NEW DRUGS

Collagenolytic Protease Preparations from Invertebrates: Biochemical Aspects of Medical and Cosmetological Applications

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Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 130, No. 7, pp. 70-75, June, 2000
Original article submitted March 23, 2000

Collagenolytic proteases from invertebrates, the active matter of new structure-destroying enzyme preparations (Collagenase from hydrobionts, Polycollagenase-K, Fermenkol), ensure deep hydrolysis of polypeptide substrates (native or partially denatured collagen types, elastin, fibrin, hemoglobin, and casein) that cannot be attained by collagenases and serine proteases. Biochemical properties of collagenolytic proteases from invertebrates, techniques and doses that may be important for elaboration and employment of preparations based on this complex are described.

Key words: *enzyme preparations*; *collagenolytic proteases*; *depth of hydrolysis*; *collagenolytic activity*; *doses and techniques*

Active components of usual structure-destroying enzyme preparations (trypsin, chymotrypsin, and collagenase) are serine proteinases (predominantly, trypsin) and sometimes collagenases (collalysin), which are highly active with respect to specific substrates and exhibit low activity against other polypeptides; therefore they are ineffective for degradation of structural protein complexes. New structure-destroying enzyme preparations contain natural complexes or special compositions of synergistically acting nonspecific collagenolytic proteases from invertebrates (CLPI). The following enzyme preparations are currently produced: Collagenase from hydrobionts, Polycollagenase-K, Fermenkol (cosmetic polycollagenase), and wound drainage sorbents Collasorb and Colladiosorb. They hydrolyze various polypeptide and olygopeptide substrates to individual amino acids and ensure efficient degradation of reticular and massive multilayer structures, which can not be attained with usual enzyme preparations. The term collagenase is often used for all enzyme preparations hydrolyzing native collagen irrespective of substrate specificity and the degree of hydrolysis, which led to improper estimation of hydrolysis rate and underestimation of the efficiency of CLPI-containing preparations, the next section contains brief description of current terminology

According to **Enzyme Classification of the International Union on Biochemistry** [17], all *proteases* (or hydrolases, EC 3.4) are subdivided into *exopeptidases* (EC 3.4.11-19, grouped into *amino-* and *carboxypeptidases* by substrate specificity) and *endopeptidases* (or *proteinases*, EC 3.4.21-99) consisting of 4 main groups according to the mechanism of catalysis (active sites) and affinity for specific inhibitors: *serine* (EC 3.4.21), *cysteine* (EC 3.4.22), and *aspartic* (EC 3.4.23), and *metalloproteinases* (EC 3.4.24). There is also a class of *proteinases* (EC 3.4.99) with unknown mechanism of action possessing affinities for several standard inhibitors or to none of them (which attested

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0007-4888/00/0007-671$25.00

to heterogeneity of the preparation or existence of new classes of proteinases). *Serine proteinases*, or *trypsins* (20-40 kDa, Ser in the active site; *e.g.*: *trypsin* EC 3.4.21.4) were found in invertebrates, fungi, crustaceans, insects (trypsins from invertebrates are less studied than mammalian pancreatic proteinases). *Cysteine* (or *thiol*) *proteinases* (Cys in the active site; *e.g. cathepsin B*, EC 3.4.22.1) are present in most bacteria, animal and plant cells. *Aspartic proteinases* (previously termed as *acidic* or *carboxylic* proteinases, Asp in the active site, retain activity at low pH; *e.g.*: *pepsin A*, EC 3.4.23.1) are present only in eukaryotes. *Metalloproteinases* incorporating metal ions (usually Zn^{2+}; *e.g.*: *collagenase of vertebrates*, EC 3.4.24.7) are presented by several forms and occur in pro- and eukaryotes (plasma membrane-associated thermolysin, collagenases, gelatinase, and peptidases [11]. They varied significantly by molecular weight (17-800 kDa) and substrate specificity. By contrast to nonspecific thermolysin, collagenases are highly specific to native collagen and possess low affinity for other substrates.

Digestive enzymes of some invertebrate hydrobionts (crustaceans, cephalopods, echinoderms, and coelenterates) exhibit high collagenolytic and proteolytic activities [2,13,14]. Although belong to different types, they possess similar enzymatic properties and are included in one group referred to as CLPI. By contrast to collagenases and trypsins, CLPI are nonspecific enzymes and are capable of effective digestion of any proteins. The presence of CLPI in hydrobionts is a consequence of predominantly saprophytic nutrition (crabs and lobsters), which needs effective hydrolysis of various structural polypeptides (connective tissue, bone, cartilage, and skin), or active locomotions (squids and octopuses) requiring much energy and, hence, effective digestion. Analysis of N-terminal sequences revealed high homology (70-90% for 20 residues) of CLPI and trypsins from various sources [3]; molecular weight of CLPI and trypsins is below 40 kDa.

Biochemical properties of CLPI are unique from the viewpoint of their practical applications.

Collagenases (EC 3.4.24) specific to native collagen cleave its molecule in a single point at a distance of about 3/4 full length from its N-terminal [12], after that collagen looses substrate properties and undergoes no further hydrolysis. Serine proteinases (EC 3.4.21) hydrolyze their substrate at multiple points (into small fragments); mammalian pancreatic proteinases (trypsin, chymotrypsin, elastase) demonstrate homology in their amino acid sequence and steric structure and differ by the type of the cleaved peptide bonds formed by positively charged (Lys, Arg), hydrophobic aromatic (Phe, Tyr), and short aliphatic (Ala, Gly) amino acid residues, respectively. These proteinases possess high primary specificity for substrates, however, acting together they can hydrolyze the majority of peptides.

CLPI are similar to mammalian pancreatic trypsins by their structure and substrate specificity, but unlike trypsins they can cleave native collagen not only due to their primary and secondary specificity, but also due to interaction between the substrate and special domains in CLPI polypeptide chains responsible for binding with appropriate substrate sites.

Collagenolytic serine proteinases form an enzyme-substrate complex geometrically similar to the complexes formed by other serine proteinases. Consequently, protein-protein interactions between the enzyme and collagen cause considerable conformational changes in the collagen molecule to provide collagen hydrolysis.

Thus, collagen hydrolysis requires mutual recognition between the surface-exposed domains of the enzyme and substrate macromolecules with the formation of primary biomolecular complex and further local denaturation of collagen triple helix (amino acid residues located far from the active sites can take part

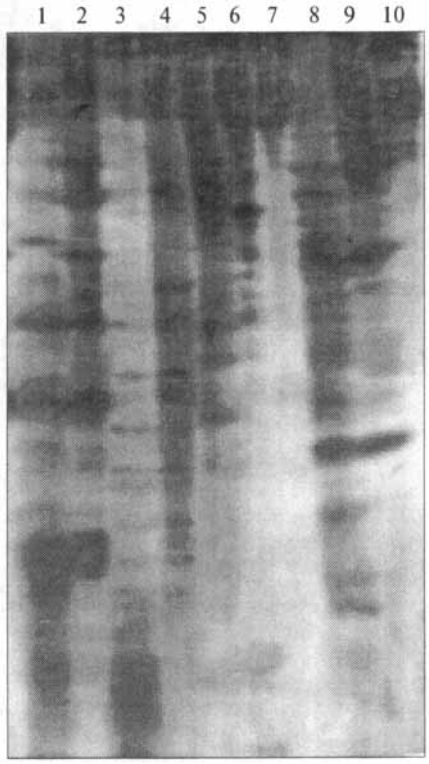

Fig. 1. Endopeptidase activity of individual components from CLPI-complex from crab *Paralithodes camtschatica* [3,4] in hydrolysis of type I collagen from rat skin. From the left to the right: entire preparation, 23 kDa, 25(I) kDa, 25(II) kDa, 25(III) kDa, 28 kDa, 32 kDa, 35(I) kDa, 35(II) kDa, and 36 kDa. Experimental conditions: 1 ml collagen solution (0.1 mg/ml) in 50 mmol Tris-HCl pH 7.5 with 3 mmol $CaCl_2$ incubated with 1-5 μg of corresponding protease at 37°C; reaction was stopped after 30 min (0.2 ml 50% trichloracetic acid).

in the domain-domain interactions between the enzyme and collagen).

Induction of this process facilitating cleavage of native collagen is a characteristic feature of CLPI and collagenases. By contrast to collagenases isolated from *Clostridium sp.*, mammalian tissues, and crab *Paralithodes camtschatica* [9,10] hydrolyzing collagen at a single point of a single chain, CLPI cleave collagen in multiple loci and in all chains of the triple helix, with collagen degradation products undergoing further hydrolysis. When individual CLPI were incubated for 5 h with radiolabeled (^{14}C [12]) or cold native collagen fragments of cleaved collagen (Fig. 1) were detected by radioassay (for radioactive collagen) or staining with Coomassie (for unlabeled collagen) as soon as after 2 min of incubation, molecular weight of these fragments gradually decreased (finally, fragments migrate together with leader dye).

Unlike collagenases recognizing three-dimensional structure of the substrate and, therefore, unable to cleave partially denatured (or modified) collagen, nonspecific CLPI exhibit pronounced endopeptidase activity against both native and denatured collagen and other polypeptide substrates (elastin, fibrin, hemoglobin, casein, *etc.*) ensuring deep (to individual amino acids) hydrolysis, which are involved *in vivo* in biosynthesis or immediately eliminated without intoxication.

CLPI cleavage short synthetic substrates of trypsin (BApNA, BAEE), chymotrypsin (BTEE) and elastase (Ac(Ala)$_3$pNA) more slowly than the corresponding pancreatic proteinases by amide and ester bonds formed by positively charged and aromatic residues, which attests to lower selectivity of CLPI substrate-binding sites. CLPI virtually do not hydrolyze collagenase substrates (PZ-peptide), *i.e.*, CLPI principally differ from collagenases by primary substrate specificity. At the same time, the rate of hydrolysis of structural tissue polypeptides by CLPI is comparable or surpasses that by collagenases (for collagen) and trypsins (for other substrates). Similar to most invertebrate, bacterial, and fish trypsins, CLPI more rapidly hydrolyze long-chain peptide substrates due to secondary bonds between the substrate and substrate-binding enzyme sites contributing to enzyme catalytic activity.

Maximum activity of CLPI-complex and its components is attained at 37°C and pH 7.5. Native CLPI-complexes retain their activity (above 20%) in a wide pH range (4.5-10.5), while activities of individual components vary with pH. Thus, 25(II) kDa protease [3,4] retained >20% of baseline activity at pH 3.7-11.0; 25(I) kDa and 28 kDa proteases >50% of activity at pH 12.1; while 32 kDa protease completely losses its activity at pH 4.1 and 10.5. Generally, optimum pH for CLPI-containing preparations is 7.5±1.0 (>50% enzyme activities, high negative charge), pH 7.5±3.0 is acceptable. In the case of individual intolerance to skin application of the preparation, CLPI can be inactivated by citric or acetic acid (pH < 3.0).

At pH 7.5, maximum activity of CLPI is attained at 37°C, it decreased to 50-90% at 45°C and to 30-70% at 4°C (synergism of CLPI components is most pronounced at 28-45°C [4]). CLPI solutions are very stable: for native CLPI complex (water, 1 mg/ml, microbiotic flora strongly inhibited [3]) half-inactivation times are 3 h at 37°C, >24 h at 20°C, and >10 days at 4°C. In 7 and 25% water solutions of DMSO, CLPI complex (10 mg/ml, 20°C) exhibits 100 and 70% activities, respectively, while 50% solution can be stored for >6 months without noticeable decrease in activity (reversible inhibition of autolysis). Lyophilized CLPI retain activity for at least 4-10 years (observation period for a variety of preparations).

The rate of autolysis of native CLPI-complexes in solutions *in vitro* increases with increasing the concentration and temperature. *In vivo* inactivation is promoted by protease inhibitors released by tissues, which protect living tissues in long-term contact with CLPI-containing preparations (wound applications). This allows to use high doses of CLPI for local injections. CLPI are tolerant to denaturating substances: the native complex [3] (water, 1 mg/ml, 20°C) retained 100% of activity in the presence of sodium dodecyl sulfate (up to 1%) or ethanol (up to 10%), and 80% of activity in the presence of EDTA (1 mmol, 25(I) kDa metalloenzyme was inhibited). Radioactive sterilization (^{60}Co, 2.0±0.5 Mrad) and local anesthetics (novocainamide, lidocaine, trimecaine — 5%) do not affect CLPI activity.

Validity of methods for comparison of collagenolytic activities (efficacy) of CLPI and collagenases was discussed earlier [4]. The rate of hydrolysis determined by radioassay using radiolabeled collagen (^{14}C [12], ^{125}I [10], ^{3}H [15]) was evaluated by the content (in mg) of *native collagen hydrolyzed by 1 mg of the enzyme per time unit under standard conditions (usually per 1 min at 37°C and pH 7.0).* By the second method, the rate of hydrolysis was evaluated by the *concentration (in μmol) of free N-terminals in native collagen hydrolysis products per 1 mg of enzyme per time unit under standard conditions* and expressed in Mandl units [16]: *Mandl unit corresponds to 1 mmol of free peptide N-terminal fragments of native collagen hydrolyzed by 1 mg of enzyme during 5 h (at 37°C and pH 7.0).*

Both methods are correct for comparing collagenolytic activities of collagenases: cleavage of a single peptide bond (at a single point in a single chain of the native collagen triple helix) yields single collagen molecule (detected by the first method) and new peptide with free amino-terminal residue (detected by the

second method). For CLPI the first method is not correct: collagen molecule first hydrolyzed at a single point and released into solution undergoes further multiple hydrolysis to small fragments (carrying free N-terminal amino groups), which can be detected by the second method. Since the degree of collagen substrate hydrolysis by preparations *in vivo* is of principle importance, the efficacy of CLPI should be evaluated by the second method [16].

Major applications of CLPI-containing preparations in medicine and cosmetology are described earlier [5] and here will be supplemented with quantitative information. Mean single therapeutic dose (TD) of CLPI for wounds of various etiology (at the stage of elimination of necrotic tissues until granulation) constitutes for small necrosis 50 mg/kg body weight or 50 mg/cm^2 wound area (or 3.5 mg/70 cm^2/70 kg). Minimum effective dose was 10 μg/cm^2 (when molecules of CLPI are distributed on the surface of inert carriers in the preparation), and maximum dose up to 25 mg in critical cases (26 years old, transosteal metallo-osteosynthesis after secondary open comminuted fracture of the shank, a month later nonhealing wound 3×3×3 cm and open bone at the bottom, without signs of granulation complicated by osteomyelitis in the initial stage). Experimental studying of CLPI-containing preparations revealed no acute (500 mg/kg, epicutaneously, which corresponds to 10^4 TD) or chronic toxicity (TD, 21 days). Body weight gain, total blood count, biochemical indices of the plasma, ECG, diuresis, and urine content after long-term treatment with therapeutic doses remained within the physiological range irrespective of the administration route. Only high doses of CLPI (100 mg/kg, 2000 TD) applied to the skin caused significant changes compared to the control [7]. It was shown that CLPI in therapeutic doses administered to the skin did not change humoral immunity. Only high doses of CPLI (50 mg/kg, 1000 TD) inhibited immune response. This can be due to inhibition of cytokine secretion by fibronectin fragments released after partial hydrolysis of extracellular collagen by CLPI and a decrease in the number of focal contacts between fibroblasts and components of extracellular matrix. It was also demonstrated that long-term skin application of CLPI (3 TD for 14 days) produce no irritation or allergic reactions [8]. For wound bandages CLPI powder (Polycollagenase-K, 0.05-0.5 mg/cm^2 depending on the size of necrotic fucos) or solutions (water, physiological saline, 1-2% lidocaine or trimecaine, polyethylene oxide, inert powder carriers) can be used. Wound drainage sorbents (10-15 g/g) on the basis of polyvinyl alcohol containing 1 mg/g CLPI (0.5-1.5 mg/g depending on individual CLPI-composition) are most effective. They can be applied to the wound (10-15 mg/cm^2) and covered with aseptic gauze bandage (Collasorb, Colladiosorb, the latter containing dioxidine). Absorbent gel slowly release CLPI, after 1-2 days it should be rinsed off with antiseptic solution. The use of higher concentrations of CLPI in sorbents (up to 5 mg/g) does not enhance their proteolytic effect.

When CLPI are employed for preparation of cell cultures instead of clostridial collagenases (tissue disintegration and separation of monolayer from the substrate) [1], it is recommended to start with final concentrations of 0.05±0.03% taking into account that CLPI do not exhibit maximum activity in the presence of phosphate buffer. These concentrations of CLPI (Collagenase from hydrobionts, Polycollagenase-K) provide maximum concentrations of proliferating keratinocytes (*in vitro* cultured by Green method or on microcarriers [6] used for the treatment of extensive and deep burns), as well as fibroblasts, hepatocytes, cardiomyocytes, and chondrocytes. Since CLPI effectively cleave both collagen and noncollagen proteins, the presence of trypsins in the dissociation solution is not necessary.

Cosmetological doses of CLPI for cutaneous injections are: 50 μg/cm^2 (1 TD) (elimination and prevention of scars, keloids, water-color effect with tattoo dye mixture), 5-50 μg/cm^2 (0.1-1 TD) for external applications (enhancement of bioavailability for active components of a composition – lifting, stimulation of regenerative processes and wrinkle prophylactics – soft peeling) and 50-500 μg/cm^2 (1-10 TD, elimination of scars and hard peeling – desquamation, removal of pigment spots and callosities). Any appropriate solvent can be used as the base for cosmetic preparations designed for skin application: water, physiological saline, water solutions of DMSO (to 25%) or ethanol (to 10%), polyethylene oxide, water-lipid emulsion, or available protein-free cosmetic compositions. CLPI should be dissolved in water before their introduction into the hydrophobic mixture, addition of $CaCl_2$ (3 mmol final concentration), but not phosphate or borate buffers, is recommended. Concentration of CLPI in compositions depends on the method of application (convenience of a procedure), skin absorption capacity (5 μl/cm^2 for water) and absorption rate (0.01-1 mg/ml for lifting and soft peeling, 1 mg/ml for scar elimination, and 1-10 mg/ml for hard peeling and tattoo "body art"). Softening of the skin is determined mainly by the efficiency of collagen hydrolysis: native CLPI-complex possesses no significant keratinolytic activity (to Keratin Azure, Sigma) [3]. A method of electrophoretic delivery of CLPI gives best results for the treatment of skin scars and keloids (Fermenkol, 0.05-0.5 mg/cm^2 in physiological saline with 3 mmol $CaCl_2$, cathode area, 0.01-0.2 mA/cm^2 current density, 10-15 min, 10-20 procedures). Phonophoresis (oil

cream, 0.1-0.4 W/cm^2 continuously, 2-5 min, 5-10 procedures) is less effective.

Thus, CLPI-containing enzyme preparations are highly effective structure-destroying enzyme preparations with unique biochemical properties of their active matter: high proteo- and collagenolytic activity in a wide range of pH and temperatures, significant stability of lyophilized forms and solutions, tolerance to denaturative agents, and lack of toxicity in therapeutic doses, which guarantee a broad area of their application in medicine and cosmetology. Both natural CLPI-complexes characterized by high synergism of individual components and individual CLPI or their compositions specially designed to potentiate their specific effects [4] are of great interest. Tritium labeling of individual CLPI (at sites not involved in group exchange) and their compositions preserving physiological activity of the enzymes and suitable for accurate pharmacokinetic investigation of CLPI-containing preparations was proposed by us previously [15].

REFERENCES

1. P. Adams, *Methods of Preparation Tissue Culture for Biochemists* [in Russian], Moscow (1983).
2. O. A. Klimova, Yu. V. Vedishcheva and A. Ya. Strongin, *Dokl. Akad. Med. Nauk SSSR*, **317**, 482-484 (1991).
3. O. A. Klimova and V. Yu. Chebotarev, *Byull. Eksp. Biol. Med.*, **128**, No. 9, 308-313 (1999).
4. O. A. Klimova and V. Yu. Chebotarev, *Ibid.*, No. 10, 391-396 (1999).
5. O. A. Klimova and V. Yu. Chebotarev, *Ibid.*, No. 12, 670-672 (1999).
6. B. A. Paramonov, *Perspectives and Modern Approaches of Skin Recovery in Patients with Severe Burns*, Abstracts of Doct. Med. Sci. Dissertation, St. Petersburg (1997).
7. B. A. Paramonov, O. A. Klimova and V. Yu. Chebotarev, *Byull. Eksp. Biol. Med.*, **129**, No. 5, 556-559 (2000).
8. B. A. Paramonov, O. A. Klimova and V. Yu. Chebotarev, *Ibid.*, **129**, No. 6, 643-645.
9. I. Yu. Sakharov and F. E. Litvin, *Biokhimiya*, **54**, 1913-1918 (1989).
10. I. Yu. Sakharov, F. E. Litvin, A. A. Artyukov, and N. N. Kofanova, *Ibid.*, **53**, 1844-1849 (1988).
11. J. S. Bond and R. J. Beynon, *Mol. Aspects Med.*, **9**, 175-287 (1987).
12. T. E. Cawston and G. Murphy, *Methods Enzymol.*, **80**, 711-722 (1981).
13. G. A. Grant G. A, K. O. Henderson, A. Z. Eisen, R. A. Bradshaw, *Biochemistry*, **19**, 4653-4659 (1980).
14. O. A. Klimova, S. L. Borukhov, N. I. Solovyeva, *et al.*, *Biochem. Biophys. Res. Commun.* **166**, 1411-1420 (1990).
15. O. A. Klimova, Yu. A. Zolotarev, and V. Yu. Chebotarev, *Ibid.*, **195**, 7581-761 (1993).
16. I. Mandll., J. MacLennan, and E. L. Howes, *J. Clin. Invest.*, **32**, 1323-1329 (1953).
17. E. C. Webb, *Enzyme Nomenclature*, New York (1984).