Collagenolytic Protease Complex from *Paralithodes Camtschatica* Hepatopancreas: Separation into Individual Components

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Nine proteases, the main components of the collagenolytic complex from hepatopancreas of *Paralithodes camtschatica* crab have been isolated by gel filtration and FPLC, including ion-exchange chromatography on DEAE-Toyo-Pearl 650M and Mono-Q columns and hydrophobic interaction chromatography on a Phenyl-Superose column. Molecular weights of the resultant proteases were 36, 35 (2 proteins), 32, 28, 25 (3 proteins), and 23 kD (according to SDS-PAAG electrophoresis and HPLC on a Zorbax GF-250 column). N-terminal analysis showed that six proteases were trypsin-like enzymes homologous to digestive trypsins from other sources. One protease (23 kD) was related to *Astacus fluviatilis* metalloprotease and two others (32 and 35 (I) kD possess unique N-terminal sequences.

Key Words: *protein separation; collagenolytic complex; collagenolytic metalloprotease*

Collagenolytic proteases (CLP) of invertebrates have been known for a long time [10,15]. Many properties, including substrate specificities for natural and synthetic substrates, have been studied in detail, primary structures of some proteases have been completely determined [6,13], as well as structures of their active centers [7], which allows to classify these enzymes as trypsin-like. Studies of the collagenolytic complex (CLC) of *Paralithodes camtschatica* crab, particularly interesting from the viewpoint of its medical and cosmetological use, are in progress [2], but of components native complex were not separated until present because of complex structure of CLC. This paper describes the process of separation of *Paralithodes camtschatica* CLC. Although individual components of crab CLC have been described [1,8]*, as well as tritium-labeled native CLC of *Paralithodes camtschatica* crab retaining its physiological activity [9], separation of CLC has never been described yet. Preliminary electrophoregrams of crab CLC preparations indicated the presence of at least six main proteolytic components.

MATERIALS AND METHODS

"Acetone powder" containing CLC from *Paralithodes camtschatica* hepatopancreas was obtained by the acetone butanol method [5]. The following chemicals were used: acrylamide, bis-acrylamide, Tris-HCl (Sigma); EDTA, glycin, Coumassie R-250, SDS (Serva); other reagents were from Russia.

The initial preparation was obtained by gel filtration on a Sephadex G-75 column. Buffer: 50 mM Tris-HCl (pH 7.5), 0.5 M NaCl, and 1 mM $CaCl_2$. Acetone powder in buffer (5 g per 20 ml) was centrifuged for 10 min at 3000*g* and the supernatant was collected,

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*The species are confused in reports [1,8] on investigations of *Paralithodes Camtschatica* and *Chionoecetes opilio* because of an error made by the crab preparation supplier. All results attributed to *Chionoecetes opilio* should be referred to *Paralithodes Camtschatica* and *vice versa*. Enzyme complexes of both crabs have enzymes with the same molecular weight (28 and 36 kD) differing from each other by other characteristics.

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after which the precipitate was extracted with buffer and centrifuged again. Supernatants were pooled, a 50-ml aliquot with protein concentration of 20 mg/ml was layered onto a Sephadex G-75 column (2.6×100 cm, Pharmacia) and fractionated at 10°C and elution rate of 30 ml/h. The separation was controlled by an UV-1 detector (280 nm, Pharmacia). The fractions were tested for proteolytic activity by gelatin film hydrolysis [12]: 10-15 μl of each fraction was layered onto exposed and developed Orwo-Chrom U18 (Orwo) reversible film and incubated at 37°C for 20 min, after which proteolytic activity of a fraction was evaluated by the presence of gelatin hydrolysis zones. Active fractions were pooled, dialyzed, and lyophilized, to obtain the initial preparation.

Ion-exchange FPLC on DEAE-Toyo-Pearl 650M column. Buffer: 50 mM Tris-HCl (pH 7.5), 3 mM $CaCl_2$. Initial preparation (0.5 g in 20 ml buffer) was

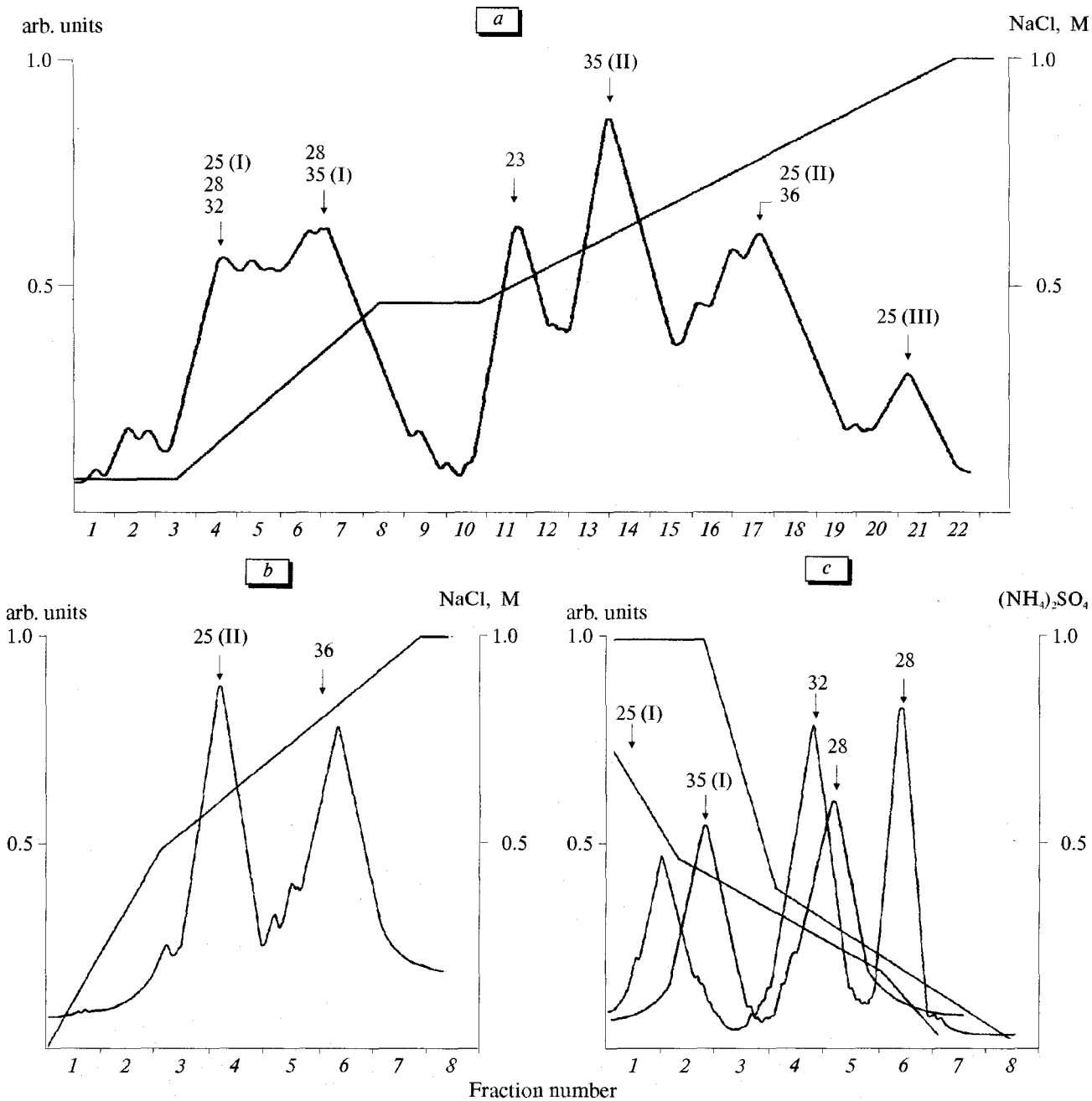

Fig. 1. Chromatograms. Ordinate (left): absorption at 280 nm; figures above the peaks: molecular weights of individual proteins (kD) inorder of their elution from DEAE-Toyo-Pearl 650M. *a*) ion-exchange FPLC of the initial preparation on DEAE-Toyo-Pearl 650M column, fraction volume 5.6 ml. *b*) ion-exchange FPLC (rechromatography) of fractions *18*, *19* (Fig. 1, *a*) on Mono-Q column, fraction volume 10 ml. *c*) hydrophobic interaction FPLC (rechromatography) on Phenyl-Superose column equlibrated with ammonium sulfate: 1 M (3-peak curve) for fractions *4*, *5* (Fig. 1, *a*) and 0.75 M (2-peak curve) for fractions *6*, *7* (Fig. 1, *a*); fraction volume 14.5 ml.

centrifuged for 15 min at 8000g and 4°C, filtered through a 0.2-μ membrane (Millipore), layered onto a DEAE-Toyo-Pearl 650M column (2.5×20 cm, Pharmacia), washed with buffer (2 ml/min), and eluted with 0-1 M linear NaCl gradient in 100 ml buffer at a rate of 2 ml/min. The separation was controlled as in gel filtration.

Ion exchange FPLC on Mono-Q column. Buffer: as for FPLC on DEAE-Toyo-Pearl 650M. Fractions obtained by ion-exchange FPLC on DEAE-Toyo-Pearl 650M were filtered through a 0.2-μ membrane, diluted 10-fold with buffer reducing ionic strength of the solution, layered onto a Mono-Q column (5×50 mm, Pharmacia), washed with 15 ml buffer (0.2-0.3 ml/min), and eluted with 0-1 M linear NaCl gradient in 60 ml buffer at a rate of 0.2-0.3 ml/min. Separation was controlled as in gel filtration.

Hydrophobic interaction FPLC on Phenyl-Superose column. Buffer: as for FPLC on DEAE-Toyo-Pearl 650M with 1 M (0.75 M) ammonium sulfate. Saturated ammonium sulfate solution in a final concentration of 1 M (0.75 M) was added to fractions obtained by ion-exchange FPLC on DEAE-Toyo-Pearl 650M and layered onto a Phenyl-Superose column (5×50 mm, Pharmacia) equlibrated with buffer containing the corresponding concentration of ammonium sulfate. The preparation was eluted with inverse 1-0 M ammonium sulfate gradient in 90 ml (from 0.75 to 0 M in 70 ml) buffer. Separation was controlled as in gel filtration.

HPLC of resultant proteases on a Zorbax GF-250 column (9.4×250 mm, DuPont) was carried out by gel filtration in 50 mM Tris-HCl buffer (pH 7.5) containing 0.2 M Na_2SO_4 at a flow rate of 1 ml/min.

SDS-polyacrilamide gel electrophoresis (SDS-PAGE) of protein fractions was carried out at 400 V for 4 h after preinactivation of proteins with trichloroacetic acid (5%). Pharmacia electrophoretic marker kit was used: 94 kD (phosphorylase B), 67 kD (BSA, monomer), 43 kD (ovalbumin), 30 kD (carbonylanhydrase), 20.1 kD (soybean trypsin inhibitor), and 14.4 kD (α-lactalbumin).

N-terminal sequences of individual proteases were analyzed by automated Edman degradation on a Beckman-890C sequencer with 1 M quadrolic buffer. PTH-amino acids were separated and identified by HPLC on a Zorbax PTH (4.6×250 mm, DuPont) equlibrated with 16.7 mM NaAc buffer (pH 5.5). PTH-amino acids were eluted with a methanol:acetonitryl mixture (17:3). Protein preparations were preliminarily denatured with phenol and 10% trichloroacetic acid; phenol was removed by 6-fold extraction with ice-cold acetone and dried.

RESULTS

The initial stage of CLP purification from acetone powder eliminated insoluble admixtures, high-molecular-weight polysaccharide components, and polypeptides with molecular weight above 60 kD and partially eliminated pigments present in acetone powder. Some known properties of proteolytic enzymes from crab hepatopancreas (low isoelectric points, stability at alkaline pH) suggested that preliminary fractionation of the initial preparation should be carried out by FLPC on a DEAE-Toyo-Pearl 650M column. Under these conditions proteins were eluted by increasing ionic strength of the eluent (Fig. 1, *a*).

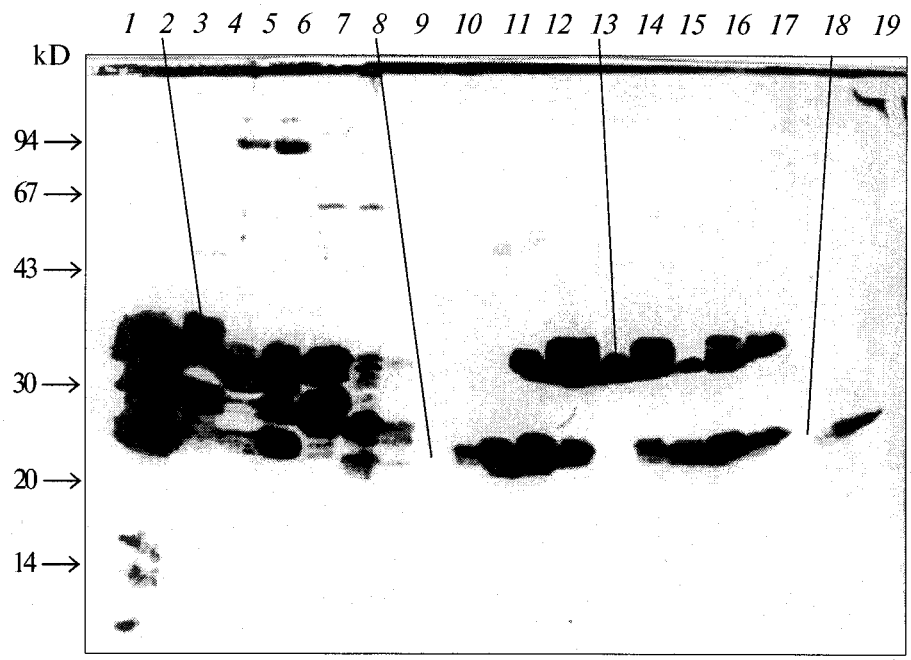

Fig. 2. Electrophoregram of active fractions (by gelatin film hydrolysis) obtained by FPLC of the initial preparation on DEAE-Toyo-Pearl 650M column. *1*) initial preparation; *2-19*) fractions *4-21*, respectively (Fig. 1, *a*).

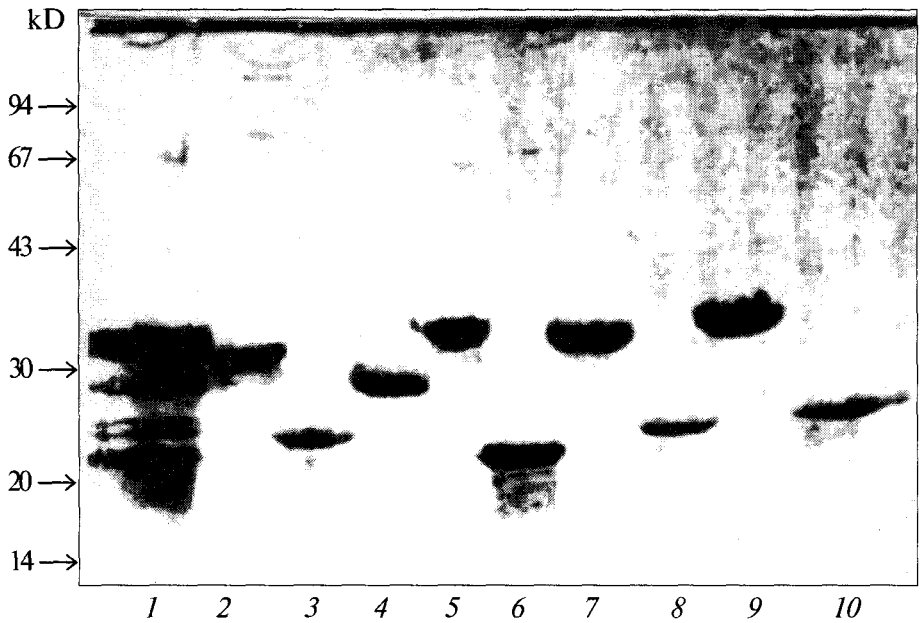

Fig. 3. Electrophoregram of nine individual proteases in order of their elution from DEAE-Toyo-Pearl 650M. *1*) initial preparation; *2*) 32 kD; *3*) 25 (I) kD; *4*) 28 kD; *5*) 35 (I) kD; *6*) 23 kD; *7*) 35 (II) kD; *8*) 25 (II) kD; *9*) 36 kD; *10*) 25 (III) kD.

SDS-PAGE of active fractions (Fig. 2) showed the presence of several proteins with the same molecular weight, but different chromatographic properties in the initial preparation: two 35-kD and three 25-kD proteases. These proteins numbered in order of their elution from DEAE-Toyo-Pearl 650M column will be referred to as 35 (I), 35 (II), 25 (I), 25 (II), and 25 (III) kD. The 25 (I), 28, 32, and 35 (I) kD proteases were poorly separated and desorbed from the column in one peak; proteases more tightly bound to ion-exchanger (23, 35 (II), and 25 (III) kD) were desorbed homogenous; and fractions containing proteases 25 (II) and 36 kD were released from the column as overlapping peaks and rechromatography was needed.

Proteases 25 (II) and 36 kD were separated by FPLC on a Mono-Q column by rechromatography of fractions 18 and 19 (Fig. 1, *a*). Desorption of proteases was performed at NaCl concentration 0.6-0.8 M and yielded homogenous 25 (II) and 36 kD protein fractions (Fig. 1, *b*).

Mono-Q was ineffective for separation of less charged proteins 25 (I), 28, 32, and 35 (I) kD. These proteases were separated by hydrophobic interaction FPLC on a Phenyl-Superose column: ammonium sulfate was added to fractions 4, 5, and 6, 7 (Fig. 1, *a*) to concentrations 1 and 0.75 M, respectively, after which the preparations were eluted in inverse gradient from the above concentrations to zero. Gradient 1-0 M helped to separate 25 (I), 32, and 28 kD proteases; 35 (I) and 28 kD proteases, also homogenous, were obtained in a smoother gradient (starting from 0.75 M) (Fig. 1, *c*).

Electrophoregram of nine individual CLP isolated from crab CLC is shown on Fig. 3. The homogeneity of individual proteases was confirmed by their HPLC on a Zorbax GF-250 column and ion-exchange FPLC on a Mono-Q column: during rechromatography individual CLP were desorbed as symmetrical peaks, absorption at 280 nm coinciding with proteolytic and collagenolytic activities.

Comparison of amino acid sequences of 20 N-terminal amino acid residues (Table 1) showed that 6 of 9 proteases isolated from *Paralithodes camtschatica* hepatopancreas are trypsin-like, most similar to *Uca pugilator* crab proteases (although they are homologous to all trypsins). Two proteases (32 and 35(I) kD) are characterized by unique N-terminal sequences which have no analogs, and one protease (23 kD) is analogous to a metalloprotease enzymes first found in *Astacus fluviatilis* lobster [13]. Though lobster protease was not tested for collagenolytic activity, the similarity of both enzymes (mol. weights, N-terminal sequences, inhibitors) suggests the presence of such activity in lobster enzyme, as well as a structural similarity of these enzymes effectively hydrolyzing various types of native and denatured (gelatinated) collagen.

Hence, we isolated homogeneous individual components of *Paralithodes camtschatica* CLC and identified them by N-terminal sequences.

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Table 1. N-Terminal Sequences of Proteases from *Paralithodes Camtschatica* Crab and Digestive Proteases from Other Sources

Enzyme	Amino acid residue No.																					
	-2	-1	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Bovine chymotrypsin A [14]			I	V	N	G	E	E	A	V	P	G	S	W	P	W	Q	V	S	L	Q	D
Bovine trypsin [14]			I	V	G	G	Y	T	C	G	A	N	T	V	P	Y	Q	V	S	L	N	S
Porcine trypsin [4]			I	V	G	G	Y	T	C	A	A	N	S	I	P	Y	Q	V	S	L	N	S
A. fluviatilis trypsin [15]			I	V	G	G	T	D	A	T	L	G	E	F	P	Y	Q	L	S	F	Q	N
H. lineatum trypsin [10]			I	I	N	G	Y	E	A	Y	T	G	L	F	P	Y	Q	A	G	L	D	I
S. griceus protease B [3]			I	S	G	G	D	A	I	Y	S	S	T	G	R	C	S	L	G	F	N	Y
Mixobacter 495 protease [11]	A	N	I	V	G	G	I	E	Y	S	I	N	N	A	S	L	C	S	V	G	F	S
Uca pugilator crab:																						
23 (I) kD [6]			I	V	G	G	V	E	A	V	P	D	S	W	P	H	Q	A	A	L	F	I
24 (II) kD [7]			I	V	G	G	Q	D	A	T	P	G	Q	F	P	Y	Q	L	S	F	Q	D
Chinoecetes opilio crab [8]:																						
28 kD			I	V	G	G	Q	E	A	S	P	G	S	W	P	X	Q	V	G	L	F	F
36 kD — A			I	V	G	G	T	E	V	T	P	G	E	I	P	Y	Q	L	S	L	Q	D
36 kD — B			I	V	G	G	T	E	V	T	P	G	E	I	P	Y	Q	L	S	F	Q	D
36 kD — C			I	V	G	G	S	E	A	T	S	G	Q	F	P	Y	Q	X	S	F	Q	D
Paralithodes camtschatica crab:																						
36 kD			I	V	G	G	T	E	V	T	P	G	E	I	P	Y	Q	L	S	L	Q	D
35 (II) kD			I	V	G	G	T	E	V	T	P	G	E	I	P	Y	Q	L	S	F	Q	D
28 kD			I	V	G	G	Q	E	A	S	P	G	S	W	P	X	Q	V	G	L	F	F
25 (I) kD			I	V	G	G	S	E	A	T	S	G	Q	F	P	Y	Q	X	S	F	Q	D
25 (II) kD			I	V	G	G	Q	E	A	T	P	H	T	W	V	H	Q	V	A	L	F	I
25 (III) kD			I	V	G	G	Q	E	A	T	P	H	T	W	V	H	Q	V	A	L	F	I
32 kD			A	M	D	X	T	A	Y	X	D	Y	D	E	I	Q	A	X	L	K	G	L
35 (I) kD			A	F	D	X	T	N	Y	N	T	F	E	E	I	N	S	I	L	D	G	V
23 kD			A	A	I	L	G	D	E	Y	L	X	S	G	G	V	V	P	Y	V	F	G
A. fluviatilis protease [13]			A	A	I	L	G	D	E	Y	L	W	S	G	G	V	V	P	Y	T	F	G

Note. Conservative and low-variable amino acid residues are shown with a dark background; X denotes amino acid residues not identifed by sequencer.

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