Properties of two chymotrypsins from the digestive gland of prawn *Penaeus monodon*

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For the first time chymotrypsins have been isolated from the hepatopancreas of prawn. Two purified chymotrypsins from *Penaeus monodon* were found to be single-chained with molecular masses of 27 and 26 kDa, respectively. They have chymotrypsin-like specificities, and cleave the insulin chain B preferentially on the carboxyl side of tyrosyl, phenylalanyl and leucyl residues. They were effectively inhibited by soybean trypsin inhibitor, turkey and chicken ovomucoid, chymostatin, PMSF, Z-AlaGlyPhe chloromethyl ketone but not by Z-Phe chloromethyl ketone and many other small protease inhibitors. The shrimp enzymes hydrolyze protein substrates as rapidly as but small substrates much slower than bovine chymotrypsin, suggesting the importance of an extended substrate interacting site.

(Shrimp) Chymotrypsin Protease Substrate specificity Insulin cleavage site Inhibitor specificity

1. INTRODUCTION

Chymotrypsin-like enzymes are considered to be ubiquitous and indeed, crustacean chymotrypsins have been found in krill [1] and the crab *Uca pugilator* [2]. Nevertheless, low amounts or the absence of digestive chymotrypsin has been reported for many species of crayfishes [3] and shrimps [4–7]. It was puzzling as to whether chymotryptic specificity was lost during the evolution of Crustacea once, or more often. We have studied the digestive proteases of locally important shrimp species including *Penaeus monodon, P. penicillatus, P. japonicus* and *Metapenaeus monoceros*. For all of them we found that 10–30% of the initial proteolysis of protein substrates was carried out by chymostatin-sensitive enzymes and 50–70% by leupeptin-sensitive enzymes [8]. Therefore, both trypsins and chymotrypsin-like enzymes are important for the digestion of food proteins by these shrimps. Here, we have isolated two major chymotrypsins from *P. monodon* and studied their specificities.

Abbreviations: Z-, benzyloxycarbonyl-; Boc-, *t*-butoxycarbonyl-; Suc-, succinyl-; Bz-, benzoyl-; -NA, *p*-nitroanilide; BzTyrEE, *N*-benzoyl-L-tyrosine ethyl ester; AcTyrEE, *N*-acetyl-L-tyrosine ethyl ester; PMSF, phenylmethanesulfonyl fluoride; ZPCK, Z-L-phenylalanine chloromethyl ketone; ZAGPCK, Z-L-alanylglycyl-L-phenylalanine chloromethyl ketone; α-CT, bovine α-chymotrypsin

2. MATERIALS AND METHODS

2.1. *Purification of chymotrypsins*

The detailed procedures of purification will be described elsewhere. Briefly, shrimp hepatopancreas extract [8] was chromatographed on a DEAE-cellulose ion exchanger (Whatman) at pH 7.0 and eluted with a salt gradient. Each of the two major chymotrypsin fractions was subsequently purified by gel filtration on a Fractogel TSK HW-55 column (Merck) and then a Sephacryl S-200 column (Pharmacia).

2.2. *Enzyme assays*

The hydrolysis of *p*-nitroanilide substrates was followed spectrophotometrically at 410 nm [2,9].

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The hydrolysis of *p*-nitrophenyl esters, BzTyrEE, and AcTyrEE, was followed at 400 [10], 256 [11] and 237 nm [12], respectively. The concentrations of chymotrypsin were titrated with the soybean inhibitor. All kinetic studies were performed in 50 mM phosphate (pH 7.6) at 25°C. The values of K_m and k_{cat} were determined according to Lineweaver-Burk.

2.3. *Cleavage sites of insulin chain B*

Bovine insulin oxidized chain B (0.35 mg) was incubated with shrimp chymotrypsins at an enzyme to substrate ratio of 1:100 (w/w) in 0.5 ml of 50 mM phosphate buffer (pH 7.6) at 37°C for 2 or 18 h. The reaction was stopped by adding 0.5 mM PMSF. The digested products were fractionated by HPLC using a μBondapak-C18 column (4.6 × 300 mm, 10 μm, Waters). A linear gradient of 1-propanol (0.33% per min) in 15 mM Tris-HCl (pH 7.1) at a flow rate of 1 ml/min was run at 30°C. Each fraction corresponding to peaks manually collected was dried in vacuo, hydrolyzed with 6 N HCl and analyzed on an LKB 4150 amino acid analyzer.

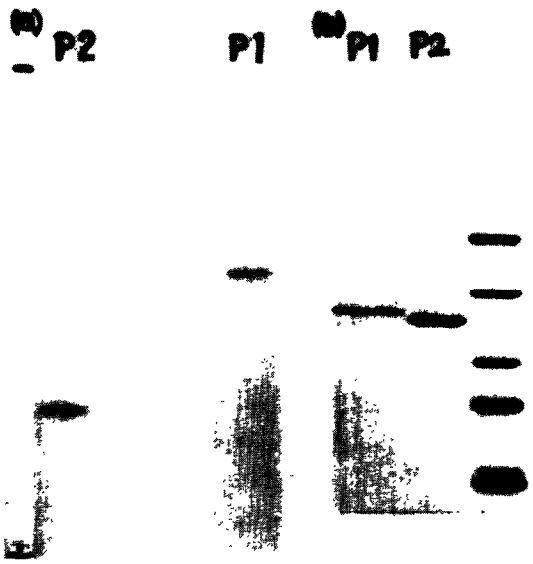

Fig.1. Gel electrophoresis of shrimp chymotrypsins P1 and P2. (a) Disc gel (12%) under nondenaturing condition [14]. (b) SDS-PAGE [15] of the enzymes in 12% gel; the right-most lane shows molecular mass markers: aldolase (36 kDa), carbonic anhydrase (29 kDa), soybean trypsin inhibitor (20 kDa), phospholipase A_2 (14 kDa) and cobra cardiotoxin (7 kDa). The molecular masses of P1 and P2 were estimated to be 27 and 26 kDa.

2.4. *Chemicals*

Suc-AlaAlaProPhe-NA and Z-PheLeuGlu-NA were from Boehringer Mannheim. All the *p*-nitrophenyl esters used, bovine α-chymotrypsins, insulin chain B (oxidized), Suc-AlaAlaProLeu-NA, Bz-ValGlyArg-NA, AlaAlaPhe-NA, Ac-TyrEE, BzTyrEE, ZPCK, TPCK, PMSF, chymostatin, leupeptin, trypsin inhibitors (soybean, turkey and chicken ovomucoid) are products of Sigma. ZAGPCK was a generous gift from Dr K. Morihara [13].

3. RESULTS AND DISCUSSION

We have found Suc-AlaAlaProPhe-NA and Bz-Arg-NA to be good specific substrates for assay of shrimp chymotrypsin and trypsin [8]. Two major chymotrypsin peaks (P1, P2) were eluted immediately before trypsin peaks during DEAE-cellulose chromatography. The enzymes purified after the final gel filtration column were homogeneous (fig.1a) and their molecular masses were shown by SDS gel electrophoresis to be 27 and 26 kDa (fig.1b). They are acidic proteins and are not stable at pH <5 or >9 but, in contrast to bovine chymotrypsin, they are stable for 50 h at pH 7 and 25°C without autodigestion. Calculation

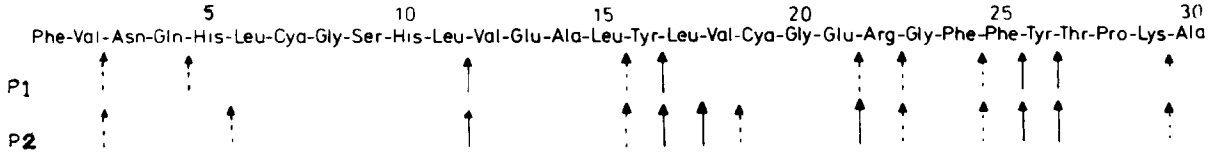

Fig.2. Sites of cleavage of the B chain of oxidized insulin by shrimp chymotrypsin P1 and P2 (1%, w/w). Solid arrows denote major cleavages and dashed arrows denote minor cleavages. Major cleavages are those which appear early (2 h) and in relatively high yield.

Table 1

Hydrolysis of *N*-acylamino acid *p*-nitrophenyl esters

p-Nitrophenyl ester	k_{cat}/K_m ($\times 10^{-4}$) ($s^{-1} \cdot M^{-1}$)		
	Shrimp		Bovine chymotrypsin
	P1	P2	
Z-Phe	86	139	258
Z-Tyr	62	290	233
Z-Trp	36	145	163
Z-Leu	20	70	95
Z-Lys	7	12	6
Boc-Gln	2	5	2.2
Z-Ile	0.1	0.1	0.1
Z-Ala	1	0.7	3

Reactions were in 50 mM phosphate (pH 7.6) with 1×10^{-5} M substrate and 5% *N,N*-dimethylformamide. Values of k_{cat}/K_m were the average of triplicate determinations and were corrected for nonenzymatic rates

based on the specific activities of purified P1 and P2 and that of the crude extract indicated that P1 and P2 together represent about 6–8% of the proteins in the hepatopancreas extract.

The cleavage sites of the shrimp chymotrypsins on oxidized bovine insulin chain B (fig.2) suggest that their peptide-bond specificities are similar to that of chymotrypsins from crab [2], hornet and *Streptomyces griseus* [16]. They prefer bonds at the carboxyl side of tyrosyl, phenylalanyl and leucyl residues, and to a lesser extent, those of lysyl, arginyl and glutaminyl residues.

The specificity of the shrimp chymotrypsins was further studied with small synthetic substrates. For activated substrates such as the *p*-nitrophenyl esters (table 1), the shrimp enzymes showed primary specificity for the aromatic amino acid esters. Although active toward BTEE and ATEE, their rates were much slower than those of α-chymotrypsin (table 2). The shrimp enzymes favor substrates with extended polypeptide chains; they

Table 2

Hydrolysis of synthetic substrates by shrimp P1, P2 and bovine chymotrypsin

Substrate	Enzyme	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\times 10^{-3}$) ($s^{-1} \cdot M^{-1}$)
Suc-Ala_2ProPhe-NA	P1	8.9	250	28
	P2	0.56	7.8	14
	αCT	0.08	26	330
Suc-Ala_2ProLeu-NA	P1	0.45	6.2	14
	P2	1.0	18	18
	αCT	0.97	8.2	8.5
BTEE	P1	1.1	27	25
	P2	0.80	41	51
	αCT	0.11	35	320
ATEE	P1	1.6	12	7.6
	P2	9.1	80	8.8
	αCT	0.7	71	101
Bz-ValGlyArg-NA	P1		<0.1	<0.1
	P2	0.29	0.72	2.5
	trypsin	0.16	440	2650

Assays of P1 and P2 with Bz-ValGlyArg-NA were performed in the presence of 4.0 μM leupeptin to eliminate contaminating trypsin activity. The values are averages of triplicate determinations

did not hydrolyze the *p*-nitroanilide of various *N*-acyl amino acids or dipeptides tested, but did hydrolyze those of the acyl tripeptides or longer substrates of the correct specificity including: Suc-AlaAlaProPhe-NA, Suc-AlaAlaProLeu-NA and Bz-ValGlyArg-NA (table 2). However, the K_m values for the shrimp enzymes are higher than those for the bovine enzyme. AlaAlaPhe-NA and Z-PheLeuGlu-NA are not substrates for P1 or P2.

The preference for extended polypeptide chain also manifested itself in the inhibitor specificities. While PMSF and ZAGPCK irreversibly and effectively inhibited P1 and P2, TPCK, ZPCK and phenylboronic acid, which are inhibitors of bovine chymotrypsin, were without effect (fig.3). The small titrants, *p*-nitrophenyl guanidinobenzoate and *t*-cinnamoylimidazole, were not reactive with P1 and P2. Oligopeptide inhibitors of microbial origin, chymostatin and leupeptin, were also tested for their ability to inhibit shrimp chymotrypsins, and we found chymostatin to be far more effective

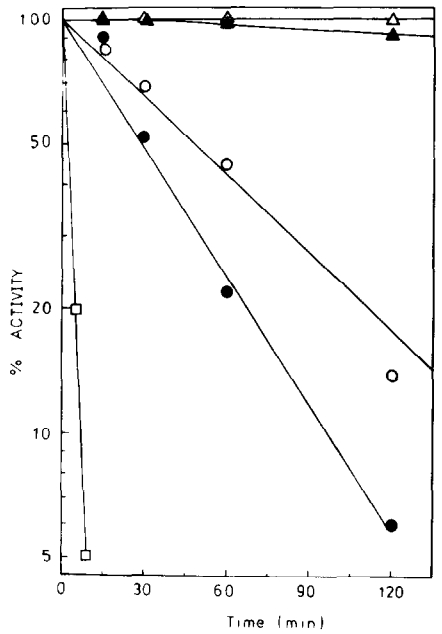

Fig.3. Inhibition of shrimp and bovine chymotrypsins. The enzymes (5×10^{-7} M) were incubated with 1.0×10^{-5} M ZAGPCK or 5.0×10^{-5} M ZPCK in 50 mM phosphate (pH 7.6) at 25°C. Shrimp P1 and ZPCK (▲); shrimp P2 and ZPCK (△); shrimp P1 and ZAGPCK (●); shrimp P2 and ZAGPCK (○); bovine chymotrypsin and ZAGPCK (□).

($K_i \simeq 8 \times 10^{-8}$ M) than leupeptin ($K_k \simeq 1.8 \times 10^{-5}$ M). The shrimp enzymes could also be effectively inhibited by soybean trypsin inhibitor, turkey and chicken ovomucoid, and bovine pancreatic inhibitor. Thus, the shrimp enzymes probably have an active site homologous to the chymotrypsin family.

In conclusion, shrimps have chymotrypsins with rather broad specificities to complement trypsins in digestion of food. The reactivities of synthetic substrates and inhibitors increase with increasing peptide length, and the shrimp enzyme hydrolyzed gelatin and hemoglobin as fast as bovine chymotrypsin did (not shown), suggesting the importance of secondary interactions between the enzymes and their peptide substrates. These interactions probably involve several residues on each side of the scissile bond. The previously reported absence of chymotrypsin in shrimps [4–7] might be explained by the limitation of the substrates (ATEE or BTEE) and inhibitors (such as TPCK and ZPCK) applied in most of the investigations.

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