membrane was modified from the original description of Matsudaira (1987) and incorporated milder staining conditions using 0.2% Ponceau 'S' solution in 1% acetic water for 1 min, followed by destaining in Milli-Q Water (Millipore) at room temperature for 3–5 min. The membrane was air-dried and the protein band revealed was excised with a clean razor blade and stored in an Eppendorf tube at –20 °C. The stained PVDF membrane-protein band was cut into approx. 2 mm squares and carefully aligned around the periphery in the recess of the upper cartridge block, care being taken to avoid occluding the reagent/solvent inlet port. The membrane pieces were held stationary by insertion into the recess of a glass-fibre disc containing 1.5 mg of precycled Biobrene. Automated gas-phase sequencing was performed on an Applied Biosystems 475A protein-sequencing system (Foster City, CA, U.S.A.), incorporating a model 470A gas-phase sequencer equipped with an on-line model 120A PTH analyser under the control of a model 900A control/data analysis module.

Enzyme assays

Enzyme activity was routinely measured with 0.1 mM-benzoyloxycarbonyl-lysine *p*-nitrophenyl ester (Cbz-Lys-ONp) in 100 mM-sodium acetate buffer, pH 5.0, containing 1 mM-EDTA and 1 mM-DTT at 22 °C. Absorbance of the released *p*-nitrophenol was measured at 340 nm and one enzyme unit gave a change of 1.0 *A* unit · min^{–1}. The casein assay used for preliminary studies of proteolytic activity was carried out in 1 ml of 100 mM-Tris/HCl buffer, pH 8.0, containing 1 mM-EDTA, 10 mM-2-mercaptoethanol and 0.4% casein. Enzyme was added, and the solution was incubated for 1 h at 37 °C. Undigested protein was precipitated by adding 1 ml of 5% (w/v) trichloroacetic acid, then centrifuged (3000 *g*, 10 min). The absorbance of the supernatant was determined at 280 nm, the appropriate blank being used. One casein unit was taken to be a change of 0.001 *A* unit · min^{–1}.

Purification

All buffer solutions contained 1 mM-EDTA and, except

for regeneration of the thiopropyl-Sepharose 6B, all procedures were performed at 4 °C.

Covalent chromatography. Thiopropyl-Sepharose 6B (15 g) was activated before use by washing with 1 mM-DTT and 20 mM-cysteine in 100 mM-Tris/HCl buffer, pH 8.0 (200 ml), followed by 1.5 mM-2,2'-dithiodipyridine in pH 8.0 buffer (750 ml), then with 100 mM-sodium acetate buffer, pH 4.5.

Freshly collected gastric juice (60 ml) was cooled to 2 °C and filtered through glass wool to remove food particles. The thiopropyl-Sepharose 6B was added in a small volume of pH 4.5 buffer and the suspension stirred for 2 h. The adsorbant was then washed with pH 4.5 buffer (500 ml), followed by pH 8.0 buffer (500 ml), then packed into a 2 cm × 14 cm column and eluted with pH 8.0 buffer containing 1 mM-DTT and 20 mM-cysteine (200 ml).

Ion-exchange chromatography. The eluate from the affinity column was adjusted to pH 7.0 and applied to a column of (2.5 cm × 40 cm) of QAE-Sephadex A-25 equilibrated with 100 mM-Tris/HCl, pH 7.0, containing 1 mM-EDTA and 10 mM-2-mercaptoethanol. The column was eluted with a linear gradient of 0–2 M-NaCl, pH 7.0 (1 litre).

Gel filtration. Pooled fractions containing the main peak of proteinase activity were concentrated on a 76 mm YM-10 Amicon ultrafiltration membrane to about 10 ml and applied to a 2.5 cm × 90 cm column of Sephadex G-50 equilibrated with the same pH 7.0 buffer used for ion-exchange chromatography. Pooled active fractions were freed of buffer compounds by ultrafiltration. Purified enzyme was then either freeze-dried or kept in solution at 2 °C for up to 48 h.

Kinetic studies

A Cary 219 spectrometer was used for all kinetic studies. For amidomethylcoumarin substrates a fluorescence attachment was used with a 460 nm filter, an excitation wavelength of 370 nm and a slit width of 3.6 nm. The gain was adjusted so that 1 µM-coumarin released = 100% fluorescence. For determinations of active lobster enzyme, it was first inhibited with an excess of E-64 [*L*-trans-epoxysuccinyl-leucylamido(4-guanidino)butane] and the excess determined by titration with papain. Titration with 5,5'-dithiobis-(2-nitrobenzoic acid) (Nbs₂) was used to determine papain activity. Active lobster enzyme concentrations were then calculated as the difference between total and excess E-64 concentrations.

All kinetic buffer solutions contained 1 mM-EDTA and 200 mM-NaCl, and were as follows: pH 3.5–5.5, sodium acetate (100 mM); pH 6.0–8.0, sodium phosphate (50 mM); pH 8.5, sodium carbonate (50 mM); and pH 9.0, sodium borate (50 mM).

RESULTS

Purification

From 5 to 10 ml of gastric juice was obtained from each lobster. Protein content varied, but averaged 60 mg · ml^{–1} and 2–3 mg · ml^{–1} for the cysteine proteinase. Fresh juice was used whenever possible; however, the

Table 1. Purification of lobster proteinase

For experimental details, see the text. Activity was measured as esterase activity with Cbz-Lys-ONp (10^{-4} M) at pH 5.0.

Step	Total protein (mg)	Total activity ($\Delta A_{340} \cdot \text{min}^{-1}$)	Specific activity ($\Delta A_{340} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$)	Purification (fold)	Yield (%)
Fresh gastric juice (60 ml)	3248	16300	5	—	100
Thiopropyl-Sepharose	350	13000	37	7.4	80
QAE-Sephadex	78	7670	98	19.6	47
Sephadex G-50	65	5400	83	16.6	33

freeze-dried juice could be stored at -20°C and used after long periods without significant loss of activity.

Initially, the proteinase was purified by gel filtration on Sephadex G-50 and ion-exchange chromatography on DEAE-Sepharose columns. It was found that, although EDTA and 2-mercaptoethanol were present in the buffer solutions, enzyme activity declined rapidly and irreversibly, so that recoveries were often less than 1 or 2% of original activity. Purification of mercury-inactivated enzyme gave similarly low yields.

Significant improvement in recovery was achieved by a more rapid purification procedure using covalent chromatography. The results of purification by this procedure are presented in Table 1. Thiopropyl-Sepharose 6B (15 g) with 2-thiopyridyl protecting groups was added directly to the cold, fresh digestive juice (60 ml), requiring no pH adjustment to the natural pH of 4.5. Used in a batchwise fashion, this step took about 3 h to complete and gave a 7-fold increase in specific activity with 80% recovery. Despite the high specificity of the method for cysteine proteinases, at least six bands were visible by SDS/PAGE, although the affinity column had been well washed with sodium acetate buffer before elution. The eluate was applied directly to the ion-exchange column and eluted overnight with a salt gradient, resulting in a further 2.6-fold purification with a recovery of 60% of the original activity. Two or three minor contaminants were still detectable by electrophoresis, but were removed by gel filtration on a Sephadex G-50 column. During the final purification step there was a decrease in specific activity due to gradual irreversible inactivation.

Stability

The specific activity of freeze-dried gastric juice was similar to that of the fresh material. It is possible that the high protein concentration and (or) a non-volatile low- M_r thiol compound protect the enzyme. The purified enzyme was considerably more sensitive to freeze-drying, which occasionally resulted in as much as 80% loss of activity. Solutions of the purified enzyme kept at 2°C and pH 5–8 in the presence of 1 mM-EDTA gradually lost activity over several days. Most of the lost activity was recoverable on incubation with 10 mM-DTT or 50 mM-2-mercaptoethanol. Recovery of activity took several hours with 2-mercaptoethanol and was thiol-concentration-dependent. With 10 mM-DTT, however, maximum activity was recovered in 10 min.

Stability of the purified proteinase to high temperatures was tested in Tris/HCl buffer, pH 6.5, containing 1 mM-EDTA and 10 mM-2-mercaptoethanol. At 60°C the half-

life was 18 min and at 80°C about 2 min. The lobster enzyme, therefore, is less temperature-stable than papain, which, under the same conditions at 60°C , lost half of its activity in 2 h.

Inhibitors

Enzyme activity was strongly inhibited by some heavy-metal ions, all at 1 mM concentrations. Cu^{2+} , Hg^{2+} and Ag^{+} were the strongest inhibitors. Zn^{2+} , Cd^{2+} and Pb^{2+} were less effective, and Ca^{2+} , Mg^{2+} and Mn^{2+} produced little or no inhibition of proteinase activity. Nine organic proteinase inhibitors were tested. The cysteine proteinase inhibitors E-64, antipain, leupeptin, dithionitrobenzene, *p*-hydroxymercuribenzoate and iodoacetic acid, all at 1 mM concentrations, totally eliminated proteinase activity. Neither EDTA nor the serine-proteinase inhibitor phenylmethanesulphonyl fluoride had any effect. Owing to the specificity of E-64 for papain-like cysteine proteinases (Barrett *et al.*, 1982), its inhibition of the crude and purified lobster digestive proteinase was tested further. With purified enzyme, stoichiometric amounts of E-64 eliminated activity completely. With crude gastric juice, 10^{-4} M-E-64 eliminated 80% of the total proteolytic activity when the caseinolytic assay was used. Thus most of the proteolytic activity of the digestive juice was associated with the cysteine proteinase.

Table 2. Comparison of the rates of hydrolysis of Cbz-amino acid-ONp substrates by the lobster proteinase

Measurements were made at 25°C , pH 7.0, in 20% (v/v) acetonitrile/20 mM-phosphate/1 mM-EDTA buffer at 0.1 mM substrate concentrations. All rates have been converted into percentages, taking the rate with the lysine-based substrate as 100%.

Amino acid in the Cbz-amino acid-ONp substrate	Rate (%)
Lysine	100
Alanine	83
Asparagine	40
Glycine	13
Leucine	13
Tyrosine	11
Glycylglycine	5
Valine	4
Isoleucine	2
Proline	0.5

Specificity for synthetic substrates

The results of a screening study of the hydrolysis rates of several *p*-nitrophenyl esters (ONp) of benzyloxycarbonyl (Cbz) amino acids by the lobster proteinase (Table 2) indicated that, like papain, the lobster enzyme has a preference for lysine in the P₁ position of the substrate and a low affinity for proline or bulky hydrophobic amino acids (i.e. tyrosine, valine, leucine and isoleucine) in this position. Kinetic constants for the three good substrates Cbz-Lys-ONp, Cbz-Ala-ONp and Cbz-Gly-ONp were determined at pH 6 and 25 °C. The values obtained were: for k_{cat}/K_m 310 ± 50 , 110 ± 6 and $8.2 \pm 0.3 \text{ mM}^{-1} \cdot \text{s}^{-1}$, and for k_{cat} 6.1 ± 0.5 , 6.5 ± 0.3 and $1.6 \pm 0.1 \text{ s}^{-1}$ for the lysine, alanine and glycine substrates respectively. For the dipeptide substrates, Cbz-Arg-Arg-7-amido-4-methylcoumarin and Cbz-Phe-Arg-7-amido-4-methylcoumarin at pH 5.0 the specificity constants, K_{cat}/K_m , were 2290 ± 250 and $2050 \pm 20 \text{ M}^{-1} \cdot \text{s}^{-1}$ respectively.

Effect of pH and temperature

Using Cbz-Ala-ONp as the substrate, the effects of pH and temperature on the catalytic activity of the lobster proteinase were investigated. The pH-dependency results are shown in Fig. 1. The continuous line through the k_{cat}/K_m data points was drawn by using the equation:

$$k_{\text{cat}}/K_m = k_1 \left/ \left[\frac{h}{K_1} + 1 + \frac{K_2}{h} + \frac{K_2 K_3}{h^2} \right] \right. + k_2 \left/ \left[\frac{h^2}{K_1 K_2} + \frac{h}{K_2} + 1 + \frac{K_3}{h} \right] \right.$$

where h is the hydrogen-ion concentration and $\text{p}K_1 = 4.4$, $\text{p}K_2 = 5.05$, $\text{p}K_3 = 8.6$, $k_1 = 425 \text{ mM}^{-1} \cdot \text{s}^{-1}$ and $k_2 = 85 \text{ mM}^{-1} \cdot \text{s}^{-1}$. The pH-dependence of k_{cat} is less well defined.

Fig. 1. Plots of the pH-dependencies of k_{cat}/K_m and k_{cat} values for the lobster-proteinase-catalysed hydrolysis of Cbz-Ala-ONp

Conditions: 25 °C; 0.2 M-NaCl/1 mM-EDTA/20% acetonitrile. Buffers were as follows: for pH 3.5–5.5, sodium acetate (100 mM); for pH 6.0–8.0, sodium phosphate (50 mM); and for pH 8.5, sodium carbonate (50 mM).

Fig. 2. Arrhenius plots of k_{cat}/K_m and K_{cat} for the lobster-proteinase-catalysed hydrolysis of Cbz-Ala-ONp

Conditions: acetonitrile, 20%; sodium acetate, 0.1 M; NaCl, 0.2 M, pH 5.0; EDTA, 1 mM.

Table 3. Amino acid composition of lobster digestive proteinase

For experimental details, see the text.

	Amino acid composition (residues/molecule)		
	Lobster proteinase		Papain (nearest integer)
	Analysis values	Nearest integer	
Cys*	11.21	11	7
Asp	28.69	29	19
Thr†	18.85	19	8
Ser†	24.63	25	13
Glu	25.56	26	20
Pro	7.32	7	10
Gly	30.21	30	28
Ala	21.85	22	14
Val‡	17.97	18	18
Met	2.38	3	0
Ile‡	10.94	11	12
Leu‡	11.45	12	11
Tyr‡	12.05	12	19
Phe‡	6.94	7	4
His	4.52	5	2
Lys	7.52	8	10
Arg	9.93	10	12
Trp§	6.36	6	5
Total no. of residues...	261	212	
M_r ...	28048	23431	

* Measured as cysteic acid after performic oxidation.

† Values from 24 and 72 h hydrolysates were extrapolated to zero time.

‡ Only the 72 h hydrolysis values taken.

§ After hydrolysis in 4 M-methanesulphonic acid.

|| From Cohen *et al.* (1986).

Enzyme	Sequence
Lobster	Ser-Thr-Glu-Val-Asp-Trp-Arg-Thr-Glu-Gly-Ala-Val-Thr-Pro
Papain	Ile-Pro-Glu-Tyr-Val-Asp-Trp-Arg-Gln-Lys-Gly-Ala-Val-Thr-Pro
Lobster	Val-Lys-Asp-Gln-Gly-Gln-Cys-Gly-Ser-Cys-Trp-Ala-Phe-Ser
Papain	Val-Lys-Asn-Gln-Gly-Gly-Ser-Cys-Gly-Ser-Cys-Trp-Ala-Phe-Ser

$$\text{EH}_3 \xrightleftharpoons{pK_{a_1}} \text{EH}_2 \xrightleftharpoons{pK_{a_2}} \text{EH} \xrightleftharpoons{pK_{a_3}} \text{E}$$

$$\begin{array}{ccc} & \downarrow k_1 & \downarrow k_2 \\ & & \end{array}$$

Cycle no.	Identification	Amino acid PTH derivative recovered (pmol)
1	Ser	125
2	Thr	133
3	Glu	51
4	Val	40
5	Asp	61
6	Trp	46
7	Arg	24
8	Thr	121
9*	Glu	31
10	Gly	115
11	Ala	89
12	Val	33
13	Thr	72
14	Pro	28
15	Val	32
16	Lys	14
17	Asp	28
18	Gln	28

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From the data in Fig. 1, $pK_{a_1} = 4.4$, $pK_{a_2} = 5.05$, $pK_{a_3} = 8.6$, $k_1 = 425 \text{ mM}^{-1} \cdot \text{s}^{-1}$ and $k_2 = 85 \text{ mM}^{-1} \cdot \text{s}^{-1}$. The pK_a values that define the pH-dependency of $k_{\text{cat.}}/K_m$ are due to the ionization of groups on the free enzyme, and k_1 and k_2 in Scheme 1 above represent $k_{\text{cat.}}/K_m$ values for the two active ionization states. For papain, $k_{\text{cat.}}/K_m$ displays a straightforward bell-shaped pH-dependency, indicating a single reactive ionization state (Williams & Whitaker, 1967). The bell-shaped pH-dependency is defined by two acid pK_a values, both near 4, and a single alkaline pK_a near 8.5 (Sluyterman & Wijdsnes, 1973; Lewis *et al.*, 1978). However, Brocklehurst and co-workers [see Brocklehurst *et al.* (1987) and references cited therein] have reported that $k_{\text{cat.}}/K_m$ for actinidin, papaya peptidase A and cathepsin B is enhanced by a deprotonation with a pK_a of approx. 5.5. In particular, a scheme similar to Scheme 1 above has been proposed by Salih *et al.* (1987) for the actinidin-catalysed hydrolysis of *N*-benzoyl-L-arginine *p*-nitroanilide. For this enzyme-substrate combination, $pK_{a_1} = 3.1$, $pK_{a_2} = 5.5$ and $pK_{a_3} = 9.7$, and the magnitudes of k_1 and k_2 are reversed, i.e. for actinidin $k_1 < k_2$ ($k_1 = 2.75 \text{ M}^{-1} \cdot \text{s}^{-1}$ and $k_2 = 8.5 \text{ M}^{-1} \cdot \text{s}^{-1}$). The nature of these $k_{\text{cat.}}/K_m$ -pH profile differences between enzymes of the same class has been discussed by Brocklehurst *et al.* (1987); however, a full understanding awaits further study. Values for $k_{\text{cat.}}/K_m$ with dipeptide coumaride substrates were similar, indicating no significant preference for arginine over phenylalanine in the P_2 subsite of the lobster enzyme. It is possible that the charge is not important, and that it is the long aliphatic side chain of arginine and the aromatic ring of the phenylalanine that interact with the binding site.

Owing to its unusual origin and role, a more detailed study of the lobster digestive cysteine proteinase will provide useful data on the variability among cysteine proteinases in general and, in particular, on the effect of pH on activity. The apparent lack of effect of temperature on $k_{\text{cat.}}/K_m$ may be an adaptation of the enzyme for its role in a cold-blooded animal which must function at temperatures that range from 2 to 20 °C at different times of the year. Furthermore, the fact that the lobster's ability to digest proteins depends largely on an enzyme that is readily inactivated by heavy-metal ions could be significant in physiological studies of this commercially important species.

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