Biological activity of extracellular protease preparations of Aspergillus ochraceus micromycete on the Paramecium caudatum model

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Abstract. The research was carried out to study the biological activity of proteases of filamentous fungi, in particular the recombinant protease of Aspergillus ochraceus BMKF41040 micromycete, on a biological model of a unicellular organism - a culture of Paramecium caudatum ciliates. The test object Paramecium caudatum is characterized by a short life cycle, rapid reproduction, it is a convenient model for studying the response of an organism to the effects of preparations. The experiment consisted of 3 stages: rapid assessment of the biological activity of the recombinant protease of Aspergillus ochraceus BMKF41040 micromycete on the culture of infusoria in the stationary growth phase; the assessment of the biological effect of the studied object on the mechanisms of adaptation and cell resistance under the damaging effect of an unfavorable factor; the assessment of the biological activity of the studied objects by the reproduction intensity of ciliates. The results of the studies have shown that the recombinant protease of Aspergillus ochraceus BMKF41040 micromycete does not have a negative and stimulating effect on ciliates and does not affect their reproduction rate. It exhibits moderate biological activity in the test with the action of an unfavorable factor.

1. Introduction
One of the research methods used in pharmacotoxicology is biotesting, which allows an express assessment of the reaction of test organisms to the studied object to be realized. As a biological model, lower organisms are usually used, in particular Paramecium caudatum, belonging to the sub-kingdom of protozoa (Protozoa), the ciliate type (Ciliopliora), Paramecium genus, Paramecium caudatum species. Protozoa are a unique multifunctional biological object, since they are both a cell and a full-fledged organism. Consequently, the reactions of protozoa to the effect of the external environment can be considered as a simple model in comparison with multicellular organisms [1-6].

The ability to obtain quickly an integral assessment of toxic and biostimulating properties makes biotesting very attractive as a method for screening studies in experimental pharmacology [7-9]. Ciliates have a high degree of adaptability and are able to develop protective reactions aimed at weakening the damaging effects of various stimuli, which is associated with their ability to self-regulate [10].

At present, in connection with the urgency of the problem of inflammatory diseases of the uterus and mammary gland in productive animals, it becomes necessary to design a new class of complex highly effective veterinary preparations based on proteolytic enzymes. Proteases of natural origin are actively used as independent medicines and components of preparations for the treatment of mastitis and metritis.
To obtain a more stable effect and minimize side effects, it is desirable to use proteases with increased affinity for heterogeneous substrates, in particular fibrin. Many secreted proteases of filamentous fungi, including the recombinant protease of Aspergillus ochraceus BMKF41040 micromycete, have the necessary properties, which has been shown in a number of studies [11-13].

The objective of our research was to study the biological activity of the recombinant protease of Aspergillus ochraceus BMKF41040 micromycete on a biological model of a unicellular organism - the culture of Paramecium caudatum ciliates.

2. Materials and methods

All manipulations were carried out in accordance with the Guidelines for preclinical trials of the preparations [14], were reviewed and approved by the bioethical commission of FSBSI “ARVRIPP&T”, complied with the state standard GOST 33044-2014, European Convention for the Protection of Vertebrate Animals Used for Experimental and other Scientific Purposes (ETS 123), Strasbourg, 1986 and Directive 2010/63/EU (2010).

The experiment consisted of 3 stages. The objects of the study were the recombinant protease preparation of Aspergillus ochraceus BMKF41040 micromycete, the extracellular proteinases preparation of Aspergillus ochraceus (not recombinant). Trypsin, a proteolytic preparation (crystalline trypsin, lyophilisate for solution preparation, 10 mg, Samson-Med), was chosen as the reference preparation.

The model at the level of a unicellular organism (monoclonal lines of Paramecium caudatum ciliates) was used in the experiment. For the cultivation of paramecium, Lozin-Lozinsky medium was used at pH 6.2-7.8 and a temperature optimum of 20 to 26 °C. Rhodotorula gracilis live yeast supplemented with wheat flour served as the food for the paramecia. Consecutive dilutions of the studied preparation and the assessment of the state of the ciliates were carried out in accordance with the methodical manual [15, 16].

At the first stage of the experiment, an express assessment of the biological activity of the recombinant protease of Aspergillus ochraceus BMKF41040 micromycete on the culture of ciliates in the stationary growth phase was carried out. The motor activity of paramecia is largely formed on the basis of the work of ion channels built into the membrane of the cilia, and it is a characteristic that reflects the functional state of the cell. At the same time, Paramecium caudatum functions in the direction of maintaining the membrane potential. As a result of the decreased membrane potential, cells move more slowly or rotate on the spot around one end.

9.9 ml of the culture of ciliates in the stationary growth phase were poured into 10 test tubes. 0.1 ml of distilled water was added to the first tube and mixed. 0.1 ml of the test object was added to the second tube and mixed. Its dilution of 1:1000 was obtained. Transferring 1 ml of liquid from the second tube to the third one, and so on, the dilutions of 1:10000, 1:100000, 1:1000000, etc. were obtained. A rack with test tubes was placed in a thermostat at \( t = 25.0 \pm 0.5 \) °C. In 30 minutes, 1.0, 3.0, 6.0 and 24.0 hours, 0.1 ml of liquid with infusoria was taken from each tube and micro aquariums were filled with it. There should be at least 400 cells in the micro aquarium. The state of the ciliates was assessed under a binocular loupe.

At the second stage of the experiment, the biological effect of the studied object on the mechanisms of adaptation and resistance of the cell under the damaging effect of an unfavorable factor (sodium chloride solution 8%) were assessed. The culture of ciliates from the stage I, which was in contact with various concentrations of the test object for 24 hours, was used.

From the control tube, 1 ml of culture was taken into 3-4 tubes and 0.1-0.5 ml of the above mentioned solutions were added there so that 100% of the cells died within 5 minutes. Then, 1 ml of liquid was taken from the test tubes, the tested amount of toxic agents was added there, and the cell lifespan was measured up to 100% death. The experiment was repeated the required number of times, and the arithmetic mean was used for the further work.

The calculation was carried out according to the formula 1:

\[
IBA = \frac{To}{Tk}
\]
where IBA is the index of biological activity of the studied object; To - life span in seconds under the effect of the resolving factor of cells that have lived for 24 hours in a medium with a tested concentration of the test object; Tk - life span in seconds under the effect of the resolving factor of cells that have lived for 24 hours in a control medium.

At the third stage of the experiment, the biological activity of the studied objects was assessed by the intensity of reproduction of ciliates. For this, Paramecium caudatum culture was used in the exponential growth phase (from 2 days to 7-10 days of the age of the culture). The cells were counted in 1 ml of culture to determine the density of the inoculum. Lugol's solution was used to fix the ciliates, the Fuchs-Rosenthal counting chamber was used for cell counting, counting was carried out in 10 squares. The samples of Paramecium caudatum culture had been incubated for 72 hours at a temperature of 27.0 ± 1.0 °C with various concentrations of the test object, carrying out aeration 2 times a day. In three days, the density of the inoculum was determined by the above given method. The reproduction rate index (RRI) was calculated according to the formula 2:

\[
RRI = \frac{DIEB \times DICB}{DIEE \times DIEB},
\]

where DIEE is the density of the inoculum in the experiment at the end of incubation, DICB is the density of the inoculum in the control at the beginning of incubation, DICE is the density of the inoculum in the control at the end of incubation, and DIEB is the density of the inoculum in the experiment at the beginning of incubation.

### 3. Study results and discussion

At the first stage of the experiment, an express assessment of the biological activity of the studied objects on the culture of ciliates in the stationary growth phase was carried out according to the following criteria:

1. ID - indifference - cells perform uniform Brownian movements; the investigated object is biologically inactive;
2. BA - cell movements are changed, the studied object is biologically: 1:1000 - weakly active; 1:10000 - moderately active; 1:100000 - active; 1:1000000 - highly active. BA-24 hours - biological activity is very stable; BA-3-6 hours - stable biological activity; BA-0.5-1 hour - biological activity is poorly stable.
3. BCA - biocidal activity - BCA-50 (death of 50 ± 10% of cells), BCA-100 (death of 90 ± 10% of cells) - toxic effect: 1:1000 - weak; 1:10000 - moderate; 1:100000 - strong; 1:1000000 - very strong. BCA-24 hours - slow damage of vital mechanisms; BCA-3-6 hours - gradual damage of vital mechanisms; BCA-0.5-1 hour - rapid damage of vital mechanisms.

The results presented in Table 1 indicated that the test samples in all dilutions had an indifferent effect. In all dilutions of the preparations, the death of ciliates was not observed, which indicated the absence of a toxic effect at the level of the unicellular organism.

### Table 1. Rapid express assessment of the biological activity of the studied preparations (stage I).

| Observation time | Dilution of the preparation | Recombinant protease of Aspergillus ochraceus BMKF41040 micromycete | Extracellular proteinases preparation of Aspergillus ochraceus (not recombinant) |
|------------------|----------------------------|---------------------------------------------------------------|------------------------------------------------------------------|
|                  | 10⁻² 10⁻³ 10⁻⁴ 10⁻⁵ 10⁻⁶ 10⁻⁷ 10⁻⁸ 10⁻⁹ 10⁻¹⁰ | ID ID ID ID ID ID ID ID ID ID ID ID ID ID ID ID ID ID ID ID ID ID ID ID ID ID ID ID ID ID ID ID ID ID ID ID ID ID ID ID ID ID ID ID ID ID ID ID ID ID ID ID ID ID ID ID ID ID ID ID ID ID ID |
At the second stage of the experiment, the biological effect of the studied enzyme samples on the mechanisms of adaptation and cell resistance under the effect of an unfavorable factor were assessed. The results of the experiment were evaluated according to the following criteria: IBA * = 1.000 ± 0.100 - the object is biologically inactive; IBA > 1.000 ± 0.1000 - the object increases cell viability; IBA < 1.000 ± 0.1000 - the object reduces cell viability (*IBA - index of biological activity). The results are shown in Table 2.

**Table 2.** The index of biological activity of proteinase samples on the Paramaecium caudatum culture under the effect of a resolving unfavorable factor (stage II).

| Dilution of the preparation/ IBA^1 | Sample name | Sample name | Sample name | Sample name |
|------------------------------------|-------------|-------------|-------------|-------------|
|                                    | Recombinant protease preparation of Aspergillus ochraceus BMKF41040 micromycete | Extracellular proteinase preparation of Aspergillus ochraceus (not recombinant) | Crystalline trypsin | Control |
| 10^2                               | 1.170±0.112 | 1.160±0.111 | 1.260±0.092 | 1.000 |
| 10^3                               | 1.210±0.094 | 1.100±0.095 | 0.980±0.074 | 1.000 |
| 10^4                               | 0.980±0.078 | 0.910±0.082 | 0.820±0.079 | 1.000 |
| 10^5                               | 0.870±0.062 | 0.790±0.064 | 0.740±0.013 | 1.000 |
| 10^6                               | 0.960±0.088 | 0.850±0.073 | 0.780±0.064 | 1.000 |
| 10^7                               | 0.920±0.082 | 0.970±0.079 | 0.760±0.067 | 1.000 |
| 10^8                               | 0.900±0.079 | 1.050±0.098 | 0.730±0.061 | 1.000 |
| 10^9                               | 0.910±0.075 | 1.070±0.102 | 0.770±0.069 | 1.000 |
| 10^10                              | 0.900±0.086 | 1.070±0.095 | 0.770±0.025 | 1.000 |

^1IBA - index of biological activity

The extracellular proteinases preparation of Aspergillus ochraceus (not recombinant) exhibits a maximum biostimulating effect at a dilution of 1·10^−2, the recombinant protease of Aspergillus ochraceus BMKF41040 micromycete - at a dilution of 1·10^−3. At the same time, with a decrease in their concentration, the index of biological activity remains close to the control value. The comparison preparation trypsin exhibits the maximum biostimulating effect at a dilution of 1·10^−2, with a decrease in the concentration of the preparation, its bioactivity decreases and the preparation does not show bioactive properties.

At the third stage of the experiment, the data were obtained for assessing the biological activity of the studied samples by the intensity of reproduction of ciliates.

The assessment criteria were the following: RRI* = 1.000 ± 0.100 - the object is biologically inactive; RRI > 1.000 ± 0.1000 - the object stimulates cell reproduction; RRI < 1.000 ± 0.1000 - the object inhibits the reproduction of cells (*RRI - reproduction rate index). The results obtained are represented in Figure 1.
The value of RRI in combination with the concentration of a given object in the medium characterizes the degree of its effect on the mechanism of cell reproduction. The studied samples do not have a stimulating effect on ciliates, since the reproduction rate index is in the range of 1.025-1.100.

![Figure 1. The effect of Proteinase Samples on the Reproduction Rate of P. caudatum.](image)

4. Conclusion
Our three-stage biotesting of the extracellular proteinase preparations of Aspergillus ochraceus (not recombinant) and the recombinant protease of Aspergillus ochraceus BMKF41040 micromycete on the model of a unicellular organism allowed us to draw the following conclusions: 1) the preparations did not have a negative effect (toxic effect) on infusoria; 2) the assessment of the biological effect of the studied enzyme samples on the mechanisms of adaptation and resistance of the cell under the effect of an unfavorable factor showed that the preparations reached a maximum biostimulating effect at a dilution of \(1 \times 10^{-2} - 1 \times 10^{-3}\), then their activity decreased, but remained higher than that one of the reference preparation; 3) the studied preparations did not have a stimulating effect on the intensity of reproduction of ciliates and therefore did not show biological activity in this test.

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