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PURIFICATION AND PHYSICOCHEMICAL PROPERTIES OF *BACILLUS LICHENIFORMIS* 249 PROTEASE WITH ELASTOLYTIC AND FIBRIN(OGEN)OLITIC ACTIVITY

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

[1]*Danylo Zabolotny Institute of Microbiology and Virology, 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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Background. *Proteolytic enzymes with a broad substrate specificity are of considerable interest for potential application in biotechnology and medicine. Proteases with specificity for elastin, fibrinogen and fibrinogen are of particular interest, in particular as antithrombotic agents. Marine microorganisms due to adaptive mechanisms to environment could be a potential producers of enzymes capable of maintaining catalytic activity in a wide range of physicochemical conditions.* ***Objective.*** *The aim of this work was to purify the protease produced by Bacillus licheniformis 249, isolated from the bottom sediments of the Black Sea and to study the physicochemical and catalytic properties as well as substrate specificity of enzyme.* ***Methods.*** *The enzyme was obtained from the supernatant of the bacterial culture liquid by precipitation with ammonium sulfate (90% saturation) with subsequent gel-permeation chromatography on Toyopearl HW-65F and ion-exchange chromatography on Toyopearl DEAE-650M.* ***Results.*** *The purified enzyme had a molecular weight of about 30.0 kDa, maximal activity at 37°C, pH 8,0 and 11,0 and was characterized by high elastolytic, fibrinogenolytic and fibrinolytic activities with a values of 2697.3; 1620 and 1615 U/mg protein, respectively. Electrophoretic analysis of fibrinogen hydrolysis products revealed selective cleavage of the Aα chain, which allows us to attribute the studied enzyme to α-fibrinogenases.* ***Conclusion.*** *The combination of elastolytic and fibrin(ogen)olytic activities, as well as stability in a wide range of physicochemical conditions, indicate the promising potential of this enzyme for use in medicine and various biotechnological processes.*

K e y w o r d s: Bacillus licheniformis, protease purification, pH and temperature optimum, substrate specificity, elastolytic and fibrin(ogen)olytic activity.

Proteolytic enzymes are one of the most important groups of biocatalysts that hydrolyze peptide bonds in proteins and play a key role in the regulation of numerous physiological processes [1, 2]. Among them, proteases with specificity for elastin, fibrinogen and fibrinogen, which are involved in the degradation of structural and plasma proteins, are of particular interest. Elastases catalyze the hydrolysis of elastin, the main component of connective tissue, which provides its elasticity, and play important roles in both normal physiological processes and the pathogenesis of a number of diseases, in particular pulmonary emphysema, cystic fibrosis, etc. [3, 4]. Fibrinolytic enzymes, in turn, cleave fibrin, the key protein of the thrombus, which determines their importance in the hemostasis system and their potential as thrombolytic agents. Fibrinogenolytic proteases are able to hydrolyze fibrinogen, a precursor of fibrin, which expands their potential in the regulation of coagulation processes [5, 6]. Most known proteases are characterized by limited substrate specificity, showing activity mainly towards one type of protein substrate. Enzymes that combine elastase, fibrinolytic and fibrinogenolytic activities are poorly studied, although such multifunctionality may indicate unique structural features of the active center and mechanism of action and determine their high biotechnological potential. Microorganisms isolated from extreme sources are of particular interest, since they are able to synthe-

size enzymes with unique properties adapted to various conditions of existence. Identification and characterization of such enzymes open new prospects for their use in medicine and in various biotechnological processes. Previously [7], as a result of our screening of bacteria isolated from Black Sea bottom sediments at different depths, the *Bacillus licheniformis* 249 strain was selected, which showed a wide range of proteolytic activities, in particular elastolytic, fibrinogenolytic, fibrinolytic, collagenase and caseinolytic. In this regard, the aim of this work was to purify and study the physicochemical, catalytic properties and substrate specificity of *Bacillus licheniformis* 249.

Materials and Methods

The object of the investigation was the strain of *Bacillus licheniformis* 249, 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. The selected strain was identified by the authors by fatty acid spectra using the Sherlock Microbial Identification System.

For the accumulation of the enzyme *B. licheniformis* 249 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-65F (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 at 280 nm. The molecular mass of the *B. licheniformis* 249 enzyme was 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 solution (0.01% Coomassie G–250 25% isopropanol and 10% acetic acid) for 15 min. A 2–8% solution of acetic acid was used to remove dye residues.

Purified enzyme preparations were used to study the effects of pH and temperature on enzyme activity. The enzyme activity was investigated 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.

Protein content was evaluated by the Lowry method [9]. Bovine serum albumin (1 mg/ml) was used as a standard.

Enzymatic activities were determined in the culture liquid supernatant and in the preparations after each stage of purification. Caseinolytic (total proteolytic) activity was determined by the Anson method [10], and elastase activity colorimetrically by the intensity of the solution color upon enzymatic hydrolysis of elastin stained with Congo red [11]. The incubation mixture contained 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 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 [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 at 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 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. licheniformis* 249 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.

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 graphs, were processed using Microsoft Excel 2007.

Results

As a result of our previous [7] screening of bacteria isolated from bottom sediments of the Black Sea at different depths, a strain was selected that was characterized by a wide spectrum of proteolytic activities (Table 1). In the culture liquid supernatant (CLS) of *Bacillus licheniformis* 249, a rather wide spectrum of proteolytic activities was detected, including fibrinogenolytic, fibrinolytic, elastolytic, caseinolytic, and collagenolytic activities (Table 1).

As a result of precipitation of *B. licheniformis* 249 CLS with 90% ammonium sulfate saturation, elastolytic, fibrinogenolytic and fibrinolytic activities were increased by 4.45, 7.0 and 7.29 times, respectively. Further purification by gel permeation chromatography allowed to obtain one fraction, in which elastolytic (666.6 U/mg protein), fibrinogenolytic (600.0 U/mg protein) and fibrinolytic (598.5 U/mg protein) activities were detected, which are 20, 30 and 31.5 times higher, respectively, compared to the initial activity (Fig. 1, Table 2). The fraction obtained after TSK-HW-65F (pH 7.8) was subjected to ion-exchange chromatography on Toyopearl DEAE 650(M) TSK-gel. The separation conditions were selected so that only ballast proteins bound to the ion exchanger (Fig. 2, Table 2). It was shown that using 0.01 M Tris-HCl buffer pH 7.8 in a salt gradient (Fig. 2) it was possible to remove of protein impurities in the preparation, while the elastolytic,

Table 1. Proteolytic activities in the culture liquid supernatant (CLS) B. licheniformis 249

Type of activity	Activity indicators, U/ml
Elastolytic	33.30 ± 0.05
Fibrinogenolytic	20.00 ± 0.05
Fibrinolytic	19.00 ± 0.07
Collagenolytic	0.25 ± 0.09
Caseinolytic	0.09 ± 0.09

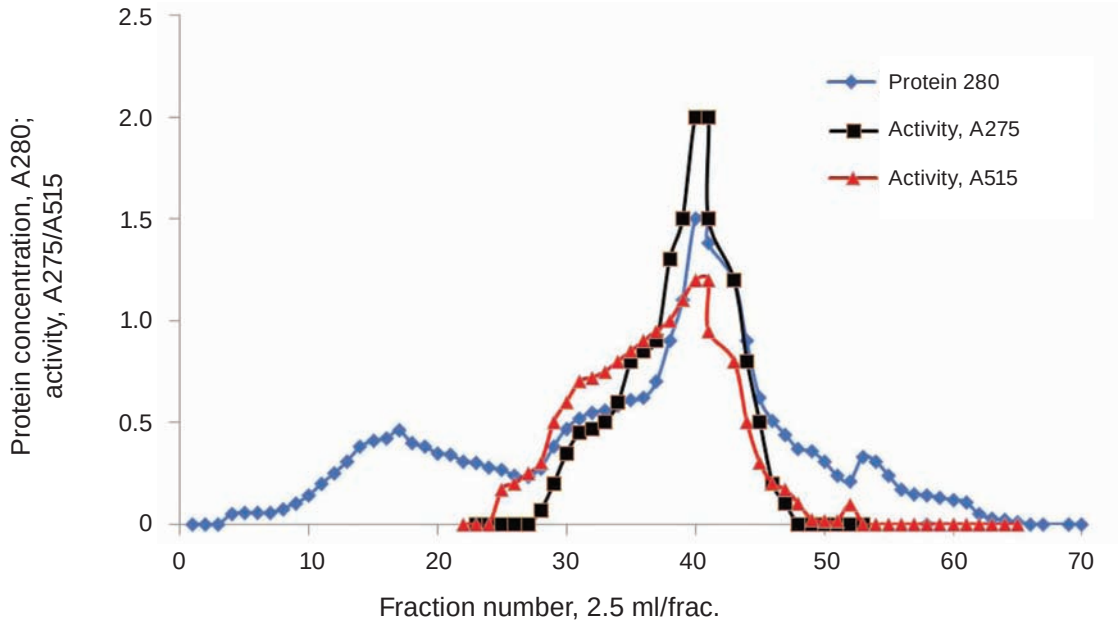

Fig. 1. Elution profile of the B. licheniformis 249 enzyme preparation on TSK-HW-65F (pH 7.8)

Table 2. Purification steps of B. licheniformis 249 enzyme preparation

Purification steps	Total protein, mg	Total activity, U	Specific activity, U/mg of protein	Yield, %	Degree of purification
Supernatant of culture liquid (0.5 l)	550	a-18315 b-11000 c-10450	a-33.3 b-20.0 c-19.0	100	1
90% ammonium sulfate	357.5	a-52500 b-50000 c-49500	a-146.85 b-139.86 c-138.46	65.0	a-4.45 b-7.0 c-7.29
TSK-gel Toyopearl HW-65F	220	a-435380 b-219780 c-219780	a-666.6 b-600.0 c-598.5	43.84	a-20.0 b-30.0 c-31.5
Toyopearl DEAE-650M	38.5	a-103854 b-62370 c-62177.5	a-2697.3 b-1620 c-1615	7	a-81 b-81 c-85

Note. a - Elastolytic, b - fibrinogenolytic, c - fibrinolytic activity.

fibrinogenolytic and fibrinolytic activities increased by 81 times (2697.3 U/mg protein, 1620 U/mg protein and 1615 U/mg protein), and 85 times (1615 U/mg protein), respectively (Table 2) and were detected in one fraction.

It was shown that the enzyme preparation of *B. licheniformis* 249 was homogeneous and represented by a single line with a molecular weight of about 30 kDa (Fig. 3).

An important characteristic of enzyme preparations is the optimal conditions of their action, in particular pH and temperature. It was shown (Fig. 4) that the purified enzyme preparation *B. licheniformis* 249 was active in a wide pH range from 2.0 to 12.0, while when splitting elastin and fibrin(ogen) it demonstrated two pH optima – 8.0 and 11.0 (Fig. 4). That is, the maximum values of activities were noted in the alkaline region. The first maximum activi-

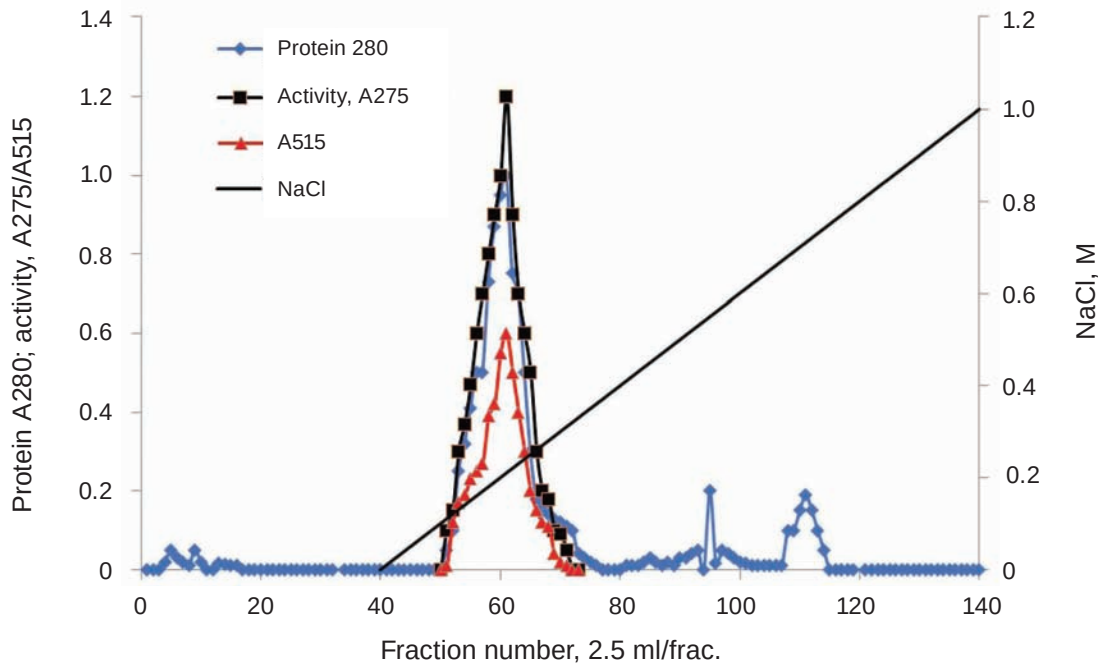

Fig. 2. Elution profile of the B. licheniformis 249 enzyme preparation on Toyopearl DEAE-650M in a 0-1 M NaCl gradient

ty was observed at pH 8.0, where elastase activity reached about 100%, and fibrin(ogen)olytic activity – about 97%. With a further increase in pH to 9, the activity of the preparation decreased (to ~ 65–70% for elastase and ~ 55% for fibrin(ogen)ase). The second maximum of activity was recorded at pH 11.0: elastase activity was about 99%, and fibrin(ogen)olytic activity reached a maximum value of 100%. With a further increase in pH to 12.0 a decrease in activity to 80–85% for elastase and approximately 60% for fibrin(ogen)olytic activity was observed. In an acidic environment (pH 2.0–4.0) all activities remained relatively low and amounted to approximately 25–35% of the optimal values for elastase and 30–35% for fibrin(ogen)ase. With an increase in pH to neutral values (pH 6.0–7.0), a gradual increase in activities to the level of 45–55% was observed.

The enzyme preparation was active in the temperature range from 4 to 60°C (Fig. 5), with a thermooptimum at 37°C. It was found (Fig. 5) that all activities increase with increasing temperature in the range of 4–37°C. However, at 4°C, fibrin(ogen)olytic activity increases from 57%, and elastolytic from 21%, but all activities reach a maximum at 37°C. After reaching the optimum, a significant decrease in activities is observed, but the dynamics are some-

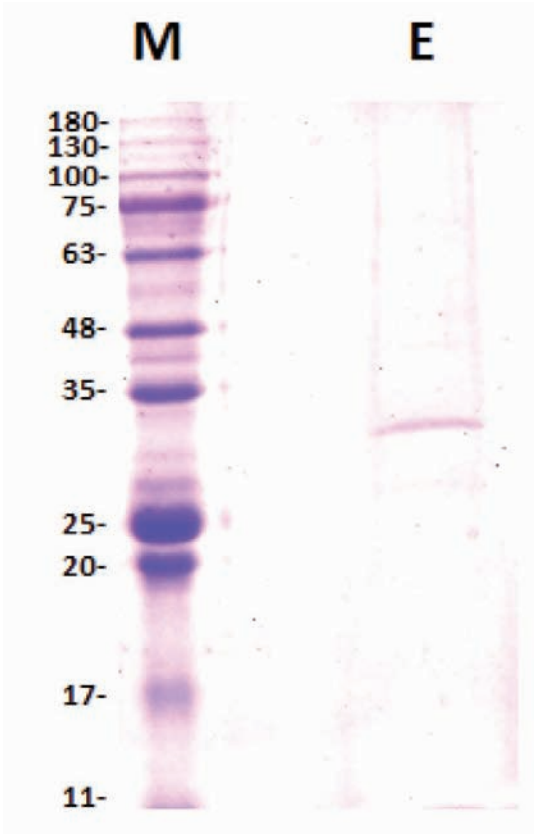

Fig. 3. SDS-PAAG electrophoregram of the enzyme preparation Bacillus licheniformis 249. M – molecular weight markers (kDa); E – enzyme preparation

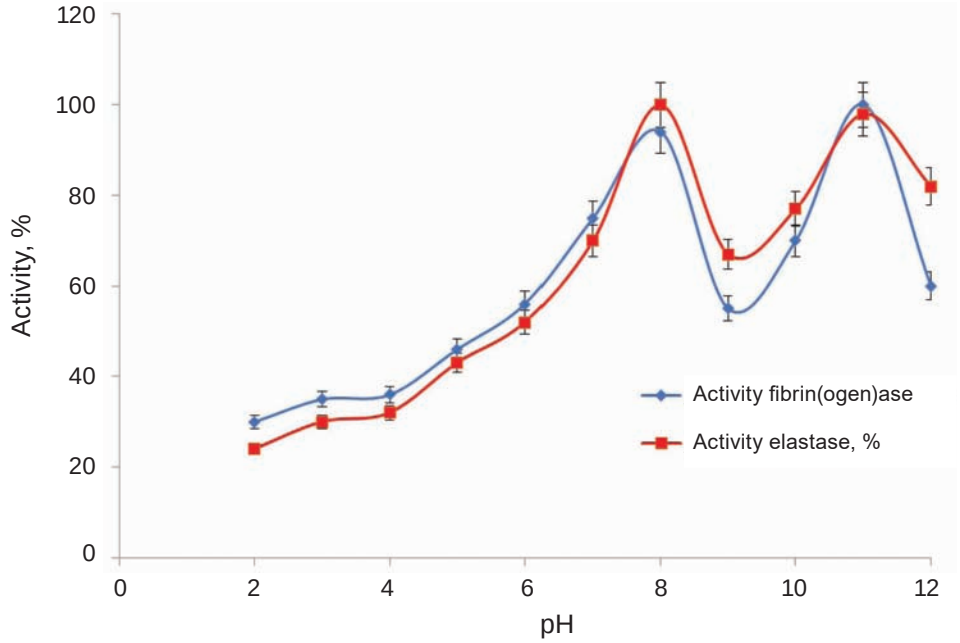

Fig. 4. Effects of pH on fibrin(ogen)olytic and elastase activities of B. licheniformis 249 enzyme preparation (40°C)

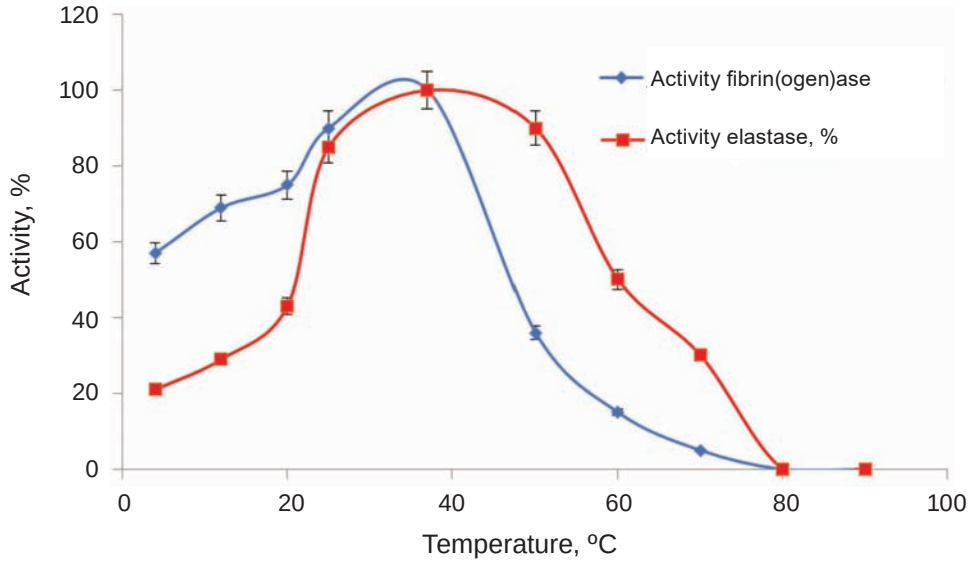

Fig. 5. Effects of temperature on the B. licheniformis 249 enzyme preparation with fibrin(ogen)olytic and elastase activities (pH 8.0)

what different. Fibrin(ogen)olytic activity decreases sharply already at 50°C (to ~36%) and is practically lost at 70–80°C. In contrast, elastase activity is more thermostable: at 50°C, it remains at the level of about 90%, decreasing to 50% at 60°C and 30% at 70°C. Complete inactivation of all enzyme activities occurs at a temperature of 80°C.

Studies of the substrate specificity of the purified enzyme *B. licheniformis* 249 showed (Table 3) that it hydrolyzed elastin best and hydrolyzed fibrinogen and fibrin to almost the same extent, and almost did not hydrolyze casein and had no effect on collagen at all.

Table 3. Substrate specificity of B. licheniformis 249 enzyme preparation

Substrate	Specific activity, U/mg protein
Elastin	2697.30 ± 0.25
Fibrinogen	1620.00 ± 0.07
Fibrin	1615.0 ± 0.5
Collagen	0
Casein	0.12 ± 0.01

To identify the region of fibrinogen and fibrin on which fibrin(ogen)ase *B. licheniformis* 249 acts, their hydrolysis products were analyzed by SDS-PAAG electrophoresis. During the hydrolysis of fibrinogen, cleavage of the Aα chain was observed, while the Bβ and γ chains remained intact. This allows us to attribute the studied enzyme to α-fibrinogenases. During the reaction, the accumulation of hydrolysis products with molecular weights from 11 to 48 kDa occurs, which indicates several sites of action on the fibrinogen molecule (Fig. 6).

Electrophoretic study of fibrin hydrolysis revealed a similar result in comparison with the effect on fibrinogen. *B. licheniformis* 249 enzyme preparation. cleaved mainly the α-chain of fibrin with an efficiency corresponding to the effect on the Aα-chain of fibrinogen (Fig. 7). During the purification of fibrinogen, a small amount of factor XIII (transglutaminase) remains associated with it. After fibrin formation and its self-association, factor XIII, activated by thrombin, catalyzes the formation of covalently cross-linked fibrin chains, predominantly γ–γ. Therefore, during fibrin electrophoresis, we observe a somewhat different pattern in the control samples compared to that seen in the analysis of fibrinogen.

Using Western blot with antibodies II-5C to the N-terminal region of the Aα chain of fibrinogen, it was found that high-molecular hydrolysis products with masses of about 33, 35 and 48 kDa contain the native N-terminus of the chain. Another reaction product with a mass of about 55 kDa was also detected, which was not detected electrophoretically. This is explained by the fact that this fragment of the Aα chain of fibrinogen is close in mass to the Bβ chain, therefore it visually merges with it (Fig. 8). Therefore, the detected low-molecular fragments of fibrinogen are cleaved from the C-terminus of the Aα chain. A fragment with a mass of 55 kDa is formed when acting on the αC domain of fibrino-

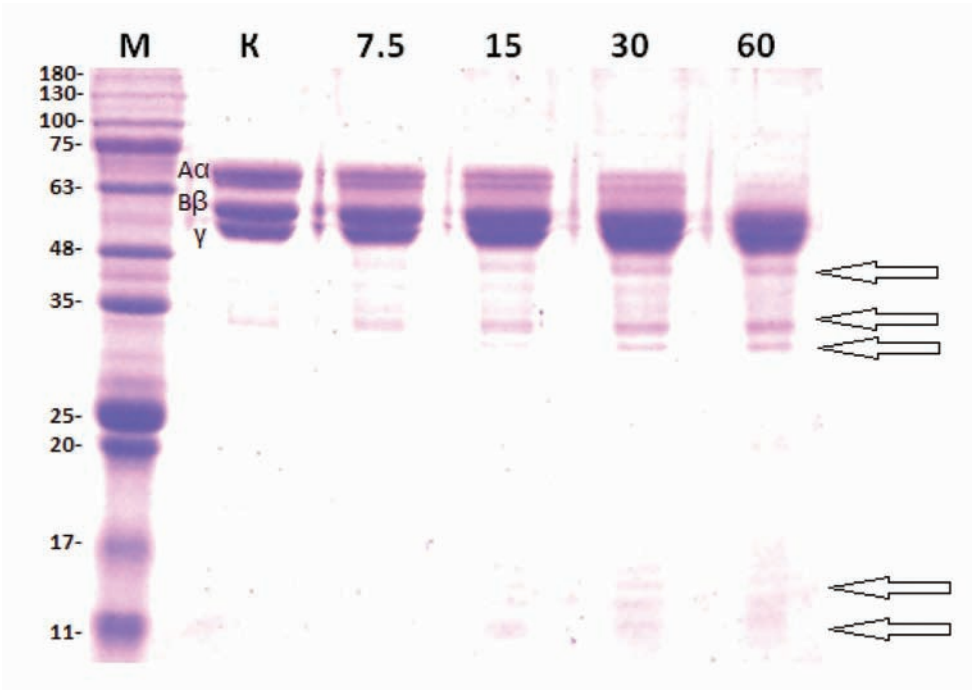

Fig. 6. Electrophoresis of fibrinogen hydrolysis products of B. licheniformis 249 enzyme preparation. M – molecular weight markers (kDa), K – native fibrinogen; 7.5, 15, 30, 60 – incubation time with the enzyme; Aα, Bβ, γ – chains of the fibrinogen molecule; arrows indicate the products of fibrinogen hydrolysis. Samples were prepared in the presence of 0.2% β-mercaptoethanol

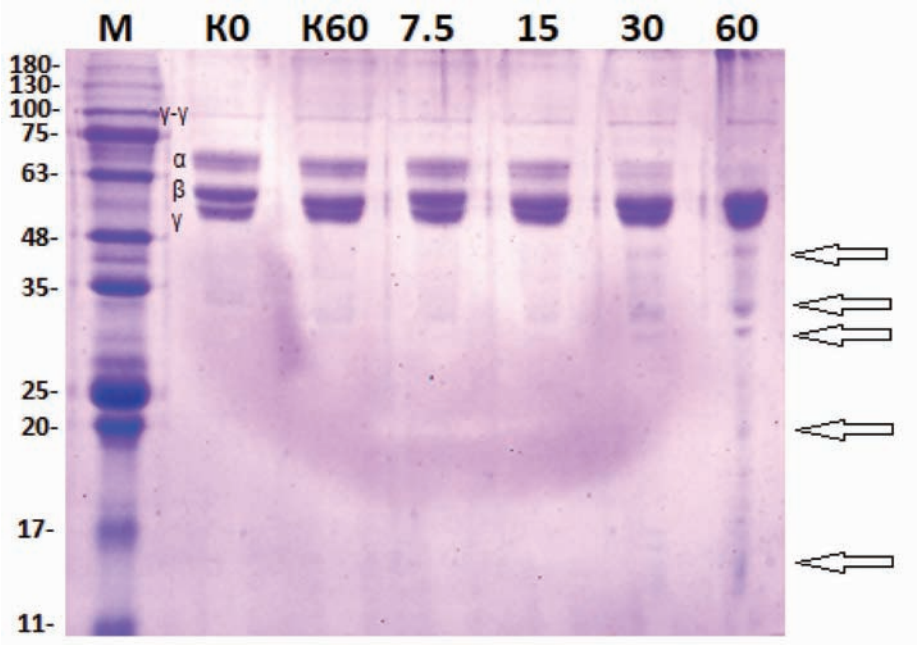

Fig. 7. Electrophoresis of fibrin hydrolysis products of B. licheniformis 249 enzyme preparation. M – markers, K0 – native fibrin; K60 – native fibrin after 60 min of incubation with thrombin; 7.5, 15, 30, 60 – incubation time with enzyme; α, β, γ – chains of fibrin molecule; γ-γ – covalently stabilized γ chains of fibrin; arrows indicate fibrin hydrolysis products. Samples were prepared in the presence of 0.2% β-mercaptoethanol

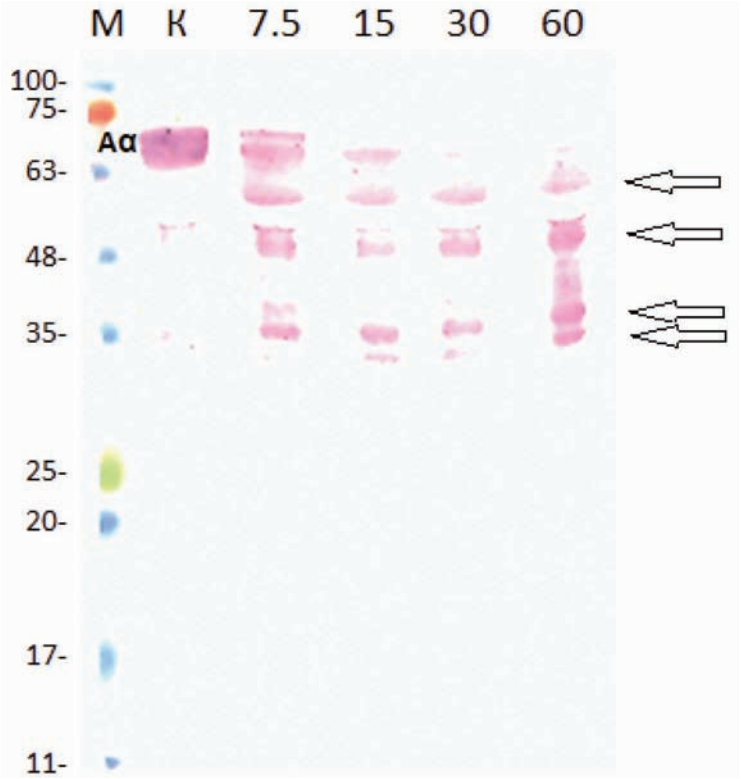

Fig. 8. Western blot with antibodies II-5C to the N-terminus of the Aα chain of fibrinogen. M – molecular weight markers (kDa); K – native fibrinogen; 7.5, 15, 30, 60 – incubation time with the enzyme; Aα – chain of the fibrinogen molecule; arrows indicate the products of fibrinogen hydrolysis. Samples were prepared in the presence of 0.2% β-mercaptoethanol

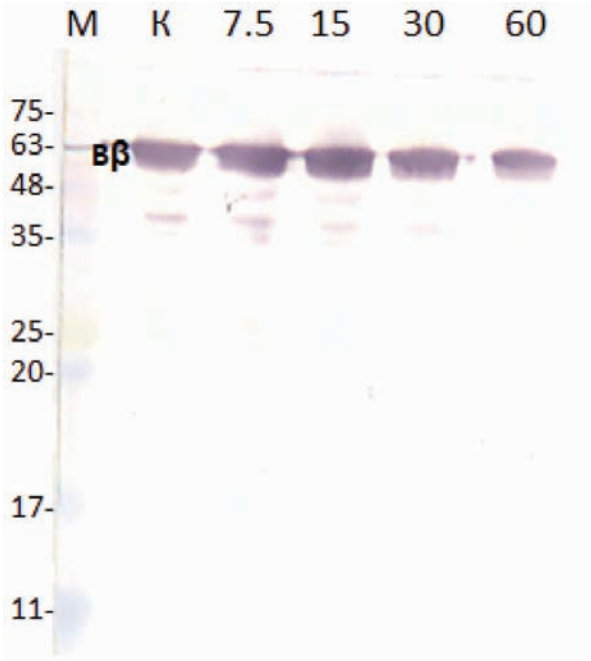

Fig. 9. Western blot with antibodies 2d2a to the N-terminus of the Bβ-chain of fibrinogen. M – molecular weight markers (kDa); K – native fibrinogen; 7.5, 15, 30, 60 – incubation time with the enzyme; Bβ – chain of the fibrinogen molecule. Samples were prepared in the presence of 0.2% β-mercaptoethanol

gen, with the cleavage of a region with a mass of about 12 kDa. The product with a mass of 48 kDa corresponds to the Aα chain almost completely devoid of the αC domain. The protein components with masses of 33 and 35 kDa are formed by the action of the studied enzyme on the connector site of the αC region of fibrinogen.

The Western blot performed with antibodies to the Bβ chain of fibrinogen revealed a slight effect on this part of the fibrinogen molecule. A slight decrease in the content of the native chain was observed only after 60 min of incubation with the enzyme. The antibodies used are specific to the N-terminus, and no reaction products are formed during the reaction. From this, we can conclude that under the action of this protease, a small section is cleaved from the N-terminus of the Bβ chain (Fig. 9).

Discussion

The search for new biocatalysts with defined functional properties is one of the priority areas of modern biotechnology. In this context, various ecosystems, including forests, savannas, polar regions, deserts and marine biotopes, are considered as potential sources of microorganisms - producers of enzymes with unique properties. Marine ecosystems, which are often characterized as extreme due to significant variations in salinity, hydrostatic pressure, temperature and limited availability of nutrients, attract the special attention of researchers. Adaptive mechanisms of marine microorganisms determine the synthesis of enzymes capable of maintaining catalytic activity in a wide range of physicochemical conditions.

Previously [7], we showed that strains of *Bacillus* sp. isolated from the bottom sediments of the Black Sea exhibit a wide range of proteolytic activities. Among them, the strain *B. licheniformis* 249 was of particular interest, in the supernatant of which high enzymatic activity was found against various protein substrates: elastin (33.3 U/ml), fibrinogen (20.0 U/ml), fibrin (19.0 U/ml), collagen (0.25 U/ml) and casein (0.09 U/ml). As part of the conducted studies, a purified enzyme preparation of *B. licheniformis* 249 was obtained. It was homogeneous and characterized by three types of proteolytic activity: fibrinogenolytic, fibrinolytic and elastolytic (1620 U/mg protein, 1615 U/mg protein and 2697.3 U/mg protein, respectively) with a molecular weight of about 30 kDa, which is consistent with the literature data for bacillary proteases.

In particular, the molecular weight of elastase isolated from *Priestia megaterium* was about 30 kDa [16], while the molecular weight of velefibrinase was approximately 32.3 kDa, which corresponds to the range of 18–38 kDa typical for fibrinolytic enzymes. The studied enzyme preparation was characterized by the ability to maintain high activity in a wide range of pH (2,0–12,0) and temperature (4–37°C) with an optimum of action at 8.0 and 11.0 and a temperature of 37°C. Similar properties have been described for a number of fibrinolytic enzymes of marine origin, in particular *Streptomyces radiopugnans* VITSD8 [17] and *Serratia marcescens* [18]. An important characteristic of enzymes is their substrate specificity, which determines possible directions of practical application. Substrate specificity studies showed preferential hydrolysis of elastin, as well as efficient cleavage of fibrinogen and fibrin, while collagen and casein were hydrolyzed slightly or not at all.

Since both elastase and fibrin(ogen)olytic activities had similar pH and temperature profiles, this may indicate that they are carried out by a single enzyme with broad substrate specificity. It is known [19] that the substrate binding specificity

of different enzymes varies significantly: some enzymes catalyze reactions involving only one substrate, while others are able to interact with several chemically related compounds. Hamiche et al. [20] described two extracellular proteases with keratinolytic and elastolytic activity, which were obtained from the *Bacillus amyloliquefaciens* S13 strain isolated from the brown alga *Zonaria tournefortii*. The purified enzymes (KERZT-A and KERZT-B) were monomers with molecular weights of 28 and 47 kDa, respectively, and their N-terminal amino acid sequences showed high homology with keratinases of the genus *Bacillus*. However, they differed in physicochemical properties: KERZT-A showed maximum activity at pH 6.5 and 50°C, while KERZT-B at pH 8.0 and 60°C. In contrast to the above data, the enzyme preparation *B. licheniformis* 249 was characterized by the ability to hydrolyze three substrates. The ability of the *B. licheniformis* 249 enzyme preparation to show high substrate specificity to elastin (2697.3 U/mg protein), and also to fibrinogen (1620 U/mg protein) and fibrin (1615 U/mg protein) may be due to the fact that 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 [21].

According to the nature of fibrinogen hydrolysis, the studied enzyme *B. licheniformis* 249 mainly cleaves the Aα chain. Such specificity is characteristic of a number of bacterial fibrinolytic enzymes [22] and is of great importance from the point of view of their potential application in thrombolytic therapy. The highly selective cleavage of the Aα-chain represents an important advantage compared with human plasmin, which non-selectively degrades the Aα-, Bβ-, and γ-chains of fibrinogen, causing depletion of plasma clotting factors and increasing hemorrhagic risk [23]. In contrast, α-fibrinogenases of bacillary origin preserve the structural integrity of the Bβ- and γ-domains, thereby limiting systemic bleeding complications.

The multifunctionality of the purified protease from *B. licheniformis* 249 becomes especially evident when compared with conventional thrombolytic agents. Modern clinical thrombolytic therapy relies heavily on plasminogen activators, such as Tissue plasminogen activator, Urokinase, and Streptokinase [24, 25]. However, these conventional agents act indirectly, requiring the conversion of endogenous plasminogen into active plasmin, which often induces systemic lytic states and elevates hemorrhagic risks [26]. In contrast, the *B. licheniformis* 249 protease exhibits direct fibrin(ogen)olytic action, actively degrading fibrin polymers independently of host plasminogen. Such direct-acting microbial proteases mimic the catalytic behavior of human plasmin but bypass the slow activation cascade, rendering them highly efficient for rapid clot dissolution [26]. In addition, the combination of elastolytic and fibrin(ogen)olytic activities, together with stability across a broad pH and temperature range, further highlights the biotechnological and biomedical potential of this enzyme preparation. Given its outstanding specific activities (2697.3 U/mg for elastin and 1615.0 U/mg for fibrin), stability under a wide pH spectrum, and precise plasmin-like mechanism with minimized hemorrhagic side effects, the *B. licheniformis* 249 protease stands out as a highly promising, biocompatible candidate for advanced antithrombotic and topical debriding biomedical applications.

When using proteases with fibrin(ogen)olytic activity in medicine, a critically important characteristic is the high specificity of the enzyme toward the fibrin(ogen) molecule. If the studied protease efficiently cleaves other blood proteins, its use as an antithrombotic agent would not be feasible. Therefore, future studies will assess the ability of the enzyme from *B. licheniformis* 249 to degrade other substrates of blood.

Limitations of the study. This study has several limitations that should be acknowledged. First, although the enzyme exhibited pronounced fibrin(ogen)olytic activity, the mechanism underlying the formation of covalently cross-linked γ–γ fibrin chains remains unclear and requires further biochemical and structural investigation. Elucidating this mechanism would provide a deeper understanding of the enzyme–substrate interactions and its mode of fibrin degradation. Second, the present work focused primarily on the fibrin(ogen)olytic properties of the purified protease. The substrate specificity toward other components of the hemostatic system and major plasma proteins was not investigated. In

particular, it remains to be determined whether the enzyme is capable of hydrolyzing albumin, immunoglobulins, coagulation factors, or other structurally related plasma proteins. Such studies are essential for evaluating the enzyme's substrate selectivity, safety profile, and potential therapeutic applicability. Future investigations should therefore include comprehensive substrate specificity analyses, mechanistic studies of fibrin degradation, and i*n vivo* assessments of efficacy and safety to better define the enzyme's potential as a thrombolytic agent.

Conclusions. A protease produced by the deep-sea strain *B. licheniformis* 249 was purified to homogeneity by ammonium sulfate precipitation followed by gel filtration and ion-exchange chromatography. The purified enzyme had an apparent molecular mass of approximately 30 kDa and exhibited high elastolytic, fibrinogenolytic and fibrinolytic activities, with an 85-fold increase in specific activity compared with the native culture supernatant.

The enzyme was active over a broad pH range (2.0–12.0), with two activity optima at pH 8.0 and 11.0 and a temperature optimum of 37°C. The identical pH and temperature dependence of elastolytic and fibrin(ogen)olytic activities, together with their co-purification during all purification steps, indicates that these activities are associated with a single protease possessing broad substrate specificity.

The purified enzyme preferentially hydrolyzed elastin and efficiently degraded fibrinogen and fibrin, whereas collagen and casein were hydrolyzed only slightly or not at all. Electrophoretic and immunoblot analyses demonstrated selective cleavage of the fibrinogen Aα chain with minimal degradation of the Bβ chain, allowing the enzyme to be classified as an α-fibrinogenase.

The combination of broad substrate specificity, selective fibrinogen degradation, and high catalytic activity indicates that the protease from *B. licheniformis* 249 represents a promising enzyme for further studies aimed at the development of fibrinolytic agents and other biotechnological applications.

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ОЧИСТКА ТА ФІЗИКО-ХІМІЧНІ ВЛАСТИВОСТІ ПРОТЕАЗИ *BACILLUS LICHENIFORMIS* 249 З ЕЛАСТОЛІТИЧНОЮ ТА ФІБРИН(ОГЕН)ОЛІТИЧНОЮ АКТИВНІСТЮ

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

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

Вступ. Протеолітичні ензими з широкою субстратною специфічністю становлять значний інтерес для потенційного застосування в біотехнології та медицині. Протеази зі специфічністю до еластину, фібриногену та фібрину становлять особливий інтерес, зокрема як антитромботичні агенти. Морські мікроорганізми завдяки адаптивним механізмам до навколишнього середовища можуть бути потенційними продуцентами ензимів, здатних зберігати каталітичну активність у широкому діапазоні фізико-хімічних умов. **Мета.** Метою цієї роботи було очищення протеази, що продукується *Bacillus licheniformis* 249, виділеної з донних відкладень Чорного моря, а також дослідження фізико-хімічних та каталітичних властивостей ензиму та його субстратної специфічності. **Методи.** Ензим от-

римували з супернатанту культуральної рідини бактерій шляхом осадження сульфатом амонію (90% насичення) з подальшою гель-проникною хроматографією на Toyopearl HW-65F та іонообмінною хроматографією на Toyopearl DEAE-650M. **Результати.** Очищений ензим мав молекулярну масу близько 30,0 кДа, максимальну активність за 37°C та значень pH 8,0 і 11,0. Ензим характеризувався високою еластолітичною, фібриногенолітичною та фібринолітичною активністю зі значеннями 2697,3; 1620 та 1615 од/мг протеїну відповідно. Електрофоретичний аналіз продуктів гідролізу фібриногену виявив селективне розщеплення ланцюга Aα, що дозволяє віднести досліджуваний ензим до α-фібриногеназ. **Висновки.** Поєднання еластолітичної та фібрин(оген)олітичної активностей, а також стабільність у широкому діапазоні фізико-хімічних умов свідчать про перспективний потенціал цього ензиму для використання в медицині та різних біотехнологічних процесах.

Ключові слова: *Bacillus licheniformis*, очищення протеази, оптимальні значення pH та температури, субстратна специфічність, еластолітична та фібрин(оген)олітична активність.

References

1. Mótyán JA, Tóth F, Tőzsér J. Research applications of proteolytic enzymes in molecular biology. *Biomolecules.* 2013; 3(4): 923-942.
2. Vorob'ev MM. Towards a Quantitative Description of Proteolysis: Contribution of Demasking and Hydrolysis Steps to Proteolysis Kinetics of Milk Proteins. *Foods.* 2025; 14(1): 93.
3. Wang K, Meng X, Guo Z. Elastin Structure, Synthesis, Regulatory Mechanism and Relationship With Cardiovascular Diseases. *Front Cell Dev Biol.* 2021; 9: 596702.
4. Demkow U, van Overveld FJ. Role of elastases in the pathogenesis of chronic obstructive pulmonary disease: implications for treatment. *Eur J Med Res.* 2010; 15(Suppl 2): 27-35.
5. Sanz-Horta R, Matesanz A, Gallardo A, Reinecke H, Jorcano JL, Acedo P, Velasco D, Elvira C. Technological advances in fibrin for tissue engineering. *J Tissue Eng.* 2023; 14: 20417314231190288.
6. Risser F, Urosev I, López-Morales J, Sun Y, Nash MA. Engineered Molecular Therapeutics Targeting Fibrin and the Coagulation System: a Biophysical Perspective. *Biophys Rev.* 2022; 14(2): 427-461.
7. Gudzenko OV, Varbanets LD, Ivanytsia VO, Shtenikov MD. Representatives of *Bacillus* from deep-water bottom sediments of the Black Sea – producers elastase, fibrin(ogen)ases, and collagenases. *Mikrobiol Zh.* 2024; 86(3): 51-57.
8. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* 1970; 227(5259): 680-685.
9. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem.* 1951; 193(1): 265-275.
10. Anson ML. The estimation of pepsin, trypsin, papain, and cathepsin with hemoglobin. *J Gen Physiol.* 1938; 22(1): 79-89.
11. Trowbridge JO, Moon HD. Purification of human elastase. *Proc Soc Exp Biol Med.* 1972; 141(3): 928-931.
12. Sharma C, Osmolovskiy A, Singh R. Microbial Fibrinolytic Enzymes as Anti-Thrombotics: Production, Characterisation and Prodigious Biopharmaceutical Applications. *Pharmaceutics.* 2021; 13(11): 1880.
13. Masada M. Determination of the thrombolytic activity of Natto extract. *Food Style.* 2004; 8(1): 92-95.
14. Moore S, Stein WH. Photometric ninhydrin method for use in the chromatography of amino acids. *J Biol Chem.* 1948; 176(1): 367-388.
15. Iskandarov E, Gryshchuk V, Platonov O, Kucheriavyi Y, Slominskyi O, Stohnii Y, Vartanov V, Chernyshenko V. Fractionation of *Vipera berus berus* snake venom and detection of bioactive compounds targeted to blood coagulation system. *Southeastern Eur Med J.* 2022; 6(2): 20-31.
16. AlShaikh-Mubarak GA, Kotb E, Alabdalall AH, Aldayel MF. A survey of elastase-producing bacteria and characteristics of the most potent producer, *Priestia megaterium* gasm32. *PLoS One.* 2023; 18(3): e0282963.
17. Dhamodharan D, Jemimah Naine S, Merlyn Keziah S, Subathra Devi C. Novel Fibrinolytic Protease Producing *Streptomyces radiopugnans* VITSD8 from Marine Sponges. *Mar Drugs.* 2019; 17(3): 164.

18. Krishnamurthy A, Belur PD. A novel fibrinolytic serine metalloprotease from the marine *Serratia marcescens* subsp. sakuensis: Purification and characterization. *Int J Biol Macromol.* 2018; 112: 110-118.
19. Gudzenko OV, Varbanets LD, Chernyshenko VO, Stognii YM, Ostapchuk A M, Ivanytsia VO. Purification and physico-chemical properties of *Bacillus atrophaeus* protease with elastolytic and fibrinogenolytic activity. *Ukr Biochem J.* 2024; 96(6): 36-46.
20. Hamiche S, Mechri S, Khelouia L, Annane R, El Hattab M, Badis A, Jaouadi B. Purification and biochemical characterization of two keratinases from *Bacillus amyloliquefaciens* S13 isolated from marine brown alga *Zonaria tournefortii* with potential keratin-biodegradation and hide-unhairing activities. *Int J Biol Macromol.* 2019; 122: 758-769.
21. Lugovskoi EV, Makogonenko EM, Komisarenko SV. Molecular mechanisms of formation and degradation of fibrin. K.: Nauk dumka, 2013. 225 p. (In Ukrainian).
22. Zhou Y, Chen H, Yu B, Chen G, Liang Z. Purification and Characterization of a Fibrinolytic Enzyme from Marine *Bacillus velezensis* Z01 and Assessment of Its Therapeutic Efficacy *In Vivo. Microorganisms.* 2022; 10(5): 843.
23. Risser F, Urosev I, López-Morales J, Sun Y, Nash MA. Engineered Molecular Therapeutics Targeting Fibrin and the Coagulation System: a Biophysical Perspective. *Biophys Rev.* 2022; 14(2): 427-461.
24. Matesanz A, Sanz-Horta R, Gallardo A, Quílez C, Reinecke H, Acedo P, Velasco D, Martínez-Campos E, Jorcano JL, Elvira C. Succinimidyl Alginate-Modified Fibrin Hydrogels from Human Plasma for Skin Tissue Engineering. *Gels.* 2025; 11(7): 540.
25. Gudzenko O, Varbanets L, Butsenko L, Pasichnyk L. Soilderived *Bacillus* strains producing proteolytic enzymes for food industry applications. *Ukr Food J.* 2026; 15(2): 532-549.
26. Barzkar N, Jahromi ST, Vianello F. Marine Microbial Fibrinolytic Enzymes: An Overview of Source, Production, Biochemical Properties and Thrombolytic Activity. *Mar Drugs.* 2022; 20(1): 46.