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Preparation of Proteolytic Enzymes from Kamchatka Crab *Paralithodes camchatica* Hepatopancreas and Their Application

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Abstract

A method was elaborated to obtain "Moricrase," a complex of collageno and fibrinolytic proteinases from *Paralithodes camchatica* hepatopancreas. Moricrol, a Moricrase-containing ointment, was shown to exhibit wound-healing activity in the treatment of burns and trophic ulceration as well as postoperative scars. The following homogeneous proteinases were isolated from Moricrase: collagenolytic serine proteinase, trypsin, metalloproteinase, carboxypeptidase, and aminopeptidase. An efficient collagenolytic activity of Moricrase is a result of cooperative action of these enzymes.

Index Entries: Collagenolytic complex of proteases; king crab *Paralithodes camchatica*; fibrinolytic properties; enzymatic débridement; purulent wounds; scarry tissue.

Introduction

Proteolytic enzymes are one of the major participants of cell metabolism. They play a crucial role providing the proteolytic modification of proteins, the activation of enzyme precursors and hormones, elimination of mutant or damaged polypeptides, and the development of immune response to various pathogens. Therefore, the study of proteolytic enzymes is of great theoretical and practical interest for medicine. One of the features of the digestive system of Arthropoda is a multiplicity of proteinases presenting in hepatopancreas. Seven proteinases from Kamchatka crab *(1–3)*

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and nine proteinases from *Chinoecetes opilio* crab *(4)* are known. These include trypsin, chymotrypsin, and mammalian elastase analogs. The proteolytic enzyme isolated from hepatopancreas of the fiddler crab, *Uca pugilator (5,6)*, is capable of degrading collagen by cleavage of native collagen helix under conditions of protein denaturation. In the present paper we describe a method for preparation, some properties and possible medicinal application of the Moricrase proteolytic complex from *Paralithodes camchatica* crab.

Materials and Methods

Enzyme Assay

The proteolytic activity of the enzyme was assayed toward Bz-Arg-pNA, Leu-pNA, BOC-Ala-Ala-Ala pNA (Sigma, Germany), Glp-Phe-Ala-pNA, DNP-Ala-Ala-Leu- Arg-NH_2 DNP-Ala-Ala-Arg (Dia-M-Co, Russia). A unit of the enzyme catalytic activity (U) was defined as the amount of enzyme that splits 1 mmol of substrate per minute under defined conditions *(7).* Proteolytic activity toward azocasein was determined by modification of the method *(8)* using 0.5% solution of azocasein in 0.05 *M* Tris-HCl buffer pH 7.5. A unit of the enzyme activity was defined as the amount of the enzyme increasing the absorbance at 440 nm by one optical density unit per minute. Fibrinolytic activity was measured by the Astrup–Mullerz procedure *(9).* A unit of fibrinolytic activity was defined as the amount of the enzyme capable of forming a 10-mm zone of fibrin hydrolysis after 5 h incubation. Collagenolytic activity was assayed using acid-soluble rat skin type I collagen as a substrate *(10).* Changes in collagen solution viscosity under the action of the enzymes were followed using an Ostwald viscosimeter *(11).* Moricrase was incubated with 0.02% collagen solution in 0.05 *M* Tris-HCl buffer, pH 7.8, containing, 10 m*M* $CaCl_2$, 30°C.

Preparation of Moricrase

Place 50 kg of frozen hepatopancreas at 40°C in a tank and add 75 L of 0.1 *M* sodium acetate, pH 6.0. After stirring, a homogenate was filtrated with PTTK membranes (Millipore, Sweden). Finally, 150 L of a limpid solution was left in a cold room at 2°C for 12 h. The layer of fat was removed and the remaining solution was subjected to microfiltration using a KK4425 apparatus (Kylola, Finland) and a 22-μm filter with a flow rate of 400 mL/min. The solution was then subjected to ultrafiltration on a Pellicon apparatus. Add 65 kg of ammonium sulfate (80% saturation) by small portions to 150 L of the ultrafiltrate under continuous stirring for about 1 h. The suspension was left in a cold room at 5°C for 2 d. The supernatant was removed by decantation of the precipitate formed. The suspension was subjected to centrifugation with a Beckman J-6M apparatus for 10 min at 5°C (3000*g*). The precipitate was dissolved in water and desalted by dialysis. Freeze 4 L of its solution and lyophilized. Finally, 260 g of a dry preparate, Moricrase, was obtained.

Results and Discussion

Purification of Moricrase

The activity of proteinases in the course of purification was checked toward chromogenic peptide substrates: DNP-Ala-Ala-Leu-Arg-NH_2 (metalloproteinase) and Bz-Arg-pNA (trypsin). Table 1 demonstrated that high proteinase activity retained after microfiltration and ultrafiltration. The significant loss was observed during precipitation by ammonium sulfate and dialysis in the absence of Ca^{2+}ions needed for metalloproteinase. Moricrase preserved activity in solution within the pH range of 6.5–8.5 in the presence of Ca^{2+} ions for 3 wk at 4°C and during 2 yr in a dry form.

Substrate Specifity

The characteristics of the complex preparation are presented in Table 2. Moricrase is able to split different peptides specific for endo- and exopeptidhydrolases. In addition, Moricrase hydrolyzed azocasein and fibrin, activated plasminogen with generation of plasmin, and also exhibited remarkable collagenolytic properties. Figure 1 demonstrates the kinetics of collagen hydrolysis by Moricrase, PC serine proteinase, an individual enzyme obtained from Moricrase *(12)*, and collagenase from *Clostridium histolyticum.* The character of collagen digestion by Moricrase is similar to that of subtilisin-type serine proteinases *(13).*

Presumably, the crab enzyme initially split the telopeptide fragments in the collagen molecule that leads to a rapid decrease in viscosity. After this step, the enzymes slowly digest α-chains. This mechanism differs significantly from that of classic collagenase, such as clostridiopeptidase which attacks the triple helix of collagen in a number of positions.

Enzymes Composition of Moricrase

Several homogeneous proteases have been obtained from Moricrase in our laboratory. Table 3 demonstrates some properties of these enzymes. Unlike mammalian pancreatic enzymes, these proteins have isoelectric points in acidic medium and, in the absence of calcium ions, lose the activity below pH 5.0. PC serine proteinase is not inhibited by many inhibitors typical for trypsin and chymotrypsin *(12)*. The total loss of activity caused by the most specific serine proteinase inhibitors, diisopropylfluophosphate (DFP) and phenylmethylsulfonylfluoride (PMSF), is the only reason for classifcation of this enzyme as a serine proteinase. Similar inhibition was found for serine collagenase from the *Uca pugilator* crab *(5)*. The main difference between PC serine proteinase and typical mammalian chymotrypsins is the ability of the former enzyme to cleave peptide bonds formed by hydrophobic and basic amino acids, especially those by Arg and Lys with comparable rates and to cleave native collagen and fibrin.

PC trypsin (*14)* is completely inactivated by DFP, PMSF and the specific inhibitor of trypsin, Tosylamino-2-phenylethylchloro-methyl ketone

Table 1
Preparation of Moricrase

Purification step	Protein content (g)	Activity					
		DNP-Ala-Ala-Leu-Arg-NH_2			Bz-Arg-pNA		
		Total ($U \times 10^{-3}$)	Specific U/g	Yield %	Total ($U \times 10^{-3}$)	Specific U/g	Yield %
Extract	260	1104	4250	100	134.7	522	100
Microfiltration	220	1064	4840	96	115.2	520	85
Ultrafiltration	130	987	7600	89	96.2	740	71
Precipitation of ammonium sulfate, lyophilization	25	250	10000	22.6	55	2200	40

Table 2
Activity of Moricrase Toward Specific Substrate of Protease

Substrate	Specific activity U/g	Enzyme
Bz-Arg-pNA	270	Trypsin
Z-D-Ala-Arg-pNA	6730	Trypsin
Z-D-Ala-Leu-Arg-pNA	4020	Trypsin
Z-Ala-Ala-Phe-Arg-pNA	4260	Trypsin
Glp-Phe-pNA	40	Chymotrypsin
Glp-Phe-Ala-pNA	270	Chymotrypsin
Glp-Phe-Leu-pNA	110	Chymotrypsin
Glp-Ala-Ala-Leu-pNA	340	Chymotrypsin
Boc-Ala-Ala-Ala-pNA	25	Elastase
DNP-Gly-Gly-Arg	57	Carboxypeptidase
DNP-Gly-Gly-Val-Lys	100	Metalloproteinase
DNP-Gly-Gly-Ile-Arg	230	Metalloproteinase
Leu-pNA	2	Aminopeptidase
Arg-pNA	3	Aminopeptidase
Azocasein	4200	Total proteolytic activity

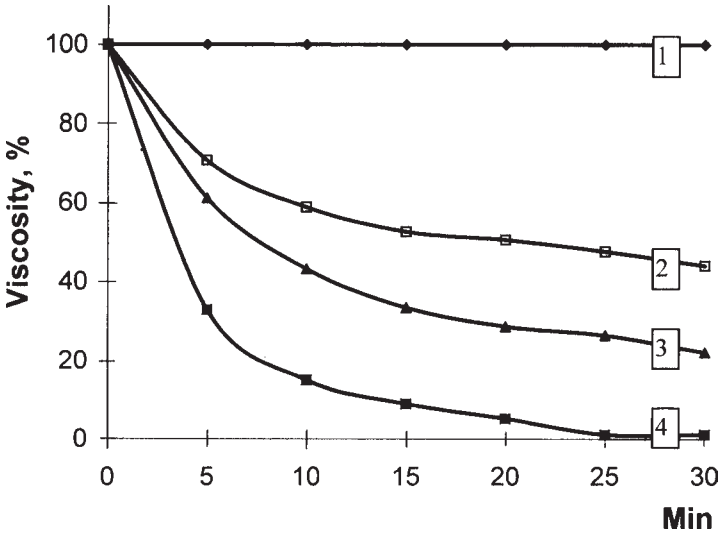

Fig. 1. Changes in viscosity of type I collagen solution in 0.05 *M* Tris-HCl buffer, pH 7.5, 25°C, containing: 1, no enzyme; 2, Moricrase (100 μg); 3, serine proteinase PC (100 μg); and 4, clostridiopeptidase (10 μg).

(TPCK). The Kunitz and Bowman–Birk soybean inhibitors and the potato inhibitor are the most potent among the protein inhibitors of trypsin. Based on these results, PC trypsin from the king crab may be assigned to typical trypsins. Like mammalian trypsins, PC trypsin did not hydrolyze native type 1 collagen. However, it exhbited remarkable gelatinase activity. Similar to other trypsins, PC trypsin hydrolyzed the bonds formed by carboxylic groups of Lys and Arg.

Table 3
Enzymatic Properties of Proteases from Moricrase

Protease	M_r, kDa	p*I*	pH-Optimum of activity	pH-Optimum of stability	T-Optimum of activity, °C	K_m, m*M*	Substrate
Collagenolytic serine proteinase *(12)*	28	3.0	7.5	4.2–8.5	47–55	0.83	Glp-Phe-Ala-pNA
Trypsin *(14)*	29	<2.5	7.5–8.0	5.8–9.0	55	50	Bz-Arg-pNA
Metalloproteinase	22	4.3	8.5	—	55–60	—	DNP-Ala-Ala-Leu-Arg-NH_2
Carboxypeptidase *(16)*	34	3.1	6.5	5.0–8.0	50–60	0.4	DNP-Ala-Ala-Arg
Aminopeptidase	220 (2 × 110)	4.1	6.0	5.5–8.0	36–40	0.075 0.078	Leu-pNA Arg-pNA

Table 4
Fibrinolytic Properties of Moricrase and Some of Its Components

Preparation	Fibrin, unheated, U	Fibrin, heated, U	Plasmin activity, U	Protein, A_{280}	Specific activity, U/A_{280}
Moricrase	1800	832	968	0.26	3723
Serine proteinase	2125	550	1575	0.14	11250
Trypsin	900	500	400	0.18	2250
Elastase	520	400	120	0.19	1000

PC metalloproteinase has no analogs in the pancreatic gland of mammals. It does not exibit any collagenolytic activity, and its properties are similar to those of fluvial cancer metalloproteinase astacin *(15)*.

PC carboxypeptidase *(16)*, unlike mammalian carboxypeptidases A and B, has a mixed substrate specificity. The enzyme can effectively split off C-terminal Phe and Tyr residues, as well as Arg and Lys. The presence of Pro, Glu, and Asp terminate the cleavage of a preceding bond.

PC aminopeptidase easily splits N-terminal Phe and Leu residues as well as Arg and Lys. Aminopeptidase such as metalloproteinase and carboxypeptidase of king crab are inactivated by EDTA and *o*-phenantroline. Aminopeptidase contained two Zn atoms in the active site. Probably, hydrolysis of proteins by Moricrase is the result of the cooperative effect of its proteolytics enzymes.

Application of Moricrase in Medicine

Moricrase and some endopeptidases PC hydrolyzed both heated and unheated plasminogen-containing fibrin patches. Its fibrinolytic activity toward unheated patches is significantly higher than toward heated patches (Table 4), apparently because fibrin in the former case is split not only by PC enzyme, but also by plasmin formed from plasminogen under the action of PC proteinases. Moricrase within the concentration range from 0.025 to 2 mg/mL shows a pronounced thrombolytic effect on the clots formed in vitro from both plasma human and rat blood. On the basis of the experiments carried out we applied the Moricrol ointment, including Moricrase, to eliminate necrotic debris from wound surfaces *(17)* and attenuation of scarry tissue in vitro. The enzymatic débridement by Moricrol was studied by its application to rat trophic pests. A partial removal of necrotic debris, the formation of red granulation tissue, and the beginning of wound edge reepithelialization were observed on d 3 after a single application of Moricrol to rat purulent wounds. Complete débridement was observed as early as d 10. By this time the surface of untreated wounds (control) was completely covered with hard necrotic tissue.

Currently, Moricrol ointment is being tested in reconstructive surgery. The conservative treatment was carried out with patients having posttraumatic and postoperative scars (Table 5). No side effects were observed

Table 5
Effect of Moricrol Application

Scar tissue	Total amount of patients	Considerable effect[a]	Moderate effect[b]	No effect
Cheloid scars:				
Posttraumatic	17	12	2	3
Postoperative	15	10	5	—
Hypertrophycal	7	5	2	—

[a]Considerable effect: a redden and itch of scar tissue were decreased, the scar became more plane and soft.

[b]Moderate effect: reddening and itch of scar tissue were decreased, but softening of the scar was not observed.

after ointment application during 1–3 mo. Moricrol assists in successful clinical treatment of ail and contributes to disappeance of scars when applied for sufficiently long time.

Conclusions

The results obtained enable us to conclude that the proteolytic complex Moricrase isolated from the king crab, *P. camchatica*, is a valuable source of proteases. Collagenolytic activity of Moricrase and Moricrol ointment can find medical applications in the treatment of trophic ulcers and scars.

Acknowledgments

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