Collagenolytic Protease Complex from Hepatopancreas of Kamchatka Crab: Enzyme Activity of Individual Components

O. A. Klimova and V. Yu. Chebotarev

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 128, No. 10, pp. 391-396, October, 1999
Original article submitted September 21, 1999

We determined primary substrate specificity and proteolytic activity with respect to polypeptides and short synthetic substrates for 9 individual proteases, components of collagenolytic complex of Kamchatka crab, and their dependence on temperature and pH. It was shown that the enzyme complex containing at least one metalloprotease and two serine proteases exhibits a variety of enzyme activities acting synergistically, which is of great importance for medical and cosmetological applications.

Key Words: *collagenolytic proteases; substrate specificity; proteolytic activity; interaction with inhibitors*

Collagenolytic protease complex (CLC) from Kamchatka crab is a highly effective protein-hydrolyzing system, which cleaves collagens and other proteins, as well as short synthetic substrates. CLC hydrolyzes substrates by the carboxylic end of peptide bonds formed by amino acids with positively charged (Lys, Arg), hydrophobic (Phe, Tyr), and short aliphatic (Ala, Gly) side chains. Broad substrate specificity [1] and high proteolytic and collagenolytic activity of crab CLC are due to the presence of many enzyme components with broad substrate specificity and variety of individual collagenolytic protease (CLP) activities.

MATERIALS AND METHODS

The procedure of separation of Kamchatka crab CLC into individual components (9 proteins) is described in detail [2]. Bovine trypsin was from Sigma, N-benzoyl-L-arginine p-nitroanilide (BApNA), benzoyl-L-arginine ethyl ester (BAEE), benzoyl-tyrosyl ethyl ester (BTEE), N-acetyl-(L-alanyl-)$_3$ p-nitroanilide (Ac(Ala)$_3$pNA), 4-phenylazobenzyloxycarbonyl-Pro-Leu-Gly-Pro-D-Arg (PZ-peptide), tosyl-L-lysine chloromethyl ketone (TLCK), tosylamine-L-phenylethyl chloromethyl ketone (TPCK), 4-chloromercuribenzoate (CMB), iodoacetic acid (IAA), dithiothreitol (DTT), EDTA, phenylmethylsulphonyl fluoride (PMSF) and azocaseine were from Serva.

Activity of CLC with respect to synthetic substrates of trypsin (BApNA and BAEE), chymotrypsin (BTEE), elastase (Ac(Ala)$_3$pNA), and clostridial collagenases (PZ-peptide) was assayed spectrophotometrically by the decrement of specific absorbance during enzymatic hydrolysis in buffer solutions containing 3 mM $CaCl_2$. K_m and V_{max} were calculated by Michaelis-Menten equation using the method of least squares.

Proteolytic and collagenolytic activities of CLP were assayed by hydrolysis of azocasein [7] and ^{14}C-acetylated collagen [4] in the presence of 3 mM $CaCl_2$. The effect of inhibitors was evaluated by residual proteolytic activity (azocasein hydrolysis) after preincubation (15 min, 37°C) with inhibitors (final concentra-

St. Petersburg-Tekhnologiya, St. Petersburg

*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.

0007-4888/99/0010-0994$22.00

tions per 0.1-10 μg enzyme): 1 mM EDTA, 2 mM IAA, 3 mM DTT and PMSF, 5 mM CMB, TLCK, and TPCK.

RESULTS

Proteolytic activity of crab CLP with polypeptide substrates (Table 1) was similar to and sometimes even surpassed that of more specific proteases; with respect of synthetic substrates CLP were inferior to specific proteases (Table 2), although K_m in reactions with short-chain substrates (Table 3) for most CLP were only 3-5-fold lower than the corresponding values for pancreatic trypsin-like proteases. The highest chymotrypsin-like activity (by BTEE hydrolysis) was exhibited by 25(II) and 25(III) kD proteases, while 36 and 35(II) kD enzymes were most active in hydrolysis of trypsin-specific substrates (BAEE and BApNA). Elastase substrate ($Ac(Ala)_3pNA$) was hydrolyzed by almost all CLP, but by 1-2 orders of magnitude less effective than trypsin and chymotrypsin substrates. Neither native CLP, nor its individual components hydrolyze PZ-peptide, clostridial collagenases test substrate (only minor activity was noted for 35(II) and 23 kD components), which indicates principal differences between crab CLP and microbial collagenases. All CLP components hydrolyzed azocasein and collagen. Unlike most collagenases, they also hydrolyzed type IV collagen, hemoglobin, fibrin, and some other substrates, which are interesting for applied studies). Seven of 9 proteases exhibit azocasein-hydrolyzing activity similar to that of trypsin. Moreover, a pronounced synergetic effect of crab CLP was observed in native CLC.

Kamchatka crab CLP primarily hydrolyze substrates containing long polypeptide chains (similarly to protease II from *Uca pugilator* [6]), which attests to a considerable contribution of enzyme-substrate interaction in secondary sites into catalytic activity of these proteases.

Noteworthy also are the question about the adequacy of applied methods for measuring the rate of hydrolysis. Introduction of as much as possible insoluble collagen molecules into solution for a short time (attaining the maximum hydrolysis rate of native collagen *in vitro*) and hydrolysis of insoluble collagen into small fragments and further to individual amino

TABLE 1. Activity of CLP from Hepatopancreas of *Paralithodes Camtschatica* Crab Assayed by Hydrolysis of Protein Substrates at pH 7.5 and 37°C

Enzyme	Type I collagen, μg/(mg×h)	Azocasein, rel. units/(mg×h)
36 kD	1860	170
35(I) kD	205	35
35(II) kD	161	150
32 kD	40	26
28 kD	640	320
25(I) kD	1463	180
25(II) kD	230	370
25(III) kD	659	141
23 kD	2038	320
Native CLP	650	450
Bovine trypsin	25	420
Cl. histolyticum		
collagenase I	1416	0
collagenase II	1854	0

TABLE 2. Activity of CLP from *Paralithodes Camtschatica* Crab Assayed by Hydrolysis of Synthetic Substrates at pH 7.5 and 24°C

Enzyme, kD	Activity, μmol/(mg×min)				
	BTEE	BApNA	BAEE	$Ac(Ala)_3pNA$	PZ-peptide
Native CLC	*	*	*	0.08	0
36	*	0.61	235	*	0
35(I)	*	*	0.90	0.18	0
35(II)	*	0.40	102	0.20	*
32	*	*	*	0.15	0
28	*	*	*	0.30	0
25(I)	*	*	*	0.12	0
25(II)	6.4	*	5.2	*	0
25(III)	6.0	*	0.60	*	0
23	*	*	14.2	0.29	*

Note. *Weak activity.

TABLE 3. Kinetic Characteristics of CLP from *Paralithodes Camtschatica* Crab Assayed by Hydrolysis of Synthetic Substrates at pH 7.5 and 24°C

Enzyme, kD	BTEE		BApNA		BAEE		$Ac(Ala)_3pNA$	
	K_m, mM	V_{max}	K_m, mM	V_{max}	K_m, mM	V_{max}	K_m, mM	V_{max}
36	—	—	0.02	2	0.2	100	—	—
35(I)	—	—	—	—	0.008	0.2	—	—
35(II)	—	—	0.01	0.1	0.02	7	—	—
32	—	—	—	—	0.03	0.2	—	—
28	—	—	—	—	0.01	0.03	50	3
25(I)	—	—	—	—	0.007	0.01	—	—
25(II)	0.2	8	—	—	0.2	20	—	—
25(III)	0.2	1.2	—	—	0.4	1	—	—
23	—	—	—	—	0.1	0.01	1	0.05

Note. V_{max}: mmol/(mg×min).

acids (attaining the maximum degree of hydrolysis, especially during purulent inflammation and necrosis *in vivo*) are different problems. There are no universal methods for comparing collagenolytic activities of CLP and collagenases, since their only common feature is the ability to hydrolyze native collagen. Indeed, collagenases hydrolyze native collagen in a single point and this partially denaturated collagen is no longer the substrate for collagenases, while collagen molecule once hydrolyzed by CLP retains its substrate properties and undergoes further hydrolysis.

Collagenolytic activity measured with ^{14}C-acetylated collagen reflects the number of cleaved molecules, but not the number of cleavage cycles. Being adequate for monocleaving collagenases from *Clostridium histolyticum*, this method allows to determine only the lower limit of CLP hydrolysis efficiency. This method was used for primary characterization of individual CLC components; however, when considering medical and cosmetological usage of CLP (when deep hydrolysis is of crucial importance), their activity should be assayed by method [9], i.e. by the number of N-terminal amino acids appeared during peptide hydrolysis (activity of native CLC and its individual components expressed in Mandl units several times surpassed that of clostridial collagenases).

The data on interaction of CKC components with site-specific inhibitors are summarized in Table 4. PMSF partially inhibits 28 kD protease and completely blocks 25(II) and 25(III) kD proteases, which allowed us to assign these enzymes to serine proteases. EDTA inhibits 25(I) protease and, consequently, be-

TABLE 4. Activity of CLP from *Paralithodes Camtschatica* Crab Assayed by Hydrolysis of Azocasein after Preincubation with Site-Specific Inhibitors at pH 7.5 and 37°C

Enzyme, kD	Residual activity, %						
	EDTA	PMSF	IAA	DTT	CMB	TLCK	TPCK
Native CLC	80	38	75	68	108	—	—
36	110	100	100	92	100	11	56
35(I)	65	50	55	50	110	54	77
35(II)	87	65	87	82	84	59	78
32	60	42	60	46	70	30	61
28	78	13	79	70	85	92	80
25(I)	11	100	83	39	145	107	25
25(II)	100	2	81	90	96	90	90
25(III)	96	3	65	87	89	97	33
23	85	66	97	70	82	84	86

Note. The data are means of 5 measurements, Initial enzyme activity (without inhibitors) was taken as 100%.

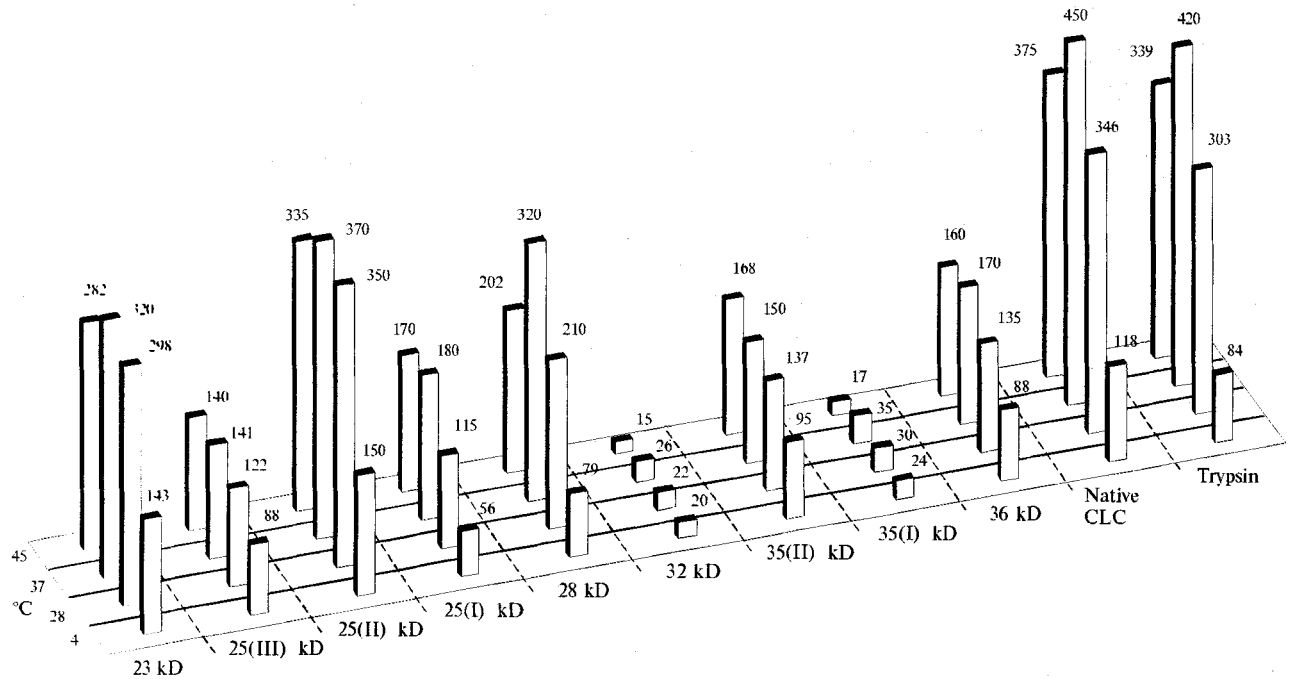

Fig. 1. Protease activity (rel. units/mg×h) from hepatopancreas of *Paralithodes camtschatica* crab at different temperatures assayed by azocasein hydrolysis at pH 7.5. Here and on Fig. 2: CLC, collagenolytic complex.

longs to metalloproteinases (metal-dependent enzyme). Despite marked similarity between N-terminal fragments of 23 kD component and protease from *Astacus fluviatilis* (metalloproteinase with Zn atom in active center [10]), EDTA does not inhibit this CLP component. Chloromethyl ketones only slightly inhibit CLP (similarly to *Uca pugilator* protease [5] and subtilisin [3]). Thus CLC from Kamchatka crab contains at least one metalloenzyme (25(I) kD) and two serine proteases (25(II) and 25(III) kD).

Optimum temperature (Fig. 1) for most CLP (except 35(II) kD protease) is 37°C. As the temperature

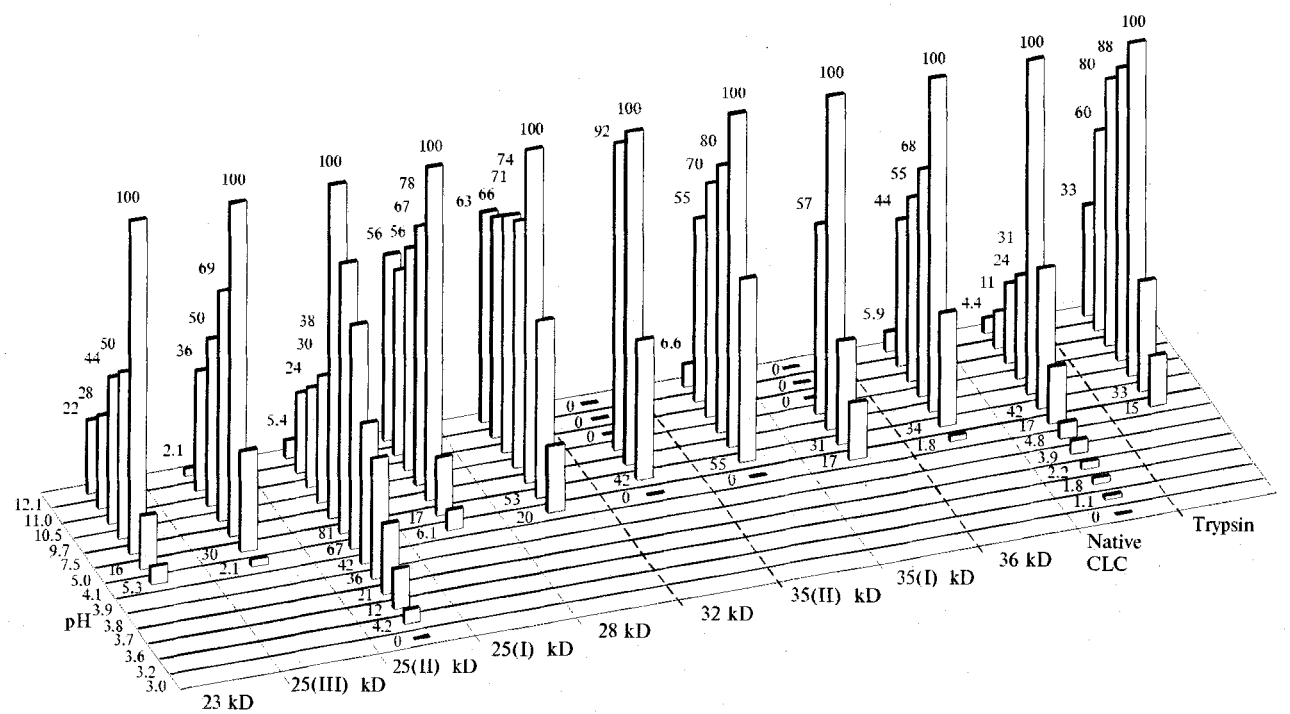

Fig. 2. Protease activity (% of maximum for each enzyme) from hepatopancreas of *Paralithodes camtschatica* crab at different pH assayed by azocasein hydrolysis at 37°C.

decreases, CLP activity decreases more slowly than trypsin activity and considerably surpasses it at 4°C (which is probably due to nutritional habits of deep-water animals). The synergism of CLC components is most pronounced at 28-45°C.

Optimum pH (Fig. 2) for all crab CLP is pH 7.5. All enzymes (except 32 kD protease) retain their activity in a wide pH range (especially 25(II) kD protease), while 32 kD protease sharply losses its activity upon pH shifts to alkaline and acidic ranges. Activity of 25(II) kD protease appears at pH 3.7, while 25(I) and 28 kD proteases retain activity at pH 12.1. All CLC components are irreversibly inactivated at pH below 3.0.

Thus, Kamchatka crab CLC is a multicomponent system of collagenolytic enzymes characterized by highly synergistic proteases activities. Further and more detailed investigations of activity and structure of individual proteins are of value not only for applied studies, but also for better understanding of the evolution of trypsin-like enzymes.

Let us emphasize again possible practical applications of these preliminary data, providing the basis for the development of some new drugs and cosmetics including CLC from various hydrobionts (enzyme preparations Polycollagenase-K and Hydrobiont Collagenase, as well as materials for wound drainage Collasorb and Colladiosorb are developed).

The use of CLP from different hydrobionts (including Kamchatka crab) as the acting factor of drugs and cosmetics was proposed in 6 inventor declarations, among them traditional (preparations of serine proteases and collagenases), novel (regulation of the rate of drug effects), and uncommon (water-color painting effect with tattooing dye mixture) applications. Apart from native CLC, of practical importance is the use of individual components from some hydrobionts and artificial CLP mixtures (for instance, 23 and 36 kD proteases with high collagenolytic activity or 25(II) kD protease retaining activity in a wide pH range), as well as native CLC enriched with some individual components potentiating their specific effects. Tritiation of individual CLP (at sites not involved in group exchange) preserving physiological activity of the enzymes and suitable for accurate pharmacokinetic studies was proposed previously by us [2].

REFERENCES

1. O. A. Klimova, Yu. V. Vedishcheva, and A. Ya. Strongin, *Dokl. Akad. Med. Nauk SSSR*, 317, 482-484 (1991).
2. O. A. Klimova and V. Yu. Chebotarev, *Byull. Eksp. Biol. Med.*, **128**, No. 9, 308-313 (1999).
3. J. S. Bond and R. J. Beynon, *Mol. Aspects Med.*, **9**, 175-287 (1987).
4. T. E. Gawston, *Anal. Biochem.*, **99**, 340-346 (1979).
5. G. A. Grant and A. Z. Eisen, *Biochemistry*, **19**, 6089-6095 (1980).
6. G. A. Grant, J. C. Saccetini, and H. C. Welgus., *Ibid.*, **22**, 354-358 (1983).
7. G. G. Hazen, *Ann. New York Acad. Sci.*, **130**, 761-768 (1965).
8. O. A. Klimova, S. I. Borukhov, N. I. Solovyeva, *et al., Biochem. Biophys. Res. Commun.*, **166**, 1411-1420 (1990).
9. I. Mandl, J. MacLennan, and E. L. Howes., *J. Clin. Invest.*, **32**, 1323-1329 (1953).
10. W. Stocker, R. L. Wolz, R. Zwilling, *et al., Biochemistry*, **27**, 5026-5032 (1988).