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PURIFICATION AND PHYSICO-CHEMICAL PROPERTIES OF *BACILLUS ATROPHAEUS* PROTEASE WITH ELASTOLYTIC AND FIBRINOGENOLYTIC ACTIVITY

O. V. GUDZENKO[1]✉, L. D. VARBANETS[1], V. O. CHERNYSHENKO[2], Y. M. STOHNII[2], A. M. OSTAPCHUK[3], V. O. IVANYTSIA[3]

[1]Institute of Microbiology and Virology named after D. K. Zabolotny, National Academy of Sciences of Ukraine, Kyiv;
[2]Palladin Institute of Biochemistry, National Academy of Sciences of Ukraine, Kyiv;
[3]Odesa I.I. Mechnikov National University, Odesa, Ukraine;
✉e-mail: alena.gudzenko81@gmail.com

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Microbial proteases, among which proteases capable of cleaving elastin, fibrin, fibrinogen, and collagen, have been a matter of interest to researchers due to their significant biotechnological potential along with low production cost. We previously showed that Bacillus atrophaeus 08 synthesizes an extracellular protease complex that exhibits high elastolytic, fibrinogenolytic, fibrinolytic activity, and minor caseinolytic and collagenase activity. The aim of the work was to isolate and purify the Bacillus atrophaeus 08 protease from the culture liquid supernatant and to study the physicochemical properties and substrate specificity of enzyme preparation. Precipitation with ammonium sulfate of 90% saturation, gel-permeation and ion-exchange chromatography were used in the experiment. According to the data obtained, the yield of the purified enzyme with a molecular weight of about 30 kDa was 6%, its elastase activity increased 30 times (420 U/mg protein), and fibrinogenolytic activity 31.8 times (350 U/mg protein). In addition, it also exhibited fibrinolytic (35.3 U/mg protein), minor caseinolytic activity (1.2 U/mg protein) and no collagenase activity. The optimum of elastin hydrolysis was at 37°C, pH 3.0 and 9.0-10.0, the optimum for fibrinogen hydrolysis was 12°C, pH 4.0. SDS-PAAG electrophoresis showed that the Bβ-chain of fibrinogen was almost not cleaved even after 1 h of incubation with the enzyme, while the Aα-chain disappeared already at the 30th min with the production of fragments with M.W. of about 30-45 kDa. The activity of the studied enzyme preparation towards fibrin was much lower than towards fibrinogen.

K e y w o r d s: Bacillus atrophaeus 08, protease, elastolytic and fibrinogenolytic activity, pH and thermooptimum, substrate specificity.

Recently, in connection with the wide use of enzymes in various branches of industry and medicine, many studies aim to search for producers capable of synthesizing enzymes with new physicochemical properties. One such enzyme is microbial proteases, which have attracted the attention of researchers due to their significant biotechnological potential along with low production cost [1-3]. These enzymes account for about 60% of total enzyme sales in the world [4, 5]. Proteases, capable of cleaving proteins such as elastin, fibrin, fibrinogen, and collagen, take the leading place among such enzymes.

The study of such enzymes can be used in the creation of effective, safe medical preparations for the prevention and treatment of heart diseases, as well as trophic ulcers, purulent wounds, burns, for dissolving fibrin clots, and others. Fibrinogenolytic enzymes can be used in the study of fibrinogen/fibrin polymerization processes, and can also be considered as a basis for the creation of drugs aimed at reducing the risk of intravascular thrombus formation by limited proteolysis of fibrinogen circulating in the patient's bloodstream. In recent years, the increased attention of researchers has attracted the proteases produced by marine microorganisms. The latter synthesize a wide range of enzymes, many of which are unique and not found in terrestrial environments. The most important bacterial sources of proteases are representatives of the genus *Bacillus*,

which are capable of producing significant amounts of neutral and alkaline proteolytic enzymes with properties such as high stability to extreme temperatures, pH, organic solvents, detergents, and oxidizing agents. It is known that bacteria are capable of synthesizing enzyme complexes of an extremely broad spectrum of action. Therefore, it is quite clear that the important stages in the preparation of drugs of targeted action are the study of the conditions of their isolation, purification from accompanying biologically active and ballast substances, a comprehensive study of their catalytic properties. The deeper our knowledge about the functioning properties of enzymes, the more effectively they can be used in certain processes. Earlier [6], we showed that *Bacillus atrophaeus* 08 synthesizes an extracellular protease complex that exhibits high elastolytic, fibrinogenolytic, and fibrinolytic activity, as well as minor caseinolytic and collagenase activity. It is known that the most important characteristics of enzyme preparations, which determine the possibility of their use in practice, include pH, temperature optimum, and substrate specificity. Therefore, the aim of the work was to isolate and purify enzymes from the supernatant of the *Bacillus atrophaeus* 08 culture liquid, as well as to study their physicochemical properties and substrate specificity.

Materials and Methods

The object of the investigation was the strain of *Bacillus atrophaeus* 08, which was isolated from bottom sediments at a depth of 1499 m in the Black Sea. The sample from which the strain was isolated was collected during the M 84/2 expedition of the University of Bremen on the ship "Meteor" in March 2011 and transferred to the Odesa National University for microbiological research by Yu.P. Zaitsev and B.G. Alexandrov (Institute of Marine Biology of the National Academy of Sciences of Ukraine). The selected strain was identified by the authors by fatty acid spectra using the Sherlock Microbial Identification System [7].

For the accumulation of the enzyme *B. atrophaeus* 08 was cultivated on a liquid medium, (g/l): KH_2PO_4 – 1.6; $MgSO_4 \cdot 7H_2O$ – 0.75; $ZnSO_4 \cdot 7H_2O$ – 0.25; $(NH_4)_2SO_4$ – 0.5; maltose – 1.0; gelatin – 10.0; yeast autolysate – 0.15, pH 7.0. The strain was cultivated for three to six days (72-144 h) in flasks on shakers (100 ml of medium, 28°C, 244 rpm). The inoculum was taken on the same medium for 24 h and inoculated in flasks at a quantity of 105-106 colony-forming units (CFU).

Cells were separated from the culture liquid medium by centrifugation at 5000 g for 30 min. Dry ammonium sulfate salt was added to the culture liquid supernatant to a final concentration of 90%. The mixture was kept for 24 h at 4°C, centrifuged at 5000 g for 30 min and the precipitate was collected, dissolved in 1.5 volumes of 0.01 M Tris-HCl buffer, pH 7.8 and applied to a column (1.8×40 cm) with neutral TSK gel - Toyopearl HW-55 (Toyosoda, Japan). The sample was eluted with the same buffer. Fractions with elastase and fibrin(ogen)olytic activity were combined and applied to a column (2.5×40 cm) with Toyopearl DEAE-650(M) (Toyosoda, Japan).

Protein content at all stages of purification was recorded on SF-26 at 280 nm. The homogeneity and molecular weight of the purified protein preparation were determined under native conditions on a Sepharose 6B column (1.5×23 cm). The calibration graph for calculating the molecular weight was constructed using marker proteins from the Pharmacia company (Sweden): α-lactalbumin (14.4 kDa), trypsin inhibitor from soybeans (20.0 kDa), carbonic anhydrase (30.0 kDa), ovalbumin (43.0 kDa), and bovine serum albumin (67.0 kDa). The molecular mass of the *B. atrophaeus* 08 enzyme was also determined under the conditions of SDS-PAAG electrophoresis using marker proteins (Thermo Fisher Scientific, USA) (250, 130, 100, 70, 55, 35, 25, 15, 10 kDa). Electrophoresis in a polyacrylamide gel (PAAG) according to the Laemmli method [8] was performed using the tris-glycine system. Protein separation was carried out at a current of 19 mA for the stacking gel and 35 mA for the separating gel. Gels were developed by staining in a staining solution (0.01% Coomassie G–250 solution in 25% isopropanol and 10% acetic acid) for 15 min. A 2–8% solution of acetic acid was used to remove dye residues.

Purified preparations of enzymes were used to study the effect of pH and temperature on enzyme activity. Determination of the influence of pH and temperature of the environment on the enzyme activity was carried out in the temperature range from 4 to 90°C and pH from 2.0 to 12.0, the latter was created with 0.05 M stock phosphate buffer and 0.05 M Tris-HCl buffer.

Enzymatic activity and protein concentration were determined in the culture liquid supernatant and in the preparations after each stage of purification. Protein content was determined by the Lowry method [9]. Caseinolytic (total proteolytic) activity was determined by the Anson method [10]. Elastase

activity was determined colorimetrically by the intensity of the solution color upon enzymatic hydrolysis of elastin stained with Congo red [4]. The incubation mixture contains 2.5 ml of 0.01 M Tris-HCl buffer (pH 7.5), 5 mg of elastin stained with 0.002% Congo red solution and 1 ml of enzyme preparation. The reaction mixture was incubated for 5 h at 37°C. The reaction was stopped by keeping the test tubes with the reaction mixture in an ice bath for 30 min. Unhydrolyzed elastin was separated by centrifugation for 10 min at 10 000 g. The color intensity was measured on an SF-26 spectrophotometer by absorption at 515 nm. The amount of enzyme that catalyzes the hydrolysis of 1 mg of substrate per hour under standard conditions was taken as a unit of elastase activity.

To determine fibrinogenolytic activity, fibrinogen was used as a substrate [12]. 1 mg of fibrinogen, 1.8 ml of Tris-HCl buffer (pH 7.5) and 0.2 ml of the studied preparation were added to the test sample and incubated for 30–45 min at 37°C. The reaction was stopped by adding 2 ml of 10% trichloroacetic acid (TCA). TCA was added to the control sample immediately. Samples were kept at room temperature for 20 min and then centrifuged at 10 000 g for 10 min to remove precipitated protein. Absorption was measured on an SF-26 spectrophotometer at a wave length of 275 nm. The amount of enzyme that under the conditions of the experiment increases absorption by 0.01 in 1 min was taken as a unit of activity. Fibrinolytic activity was determined according to the Masada method [13]. The formation of fibrin cleavage products was measured on an SF-26 spectrophotometer at 275 nm. The amount of enzyme that increased the optical density of the reaction mixture by 0.01 in 1 min was taken as a unit of fibrinolytic activity.

Collagenase activity was determined by the content of free amino acids in the reaction mixture [14]. The unit of activity was the number of micromoles of released amino acids according to the standard curve plotted for leucine.

Human blood plasma was used to isolate and purify fibrinogen [15]. $BaSO_4$ (30 g per liter of plasma) was added to the blood plasma with constant stirring to remove vitamin K-dependent proteins. The plasma was stirred for an hour. Then the plasma was centrifuged at 1300 g for 10 min. The procedure of adding $BaSO_4$ was repeated twice. Then the blood plasma was heated in a water bath to no more than 27°C for 30 min and 1/10 of the total volume of 1 M glycine buffer, pH 9.0 was added, and with constant stirring, 16% Na_2SO_4 was slowly added in small portions. The resulting precipitate was separated by centrifugation (1300 g, 30 min) at room temperature, and a 16% Na_2SO_4 solution was added to the supernatant, centrifuged at 1300 g for 30 min at 10-15°C, and a fibrinogen precipitate was obtained. The precipitate was dissolved in 0.2 M NaCl. Fibrinogen was then reprecipitated with an equal volume of 16% Na_2SO_4. Next, the redeposited fibrinogen was dissolved at room temperature in 0.15 M NaCl and stored at –20°C.

Proteolysis of fibrinogen was carried out at a final concentration of 2 mg/ml in 0.05 M Tris-HCl buffer, pH 7.4, which contained 0.13 M NaCl at 37°C. The proteolytic enzyme was added to a final concentration of 0.005 mg/ml and incubated for 5-60 min. The hydrolysis reaction was stopped by adding sample buffer containing β-mercaptoethanol, and then boiling the resulting mixture.

To study fibrin hydrolysis, a thrombin solution (to an activity of 0.25 NIH/ml) and a proteolytic enzyme purified from the culture medium of *B. atrophaeus* 08 were added to the fibrinogen solution at a final concentration of 2 mg/ml. The reaction mixtures were incubated at 37°C for 5-60 min. A buffer solution for electrophoretic samples was added to the prepared samples containing β-mercaptoethanol in a ratio of 1:1.

Elastin hydrolysis products were obtained as described in the method for determining elastase activity.

Protein concentration was determined using to the Lowry method [9]. Bovine serum albumin (1 mg/ml) was used as a standard.

All experiments were performed in 3-5 repetitions. The Student's *t*-test was used for statistical analysis. Data are presented as mean ± standard deviation error ($M \pm m$) and are considered significant at $P < 0.05$. The results, presented in the form of graphs, were processed using Microsoft Excel 2007.

Results

We previously showed that *Bacillus atrophaeus* 08 synthesizes an extracellular enzyme complex, in the supernatant of the culture liquid of which elastase (14 U/ml), fibrinogenolytic (11 U/ml), fibrinolytic (10 U/ml), as well as minor caseinolytic and collagenase activity (0.1 U/ml) were detected [6].

Isolation and purification of the *B. atrophaeus* 08 proteolytic complex were carried out by classical biochemical methods: precipitation of the supernatant of the culture liquid with ammonium sulfate of

90% saturation, gel-filtration and ion-exchange chromatography (Fig. 1, 2, Table 1). As a result of the precipitation of the culture liquid supernatant with ammonium sulfate of 90% saturation, the elastase and fibrinogenolytic activity remained practically unchanged. Further purification by gel filtration chromatography made it possible to obtain a fraction whose elastase (140 U/mg of protein) and fibrinogenolytic (120 U/mg of protein) activity exceeded the original activity by 10.0 and 10.5 times, respectively (Fig. 1, Table 1). For ion-exchange chromatography on a charged Toyopearl DEAE 650(M) TSK gel, the separation conditions were selected in a way that only ballast proteins bound to the ion exchanger (Fig. 2, Table 1). Thus, as a result of using 0.01 M Tris-HCl buffer pH 7.8 in a salt gradient, a fraction with elastase (420 U/mg protein) and fibrinogenolytic (350 U/mg protein) activities was obtained (Fig. 2), which are in 30 and 31.8 times higher, respectively, compared to the initial activity (Table 2). The yield of the purified preparation was 6% (Table 1).

The study of the substrate specificity (Fig. 3) of the purified enzyme preparation showed that, in addition to elastin and fibrinogen, it hydrolyzed fibrin to a small extent (35.3 U/mg of protein), almost did not hydrolyze casein (1.2 U/mg of protein) and did not hydrolyze collagen at all.

The homogeneity of the *B. atrophaeus* 08 enzyme was proven both in native conditions (gel filtration on a Sepharose 6B column) (Fig. 4) and in a denaturing system (SDS-PAAG electrophoresis) (Fig. 5). With the use of marker proteins, its molecular weight was established (about 30 kDa).

An important characteristic of enzyme preparations is the optimal conditions for their action, in particular pH and temperature. It was shown (Fig. 6) that the purified enzyme preparation of *B. atrophaeus* 08 was active in a wide pH range from 3.0. to 12.0, while when splitting elastin it showed two pH-optimums – 3.0 and 9.0-10.0 (Fig. 6, *A*), and when hydrolyzing fibrinogen one – at pH 4.0 (Fig. 6, *B*). Elastase activity was quite high at different pH values (Fig. 6, *A*). Thus, elastase activity at pH 4.0 was 59%, slightly lower at pH 5.0 (36%), and then again an increase in activity was noted when the pH level increased. At pH 6.0, it was 64%, at pH 7.0 it was 84%, and at pH 8.0 it was 88%. The fact that at pH 12.0 the activity was 60% was interesting. A similar pattern was noted for the fibrinogenolytic activity. It was shown (Fig. 6, *B*) that at pH 5.0, fibrinogenolytic activity was 93.5%, and at pH 3.0, it was 82.2%. Interestingly, that at pH 6.0-10.0, the activity ranged from 53 to 63%, while at pH 11.0 and 12.0, it significantly increased to 87 and 90%, respectively.

The study of the thermooptimum of the enzyme preparation showed (Fig. 7) that it differs slightly according to the optimal temperatures of substrate hydrolysis. The enzyme preparation was active in the range of temperature values from 4 to 60°C (Fig. 7, *A*, *B*), but at the same time, the optimum of elastin hydrolysis was at 37°C (Fig. 7, *A*), and of fibrinogen at 12°C (Fig. 6, *B*). The study of the thermooptimum

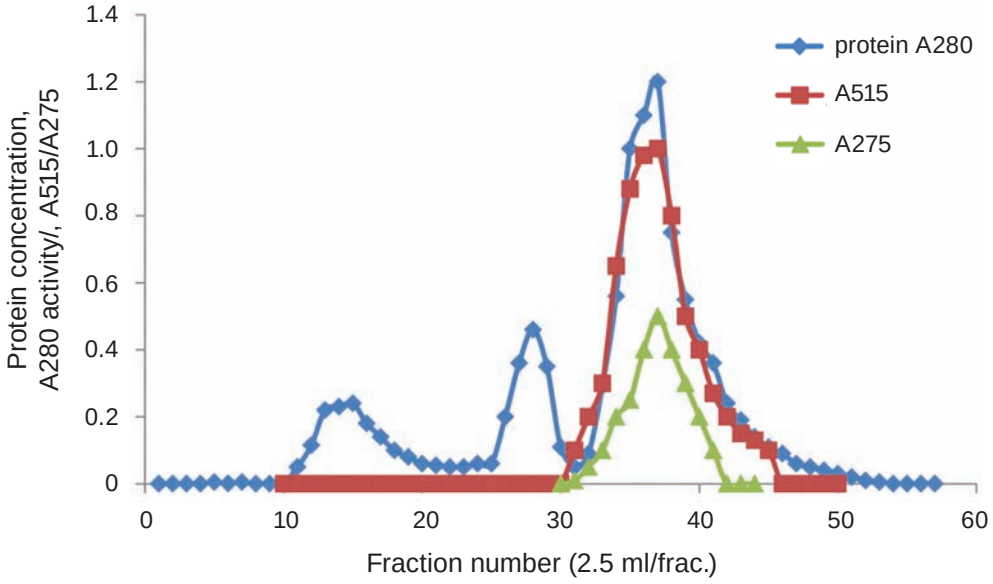

Fig. 1. Elution profile of the B. atrophaeus 08 enzyme preparation on TSK-HW-55 (pH 7.8)

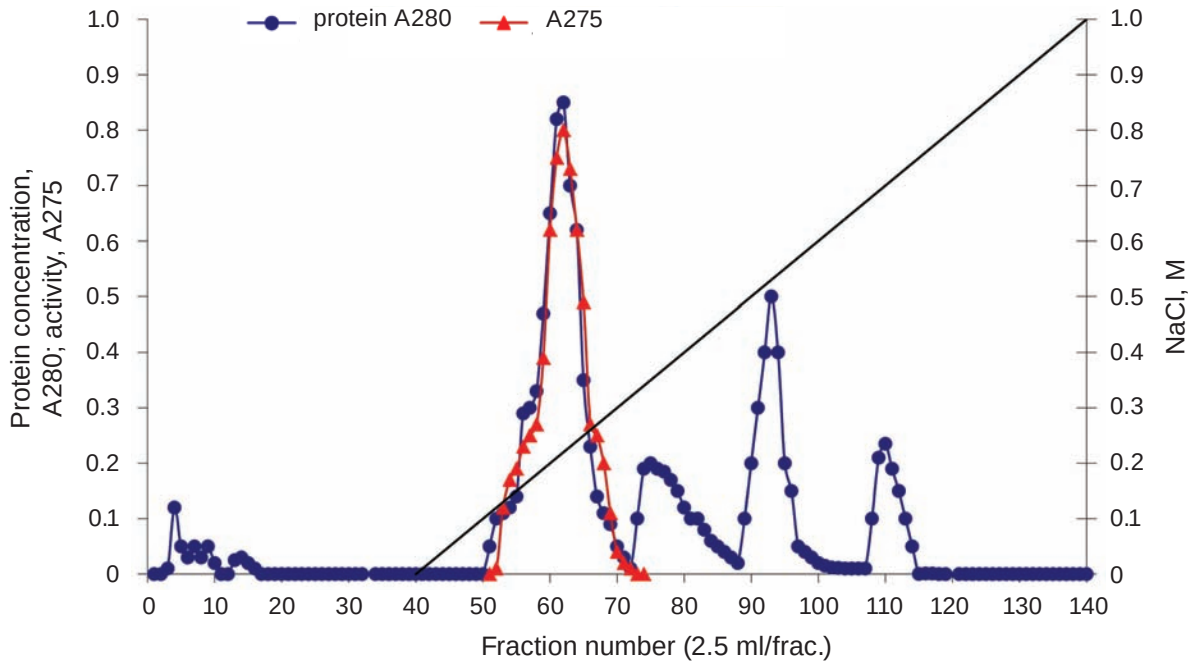

Fig. 2. Elution profile of the B. atrophaeus 08 enzyme preparation on Toyopearl DEAE-650M in a 0-1 M NaCl gradient

Table 1. Purification steps of B. atrophaeus 08 enzyme preparation

Purification steps	Total protein, mg	Total activity, U	Specific activity, U/mg of protein	Yield, %	Degree of purification
Supernatant of culture liquid	200	2800[a] 2200[b]	14[a] 11[b]	100	1
TSK-gel Toyopearl HW-55	40	5600[a] 4800[b]	140[a] 120[b]	20	10.0[a] 10.9[b]
Toyopearl DEAE-650M	12	5040[a] 4200[b]	420[a] 350[b]	6	30.0[a] 31.8[b]

Note. [a]Elastase activity, [b]fibrinogenolytic activity

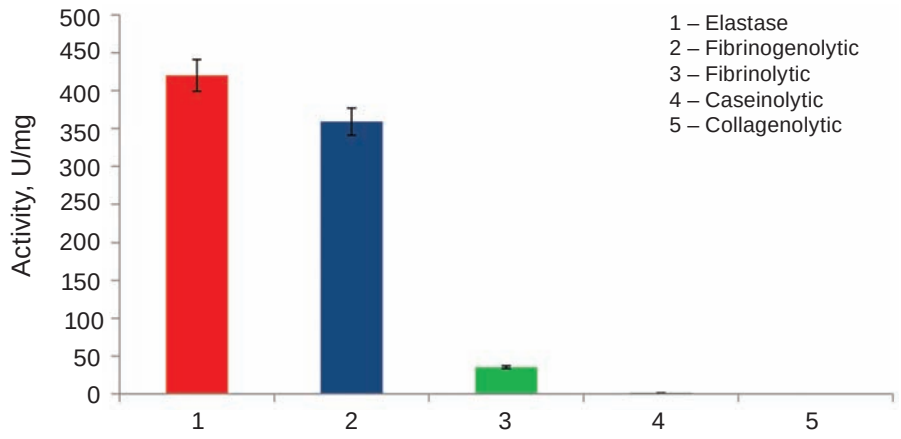

Fig. 3. Substrate specificity of B. atrophaeus 08 enzyme preparation

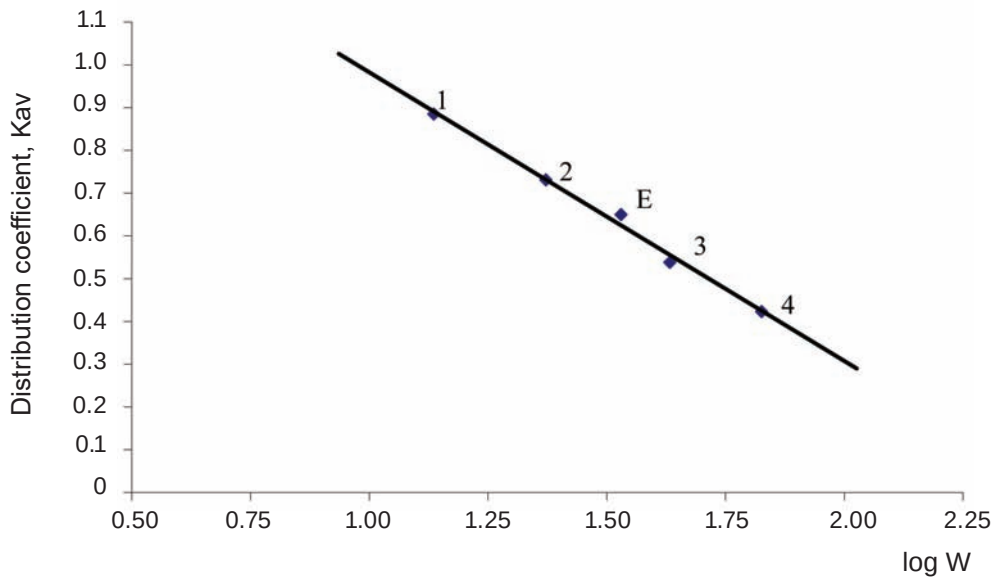

Fig. 4. Molecular weight of the B. atrophaeus 08 enzyme preparation in the native system. Molecular weight markers: ribonuclease (13.7 kDa) (1), proteinase K (25 kDa) (2), chicken egg ovalbumin (43 kDa) (3), bovine serum albumin (67 kDa) (4), E – enzyme preparation of B. atrophaeus 08

of *B. atrophaeus* 08 elastase activity showed (Fig. 7, *A*) that even at 4°C, elastase activity was 58%, at 12°C – 65%, at 15°C – 70%, at 20°C – 80%, and at 30°C – 95%. When the temperature increased above the optimum, quite high elastase activity was also noted. Thus, at 50°C it was 78%, at 60°C – 50%. However, at 70°C, the elastase activity was only 5% of the initial one, and at 80°C, the elastase activity was completely lost.

As for fibrinogenolytic activity (Fig. 7, *B*), the enzyme was quite active in the temperature range from 4 to 37°C (activity at 4°C was 70%, at 15°C – 98%, at 20 °C – 90%) , at 30°C – 85%, and at 37°C – 80%). When the temperature increased to 50°C, the activity decreased by 40%, and at 60°C by 64%. At 70°C, the enzyme was completely inhibited.

To identify the area of fibrinogen affected by the studied *B. atrophaeus* 08 enzyme, its hydrolysis products were analyzed by SDS-PAAG electrophoresis (Fig. 8). As can be seen, the Bβ-chain remained almost uncleaved even after 1 h of incubation with the enzyme, while the native Aα-chain disappears already at the 30th min of incubation. During the reactions, there was an accumulation of fibrinogen Aα-chain cleavage products with molecular weights about 30-45 kDa.

As for fibrin, the studied enzyme cleaved it much more slowly than fibrinogen. Thus, traces of

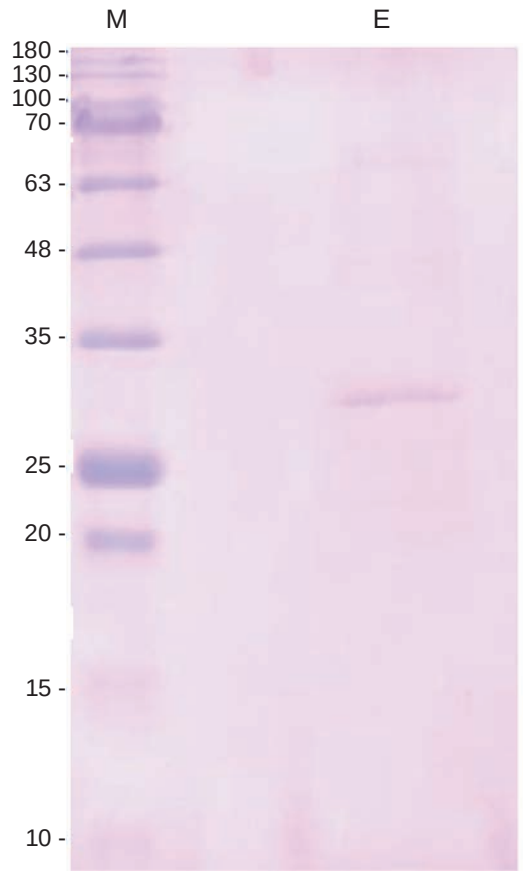

Fig. 5. Molecular weight of the B. atrophaeus 08 enzyme preparation under the conditions of SDS-PAAG electrophoresis

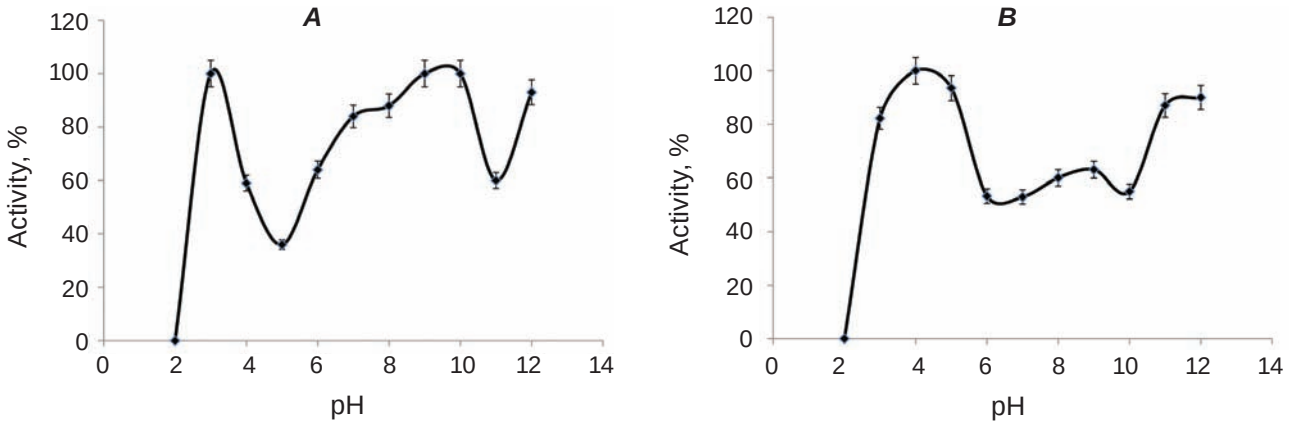

*Fig. 6. Effects of pH on elastase (**A**) and fibrinogenolytic (**B**) activity of B. atrophaeus 08 enzyme preparation (40 °C)*

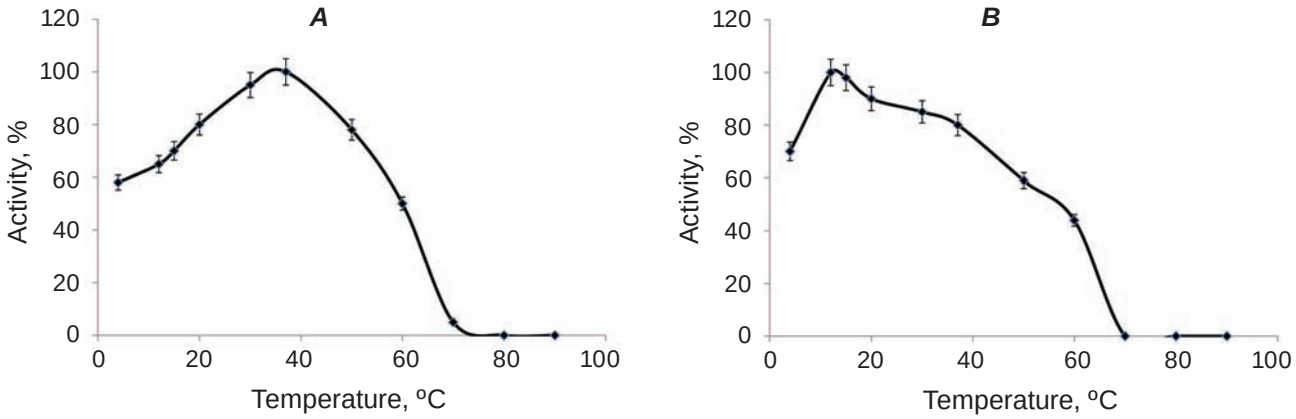

*Fig. 7. Effects of temperature on elastase (pH 9.0) (**A**) and fibrinogenolytic (pH 3.0) (**B**) activity of B. atrophaeus 08 enzyme preparation*

the native Aα-chain of fibrin were detected even after 60 min of incubation with the enzyme (Fig. 9).

During electrophoresis of elastin hydrolysis products, which were formed during its incubation with *B. atrophaeus* 08 enzyme preparation, we observed the formation of certain protein components in the solution, although this was not observed in the control (Fig. 10). Apparently, this is due to the fact that the enzyme preparation cleaved parts of elastin and they released into the solution. This was not observed in the control, since elastin is a difficult to dissolve protein, so there were no soluble protein components in the solution.

Discussion

The marine environment, as a large ecosystem, is one of the most important sources of natural biologically active compounds and one of the richest and most perfect ecosystems on earth in terms of biodiversity. The harsh conditions of marine ecosystems contribute to the production by microorganisms of various types of molecules with unique structural and functional properties. Compared to terrestrial species, marine species produce a greater number of bioactive compounds, making them a significant source of secondary metabolites, including enzymes. All over the world, the use of various enzymes, in particular proteases, for industrial purposes and in medicine is increasing every year. Among the various producers of proteases, representatives of the genus *Bacillus* are mainly used commercially. One of its representatives, *B. atrophaeus* 08, isolated from the bottom sediments of the Black Sea, showed high elastolytic, fibrin(ogen)olytic activity in the supernatant of the culture liquid, which was we established earlier [6].

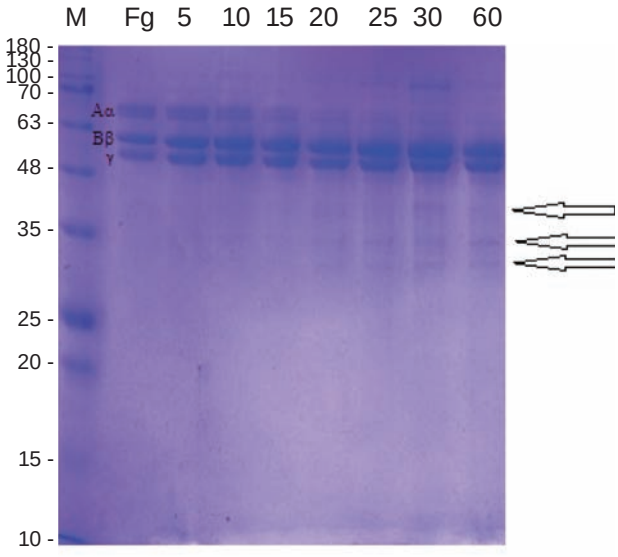

Fig. 8. Electrophoregram of fibrinogen hydrolysis products by B. atrophaeus 08 enzyme preparation. Fg is native fibrinogen; 5-60 min – incubation time of B. atrophaeus 08 enzyme (0.005 mg/ml) with fibrinogen (2 mg/ml). Fibrinogen hydrolysis products are indicated by arrows

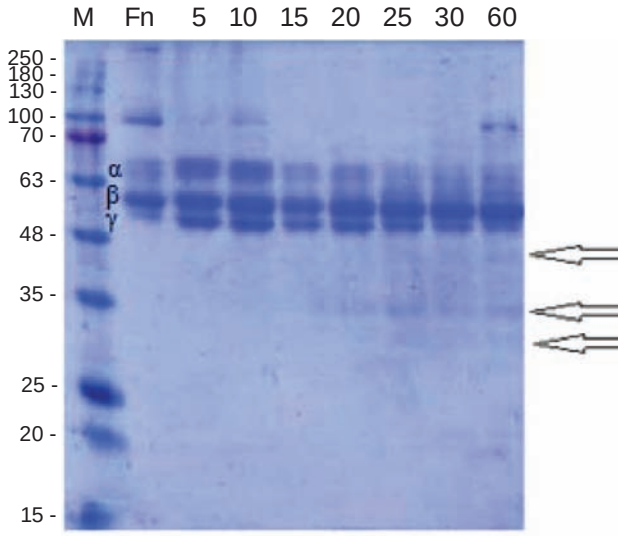

Fig. 9. Electrophoregram of fibrin hydrolysis products by B. atrophaeus 08 enzyme preparation. Fn is native fibrin; 5-60 min is the incubation time of B. atrophaeus 08 enzyme (0.005 mg/ml) with fibrin (2 mg/ml). Fibrin hydrolysis products are marked by arrows

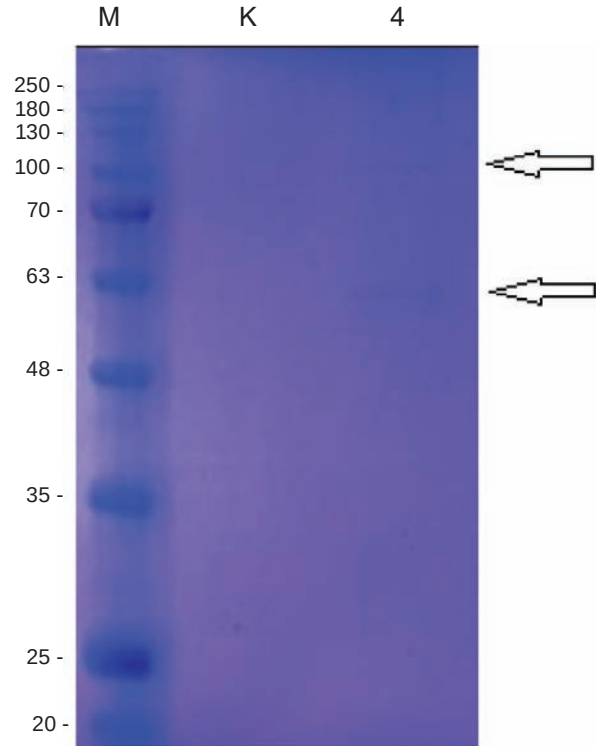

Fig. 10. Electrophoregram of elastin hydrolysis products by B. atrophaeus 08 enzyme preparation. K is native elastin; 4 – hours of the incubation time of B. atrophaeus 08 enzyme with elastin

Using classical biochemical methods, in particular precipitation with ammonium sulfate followed by gel-permeation and ion-exchange chromatography, we isolated and purified a homogeneous enzyme preparation with elastolytic (420 U/mg protein) and fibrinogenolytic (350 U/mg protein) activity, with the molecular weight about 30 kDa, which is typical for many bacterial proteases. The study of some physicochemical properties, in particular the optimal pH values and temperature of the reaction medium, showed that the elastolytic activity of the enzyme had two pH-optimums at 3.0 and 9.0-10.0, and the fibrinogenolytic one at 4.0. As for the influence of incubation temperature, it was established that the maximum value of elastolytic activity was at 37°C, and fibrinogenolytic activity was at 12°C. The presence of two pH-optimums for elastase activity may indicate a complex structure of the active center of the enzyme or the presence of several catalytic centers. Different temperature optima for the hydrolysis of elastin and fibrinogen may be associated with different conformational states of the enzyme when interacting with different substrates. The wide range of pH and temperature in which the enzyme retains its activity indicates its high stability.

The results obtained differ slightly from the literature data [16] regarding pH values but are close

regarding molecular weight. Thus, Kotb E. and co-authors [17] showed that *Bacillus subtilis* elastase had a molecular weight of 25 kDa and was active between pH 6.0 and 9.5, with an optimum of 9.0. The molecular weight of purified elastase from *P. megaterium* gasm32 was 30 kDa. The best reaction pH for gasm32 elastase was determined to be 8.0, which is the same as that of *P. aeruginosa* [18] and the *B. megaterium*-TK1 strain isolated from saltwater. This optimal pH was different from those obtained for other elastases. So, pH values were 9.3 for the alkaline elastase from *Micrococcus luteus*, 7.5 for *B. megaterium*, 7.5 for *Pseudomonas aeruginosa* ZuhP13, and 11.75 for alkalophilic *Bacillus* strain Ya-B [17].

As for the thermooptimum, for elastase from *P. megaterium* gasm32 it made 45°C, for the elastase of *C. indologenes* 37°C [19], for the elastase of *P. aeruginosa* ZuhP13 40°C, the temperature range of 57–59˚C was optimal for the elastase of *Micrococcus luteus*, and 60°C was the optimal reaction temperature for *Bacillus* Ya-B elastase. *P. megaterium* gasm32 elastase was thermally stable for 45 min at 50°C. 70% inhibition at 50°C for 4 h was observed for *Flavobacterium odoratum* elastase and 50% inhibition at 55°C for elastase of *C. indologenes*. *P. aeruginosa* ZuhP13 elastase was inhibited 50% at 60°C after 30 min of exposure [18]. These differences may be due to the source of the isolation and the type of bacteria.

Data on the physicochemical properties of fibrinogenolytic enzymes of microorganisms were not found in the available literature. However, numerous microorganisms have been identified as producers of fibrinolytic enzymes. These are representatives of the genera *Bacillus*, *Marinobacter*, *Serratia*, *Pseudomonas*, *Streptomyces*, *Arthrospir*a, etc. The fibrinolytic enzymes synthesized by them were characterized by molecular masses from 21 kDa (*Streptomyces lusitanus*) to 72 kDa (*Arthrospira platensis)* [16, 19]. Their pH optima ranged from 6.0 (*Artrospira platensis*) to 9.0 (*Bacillus subtilis* ICTF-1), but for most enzymes (mainly for various *Bacillus* species), the pH optimum was 8.0. As for the thermooptimum, it varied from 33°C (*Streptomyces radiopugnans*) to 60°C (*Bacillus flexis*).

An important characteristic of enzymes is their substrate specificity, on the basis of which it is possible to predict the main directions regarding their possible practical use. It is known that the specificity of substrate binding in different enzymes varies greatly. Thus, some enzymes can catalyze a reaction with the participation of only one substrate, while others – with several chemically related substrates. Thus, the ability of the *B. atrophaeus* 08 enzyme preparation to show high substrate specificity to both elastin (420 units/mg of protein) and fibrinogen (350 units/mg of protein) may be due to the fact that both substrates are glycoproteins that have common amino acids in their structure: glycine and proline, and have peptide regions with repetitive structures. For example, elastin contains numerous repeating regions consisting of such amino acids as glycine, alanine, and proline. The fragment of fibrinogen Aα220-390 also contains a number of repeated sequences, each of which consists of 13 amino acid residues. There are 10 such sequences in human fibrinogen that are characterized by an increased content of serine, glycine, threonine and proline [20]. The *B. atrophaeus* 08 enzyme preparation cleaves the native Aα-chain of fibrinogen, much slower – fibrin, but almost does not cleave the Bβ-chain.

Since *Bacillus atrophaeus* 08 proteinase showed high substrate specificity for elastin and fibrinogen, it can be promising both for the treatment of diseases associated with fibrinolysis disorders (thrombosis, embolism) and for fundamental research into the structure and function of the fibrin(ogen) molecule. With the help of this enzyme, it is possible to obtain unique forms of fibrinogen with a shortened Aα chain. The study of such modified fibrin(ogen) will allow obtaining new data on the function of individual sections of the fibrinogen molecule.

Conflict of interest. The authors have completed the Unified Conflicts of Interest form at http://ukrbiochemjournal.org/wp-content/uploads/2018/12/coi_disclosure.pdf and declare no conflict of interest.

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ОЧИСТКА ТА ФІЗИКО-ХІМІЧНІ ВЛАСТИВОСТІ ПРОТЕАЗИ *BACILLUS ATROPHAEUS* З ЕЛАСТАЗНОЮ І ФІБРИНОГЕНОЛІТИЧНОЮ АКТИВНІСТЮ

О. В. Гудзенко[1]✉, Л. Д. Варбанець[1], В. О. Чернишенко[2], Є. М. Стогній[2], А. М. Остапчук[3], В. О. Іваниця[3]

[1]Інститут мікробіології і вірусології ім. Д. К. Заболотного НАН України, Київ;
[2]Інститут біохімії ім. О. В. Палладіна НАН України, Київ;
[3]Одеський національний університет ім. І. І. Мечникова, Одеса, Україна;
✉e-mail: alena.gudzenko81@gmail.com

Мікробні протеази, які здатні розщеплювати еластин, фібрин, фібриноген і колаген, привертають увагу дослідників через їх значний біотехнологічний потенціал поряд з низькою вартістю виробництва. Раніше ми показали, що *Bacillus atrophaeus* 08 синтезує позаклітинний протеазний комплекс, який виявляє високу еластолітичну, фібриногенолітичну та фібринолітичну дію, а також незначну казеїнолітичну і колагеназну активність. Метою роботи було виділення та очищення протеази *Bacillus atrophaeus* 08 із супернатанту культуральної рідини та вивчення її фізико-хімічних властивостей і субстратної специфічності. В експерименті використовували осадження сульфатом амонію 90% насичення, гель-проникну та іонообмінну хроматографію. Згідно з отриманими даними, вихід очищеного ензиму з молекулярною масою близько 30 кДа становив 6%, його еластазна активність зросла в 30 разів (420 од/мг протеїну), а фібриногенолітична активність – у 31,8 раза (350 од/мг протеїну). Крім того, ензим також виявляв фібринолітичну дію (35,3 од/мг протеїну), незначну казеїнолітичну активність (1,2 од/мг протеїну) і відсутність колагеназної активності. Термооптимум гідролізу еластину – 37°C, pH-оптимум 3,0 і 9,0-10,0, термооптимум гідролізу фібриногену – 12°C, pH-оптимум – 4.0. Електрофорез SDS-PAAG показав, що Bβ-ланцюг фібриногену майже не розщеплювався навіть через 1 год інкубації з ензимом, тоді як Aα-ланцюг зникав уже на 30-й хв з утворенням фрагментів із молекулярною масою близько 30-45 кДа. Активність досліджуваного ензимного препарату щодо фібрину була значно нижчою, ніж до фібриногену.

Ключові слова: *Bacillus atrophaeus* 08, протеаза, еластолітична та фібриногенолітична активність, pH та термооптимум, субстратна специфічність.

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