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Isolation and Properties of Collagenolytic Serine Proteinase Isoenzyme from King Crab *Paralithodes camtschatica*

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Abstract—An electrophoretically homogeneous isoenzyme CSP-2 of collagenolytic serine proteinase has been isolated from the total preparation of king crab digestive enzymes. The molecular mass of the proteinase is 24.8 ± 0.3 kD, pH optimum for activity is 8.5, the temperature optimum for activity is 38-40°C, and the pH range of stability is 7-10. The enzyme has dual substrate specificity, but preference for positively charged amino acid residues in P_1-position is more pronounced than in the case of the major isoenzyme. The temperature dependence of kinetic constants for synthetic substrate hydrolysis by CSP-2 has been investigated. Inhibition specificity of the enzyme is characteristic of serine proteinases but more like that of crab trypsin than that of the major CSP isoenzyme. The isolated collagenolytic proteinase also cleaves fibrinogen and fibrin and activates plasminogen. The amino acid sequence of the CSP-2 proteinase, which has been partially determined by tandem mass spectrometry, displays some similarity to the sequence of the major CSP isoenzyme.

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Key words: king crab, psychrophilic proteinase, kinetic constants, collagenolysis, fibrinolysis

Serine proteinases of cold sea invertebrates are attractive objects for studying the mechanisms of enzyme adaptation to low temperatures. Many of these enzymes can cleave collagen and therefore are of interest for medicine, biotechnology, and food industry. Collagenolytic serine proteinases of crustaceans belong to the brachyurin subfamily of the chymotrypsin family (according to NC-IUBMB nomenclature, EC 3.4.21.32) [1].

"Moricrase" enzyme preparation from the hepatopancreas of king crab *Paralithodes camtschatica* possesses considerable collagenolytic activity and is an efficient medication for the treatment of burns and wounds [2]. Individual components of this complex—collagenolytic serine proteinase (CSP) [3], trypsin [4], amino- and carboxypeptidase [2], and metalloproteinase [5]—have been isolated and characterized in our laboratory. Klimova and Chebotarev elaborated a procedure for the chromatographic separation of *P. camtschatica* proteinases with molecular masses less than 60 kD, determined their N-terminal amino acid sequences [6], and partially characterized their properties [7]. The aim of the present work was the isolation and characterization of a collagenolytic serine proteinase isoenzyme, which will be referred to as CSP-2.

MATERIALS AND METHODS

"Moricrase", a total preparation of king crab digestive enzymes, was kindly provided by JSC RPE Trinita (Russia). Fibrinogen, soybean trypsin inhibitor (SBTI), hen ovomucoid, phenylmethylsulfonyl fluoride (PMSF), and chromogenic substrates Bz-Arg-pNA, *D*-VLK-pNA, *D*-PFR-pNA, and *D*-VLR-pNA were from Serva (Germany); leupeptin was from MP Biomedicals (USA); thrombin was from PBP (Lithuania); lysozyme was from Olaine Chemicals (Latvia). PageRuler protein markers (11-170 kD) were from Fermentas (Lithuania); protein markers (14.4-94.6 kD) were from Helicon (Russia).

Abbreviations: Bz) benzoyl; CSP) collagenolytic serine proteinase; glp) pyroglutamyl; MALDI) matrix assisted laser desorption ionization; PMSF) phenylmethylsulfonyl fluoride; pNA) *p*-nitroanilide; SBTI) soybean trypsin inhibitor; Suc) succinyl; Tos) *p*-toluenesulfonyl; Z) benzyloxycarbonyl.

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Chromogenic substrates Z-APR-pNA and Z-AFR-pNA were synthesized in the State Institute of Genetics and Selection of Industrial Microorganisms (Moscow, Russia); glp-FA-pNA, glp-FL-pNA, glp-AAL-pNA, and glp-AAFL-pNA were synthesized at the Laboratory of Protein Chemistry, Faculty of Chemistry, Moscow State University. Recombinant glutamyl endopeptidase from *Bacillus intermedius* was prepared in the State Institute of Genetics and Selection of Industrial Microorganisms. Type I collagen from rat tails was kindly provided by Dr. T. S. Kalebina (Department of Molecular Biology, Faculty of Biology, Moscow State University); other collagens, azocasein, α-antitrypsin, antipain, rabbit plasminogen, and chromogenic substrate Suc-AAPF-pNA were from Sigma (USA). Other reagents, of "chemically pure" or "pure" grade, were manufactured in Russia.

Electrophoretically homogeneous CSP-2 was obtained by ion-exchange chromatography on DEAE-Toyopearl and gel chromatography on Sephadex G-50. "Moricrase" solution (10 ml with concentration 25 mg/ml in 0.05 M Tris-HCl buffer, pH 7.5) was applied to a 20 × 2 cm column with DEAE-Toyopearl (Toyo Soda, Japan) equilibrated with the same buffer. After the elution of non-adsorbed proteins by the starting buffer, adsorbed proteins were eluted in a linear 0-1 M NaCl concentration gradient. Absorbance A_{280} and proteolytic activity of the fractions were measured (Fig. 1) and their protein composition was determined using SDS-PAGE according to the procedure of Laemmli [8] (Fig. 2). CSP activity towards glp-AAL-pNA and trypsin activity towards Bz-Arg-pNA were measured according to the procedure of Lyublinskaya et al. [9], and metalloproteinase activity was measured towards azocasein [10]. Fractions which contained CSP-2 were pooled, concentrated by ultrafiltration on a Diaflo (Amicon, USA) cell with an Amicon YM-10 membrane (molecular weight cutoff 10 kD) and subjected to gel chromatography on a 60 × 1 cm column with Sephadex G-50 (Pharmacia, Sweden) equilibrated with 0.1 M ammonium acetate buffer, pH 6.4, which contained 1 M NaCl.

In-gel cleavage of CSP-2 by glutamyl endopeptidase from *B. intermedius* or trypsin after SDS-PAGE according to the procedure of Laemmli [8], as well as the sequencing of some of the cleavage products by tandem MALDI (matrix assisted laser desorption ionization) mass spectrometry was performed at the Center of Postgenome Technologies of the V. N. Orekhovich Institute of Biomedical Chemistry, Moscow, Russia. The mass spectra were processed using the program Peaks 2.0 [11]. Theoretical *m/z* values for the products of king crab trypsin and the major CSP isoenzyme cleavage by the enzymes used for CSP-2 cleavage were calculated by applying the program Peptide Mass (www.expasy.org) to the enzyme sequences determined from cDNA sequences [12].

CSP-2 activity towards peptide *p*-nitroanilides was determined according to the procedure described by Lyublinskaya et al. [9], and activity towards azocasein was determined as described by Shaginyan et al. [10]. The action of the enzyme on fibrin plates was studied according to the procedure of Astrup and Mullertz [13].

The enzyme was incubated with inhibitors in 0.05 M Tris-HCl buffer, pH 8.2, at 22°C for 10 min, and then the initial rate of Z-AFR-pNA hydrolysis was determined (in the case of PMSF and *o*-phenanthroline the enzyme was incubated with inhibitors for 30 min and activity was measured towards azocasein).

The pH optimum of enzyme activity towards Z-AFR-pNA was determined at 37°C in 0.1 M universal buffer ($H_3PO_4/H_3BO_3/CH_3COOH$/NaOH, pH 2-12). The temperature optimum of Z-AFR-pNA hydrolysis was determined at 22-52°C in 0.05 M Tris-HCl buffer, pH 8.2. To determine the pH range of enzyme stability, proteinase solution was incubated at 22°C for 30 min or 24 h with 0.1 M universal buffer and then activity towards Z-AFR-pNA was measured at 37°C and pH 8.2 (0.1 M Tris-HCl buffer).

Collagenolytic activity towards collagens of type I-IV according to Bornstein and Traub's classification [14] was determined in the following way: the enzyme was incubated with collagen solution in 0.05 M Tris-HCl buffer, pH 7.5, for 19 h at 22°C. Bovine fibrinogen proteolysis was performed at 4 or 37°C in 0.05 M Tris-HCl buffer, pH 8.0. The enzyme/substrate ratio was 1 : 20 (w/w) in both cases. The reaction was terminated by adding TCA (final acid concentration was 10%, w/v). The precipitated proteins were washed twice with acetone, dried, and dissolved in Laemmli buffer [8], either containing or not containing β-mercaptoethanol. SDS-PAGE was performed according to Laemmli [8] in a MiniProtean®3 cell (BioRad, USA). When fibrinogen cleavage products were analyzed after reduction with β-mercaptoethanol, the stacking gel concentration was 8% and the separating gel concentration was 16%; if no reduction was performed, the stacking gel concentration was 6% and the separating gel concentration was 12%. For the analysis of collagen cleavage products, 5% stacking gel and 10% separating gel were used. Fibrinogen cleavage products of high molecular weight were identified using digestion with trypsin and MALDI mass-spectrometry (the experiments were performed at the Center of Postgenome Technologies of V. N. Orekhovich Institute of Biomedical Chemistry).

The substrate specificity of CSP-2 towards denatured lysozyme was determined using MALDI mass spectrometry after in-gel hydrolysis. After SDS-PAGE, the lysozyme band was excised, destained, and treated first with dithiothreitol and then with iodoacetamide in order to reduce the disulfide bonds and alkylate the SH-groups. After dehydrating the gel with acetonitrile and vacuum drying, 5 μl of 0.1 μg/ml protease solution were added to it. After incubation at 37°C for 20 h, MALDI mass spectra were registered.

Thromboelastograms characterizing the process of blood plasma coagulation after treatment of 250 μl of citrated human plasma with 50 μl of CSP-2 solution were recorded on a Hellige thromboelastograph (Austria). Instead of CSP-2 solution, 0.85% NaCl solution was added to the blank sample. Coagulation was initiated by adding isotonic $CaCl_2$ solution.

Plasminogen activation was performed in 0.05 M Tris-HCl buffer, pH 7.5. The reaction mixture containing CSP-2 and plasminogen in a ratio of 1 : 20 (w/w) was incubated at 37°C for 1 h, and then plasmin substrate *D*-VLK-pNA was added, the solution was incubated at 37°C, and its optical density at 410 nm was monitored.

Kinetic constants for the hydrolysis of Z-AFR-pNA (at 4 and 37°C), glp-AAL-pNA, and glp-AAFL-pNA were determined from Lineweaver–Burk plots; in other cases kinetic constants were calculated using kinetic curves [15]. Absorbance of the reaction mixture at 410 nm was recorded at 22°C and pH 8.2 (0.05 M Tris-HCl buffer) on a Genesys UV10 spectrophotometer (Thermo Spectronic, USA). Enzyme concentration for the calculation of turnover number (k_{cat}) was determined according to the method of Bradford [16]. Inhibition constants were determined from Dixon plots [15].

RESULTS AND DISCUSSION

Previously, efficient procedures for the isolation of individual king crab proteinases by affinity chromatography were designed in our laboratory. However, for comparative studies of proteinases a procedure is desirable which allows obtaining several enzymes after one purification run. Therefore ion-exchange chromatography on DEAE-Toyopearl has been used in the first stage of purification (Fig. 1). This approach produces fractions enriched in the major CSP isoform and in trypsin, as well as a highly purified metalloproteinase preparation and a highly purified preparation of CSP-2 containing a small admixture of trypsin-like protease (Fig. 2). Gel chromatography on Sephadex G-50 yielded electrophoretically homogeneous CSP-2.

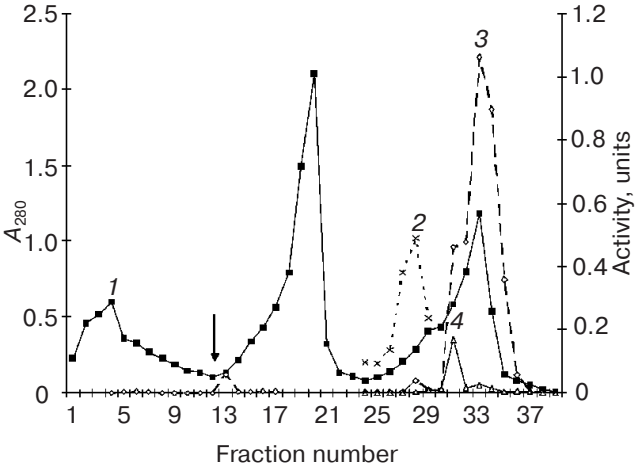

Fig. 1. Ion-exchange chromatography of "Moricrase" preparation from *P. camtschatica* hepatopancreas on DEAE-Toyopearl: *1*) A_{280}; *2*) metalloproteinase activity towards azocasein; *3*) trypsin activity towards Bz-Arg-pNA; *4*) CSP activity towards glp-AAL-pNA. Trypsin and CSP activity units, 0.1 μmol substrate/min; metalloproteinase activity unit, $\Delta A_{440} = 0.005$/min. CSP-2 was isolated from the pooled fractions 36-38. The arrow indicates the beginning of elution with a linear 0-1 M NaCl gradient.

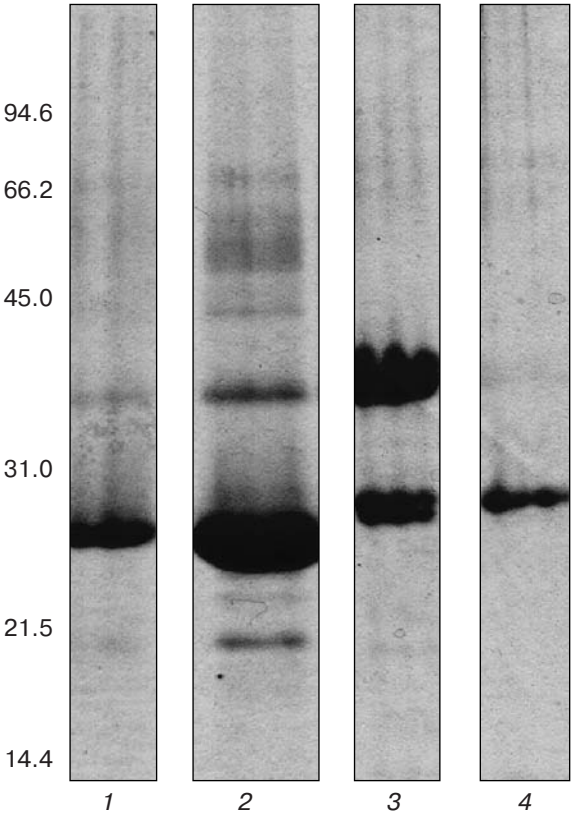

Fig. 2. SDS-PAGE of preparations after ion-exchange chromatography. Numbers under the lanes indicate the fractions: *1*) metalloproteinase (fraction 28, Fig. 1); *2*) major CSP isoenzyme (fraction 31); *3*) trypsin (fraction 34); *4*) CSP-2 (fraction 37). Molecular masses of protein markers (in kD) are indicated on the left.

The molecular mass of CSP-2 determined by SDS-PAGE is 24.8 ± 0.3 kD. The isoelectric point is probably at an acidic pH because the enzyme is retained by DEAE-Toyopearl at pH 7.5 even stronger than the major CSP isoform (pI = 3.0 [3]) and trypsin (pI < 2.5 [4]) of *P. camtschatica*.

The calculated *m/z* values of tryptic peptides of the major CSP isoform and king crab trypsin [12] do not correspond to the *m/z* values of CSP-2 tryptic peptides; the same is true in the case of peptides obtained after hydrolysis with *B. intermedius* glutamyl endopeptidase. This

Table 1. Properties of CSP-2 compared to other crustacean serine proteinases

Enzyme		Mass, kD	T-optimum, °C	Activity pH optimum	pH stability
CSP-2 from *P. camtschatica*		24.8	38-40	8.5	7.0-10.0
Major CSP isoform from *P. camtschatica* [3]		29	47-55	7.5	4-9
P. camtschatica trypsin [4]		24.8	55	7.5-8.0	5.8-9.0
Proteinase 25(III) from *P. camtschatica* [7]		25	37	7.5	>3.0
Trypsin from *Euphausia superba* krill [17]		30	37	9	6-10
Chymotrypsin from *Euphausia superba* krill [18]		29	45	8	5.5-9
Trypsin from *Euphausia pacifica* krill [19]		33	40-50	9	6-11
		32.3			
Trypsin from *Neomysis japonica* shrimp [20]		32.6	43-48	8.5-9.5	7-11
Trypsins from *Penaeus vannamei* shrimp [21]	A	32.9		7	
	B	32.9	60	9	6-11
	C	30.2		7	
Chymotrypsin from *Penaeus orientalis* shrimp [22]		24	70	7.5-9.0	6-9
Trypsins from *Procambarus clarkii* crayfish [23]	I	35.0			
	II	41.2	60-70	8-8.5	7-10
	III	37.9			
	IV	39.5			
Chymotrypsin from *P. vannamei* [24]		33.2	–	8	6-10
CSP from *Carcinus maenas* crab [25]		23	30	7	–
CSP from *Uca pugilator* crab [26]		25	–	8	3.5-9

suggests that CSP-2 is a novel protease. The presence of several isoenzymes is characteristic of many digestive serine proteases of crustaceans (Table 1).

The sequence of CSP-2 tryptic cleavage product with *m/z* 1165.6 is VDTNDVAYIR according to tandem mass-spectrometry data. This sequence displays considerable similarity to the sequences of crustacean serine proteases. The Asp residue following the Asn residue is invariable, being a part of the catalytic triad (Fig. 3).

The pH optimum of activity of CSP-2 is 8.5, a little higher than those of the major CSP isoform and trypsin from king crab (Table 1). Although proteinase 25(III) characterized by Klimova and Chebotarev and similar to CSP-2 with regard to chromatographic behavior displayed the highest activity towards azocasein at pH 7.5, its activity at pH 9.7 was only a little lower [7].

The temperature optimum of Z-AFR-pNA hydrolysis is 38-40°C, considerably lower than those of trypsin and the major CSP isoform from king crab (Table 1). The temperature optimum of azocasein hydrolysis with proteinase 25(III) was slightly higher than 37°C [7], being in good correspondence with the results of the present work. According to literature data [19], trypsins from fishes and crustaceans living at low temperatures have temperature optima at 42-53°C, while the T-optima of trypsins from tropical organisms are at 55-65°C. It is necessary to note that the observed temperature optimum depends on the time of enzyme incubation with substrate, because enzyme inactivation, as well as substrate hydrolysis, proceeds more quickly at elevated temperatures. CSP-2 is stable at pH 7-10 but quickly loses activity at acidic pH values, which is characteristic of crustacean serine proteinases (Table 1).

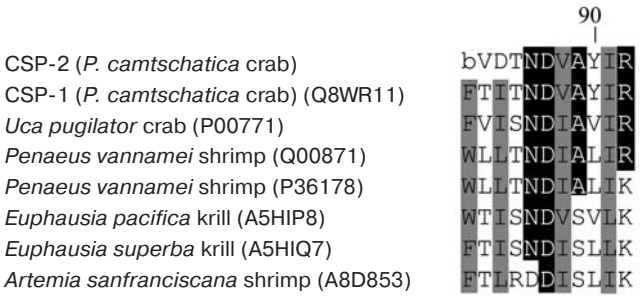

Fig. 3. Comparison of CSP-2 partial amino acid sequence to the sequences of other crustacean serine proteinases. The letter "b" denotes the amino acid residue (Arg/Lys), after which the CSP-2 peptide chain was cleaved during trypsinolysis. Amino acid sequence identifiers in the Swiss-Prot database are indicated in parentheses.

Table 2. Inhibition of CSP-2 (substrate Z-AFR-pNA)

Inhibitor	K_i, nM
Ovomucoid	78 ± 11
α-Antitrypsin	42 ± 7
Soybean trypsin inhibitor	2.3 ± 0.4
Leupeptin	5.9 ± 1.1
Antipain	4.0 ± 0.7
ε-Aminocaproic acid	200 000 ± 50 000

CSP-2 is completely inactivated after addition of PMSF but is not sensitive to *o*-phenanthroline and iodoacetamide—inhibitors of cysteine proteases and metalloproteases. When the inhibition of CSP-2 by *o*-phenanthroline and PMSF was studied, azocasein was used as a substrate to eliminate the possibility of CSP-2 contamination by a metalloprotease incapable of cleaving *p*-nitroanilides and to confirm that CSP-2 is a serine proteinase.

The interaction of CSP-2 with proteinaceous inhibitors and peptide aldehydes is more similar to that of crab trypsin than to that of the major CSP isoform: CSP-2 is inhibited by leupeptin and is more sensitive to SBTI than to ovomucoid (Table 2). The major CSP isoenzyme is more sensitive to ovomucoid than to SBTI [3]. Crustacean serine proteinase isoforms can differ markedly with regard to inhibition specificity: the isoform of a trypsin-like enzyme with p*I* 4.5 from the shrimp *E. pacifica* is much more sensitive to ovomucoid than to SBTI, and the isoform with p*I* 4.3 is nearly equally sensitive to the two inhibitors [19]. An inhibitor of plasmin, ε-aminocaproic acid, influences the activity of CSP-2 much less strongly than other inhibitors, notwithstanding its structural similarity to lysine.

When temperature increases from 22 to 37°C, k_{cat} of glp-AAL-pNA hydrolysis by CSP-2 decreases slightly (Table 3), probably due to a conformational change in the enzyme molecule. In the case of the major CSP isoenzyme, k_{cat} of glp-AAL-pNA hydrolysis is even less at 37°C than at 4°C [27]. The temperature dependence of the K_m of glp-AAL-pNA hydrolysis by CSP-2 is less pronounced than in the case of the K_m of Z-AFR-pNA hydrolysis. Weak dependence of K_m on temperature has been documented for the hydrolysis of Bz-Tyr-OEt and Bz-Tyr-pNA by psychrophilic chymotrypsins from Atlantic cod *Gadus morhua* [28].

The K_m for the reaction of Z-AFR-pNA hydrolysis decreases more than twofold when the assay temperature is decreased from 37 to 4°C (Table 3). Such temperature dependence of K_m is characteristic of anionic trypsins, both from mesophilic and psychrophilic organisms [29], including *P. camtschatica* trypsin [27]. To our knowledge, similarity of the temperature dependence of K_m for the hydrolysis of arginine-containing substrates by a dual-specificity anionic serine proteinase to the K_m temperature dependence determined for anionic trypsins has been demonstrated in the present work for the first time.

The substrate specificity of CSP-2 is mixed. Both tryptic and chymotryptic activity of the enzyme is suppressed by SBTI. The preference of substrates with positively charged amino acid residues in P_1-position is more pronounced than in the case of the major isoenzyme, which cleaves glp-AAL-pNA more quickly than Z-*D*-ALR-pNA and Z-*D*-AAFR-pNA [3] or *Uca pugilator* collagenase I. The k_{cat}/K_m value of Suc-AAPR-pNA hydrolysis with the latter enzyme is 15 times higher than that of Suc-AAPL-pNA hydrolysis [30] (Tables 3 and 4). The rate of Bz-Arg-pNA hydrolysis with CSP-2 is 22 times higher than the rate of glp-FA-pNA hydrolysis under the same conditions, while the major isoform splits glp-FA-pNA eight times faster than Bz-Arg-pNA [3]. It should be noted that proteinase 25(III) described by Klimova and Chebotarev cleaved Bz-Tyr-OEt 10 times faster than Bz-Arg-OEt [7]. CSP-2 does not cleave glp-FL-pNA or Suc-AAPF-pNA.

The K_m values for the hydrolysis of chromogenic substrates with CSP-2 are considerably lower than for the

Table 3. Temperature dependence of kinetic constants of chromogenic substrate hydrolysis by CSP-2

Substrate	T, °C	K_m, μM	k_{cat}, sec^{-1}
glp-AAL-pNA	5	200 ± 20 (960* [27])	0.09 ± 0.01
	22	220 ± 20	0.61 ± 0.04
	37	120 ± 10 (350* [27])	0.56 ± 0.04
Z-AFR-pNA	4	1.24 ± 0.13	0.078 ± 0.002
	22	1.9 ± 0.2	0.53 ± 0.02
	37	3.5 ± 0.5	0.73 ± 0.09

* Major isoform.

Table 4. CSP-2 substrate specificity (22°C). Comparison with other serine proteinases

Substrate	Enzyme	K_m, μM	k_{cat}, sec^{-1}
D-VLK-pNA	CSP-2	1.1 ± 0.1	0.84 ± 0.02
	Lys-plasmin	210 ± 20	25.6 ± 1.1 [31]
D-VLR-pNA	CSP-2	1.25 ± 0.09	0.78 ± 0.17
	porcine kallikrein, 37°C	18.3	6.6 [32]
	human kallikrein-1, 37°C	12.0 ± 0.8	0.81 ± 0.02 [33]
	thrombin, 20°C	189	38 [34]
Z-*D*-ALR-pNA	CSP-2	1.1 ± 0.2	0.46 ± 0.07
Z-APR-pNA	CSP-2	2.39 ± 0.09	0.59 ± 0.09
Z-AFR-pNA	CSP-2	1.9 ± 0.2	0.53 ± 0.02
	bovine trypsin, 25°C	67	11 [35]
	porcine kallikrein, 25°C	61	16 [36]
D-PFR-pNA	CSP-2	0.96 ± 0.07	0.68 ± 0.12
	proteinase from the snake *Trimeresurus jerdonii*	6.25	20.3 [37]
	human tissue kallikrein, 30°C	11 ± 2	1.3 ± 0.3 [38]
glp-AAFL-pNA	CSP-2	23 ± 4	0.26 ± 0.05

hydrolysis of these substrates by serine proteinases of homoiothermic animals (Table 4). Therefore, the catalytic efficiency (k_{cat}/K_m) is high, though k_{cat} values are also lower than in the case of homoiotherm proteinases. King crab trypsin binds Bz-Arg-pNA more efficiently than bovine trypsin: K_m values at 35°C are 0.053 and 0.62 mM, respectively [27]. This feature of crab proteinases probably allows effective functioning at low temperatures. Low K_m values are the main factor of adaptation to low temperatures in the case of psychrophilic fish trypsins [29]. It should be noted that trypsins from the prawn *Penaeus vannamei* bind Bz-Arg-pNA even more efficiently than crab trypsin (K_m at 35°C is 0.27-0.34 μM) [21].

The increase in substrate length influences the binding positively, regardless of the type of P_1-residue (for arginine- and lysine-containing substrates, data not shown; for leucine-containing substrates, see Tables 3 and 4).

Comparison of K_m values for substrates with an Arg residue in P_1-position shows that the presence of Pro residue in P_2 position makes substrate binding less efficient, but the presence of a *D*-Pro residue in P_3-position improves binding. The kinetic constants of *D*-VLK-pNA and *D*-VLR-pNA hydrolysis by CSP-2 do not differ very much, though trypsin [4] and the major CSP isoform from *P. camtschatica* [3], as well as collagenase I of the fiddler crab *U. pugilator* [30] and the trypsin from the shrimp *E. superba* [18] prefer substrates with arginine in P_1-position over lysine-containing substrates.

The main factor determining the preference of lysine or arginine in P_1-position by mammalian serine proteinases is the position of the negatively charged residue in the S_1-site of the molecule. Bovine duodenase (a serine proteinase with mixed substrate specificity) binds Tos-Gly-Pro-Lys-pNA more efficiently than Tos-Gly-Pro-Arg-pNA [39]. Mutant bovine trypsin [Gly189, Asp226] in which the position of Gly and Asp residues is reversed compared to the wild-type enzyme prefers lysine in P_1-position [39], while the wild-type enzyme prefers arginine [29]. However, *U. pugilator* collagenase I prefers arginine, though the position of Gly and Asp residues in the S_1-site of this enzyme is the same as in mutant trypsin [30].

The *m/z* values of most peptides formed upon cleavage of hen egg lysozyme by CSP-2 correspond to the calculated values for the products of cleavage after arginine and lysine; in some cases, dual specificity is evident (Fig. 4). Products of Lys33–Phe34 and Lys97–Ile98 bond cleavage were not detected.

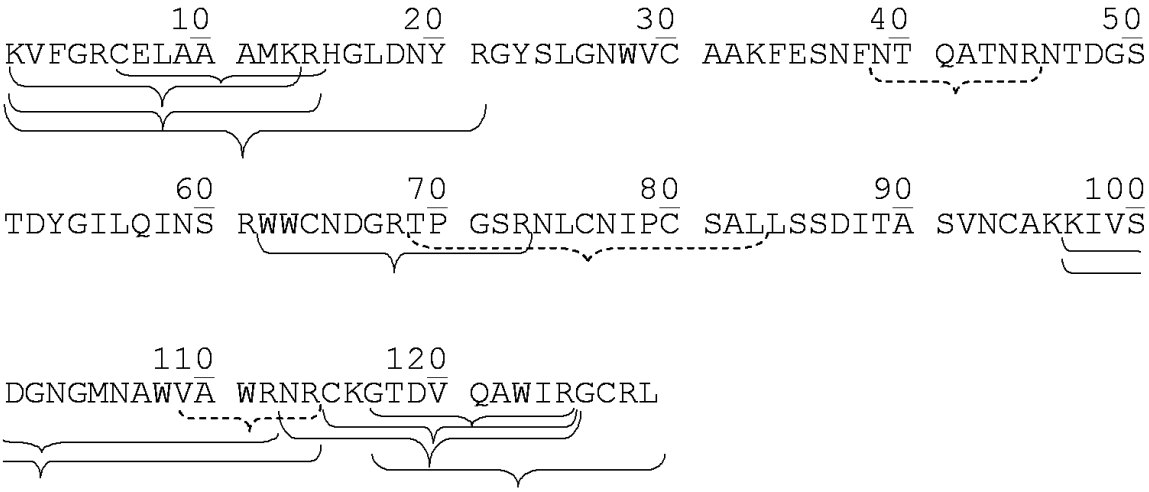

Fig. 4. Products of denatured hen egg lysozyme hydrolysis by CSP-2. Peptides corresponding to trypsin specificity are indicated by solid brackets, and peptides corresponding to dual specificity are indicated by dotted brackets.

CSP-2 cleaves both heated and unheated fibrin in fibrin plate assay. Unheated fibrin is cleaved more quickly, and this is indicative of the ability of CSP-2 to activate plasminogen. Activation of plasminogen was observed directly in an experiment involving chromogenic substrate cleavage. SDS-PAGE showed that the band of intact plasminogen disappears and the bands corresponding to heavy and light plasmin chains appear after incubation of plasminogen with the enzyme (Fig. 5).

Formation of fibrinogen fragments with molecular masses of about 150 and 250 kD is characteristic of the first stage of fibrinogen proteolysis, either by plasmin or by king crab proteases described by Sakharov et al. [40], and by CSP-2. Upon prolonged fibrinogen hydrolysis by plasmin, 95-kD D-fragments and 45-kD E-fragments are formed, which are not cleaved by plasmin, but CSP-2 degrades a fragment with molecular mass of about 95 kD, which is formed in the intermediate stage of hydrolysis. This is probably due to a broader substrate specificity of CSP-2 compared to plasmin. CSP-2 cleaves fibrinogen at both 4 and at 37°C.

At intermediate stages of fibrinogen hydrolysis by plasmin, three or four C-terminal Aα-chain fragments with molecular mass of 15-20 kD are formed. Fibrinogen Aα-chain fragments were not detected among the major products of fibrinogen cleavage by CSP-2 having molecular masses between 24 and 55 kD: CSP-2 probably splits the Aα-chain of fibrinogen into short peptides because the intensity of the corresponding band decreases quickly, as shown by SDS-PAGE (Fig. 6).

Analysis of cleavage products showed that CSP-2 cleaves the coiled-coil parts of fibrinogen Bβ- and γ-chains but leaves intact the central domain rich in disulfide bonds. It is known that some of the parts of coiled-coil fragments are packed less tightly due to the presence of bends, but the probable positions of cleavage sites do not correspond to the positions of these bends.

Many of the proteinases that cleave fibrinogen *in vitro* are incapable of cleaving this protein present in blood plasma, because plasma is rich in protease inhibitors. However, blood plasma treated with CSP-2 yields a less dense clot than untreated plasma, this being indicative of fibrinogen hydrolysis by CSP-2 in the presence of plasma inhibitors. The ability to cleave fibrinogen and fibrin was also documented for trypsin-like proteinases of krill (*E. superba*) and consequently it was suggested to use these proteinases for the lysis of thrombi [41].

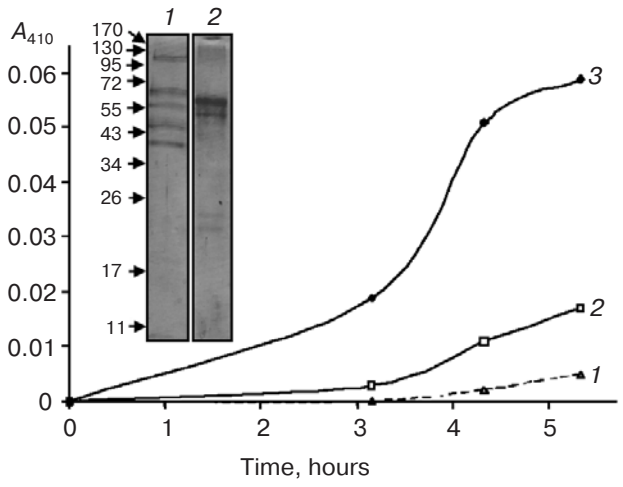

Fig. 5. Plasminogen activation: hydrolysis of the synthetic chromogenic substrate *D*-VLK-pNA in the presence of plasminogen (*1*), CSP-2 (*2*), and plasminogen and CSP-2 (*3*). Electrophoregram: *1*) plasminogen; *2*) products of plasminogen treatment with CSP-2 (molecular masses of marker proteins in kD are indicated on the left).

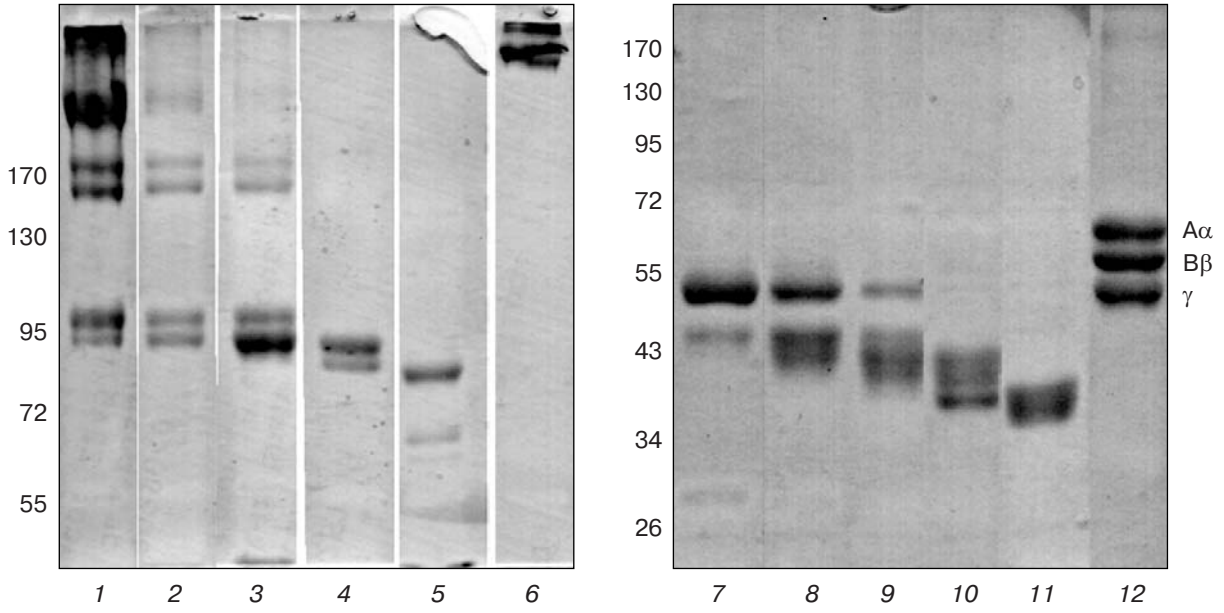

Fig. 6. Electrophoregrams of products of bovine fibrinogen hydrolysis by CSP-2 at 4°C. Lanes *1-6* (without reduction): *1-5*) 15, 30, 60, 120, and 240 min of incubation with the enzyme, respectively; *6*) intact fibrinogen sample; lanes *7-12* (after reduction): *7-11*) 15, 30, 60, 120, and 240 min of incubation with the enzyme, respectively; *12*) intact fibrinogen sample. Molecular masses of marker proteins in kD (for lanes *1-6* and *7-12*) are indicated near the lanes, and letters indicate the position of intact fibrinogen chains.

The pattern of calf skin type I collagen hydrolysis by CSP-2 resembles that of *U. pugilator* collagenase [30]: a fragment is formed which is about 3/4 of the intact molecule in length (Fig. 7). In the case of rat collagen type I, as well as type II and III collagens, cleavage products of high molecular weight were not detected, though the intensity of intact collagen bands decreased. Cleavage products are probably more prone to enzymatic degradation than intact collagen molecules. The intensity of bands corresponding to collagen chain β-dimers also decreases due to cleavage of non-helical parts of collagen chains near the intermolecular cross-link sites. Unlike proteinase II from *U. pugilator* [42], CSP-2 does not cleave type IV collagen.

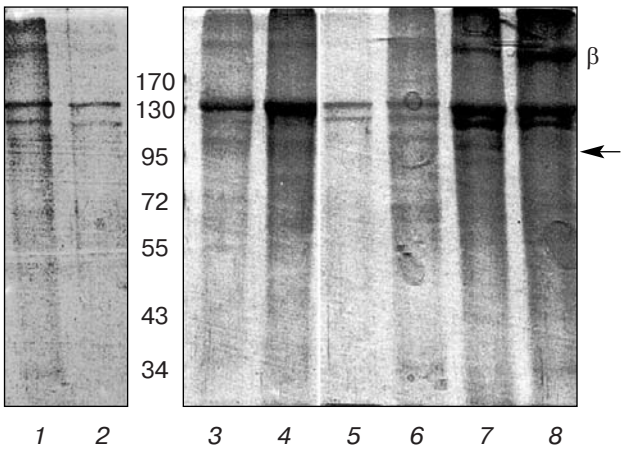

Fig. 7. Electrophoregrams of collagen hydrolysis products by CSP-2: *1*) rat type I collagen, blank; *2*) rat type I collagen hydrolyzate; *3*) bovine trachea type II collagen hydrolyzate; *4*) bovine trachea type II collagen, blank; *5*) human placenta type III collagen hydrolyzate; *6*) human placenta type III collagen, blank; *7*) calf skin type I collagen hydrolyzate; *8*) calf skin type I collagen, blank. The band corresponding to collagen cleavage products is indicated by an arrow, and the band corresponding to collagen chain β-dimers is indicated with the character β. Protein marker molecular masses in kD are indicated between lanes *2* and *3*.

Thus, CSP-2 is another collagenolytic enzyme from *P. camtschatica* that contributes to the collagenolytic action of the "Moricrase" enzyme preparation. The ability of the enzyme to cleave collagen and fibrin and to activate plasminogen opens wide perspectives of its medical application, and conservation of activity at low temperatures is of interest for biotechnology.

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REFERENCES

1. Rudenskaya, G. N. (2003) *Russ. J. Bioorg. Chem.*, **29**, 117-128.
2. Rudenskaya, G. N., Isaev, V. A., Shmoylov, A. M., Karabasova, M. A., Shvets, S. V., Miroshnikov, A. I., and

Brusov, A. B. (2000) *Appl. Biochem. Biotechnol.*, **88**, 175-183.
3. Rudenskaya, G. N., Isaev, V. A., Stepanov, V. M., Dunaevskii, Ya. E., Baratova, L. A., Kalebina, T. S., and Nurminskaya, M. V. (1996) *Biochemistry (Moscow)*, **61**, 804-814.
4. Rudenskaya, G. N., Isaev, V. A., Kalebina, T. S., Stepanov, V. M., Mal'tsev, K. V., Shvets, S. V., Luk'yanova, N. A., Kislitsin, Yu. A., and Miroshnikov, A. I. (1998) *Russ. J. Bioorg. Chem.*, **24**, 112-118.
5. Semenova, S. A., Rudenskaya, G. N., Rebrikov, D. V., and Isaev, V. A. (2006) *Protein Pept. Lett.*, **13**, 571-575.
6. Klimova, O. A., and Chebotarev, V. Yu. (1999) *Byul. Eksp. Biol. Med.*, **128**, 308-313.
7. Klimova, O. A., and Chebotarev, V. Yu (1999) *Byul. Eksp. Biol. Med.*, **128**, 391-396.
8. Laemmli, U. K. (1970) *Nature (London)*, **227**, 680-685.
9. Lyublinskaya, L. A., Haidu, I., and Balandina, G. N. (1987) *Russ. J. Bioorg. Chem.*, **13**, 748-753.
10. Shaginyan, K. A., Izotova, L. S., Iomantas, Yu. V., Strongin, A. Ya., and Stepanov, V. M. (1980) *Biokhimiya*, **45**, 2083-2095.
11. Ma, B., Zhang, K., Hendrie, C., Liang, C., Li, M., Doherty-Kirby, A., and Lajoie, G. (2003) *Rapid Commun. Mass Spectrom.*, **17**, 2337-2342.
12. Rudenskaya, G. N., Kislitsin, Y. A., and Rebrikov, D. V. (2004) *BMC Struct. Biol.*, **4**, 2.
13. Astrup, T., and Mullertz, S. (1952) *Arch. Biochem. Biophys.*, **40**, 346-351.
14. Bornstein, P., and Traub, W. (1979) *The Proteins*, **4**, 411-605.
15. Berezin, I. V., and Klyosov, A. A. (1976) *Practical Course of Chemical and Enzymatic Kinetics* [in Russian], Moscow State University Publishers, Moscow.
16. Bradford, M. M. (1976) *Analyt. Biochem.*, **72**, 248-254.
17. Yamada, K., Murakami, H., Tachibana, H., Watanabe, Y., Miyake, Y., and Omura, H. (1987) *Agric. Biol. Chem.*, **51**, 3363-3368.
18. Kimoto, K., Yokoi, T., and Murakami, K. (1985) *Agric. Biol. Chem.*, **49**, 1599-1603.
19. Wu, Z., Jiang, G., Xiang, P., Yang, D., and Wang, N. (2008) *Biotechnol. Lett.*, **30**, 67-72.
20. Wu, Z., and Jiang, G. (2008) *Int. J. Pept. Res. Ther.*, **14**, 75-80.
21. Sainz, J. C., Garcia-Carreno, F. L., and Hernandez-Cortes, P. (2004) *Comp. Biochem. Physiol. B Biochem. Mol. Biol.*, **138**, 155-162.
22. Oh, E.-S., Kim, D.-S., Kim, J.-H., and Kim, H.-R. (2000) *J. Food Biochem.*, **24**, 251-264.
23. Jeong, Y., Wei, C. I., Preston, J. F., and Marshall, M. R. (2000) *J. Food Biochem.*, **24**, 311-332.
24. Hernandez-Cortes, P., Whitaker, J., and Garcia-Carreno, F. L. (1997) *J. Food Biochem.*, **21**, 497-514.
25. Roy, P., Colas, B., and Durand, P. (1996) *Comp. Biochem. Physiol. B Biochem. Mol. Biol.*, **115**, 87-95.
26. Eisen, A. Z., Henderson, K. O., Jeffrey, J. J., and Bradshaw, R. A. (1973) *Biochemistry*, **12**, 1814-1822.
27. Papisova, A. I., Rudenskaya, G. N., Kislitsyn, Yu. A., and Semenova, S. A. (2008) *Russ. J. Bioorg. Chem.*, **34**, 479-486.
28. Asgeirsson, B., and Bjarnason, J. B. (1991) *Comp. Biochem. Physiol. B. Biochem. Mol. Biol.*, **99**, 327-335.
29. Mikhailova, A. G., Rumsh, L. D., Dalgalarrondo, M., Chobert, J. M., and Haertle, T. (2003) *Biochemistry (Moscow)*, **68**, 926-933.
30. Tsu, C. A., Perona, J. J., Schellenberger, V., Turck, C. W., and Craik, C. S. (1994) *J. Biol. Chem.*, **269**, 19565-19572.
31. Wohl, R. C., Summaria, L., and Robbins, K. C. (1980) *J. Biol. Chem.*, **255**, 2005-2013.
32. Blaber, M., Isackson, P. J., Marsters, J. C., Burnier, J. P., and Bradshaw, R. A. (1989) *Biochemistry*, **28**, 7813-7819.
33. Sousa, M. O., Miranda, T. L. S., Costa, E. B., Bittar, E. R., Santoro, M. M., and Figueiredo, A. F. S. (2001) *Braz. J. Med. Biol. Res.*, **34**, 35-44.
34. Le Bonniec, B. F., and Esmon, C. T. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 7371-7375.
35. Tanaka, T., McRae, B. J., Cho, K., Cook, R., Fraki, J. E., Johnson, D. A., and Powers, J. C. (1983) *J. Biol. Chem.*, **258**, 13552-13557.
36. Powers, J. C., McRae, B. J., Tanaka, T., Cho, K., and Cook, R. R. (1984) *Biochem. J.*, **220**, 569-573.
37. Jia, Y.-H., Jin, Y., Lu, Q.-M., Li, D.-S., Wang, W.-Y., and Xiong, Y.-L. (2003) *Acta Biochim. Biophys. Sin.*, **35**, 689-694.
38. Chagas, J. R., Portaro, F. C. V., Hirata, I. Y., Almeida, P. C., Juliano, M. A., Juliano, L., and Prado, E. S. (1995) *Biochem. J.*, **306**, 63-69.
39. Zamolodchikova, T. S., Sokolova, E. A., Alexandrov, S. L., Mikhaleva, I. I., Prudchenko, I. A., Morozov, I. A., Kononenko, N. V., Mirgorodskaya, O. A., Da, Ui, Larionova, N. I., Pozdnev, V. F., Ghoshi, D., Duax, W. L., and Vorotyntseva, T. I. (1997) *Eur. J. Biochem.*, **249**, 612-621.
40. Sakharov, I. Yu., Litvin, F. E., Mitkevitch, O. V., Samokhin, G. P., and Bespalova, Z. D. (1994) *Comp. Biochem. Physiol. B Biochem. Mol. Biol.*, **107**, 411-417.
41. Hellgren, L., Mohr, V., Vincent, J., Vincent, J., and Karlstam, B. (1995) European Patent No. WO1994SE00550 19940607.
42. Welgus, H. G., and Grant, G. A. (1983) *Biochemistry*, **22**, 2228-2233.