Collagenolytic Protease Preparations from Invertebrates: Experimental Study of Acute and Chronic Toxicity

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Toxicity of active matter of new structure-destroying enzyme preparations containing a complex of collagenolytic proteases from invertebrates (Collagenase from hydrobionts, Polycollagenase-K, Fermenkol) was studied. The absence of acute toxicity in skin application is proved, and LD_{50} for intraperitoneal administration to mice and rats were determined. A long-term treatment with clinical doses under various types of application does not affect body weight gain, biochemical indices of the plasma, ECG, diuresis, and urine content.

Key words: *enzyme preparations*; *collagenolytic proteases*; *acute toxicity*; *chronic toxicity*

Active components of structure-destroying enzyme preparations used now in clinical practice [1,2,6] are serine proteases (or trypsins, EC 3.4.21) and collagenases (EC 3.4.24), proteinases with high specificity for their substrates and low activity against other polypeptides [8,9]. They are ineffective for degradation of structural protein complexes. Novel enzyme preparations (Collagenase from hydrobionts, Polycollagenase, Fermenkol (cosmetic collagenase), and draining sorbents Kollasorb and Kolladiasorb are now produced) containing natural complexes or special compositions of sinergistically acting nonspecific collagenolytic proteases from invertebrates (CLPI) [3-5,10] ensure deep hydrolysis (to individual amino acids) of a variety of substrates (native or partially denaturated collagen, elastin, casein, fibrin, hemoglobin and other polypeptides) and efficient degradation of reticular and massive multylayer structures that can not be attained with usual enzyme preparations. We evaluated the toxicity (by a limited number of parameters) of a purified (proteins>99%) natural complex of CLPI from hepatopancreas of *Paralithodes camtschatica* crab [3,4]. Similar parameters were analyzed for preparations containing other CLPI components.

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MATERIALS AND METHODS

Acute toxicity was tested on inbred albino male mice (18-20 g) and inbred male rats (150-180 g) housed in a vivarium with free access to food and water and quarantined for 2 weeks before the experiments. CLPI were applied to the skin or injected intraperitoneally. CLPI were dissolved in water just before treatment. LD_{50} was calculated by a standard method [7]. To this end, the mortality/survival ratio was determined for all animal groups treated with increasing (in a logarithmic scale) doses of CLPI. To avoid errors, two series of experiments were performed.

Chronic toxicity was tested on 80 inbred male rats housed and fed under the same conditions as the control group. CLPI were administered daily for 21 days (in November, 1998) in doses of 10, 50, and 100 mg/kg to shaved skin in the back (4 cm^2) with a pipette, or intraperitoneally in doses of 0.1, 0.5, and 1.0 mg/kg in isotonic NaCl, which corresponded to approximately 1/1000, 1/200, and 1/100 LD_{50}, respectively. The control animals were treated with the same volumes of water or saline. This duration of treatment is based on the proposed period (3-5 days) for the treatment with CLPI-containing preparations in humans. Test parameters were determined: before and during (on days 7, 14, and 21) the treatment, and 2

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TABLE 1. Effects of CLPB on Blood Biochemical Parameters in Rats ($M\pm m$, n=10)

Parameters	Initial level	Day 21 of treatment						On day 35 (after termination of treatment)
		intraperitoneal, mg/kg			cutaneous, mg/kg			
		0.1	0.5	1.0	10	50	100	
Total protein, g/l	64.8±5.7	63.8±5.7	68.4±5.2	65.6±5.4	68.2±3.9	67.7±4.9	65.7±7.2	66±5
Glucose, mmol/l	6.9±0.2	7.7±0.5	6.8±0.5	7.6±0.8	6.1±0.5	7.0±0.9	5.5±0.7	6.6±0.9
Total cholesterol, mmol/l	1.1±0.2	1.7±0.3	1.4±0.1	1.2±0.2	1.1±0.1	1.4±0.1	1.2±0.2	1.1±0.2

weeks after termination of the treatment (on day 35). The mean and standard deviation for each group were calculated and matched test-and-control pairs were tested for coincidence (with 0.95 probability) of their actual means assuming equal variances (Student's t test). The animals were weighted weekly before feeding in the morning. The following hematological parameters were determined: erythrocyte, leukocyte and platelet counts in a Goryaev's chamber; differential leukocyte count in Romanovsky-stained blood smears, hemoglobin by Sahli hemometer, and glucose, protein, and cholesterol concentrations using Olvex Diagnosticum kits. Urine protein and sugar was qualitatively estimated with standard diagnostic strips. ECG was recorded in the standard lead II.

RESULTS

Topical application of CLPI to the skin in a dose of 500 mg/kg caused neither animal death nor behavioral changes, nor local response in the application site. Application of CLPI in higher doses was technically impossible and LD_{50} could not be measured under these conditions.

Intraperitoneal injection of 200 mg/kg CLPI caused death of all mice within 15-45 min due to respiratory insufficiency. The 4-step decrease of the injected dose allowed to calculate LD_{50} (Fig. 1). Postmortem examination revealed no visible changes in visceral organs. In survived mice, symptoms and duration of intoxication diminished with decreasing the doses. Symptoms of intoxication completely disappeared 3-5 h post-injection, while in doses of 50.1 and 63.1 mg/kg CLPI produced no physiological discomfort. We observed no latent deaths in survivors throughout the observation period (14 days). LD_{50} was 91.7 mg/kg (81-113 mg/kg) for mice and 89.0 mg/kg (79-100 mg/kg) for rats.

When studying chronic toxicity of CLPI, no visible changes in animal behavior and food consumption were found compared to the control; rectal temperature was within the normal range. There were also no significant differences between the groups in body weight gain (Fig. 2), which implies that CLPI produced no effect on this parameter.

By day 21, against the background of stable hematological indices, leukocyte counts increased in all test groups to a maximum of $18.6\pm1.8\times10^9$/liter, although it remained within physiological norm (initial value of $12.7\pm1.2\times10^9$/liter and $17.6\pm1.7\times10^9$/liter in the control on day 21).

Comparison of plasma samples from the test and control groups revealed no significant differences in biochemical indices (Table 1), which suggest no influence of CLPI on the protein, carbohydrate, and lipid metabolism.

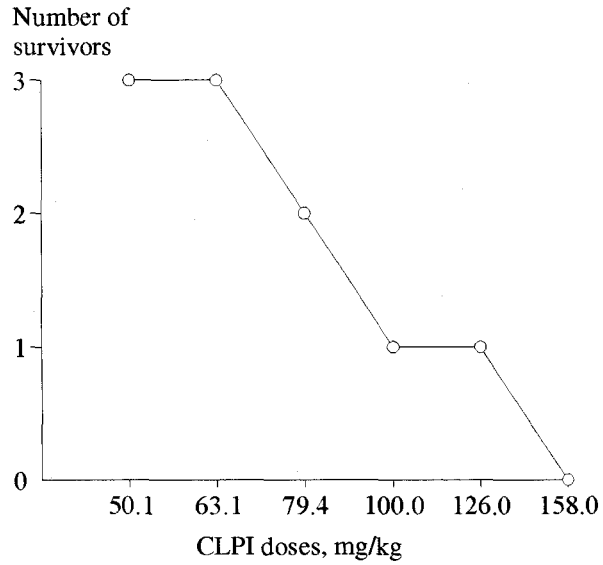

Fig. 1. Mouse survival after intraperitoneal injection of collagenolytic protease preparations from invertebrates (CLPI) in various doses (n=3).

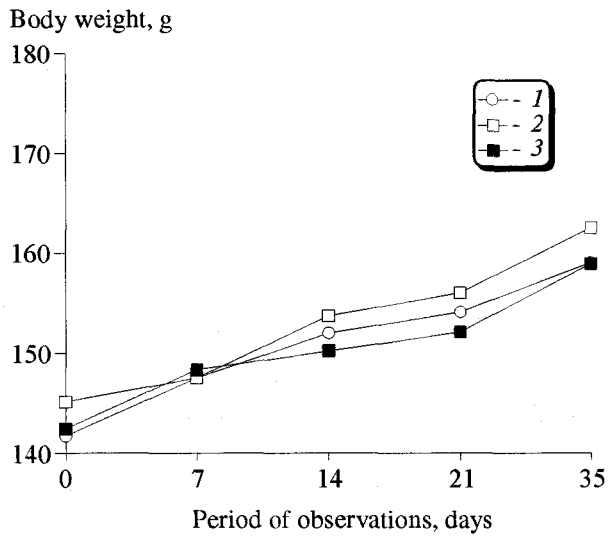

Fig. 2. Body weight gain before (*1*) and during intraperitoneal injections (1.0 mg/kg, 2) and topical applications (100 mg/kg, 3) of collagenolytic protease preparations from invertebrates for 21 days and after the end of treatment (n=10).

Changes in diuresis 1, 2, and 3 h after water load (5% of body weight), absolute and relative diurnal diuresis, urine density and pH were similar in all groups, did not depend on administration route, remained within the physiological range, and were most likely determined by changes in room temperature (diuresis and density) and the diet (pH). Statistically significant (p<0.05) differences between the test and control animals were found only for diuresis over the second hour after water load (2.0±0.1 compared with 2.7±0.3 ml), absolute and relative diurnal diuresis (9.4±0.5 and 6.1±0.3 ml/100 g compared with 12.1±0.7 and 8.4±0.5 ml/100 g, respectively) after skin application of CLPI in a dose of 100 mg/kg, which 2000-fold surpassed the therapeutic dose. Protein and sugar were absent in all tests. These results indicate that the excretory system was not influenced by CLPI.

ECG parameters did not change after CLPI administration.

Thus, topical application of CLPI produce no acute toxicity in mice and rats. We determined LD_{50} for intraperitoneal injections of CLPI. It was established that a long-term treatment with CLPI in therapeutic doses does not alter significantly the tested parameters irrespective on the administration route.

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