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PURIFICATION AND PHYSICO-CHEMICAL PROPERTIES OF *BACILLUS* SP. L9 PROTEASE WITH FIBRIN(OGEN)OLYTIC ACTIVITY

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Previously, we isolated a number of Bacillus sp. strains from the dry grass of the coastal zone of the Kinburn Spit, which may be promising for further research as producers of proteases with fibrinolytic and fibrinogenolytic activity. The aim of the work was to isolate, purify and study the properties of fibrin(ogen)ase from the Bacillus sp. L9 strain. The enzyme preparation was isolated from the supernatant of the Bacillus sp. L9 culture liquid. The yield of the purified enzyme was 1.8%, the specific fibrinogenolytic and fibrinolytic activities were 483 and 383 U/mg protein, respectively, the molecular weight of the enzyme was about 40 kDa, the optimum pH was 8.0, and the thermooptimum was 40°C. Bacillus sp. L9 fibrin(ogen)ase is a serine protease, in the active center of which is the carboxyl group of the C-terminal (aspartic or glutamic) amino acid. At some distance from the active site are localized sulfhydryl groups that do not participate in catalysis, but play an important role in maintaining the catalytically active conformation of the protein molecule. The enzyme from Bacillus sp. L9 hydrolyzed fibrin molecules much more slowly than fibrinogen, and showed the greatest specificity in the hydrolysis of bonds formed by the Aα-chain of fibrinogen. According to the specificity of action on fibrinogen, the enzyme was identified as α-fibrinogen(ogen)ase.

K e y w o r d s: fibrin(ogen)olytic enzyme, Bacillus sp. L9; purification, pH-, thermal optima, molecular weight, specificity.

Fibrin(ogen)olytic enzymes of bacterial origin are attracting increasing attention of researchers due to their potential for application in medicine, in particular in the therapy of thrombosis and in biotechnological processes. To date, fibrinolytic enzymes have been isolated and purified from various producers of microbial origin, but only some of them are used as clinical and therapeutic agents, because they have a number of disadvantages. Thus, streptokinase (*Streptococcus* spp.), although used in the treatment of thrombosis and myocardial infarction, is characterized by high immunogenicity, the risk of allergic reactions and a short half-life; staphylokinase (*Staphylococcus aureus*) exhibits high selectivity for fibrin, but is characterized by the complexity of production and possible immune complications; nattokinase (*Bacillus subtilis natto*) is widely used as a biologically active additive, but its effectiveness and safety have not been sufficiently confirmed by clinical studies [1-3]. Other bacterial proteases with fibrinolytic activity are still at the research stage due to the risk of nonspecific proteolysis and insufficient specificity of action. Therefore, the search for fibrin(ogen)olytic enzymes with high specificity, fewer side effects and those that can be mass-produced at lower cost and higher yield is relevant. It is known [4] that enzymes of bacteria isolated from different ecosystems may differ in their catalytic, physicochemical properties, and substrate specificity. Those isolated from extreme sources, in particular geothermal, marine ecosystems, and coastal zones, have aroused the greatest interest in recent years. Such microorganisms produce a wide range of enzymes characterized by higher activity and sometimes unique properties. Thus, it was shown by us [5], as well as by other researchers [6, 7], when studying the activity of a number of bacterial strains isolated from the bottom sediments of the Black Sea and the waters of the Pacific Ocean, respectively, that the most promising

producers of fibrin(ogen)olytic enzymes are representatives of the genus *Bacillus*. They are characterized by high activity, stability and ability to secrete proteins into the culture medium. As shown by our studies [8], some of them, when grown at 12°C, produce an enzyme with elastase and fibrinolytic activity that is 2 times more active compared to growing the producer at 28°C. Such cold-adapted enzymes, due to their high activity and stability at low temperatures, can be used in industrial processes, which provides economic advantages, since expensive heating of reactors is not required. As for the producers of fibrin(ogen)olytic enzymes isolated from the coastal zone of the Black Sea, we did not find any data in the available literature. However, earlier [9], we isolated a number of *Bacillus* sp. strains from dry grass of the coastal zone of the Kinburn Spit, which may be promising for further research as producers of proteases with fibrinolytic and fibrinogenolytic activity. Therefore, the aim of the work was to isolate, purify, and study the properties of fibrin(ogen)ase from one of these strains, namely *Bacillus* sp. L9.

Materials and Methods

The object of the investigation was the strain of *Bacillus* sp. L9, isolated from plants of the coastal zone of the Kinburn Spit.

For the accumulation of the enzyme *Bacillus* sp. L9 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 days (72 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 into 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 60%. 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 (Tosoh Corporation).

Protein content at all stages of purification was recorded at 280 nm.

The molecular weight of the *Bacillus* sp. L9 enzyme was determined under the conditions of SDS-PAAG electrophoresis according to the Laemmli method [10] using Tris-glycine system. Protein separation was carried out at a current of 19 mA in the concentrating and 35 mA for the distribution gels. Gels were stained in solution (0.01% Coomassie G–250 solution in 25% isopropanol and 10% acetic acid) for 15 min and 2–8% solution of acetic acid was used to remove dye residues. Proteins with molecular weights from 180 to 11 kDa (Thermo Fisher Scientific, USA) were used as markers.

Enzyme electrophoresis was used to identify proteolytically active enzymes. For this purpose, a 12% polyacrylamide gel was polymerized in the presence of fibrinogen (1 mg/ml). After electrophoresis, carried out according to the Laemmli method [10], SDS was removed from the gel by three consistent washes with 2.5% Triton X-100 for 30 min. Then the gel was incubated in 0.05M Tris-HCl with 0.13 M NaCl for 12 h to undergo a hydrolysis reaction [11].

The determination of the influence of pH and temperature of the environment on the enzyme activity was carried out in a 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 of 0.05 M Tris-HCl buffer.

To determine fibrinogenolytic activity, fibrinogen was used as a substrate [5]. 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 [12]. 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.

Elastase activity was determined colorimetrically by the intensity of the color of the solution upon enzymatic hydrolysis of elastin stained with Congo red [5]. The color intensity was determined by colorimetrically 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 [14].

Caseinolytic (total proteolytic) activity was determined by the Anson method [15]. Collagenase activity was defined by the content of free amino acids in the reaction mixture in the reaction with ninhydrin [16]. The unit of activity was the number of micromoles of released amino acids according to the standard curve constructed for leucine.

To obtain purified fibrinogen, we used human blood plasma [17]. Proteolysis of fibrinogen was carried out at a final concentration of 2 mg/ml in 0.05 M Tris-HCl buffer, pH 7.4, containing 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 purified proteolytic enzyme 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 in a ratio of 1:1, containing β-mercaptoethanol.

The effects of various cations NH^{4+}, K^{+}, Na^{+}, Li^{+}, Ag^{+}, Cd^{2+}, Ca^{2+}, Mg^{2+}, Hg^{+}, Ni^{2+}, Mn^{2+}, Zn^{2+}, Co^{2+}, Cu^{22+}, Ba^{2+}, Pb^{2+}, Fe^{2+}, Fe^{3+} (in the form of sulfates at a final concentration of 10^{-3} M) on the enzyme activities were studied. For inhibitory analysis, the following specific chemical reagents were used at a final concentration of 10^{-3} M: ethylene diammine tetra acetic acid (EDTA), o-phenanthroline, dithiothreitol (DTT), L-cysteine, β-mercaptoethanol, *p*-chlormercuribensoate (CMB), N-ethylmaleimide, 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide methiodide, phenyl methyl sulfonyl fluoride (PMSF) (Sigma-Aldrich, USA). Reactions were carried out for 60 min at 37°C and pH 7.0 in the presence or absence of the compound tested. The enzymes activities were investigated by standard assays.

Fibrinogen, fibrin, elastin, casein, collagen in concenrtrations of 1mM were used to determine fibrin(ogen)ase substrate specificity. Activities were determined by the methods described above.

Protein concentration was determined according to the Lowry method [18]. 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 $M \pm m$ and are considered significant at $P < 0.05$. The results, presented in the form of graphs, were processed using Microsoft Excel 2007.

Results and Discussion

Isolation and purification of the proteolytic complex of *Bacillus* sp. L9 was carried out by classical biochemical methods, the first of which was precipitation of the supernatant of the culture liquid with ammonium sulfate. As shown by numerous studies [19-22], the use of ammonium sulfate for protein precipitation is preferable, since it is an inexpensive reagent, water-soluble and capable of stabilizing proteins. Enzyme precipitation was carried out at both 60 and 90% saturation with ammonium sulfate. Studies have shown that the main activity of the protein is precipitated at 60% saturation, while only a small part of it was detected at 90% precipitation, so it was not taken for further studies. Precipitation with ammonium sulfate allowed the increase in the fibrinogenolytic and fibrinolytic activities of the enzyme by 10 times (96.6 U/mg protein and 76.6 U/mg protein, respectively) (Table 1). Further purification by gel permeation chromatography allowed obtaining a fraction with fibrin(ogen)olytic activity (483 and 383 U/mg protein, respectively), which exceeded the initial activity by 50 times. The yield of the purified enzyme preparation was 1.8% (Fig. 1, Table 1).

Since the enzyme preparation of *Bacillus* sp. L9 was homogeneous after purification by gel permeation chromatography, as proven by polyacrylamide gel electrophoresis in a denaturing system (SDS-PAAG) (Fig. 2), as well as by enzyme electrophoresis (Fig. 3), further purification by ion exchange chromatography was not required. Using marker proteins, the molecular weight of the enzyme was established, which was about 40 kDa.

A similar molecular weight (about 42 kDa) was also characterized by the fibrinolytic enzyme BSFE1 of *Bacillus* sp. S-3685 [19]. However, the *Bacillus* sp. L9 enzyme we studied differed from both the fibrinase of *Bacillus subtilis* K2, which had a molecular weight of 26 kDa [24], and the fibrinolytic protease of *Bacillus cereus* VMI2, which had a molecular weight of 29 kDa [25].

The substrate specificity study (Fig. 4) of the purified enzyme preparation *Bacillus* sp. L9 showed that, in addition to fibrinogen (483.0 U/mg protein) and fibrin (473.0 U/mg protein), it hydrolyzed casein (0.2 U/mg protein) to an error level and did not hydrolyze collagen and elastin at all. While the results of the substrate specificity study of the *Bacillus atrophaeus* enzyme indicate that the regularity was different: fibrin (62.3 U/mg) > fibrinogen (46.2 U/mg protein) > casein (42.1 U/mg protein) > serum albumin (6.8 U/mg protein) [23]. The *Bacillus subtilis* DC27 enzyme showed significant activity only against fibrin and fibrinogen.

The specificity of enzymes for fibrin is a critical parameter in assessing their potential as therapeutic agents. According to the literature, most microor-

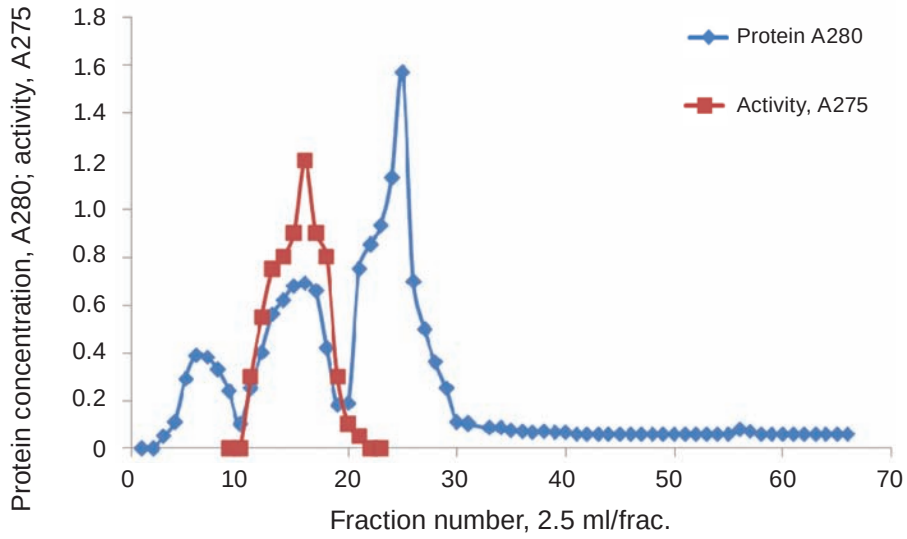

Fig. 1. Elution profile of the Bacillus sp. L9 enzyme preparation on TSK-HW-55 (pH 7.8)

Table 1. Purification steps of Bacillus sp. L9 enzyme preparation

Purification steps	Total protein, mg	Total activity, U	Specific activity, U/ mg of protein	Yield, %	Degree of purification
Supernatant of culture liquid	500	4830	9.66a 7.66[b]	100	1
60% ammonium sulfate	250	2415[a] 1915[b]	96.6[a] 76.6[b]	50	10[a] 10[b]
TSK-gel Toyopearl HW-55	9	4347[a] 3447[b]	483[a] 383[b]	1.8	50[a] 50[b]

Notes. [a]Fibrinogenolytic activity, [b]fibrinolytic activity

ganisms that synthesize fibrinolytic enzymes also exhibit fibrinogenolytic activity [1, 12, 27]. Therefore, it is important to study the ratio of fibrinolytic to fibrinogenolytic activity. For the enzyme *Bacillus* sp. L9, it is 1:1.02.

The study of fibrinogen hydrolysis products by the *Bacillus* sp. L9 enzyme was performed by SDS-PAGE. A solution of native fibrinogen with a concentration of 2 mg/ml was incubated with the enzyme for 5, 10, 20 and 40 min. The samples were prepared in the presence of 0.2% β-mercaptoethanol to cleave disulfide bonds and ensure complete separation of fibrinogen chains during electrophoresis. It was found that during the hydrolysis of the native fibrinogen molecule, protein components with a lower molecular weight are formed (indicated by arrows). Electrophoretic analysis showed that the *Bacillus* sp. L9 protease exhibits a high specificity for the Aα-chain of fibrinogen, since its degradation is detected at the early stages of incubation. Traces of the native Aα-chain disappear after 20 min of incubation of fibrinogen with the enzyme. Under the conditions of the experiment, no hydrolysis of the Bβ and γ chains of fibrinogen was detected. Therefore, the protease under study is α-fibrinogenase. Hydrolysis of the Aα chain of the fibrinogen molecule leads to the formation of stable hydrolysis products with a mass of approximately 35 and 40 kDa (Fig. 5).

In addition to the ability of the enzyme preparation *Bacillus* sp. L9 to hydrolyze fibrinogen, we assessed its ability to also cleave fibrin. It is known [28] that the fibrin polymerization process is activated by thrombin, factor XIII, or transglutaminase. Under the action of this enzyme, covalent “flashing”

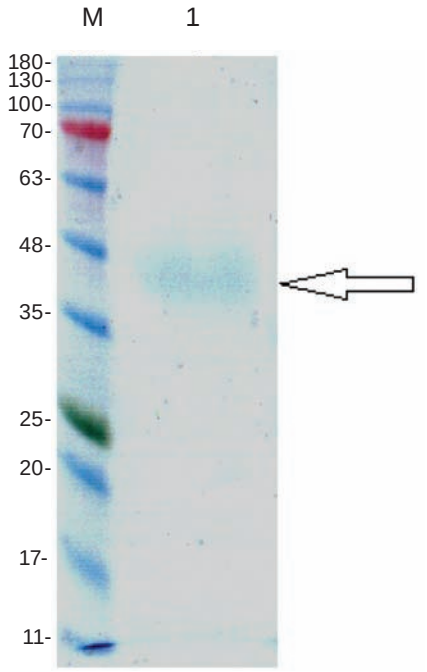

Fig. 2. SDS-PAAG electrophoregram of the enzyme preparation Bacillus sp. L9. M – molecular weight markers (kDa); 1 – enzyme preparation

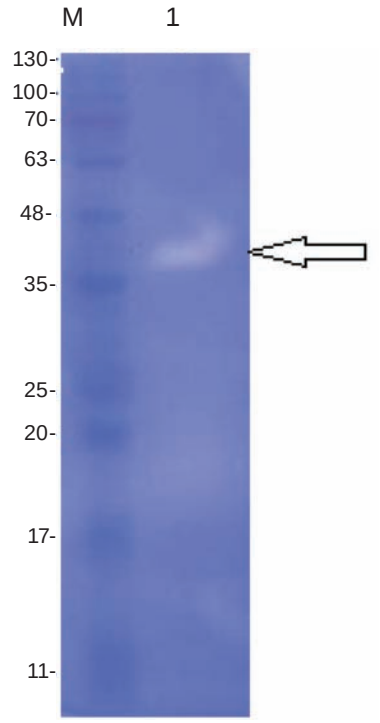

Fig. 3. Enzyme-electrophoregram of the of Bacillus sp. L9 preparation; M – molecular weight markers (kDa); 1 – enzyme preparation

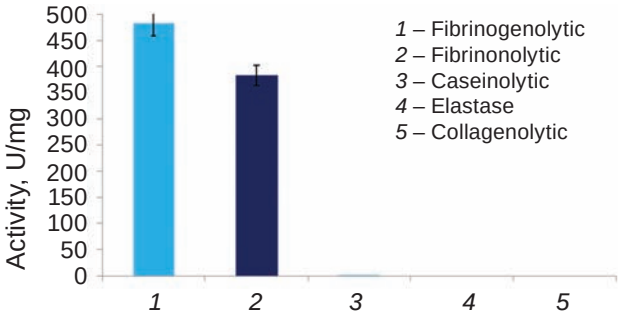

Fig. 4. Substrate specificity of Bacillus sp. L9 enzyme preparation

of fibrin molecules occurs with the formation of isopeptide bonds. Transglutaminase of human blood plasma is the most specific to γ-chains of fibrin, and when it acts on fibrin, covalently bound γ-chains with a mass of about 90 kDa are formed quite quickly. With a longer time of action, this enzyme combines α-chains of fibrin [29]. Therefore, to assess the ability of the protease to cleave fibrin, a control was set in which the transglutaminase reaction would last 40 min, i.e. the longest time of fibrin hydrolysis by the protease under study (Fig. 6).

During the study, it was determined that the ability of *Bacillus* sp. L9 protease to cleave fibrin is lower compared to the hydrolysis of fibrinogen. Even after 40 min of incubation with the enzyme, the α-chain of fibrin could be observed. During the hydrolysis of fibrin, the formation of hydrolysis products with molecular weights of about 30-35 kDa is observed (Fig. 6). The hydrolysis products are indicated by arrows.

The greater specificity of the *Bacillus* sp. L9 enzyme preparation to the fibrinogen molecule than to fibrin may be due to the screening of hydrolysis sites during the formation of fibrils from fibrin molecules. The products of fibrin hydrolysis were smaller than the products of fibrinogen hydrolysis. This can be explained by conformational rearrangements that occur during the conversion of fibrinogen to fibrin, which may slightly change the accessibility of certain parts of the molecule to the enzyme.

The specificity of the action of the studied protease on fibrinogen was also confirmed by Western blot analysis using antibodies II-5C to the N-terminal part of the Aα-chain. This experiment aimed to identify specific degradation products formed as a

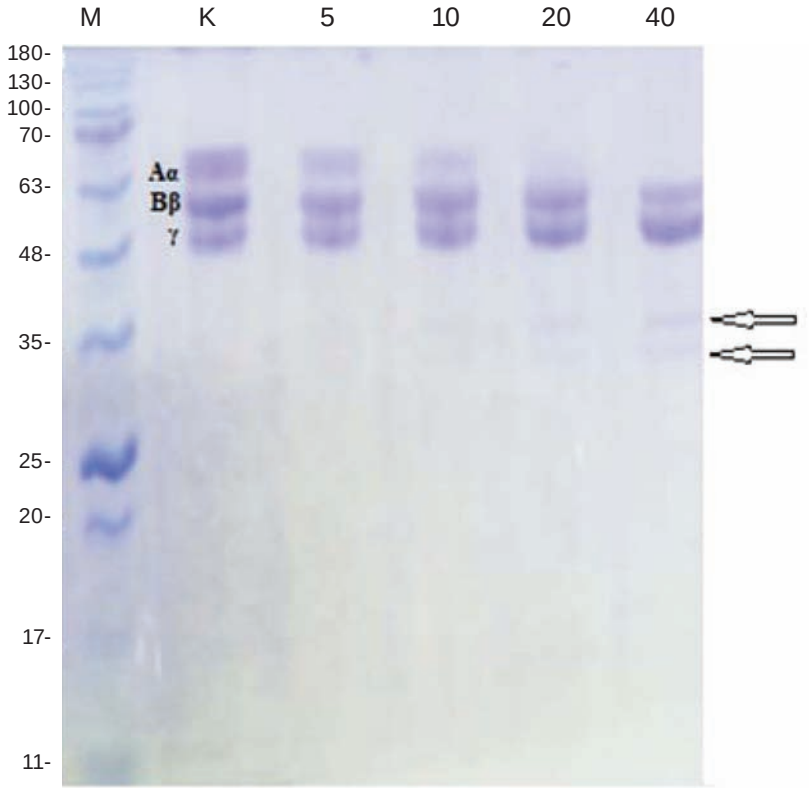

Fig. 5. Electrophoregram of the separation of fibrinogen hydrolysis products by the enzyme preparation Bacillus sp. L9. M – molecular weight markers (kDa). K – native fibrinogen; 5, 10, 20, 40 min of incubation with the enzyme, respectively; Aα, Bβ, γ – chains of the fibrinogen molecule

result of hydrolysis and establish the part of the fibrinogen molecule on which the obtained protease acts. It was found that during the hydrolysis process, small fragments are cleaved from the C-terminal part of the Aα-chain of fibrinogen. It was also found that several hydrolysis products are formed under the action of the studied enzyme. The following protein components were identified, which contained the native N-terminal parts of the Aα-chain of fibrinogen: approximately 67 kDa – full-length Aα-chain, indicating an intact, uncleaved fibrinogen molecule; 50 kDa – cleavage product, which is a stable fragment of the α-chain after cleavage of a fragment from the C-terminus of the Aα-chain with a mass of about 17 kDa (the protease action site is located in the αC-domain of the fibrinogen molecule); 40 kDa – degradation product, the formation of which can be explained either by cleavage of a area with a mass of about 10 kDa from the 50 kDa fragment, or by the action on the native fibrinogen chain with cleavage of a fragment with a mass of 27 kDa (the protease action site is located in the connector region of fibrinogen and in this case the αC-domain of fibrinogen is completely cleaved); 35 kDa – a hydrolysis product formed as a result of the action on the hydrolysis site almost in the center of the Aα-chain of fibrinogen in the connector region of the Aα-chain of the fibrinogen molecule (Fig. 7). The stepwise nature of fibrinogen degradation (Fig. 7) indicates that the Aα-chain undergoes sequential proteolytic hydrolysis, which in turn leads to the accumulation of specific fragments. Using such a protease, it is possible to obtain forms of fibrinogen devoid of the corresponding areas of the molecule, as well as to purify fragments that are cleaved from the C-terminus of the Aα-chain of fibrinogen. The study of the properties of these parts of the molecule makes it possible to establish the structural and functional role of each individual site on the C-terminus of the Aα-chain of fibrinogen.

Researchers [22] showed that the *Coprinus comatus* CFE enzyme degraded the Aα chain of fibrinogen within 1 min, while the degradation of the Bβ chain occurred within 1 h, and the γ chain – even more slowly, within 3 h. The nature of the degradation of fibrin by the CFE enzyme differed from the

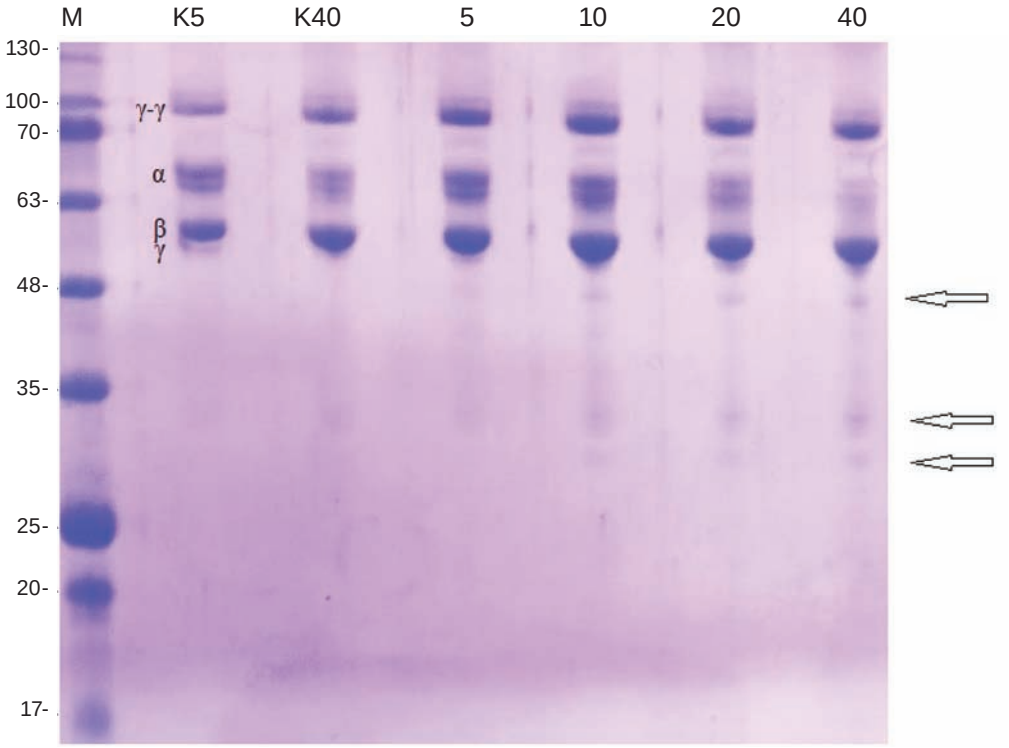

Fig. 6. Electrophoregram of fibrin hydrolysis products separated by the enzyme preparation Bacillus sp. L9. M – molecular weight markers (kDa); K5 – native fibrin after 5 min of activation of the polymerization process; K40 – native fibrin after 40 min of activation of the polymerization process; 5, 10, 20, 40 min of incubation with the enzyme, respectively; α, β, γ – chains of the fibrin molecule; γ-γ – fibrin γ-chains covalently connected with factor XIII. The samples were prepared in the presence of 0.2% β-mercaptoethanol

nature of the degradation of fibrinogen. Thus, β- and γ-chains were hydrolyzed simultaneously, leading to complete degradation of both chains within 2 h of incubation. The Aα chain of fibrin was hydrolyzed rather slowly and completely degraded within 5 h.

An important characteristic of enzyme preparations is the optimal conditions for their action, in particular pH and temperature. When studying the effect of pH on the rate of fibrinogen hydrolysis, it was found that the optimum action of fibrin(ogen)ase *Bacillus* sp. L9 was at pH 8.0 (Fig. 8). In addition, it was shown (Fig. 8) that the enzyme preparation *Bacillus* sp. L9 is active in the pH range from 3.0. to 12.0. At pH values of 3.0 and 4.0, the activity was only 25 and 33%, respectively. While at pH values close to the optimum, namely pH 9.0, 10.0, 7.0 and 6.0, the activity was 96, 85, 82 and 70%, respectively. At pH 11.0, the activity was 63%, and at pH 12.0 and 5.0, it was 54 and 52%, respectively. The results obtained are consistent with the literature data obtained for most fibrin(ogen)ases. Thus, researchers have shown that the fibrinolytic enzyme BSFE1, isolated from the marine bacterium *Bacillus* sp. S-3685, had a similar pH optimum as that obtained in our studies. In addition, it remained stable in the pH range of 7.0-10.0. However, the *Bacillus* sp. L9 enzyme differs from the *Bacillus atrophaeus* enzyme, which exhibits maximum activity at pH 7.0 [23].

The study of the thermooptimum of the enzyme preparation *Bacillus* sp. L9 showed (Fig. 9) that it hydrolyzes fibrinogen at a maximum rate at a temperature of 40°C, but was also active in the temperature range from 4 to 70°C (Fig. 8). At temperatures of 4 and 70°C, fibrin(ogen)olytic activity was only 10 and 15%, respectively, while at 15°C – 35%, at 20°C – 59%, at 30 °C – 95%. With an increase in temperature values above the optimum, a fairly high activity was also noted. Thus, at 50°C it was 75%, at 60°C – 45%. While at a temperature of 70°C, fibrin(ogeno)tic activity was only 15% of the initial one, and at a temperature of 80°C, the enzyme activity was completely inhibited.

Similar results were obtained by researchers [19], who showed that the thermooptimum of the fibrinolytic enzyme BSFE1, isolated from the ma-

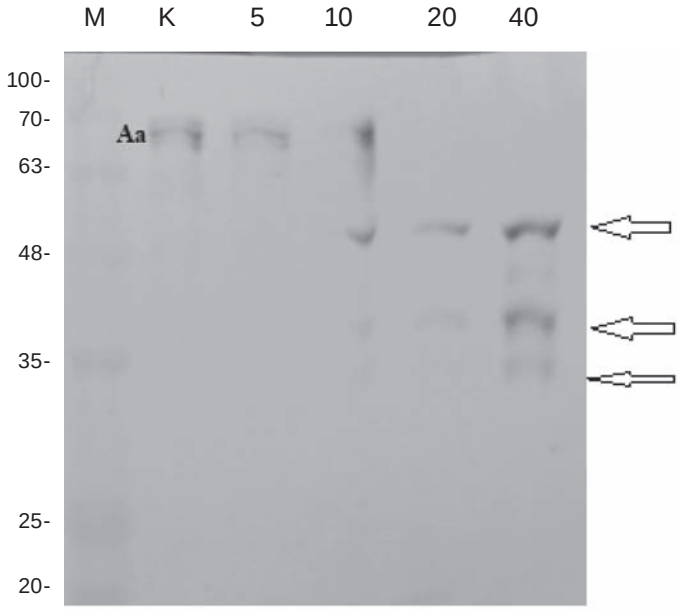

Fig. 7. Western blot analysis of fibrinogen hydrolysis products by Bacillus sp. L9 protease using II-5C antibodies to the N-terminal region of the Aα chain of fibrinogen. M – molecular weight markers (kDa); K – native fibrinogen; 5, 10, 20, 40 min of incubation with the enzyme, respectively; Aα – chain of the fibrinogen molecule. Hydrolysis products are indicated by arrows

rine bacterium *Bacillus* sp. S-3685, was 37 °C, and at 30°C, it retained 92.0% of the activity. Similar thermooptimums were established for other studied enzymes: 37 °C for the enzyme BpKJ-31 *Bacillus licheniformis* KJ-31 [30], 38°C for the fibrinolytic enzymes *Streptomyces radiopugnans* VITSD8 [31] and 40°C for velefibrinase *Bacillus velezensis* z01 [32]. Together with this, the optimal temperature of the *Bacillu*s sp. L9 enzyme we studied was lower than that of *Serratia marcescens* subsp. *sakuensi* (43°C) [33], *Chlorella vulgaris* (45°C) [34], and higher than that of *Bacillus subtili*s JS2-AprEJS2 (24°C) [35] and *Bacillus pumilus* BS15 AprEBS15 (27°C) [36].

Important regulators of enzymatic activity are metal ions, which not only ensure the possibility of the reaction but also influence its efficiency. In particular, it is known that the activity of many fibrinolytic enzymes depends on the presence of divalent metal ions, such as Zn^{2+}, Mg^{2+}, Ca^{2+}, Hg^{2+} or Co^{2+} [32], and some metals, such as Na^{+}, K^{+} and Ca^{2+}, can increase it [30, 37]. So, we have shown that the activity of the enzyme *Bacillus* sp. L9 under the influence of ions Ca^{2+} increased by 91.2%, slightly less Hg^{+} (by 29.2%), Li^{+} and Ni^{2+} (by 16%). Some ions exhibited inhibitory effects: Ag^{+} (by 65%), Na^{+} (by 54%), Zn^{2+} i NH^{4+} (by 25%), K^{+} i Mg^{2+} (by 21%). Another regularity was noted by the authors [19] in studies on the influence of metal ions on the activity of fibrinolytic enzyme BSFE1 *Bacillus* sp. S-3685, whose activity was increased by ions Na^{+}, Ba^{2+}, K^{+}, Co^{2+}, Mn^{2+}, Al^{3+} and Cu^{2+}, while ions Fe^{2+}, Fe^{3+}, Ca^{2+}, Mg^{2+} and Zn^{2+} lowered it. The activity of velefibrinase *Bacillus velezensis* Z01 was increased by the influence of ions Mg^{2+} and Ca^{2+}, but decreased under the influence of ions Zn^{2+}, Mn^{2+} and Co^{+} [32]. That is, the same ions on enzymes from different producers have different effects. Unfortunately, we cannot even speculate on the mechanism of this diverse action within the framework of our study.

One of the approaches to studying the mechanism of action of enzymes is to investigate the effect of specific chemical reagents on their activity, which allows establishing the functional groups involved in catalysis. Thus, when studying the effect of chelating agents EDTA and o-phenanthroline on the enzyme preparation *Bacillus* sp. L9 (Table 3), their inhibitory effect was established (by 70 and 50%, respectively). This suggests that functional groups containing metal atoms are involved in the catalysis performed by fibrin(ogen)ase *Bacillus* sp. L9. The resistance of enzymes to chelators would be a good characteristic of the enzyme, especially considering their use in detergent formulations as water softeners and stain removers [38].

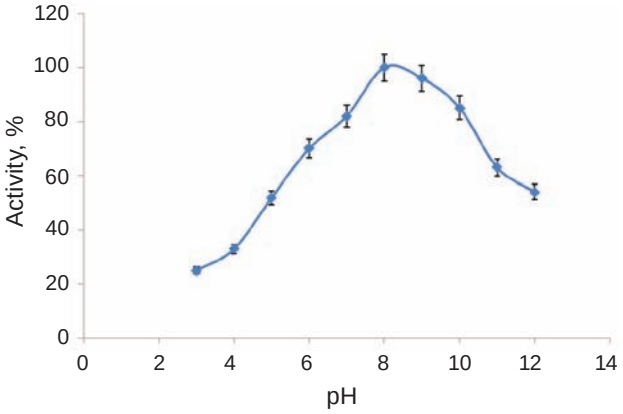

Fig. 8. Effects of pH on fibrin(ogen)olytic activity of Bacillus sp. L9 enzyme preparation(40°C)

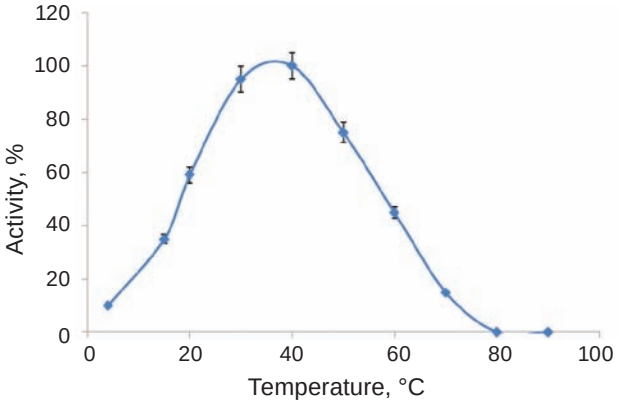

Fig. 9. Effects of temperature on the Bacillus sp. L9 enzyme preparation with fibrin(ogen)olytic (pH 8.0) activity

Table 2. Effect of metal ions and ammonium on the activity of Bacillus sp. L9 fibrin(ogen)ase (M ± m, n= 5)

Metal ions, 10^{-3} M	Activity, % after incubation with cations and ammonium, 60 min
Control	100.00
NH^{4+}	75.00 ± 0.09
K^{+}	79.00 ± 0.05
Na^{+}	46.00 ± 0.07
Li^{+}	116.00 ± 0.03
Ag^{+}	35.00 ± 0.50
Cd^{2+}	100.00 ± 0.09
Ca^{2+}	191.20 ± 0.06
Mg^{2+}	79.20 ± 0.07
Hg^{+}	129.20 ± 0.06
Ni^{2+}	116.00 ± 0.06
Mn^{2+}	100.00 ± 0.05
Zn^{2+}	75.00 ± 0.50
Co^{2+}	100.00 ± 0 01
Cu^{2+}	100.00 ± 0.05
Ba^{2+}	100.00 ± 0.03
Pb^{2+}	100.00 ± 0.09
Fe^{2+}	100.00 ± 0.08
Fe^{3+}	100.00 ± 0.07
Al^{3+}	100.00 ± 0.05

Reagents that reduce disulfide bonds – dithiothreitol, L-cysteine, β-mercaptoethanol inhibited the activity of the enzyme preparation *Bacillus* sp. L9 by 30, 30, 50%, respectively. This suggests that disulfide bonds are present in the enzyme molecule. Similar results were obtained by other researchers, who also showed that dithiothreitol and β-mercaptoethanol did not significantly inhibit the activity of subtilisin [20]. Strong thiol inhibitors, *p*-CMB and N-ethylmaleimide, inhibited the enzyme preparation *Bacillus* sp. L9 by 20 and 50%, respectively. The results obtained indicate that SH-groups, which are necessary for the manifestation of activity, are present in the enzyme molecule, and not in its active center. Apparently, they are localized at some distance from the active site, do not participate in catalysis, but play an essential role in maintaining the catalytically active conformation of the protein molecule. The reaction with 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide methiodide is the most selective method of chemical modification of carboxyl groups of amino acids. Inhibition of the activity of the enzyme preparation *Bacillus* sp. L9 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide methiodide may indicate the presence of functionally active carboxyl groups in the fibrin(ogen)ase molecule of *Bacillus* sp. L9. Since phenyl methyl sulfonyl fluoride completely inhibited the activity of fibrin(ogen)ase of *Bacillus* sp. L9, it can be assumed that it is a serine protease similar to the fibrinolytic enzyme DFE27, the producer of

Table 3. Effect of specific chemical reagents on the activity of Bacillus sp. L9 fibrin(ogen)ase

Metal ions and specific chemical reagents	Concentration, mM	Relative activity, %
Control	–	100
EDTA	5	30.00 ± 2.13
o-phenantroline	5	50.00 ± 1.25
DTT	5	70.00 ± 0.09
L-cystein	5	70.00 ± 0.05
β-mercaptoethanol	5	50.00 ± 1.46 *
p-Chlormercuribenzoate	5	80.00 ± 4.08
N-ethylmaleimide	5	50.00 ± 2.06 **
1-(3-dimethylaminopropyl)-3-ethylcarbodiimide methiodide	5	30.00 ± 3.43 **
PMSF	5	–**

Note. Values represent mean ± SD of 5 repetitions. –: The enzyme activity was totally inhibited. Significant difference with respect to control: *$P < 0.05$, **$P < 0.01$

which is *Bacillus subtilis* DC27T [36]. According to the data available in the literature [20], proteases containing serine constitute almost 1/3 of all known proteases. The fibrinolytic enzyme produced by B*acillus licheniformis* KJ-31 [38] is completely inhibited by low concentrations of PMSF, while EDTA does not affect its activity.

Thus, an enzyme preparation with fibrin(ogen) olytic activity was isolated from the supernatant of the culture liquid of *Bacillus* sp. L9. The yield of the enzyme after purification was 1.8%. The specific fibrinogenolytic and fibrinolytic activities were 483 and 383 U/mg of protein, respectively. The molecular weight of the purified enzyme was about 40 kDa, the pH optimum was 8.0, and the thermooptimum was 40°C. *Bacillus* sp. L9 fibrin(ogen)ase is a serine protease, in the active center of which there is a carboxyl group of the C-terminal (aspartic or glutamic) amino acid. At some distance from the active center, sulfhydryl groups are localized, which do not participate in catalysis, but play an important role in maintaining the catalytically active conformation of the protein molecule. The *Bacillus* sp. L9 enzyme with fibrin(ogen)olytic activity exhibits the highest specificity for bonds formed by the Aα-chain of fibrinogen, hydrolyzing fibrin molecules much more slowly. By specificity of action on fibrinogen, it is an α-fibrin(ogen)ase. With the help of the enzyme *Bacillus* sp. L9 with fibrin(ogen)olytic activity, it is possible to obtain forms of fibrinogen devoid of the corresponding parts of the molecule, as well as to purify fragments cleaved from the C-terminus of the Aα-chain of fibrinogen. Studying the properties of these parts of the molecule will make it possible to establish the structural and functional role of each individual region of the C-terminus of the Aα chain of fibrinogen. This indicates its potential for fundamental research, as well as in medicine as an antithrombolytic agent.

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ОЧИСТКА ТА ФІЗИКО-ХІМІЧНІ ВЛАСТИВОСТІ ПРОТЕАЗИ *BACILLUS* SP. L9 З ФІБРИН(ОГЕН)ОЛІТИЧНОЮ АКТИВНІСТЮ

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Раніше нами із сухої трави прибережної зони Кінбурнської коси було виділено ряд штамів *Bacillus* sp., які можуть бути перспективними для подальших досліджень як продуценти протеаз із фібринолітичною та фібриногенолітичною активністю. Метою роботи було виділення, очищення та вивчення властивостей фібрин(оген)ази зі штаму *Bacillus* sp. L9. Препарат ензиму було виділено з супернатанту культуральної рідини *Bacillus* sp. L9. Вихід очищеного ензиму становив 1,8%, питома фібриногенолітична та фібринолітична активності становили 483 та 383 од/мг протеїну відповідно, молекулярна маса ензиму становила близько 40 кДа, оптимум pH 8,0, термооптимум 40°C. Фібрин(оген)аза *Bacillus* sp. L9 є сериновою протеазою, в активному центрі якої знаходиться карбоксильна група С-кінцевої (аспарагінової або глютамінової) амінокислоти. На деякій відстані від активного центру локалізовано сульфгідрильні групи, які не беруть участь в каталізі, але відіграють важливу роль в підтриманні каталітично активної конформації протеїнової молекули. Ензим з *Bacillus* sp. L9 гідролізував молекули фібрину значно повільніше, ніж фібриногену, та виявляв найбільшу специфічність у гідролізі зв'язків, утворених Аα-ланцюгом фібриногену. За специфічністю дії на фібриноген, ензим був ідентифікований як α-фібриноген(оген)аза.

Ключові слова: фібрин(оген)олітичний ензим, *Bacillus* sp. L9; очистка, pH-, термооптимуми, молекулярна маса, функціональні групи активного центру.

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