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ISOLATION AND CHARACTERIZATION OF *BACILLUS* SP. IMV B-7883 PROTEASES

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The representatives of Bacillus are some of the best protease producers studied so far since they exhibit broad substrate specificity, significant activity, stability, simple downstream purification, short period of fermentation and low cost. Earlier, we showed that Bacillus sp. IMV B-7883 strain synthesizes an extracellular proteases, which exhibit elastolytic and fibrinogenolytic activity. The aim of the work was to isolate and purify these enzymes from the culture liquid of the Bacillus sp. IMV B-7883 strain, as well as to study their properties. Isolation and purification of proteases was carried out by precipitation of the culture liquid with ammonium sulfate, gel permeation and ion exchange chromatography and rechromatography on Sepharose 6B. As a result, proteases with elastolytic and fibrinogenolytic activity with a molecular weight of 23 and 20 kDa respectively were isolated with elastase activity increased by 63.6 and fibrinogenolytic activity by 44.1 times. The enzyme with elastase activity had a pH-optimum of 7.0 and hydrolyzed only elastin, while the enzyme with fibrinogenolytic activity was an alkaline protease with a pH-optimum of 8.0 and in addition to fibrinogen, showed specificity for fibrin and, in trace amounts, for collagen.

K e y w o r d s: Bacillus sp. IMV B-7883, elastase, fibrinogenase, pH optimum, substrate specificity.

The important industrial enzymes synthesized by different types of microbes like fungi, bacteria, yeasts and also by some plants and animal tissues are proteases. Because of their rapid growth, less space requirement for their cultivation, microbes serve as a preferred source of protease enzymes [1]. Proteases are used in the production of detergents (to remove protein contaminants); in the textile industry for processing wool, silk and leather products and improving their consumer properties, for dehairing hides in the production of leather; in various branches of the food industry to obtain protein hydrolysates, to soften meat and fish, to obtain adhesives; in agriculture − as a feed additive, as well as for the disposal of feathers and the production of feed products from them. The representatives of *Bacillus* are among the best protease producers studied so far since they exhibit properties like broad substrate specificity, significant activity, stability, simple downstream purification, short period of fermentation and low cost [2]. These properties make the *Bacillus* sp (*B. sterothermophilus*, *B. cereus*, *B. megaterium*, *B. mojavensis*, and *B. subtilis*) proteases most suitable for wider applications in industries [3]. Enzymes capable of cleaving proteins, such as elastin, fibrin, fibrinogen, and collagen, occupy a special place among the proteases of microorganisms. Earlier [4, 5], we showed that the strain *Bacillus* sp. IMV B-7883 synthesizes an extracellular protease complex, which exhibits, in particular, elastolytic and fibrinogenolytic effects. Elastases are used in industry and medicine for the hydrolysis of elastin, a natural insoluble fibrillar protein found in the tissues of most vertebrates, in biochemical medicine for isolating type II lung cells, in the food industry, cosmetics, in environmental protection, and in scientific research for establishing the structure of peptides [6]. Fibrin(ogen)olytic enzymes can be considered a basis for the creation of drugs aimed at reducing the threat of intravascular thrombus formation by limited proteolysis of fibrinogen circulating in the patient's bloodstream. In the case of targeted delivery, fibrinogenolytic enzymes can be used to break up intravascular blood clots that prevent blood supply to organs due to such pathologies as myocardial infarction, ischemic stroke, pulmonary embolism, etc. Proteases characterized by fibrinogenolytic activity are part of drugs that prevent blood clotting

and lower blood pressure [7-9]. Considering the prospects of practical application of such proteases both in medicine and in certain branches of industry (food, cosmetics), the aim of the work was to isolate and purify proteolytic enzymes from the supernatant of the culture liquid of the producer strain *Bacillus* sp. IMV B-7883, as well as to study their physical and chemical properties and substrate specificity.

Materials and Methods

The object of the investigation was the strain of *Bacillus* sp., deposited in the Ukrainian Collection of Microorganisms under the number IMV B-7883. For the accumulation of the enzyme *Bacillus* sp. IMV B-7883 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 shaken for three to six days (72-144 hours) in flasks on shakers (100 ml of medium, 28°C, 244 rpm). The inoculum was taken on the same medium for 24 hours and inoculated in flasks at a quantity of 105-106 colony-forming units (CFU).

Cells were separated from the supernatant of the culture liquid medium by centrifugation at 5000 g for 30 min. Dry ammonium sulfate salt was added to the culture 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 (fibrinogenolytic) 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). 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 were determined in the supernatant of the culture liquid and in the preparations after each stage of purification. Protein content was determined by the Lowry method [10].

Caseinolytic (total proteolytic) activity was determined by the Anson [11] method on casein (pH 8.0) for 10 min of incubation at 30°C. The activity unit expresses the millimoles of tyrosine released in 1 min of incubation by 1 ml of post-culture liquid.

Elastase activity was determined colorimetrically by the intensity of the color of the solution upon enzymatic hydrolysis of elastin stained with Congo red [12]. 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 culture liquid. The reaction mixture is incubated for 5 hours at a temperature of 37°C. The reaction is stopped by keeping the test tubes with the reaction mixture in an ice bath for 30 min. Unhydrolyzed elastin is separated by centrifugation for 10 min at 10 000 g. The color intensity is measured on a 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 [13]. 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. Incubate for 30-45 min at 37°C. The reaction is stopped by adding 2 ml of 10% trichloroacetic acid (TCA). TCA is added to the control sample immediately. Samples are kept at room temperature for 20 min and then centrifuged at 10 000 g for 10 min to remove precipitated protein. Absorption is measured on a SF-26 spectrophotometer at a wavelength of 275 nm. The amount of enzyme that, under the conditions of the experiment, increases absorption by 0.01 in 1 min is taken as a unit of activity.

Collagenase activity was defined by the content of free amino acids in the reaction mixture in the reaction with ninhydrin [14]. The unit of activity was the number of micromoles of released amino acids according to the standard curve constructed for leucine. Fibrinolytic activity was determined according to the Masada method [15]. The formation of fibrin

cleavage products was measured on a SF-26 spectrophotometer at 275 nm. The amount of enzyme that increases the optical density of the reaction mixture by 0.01 in 1 min was taken as a unit of fibrinolytic activity.

All experiments were performed in no less than 3–5 replications. Statistical processing of the results of the experimental series was carried out by standard methods using Student's *t*-test at 5% significance level.

Results

It is shown that *Bacillus* sp. IMV B-7883 synthesizes an extracellular enzyme complex, in the supernatant of the culture liquid of which there were caseinolytic, elastase, fibrinogenolytic, fibrinolytic and collagenase activities. The study of enzyme synthesis in the dynamics of culture growth showed that its maximum is observed on the 3rd day of cultivation (Table 1). Therefore, the supernatant of the culture liquid of *Bacillus* sp. IMV B-7883, which was grown for three days, was used in further studies.

Isolation and purification of the proteolytic complex of *Bacillus* sp. IMV B-7883 was carried out by classical biochemical methods: precipitation of the supernatant of the culture liquid with ammonium sulfate of 90% saturation, gel permeation and ion exchange chromatography (Fig. 1, 2, Table 2). As a result of precipitation of the supernatant of the culture liquid with ammonium sulfate of 90% saturation, the activity of elastase increased by 1.08 times, and that of fibrinogenolytic protease by 1.32 times, respectively. Further purification by gel-permeation chromatography made it possible to obtain two fractions, the first of which showed elastase activity (663.8 U/mg of protein), and the second - fibrinogenolytic activity (180 U/mg of protein), which is 10.2 and 9.52 times higher compared to the initial activity (Fig. 1, Table 2). Each of these fractions was separately subjected to ion-exchange chromatography on charged Toyopearl DEAE 650(M) TSK-gel. The separation conditions were selected so that only ballast proteins bind to the ion exchanger (Fig. 2, *A*, *B*, Table 2). It is shown that under the conditions of using 0.01 M Tris-HCl buffer pH 7.8, elastase (Fig. 2, *A*) and fibrinogenolytic protease (Fig. 2, *B*) are released in the salt gradient. As a result, it was possible to get rid of part of the protein impurities in the preparations, while the elastase activity increased by 17.4 times (1132 U/mg of protein), and the fibrinogenolytic activity by 22.01 times (416 U/mg of protein) (Table 2).

As a result of rechromatography on Sepharose 6B: the activity of the *Bacillus* sp. IMV B-7883 fractions with elastase and fibrinogenolytic activity was increased respectively by 63.6 times (4138 U/mg of protein) (Fig. 3, *A*, Table 2), and by 44.07 times (833 U/mg of protein) (Fig. 3, *B*, Table 2), the homogeneity of the fractions was shown (Fig. 3), their molecular weights were determined (23 and 20 kDa, respectively for enzyme preparations with elastase and fibrinogenolytic activity) (Fig. 4).

An important characteristic of enzyme preparations is the optimal conditions for their action, in particular pH and temperature. It is shown (Fig. 5) that purified enzyme preparations of *Bacillus* sp. IMV B-7883 are active in a fairly wide pH range from 4.0 to 11.0, with the pH optimum of the enzyme degrading elastin at 7.0 (Fig. 5, *A*), and fibrinogen at 8.0 (Fig. 5, *B*).

Determination of the thermooptimum of proteases showed that it is at 40°C (Fig. 6). Enzymes are active in the range of values from 4 to 60°C. Fibrinogenolytic protease is more stable at high temperatures.

Since in the supernatant of the culture liquid *Bacillus* sp. IMV B-7883, in addition to the dominant elastase and fibrinogenolytic activity, revealed insignificant collagenase and fibrinolytic activity, we

Table 1. Dynamics of the synthesis by Bacillus sp. IMV B-7883 enzyme with proteolytic activity

Type of activity	Activity (U/ml) per day of cultivation:					
	1	2	3	4	5	6
Caseinolytic	0.12	0.40	0.55	0.12	0.12	0.12
Elastase	5.85	13	24	11	11	8
Fibrinogenolytic	1.33	5.33	7	5	4.66	4.58
Fibrinolytic	0.3	4.0	6.0	3.12	2.2	1.5
Collagenase	0	1.3	1.7	1.35	1.25	0.35

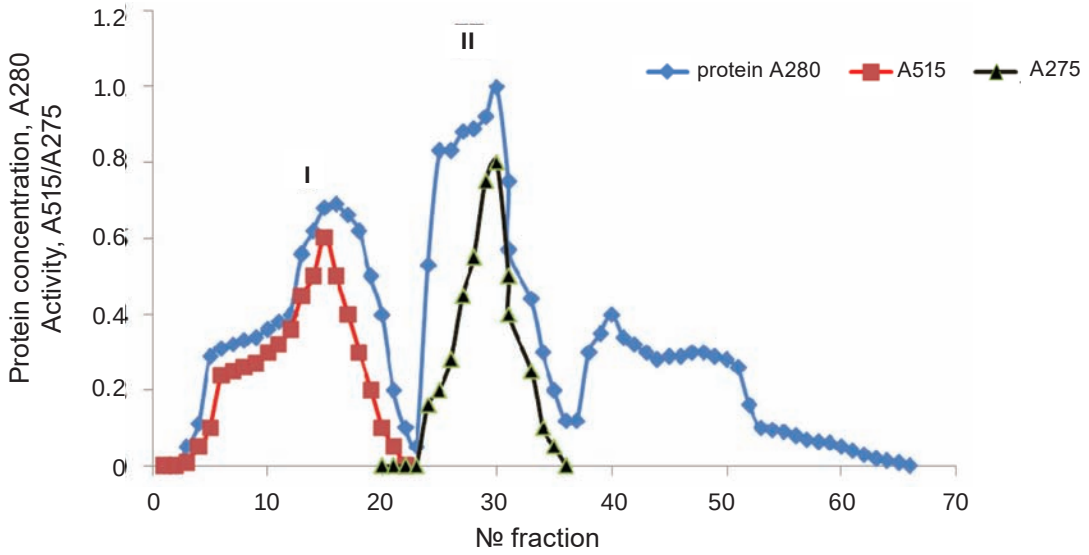

Fig. 1. Elution profile of the Bacillus sp. IMV B-7883 enzyme preparation on TSK-HW-55 (pH 7.8): I – elastase activity, II – fibrinogenolytic activity

Table 2. Purification steps of Bacillus sp. IMB B-7883 proteolytic complex

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)	200	130000a 3780b	65[a] 18.9[b]	100	1
90% ammonium sulfate	111.4	7800[a] 2785b	70[a] 25[b]	55.7	1.08[a] 1.32[b]
TSK-gel Toyopearl HW-55	7.05[a] 14.4[b]	4680 2592	663.8[a] 180.0[b]	3.5[a] 7.2[b]	10.2[a] 9.52[b]
Toyopearl DEAE-650M	3.5[a] 6.8[b]	3962[a] 61.176	1132[a] 416[b]	1.75[a] 3.4[b]	17.4[a] 22.01[b]
Rechromatography on Sepharose 6B	2.9[a] 5.4[b]	1427[a] 154.2[b]	4138[a] 833[b]	1.45[a] 2.7[b]	63.6[a] 44.07

Notes: [a]elastase activity, [b]fibrinogenolytic activity

investigated the substrate specificity of purified fractions with elastase (Fig. 7, *A*) and fibrinogenolytic activity (Fig. 7, *B*). The substance of the fraction with elastase activity (4138 U/mg protein) showed specificity only for elastin, while the substance of the fraction with fibrinogenolytic activity (833 U/mg protein) also showed specificity for fibrin (205 U/mg protein) and collagen (21 U/mg of protein) and did not show any specificity for elastin.

Therefore, from the supernatant of the culture liquid *Bacillus* sp. IMV B-7883 proteases with elastolytic and fibrinogenolytic activity were isolated. The yield of enzymes as a result of purification was 1.45 and 2.7%, respectively. Specific elastase activity was 4138 U/mg protein, and specific fibrinogenolytic activity was U/mg protein, molecular weight 23 and 20 kDa, pH-optimum 7.0 and 8.0, respectively. Termooptimum of proteases showed that it is at 40°C.

Discussion

The microbes producing extracellular proteases are of great importance. They can be synthesized enzymes in large quantities and represent one of the largest classes of industrial enzymes, accounting

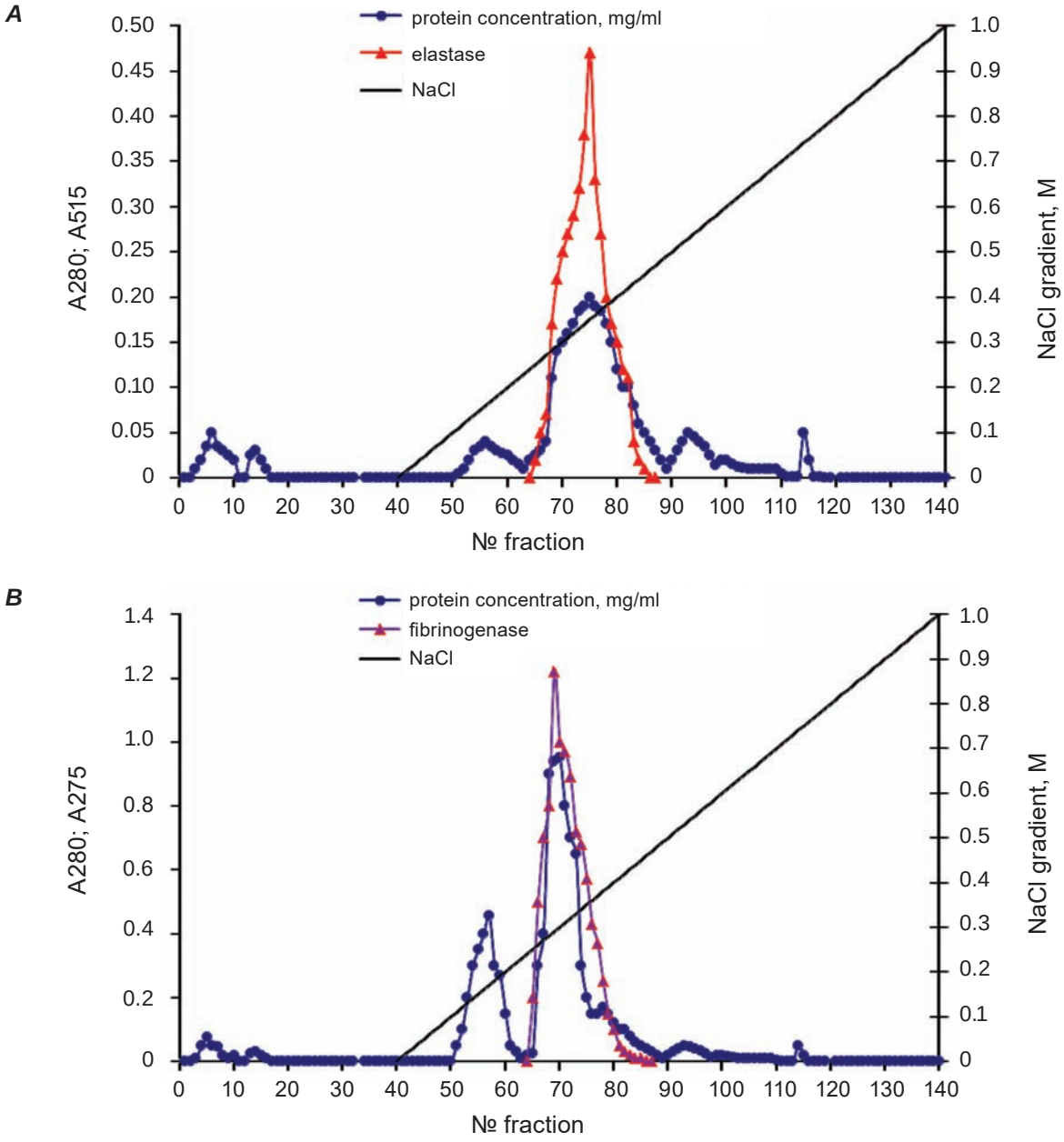

*Fig. 2. Elution profile of the Bacillus sp. IMV B-7883 enzyme preparation on Toyopearl DEAE-650M in a 0-1 M NaCl gradient: **A** – elastase activity, **B** – fibrinogenolytic activity*

for 40% of the total worldwide sales of enzymes by value. Microbial proteases have interesting characteristics in the sense of low cost of production, good stability and specificity representing a powerful tool in the development and production of new protein hydrolysates with characteristics that can be explored industrially. Proteases of microbial origin are considered the most significant hydrolytic enzymes in various industrial and pharmaceutical applications. With the passage of time, scientists have found the broad use of proteases in the medical field. So, it was shown that *Streptomyces fradiae* and *Bacillus thermoproteolyticus* elastases were among the most potent elastolytic proteinases discovered at that time because they are 4-8-fold more effective than pancreatic elastases [16]. For the treatment of various diseases, such as burns, carbuncles, furuncles, and wounds, a preparation of elastoterase immobilized on bandage was used [17]. It has been reported that fibrin degradation has been achieved by alkaline fibrinolytic proteases. The use of this fibrinolytic enzyme suggested its future application as an an-

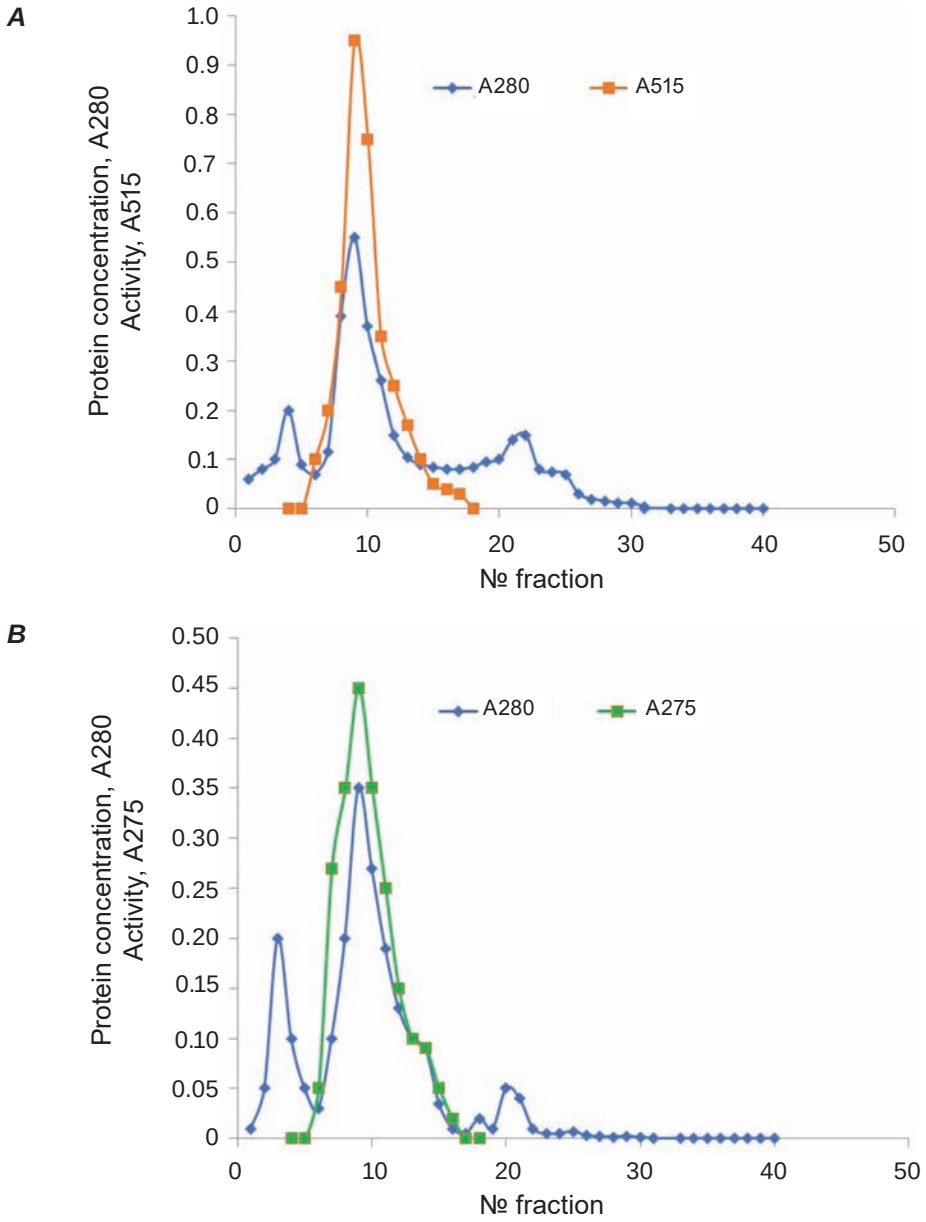

*Fig. 3. Rechromatography on Sepharose 6B: **A** – elastase activity, **B** – fibrinogenolytic activity*

ticancer drug and in thrombolytic therapy [1, 3]. Slow-release dosage form preparation containing collagenases with alkaline proteases is extensively used in therapeutic applications. The hydrolysis of collagen by the enzyme liberates low-molecular-weight peptides without any amino acid release for therapeutic use [1, 6]. Information on the synthesis and properties of elastases of microbial origin is scattered and rather limited. The majority of microbial elastases are produced by *Pseudomonas aeruginosa* [18], *Staphylococcus epidermidis* [19], *Bacillus licheniformis* [20], *Bacillus subtilis* [21], and *Chryseobacterium indologenes* [22]. Unfortunately, most of them have low stability under environmental conditions. The most common producers of fibrinogenolytic proteases are bacilli isolated from soil, fresh and seawater. Most of these enzymes are alkaline serine proteases with optimal conditions of action at pH 8.0–10.0 and temperature 30–60°C [1]. Interest in the research of proteases with fibrinogenolytic activity is related to their involvement in the development of various diseases of inflammatory origin [23].

Since the majority of microbial producers of proteases have a number of serious disadvantages, the search for new, more effective producers

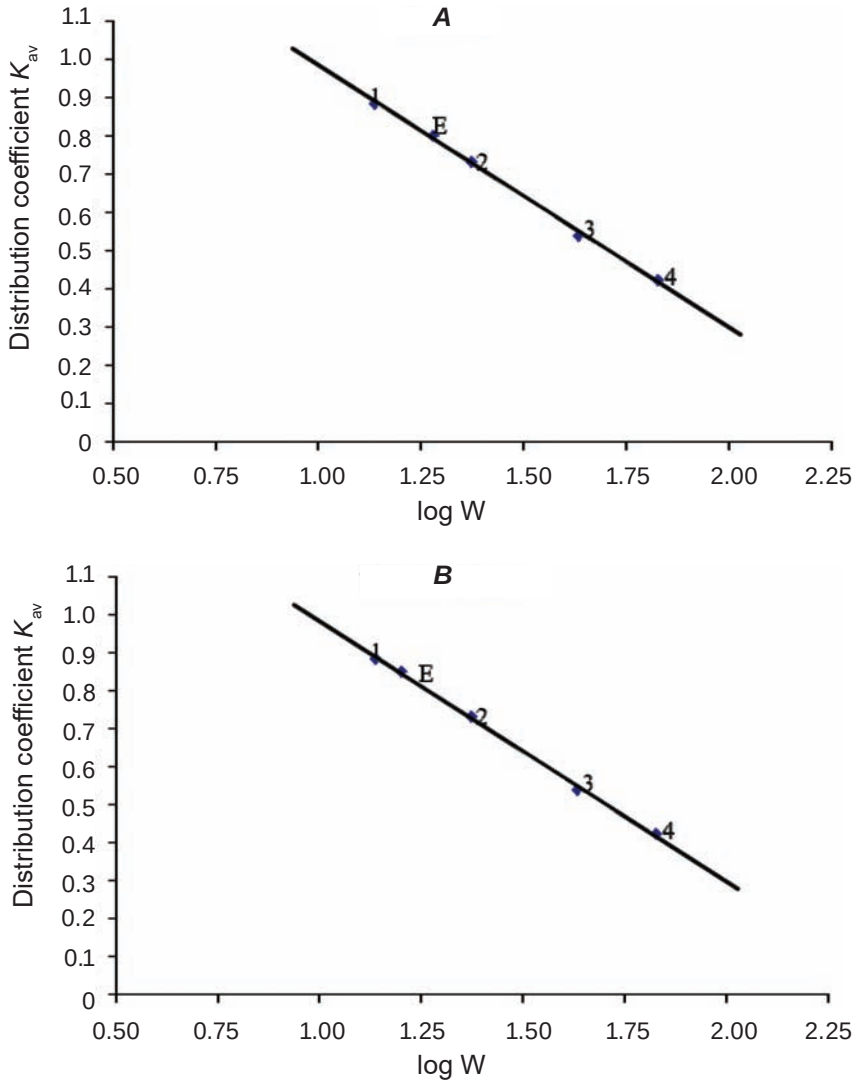

*Fig. 4. Molecular mass determination of Bacillus sp. IMV B-7883: elastase (**A**), fibrinogenase (**B**) in the native system. Molecular markers: ribonuclease (13.7 kDa) (1), proteinase K (25 kDa) (2), chicken ovalbumin (43 kDa) (3), bovine serum albumin (67 kDa), E – purified enzyme*

continues to be an urgent issue, taking into account the fact that there are no highly active producers of elastase and fibrinogenase in Ukraine. Therefore, we from the supernatant of the culture liquid *Bacillus* sp. IMV B-7883 isolated and purified proteases with elastolytic and fibrinogenolytic activity, the specific activity of which was 4138 U/mg of protein and 833 U/mg of protein, respectively. Like enzymes from other well-known producers, the investigated proteases were of low molecular weight, 23 and 20 kDa, respectively, for the protease with elastolytic and fibrinogenolytic activity. Unlike the elastases described in the literature, which are alkaline proteases, the enzyme with elastase activity had a pH-optimum of 7.0, while the enzyme with fibrinogenolytic activity was an alkaline protease with a pH-optimum of 8.0. The results of research on substrate specificity of enzymes are of interest. Thus, the protease with elastase activity hydrolyzed only elastin, while the protease with fibrinogenolytic activity, in addition to fibrinogen, showed specificity also for fibrin and, in trace amounts, for collagen. Such enzymes, which, in addition to fibrinogenolytic activity, also exhibit fibrinolytic activity, have an advantage over enzymes that have only fibrinogenolytic activity. Such are fibrolase, which is obtained from the venom of the snake *Agkistrodon contortrix contotrix* [24] or nattokinase of *Bacillus subtilis* [25].

Thus, the preparations of elastase and enzyme, which exhibit fibrinogenolytic and fibrinolytic ac-

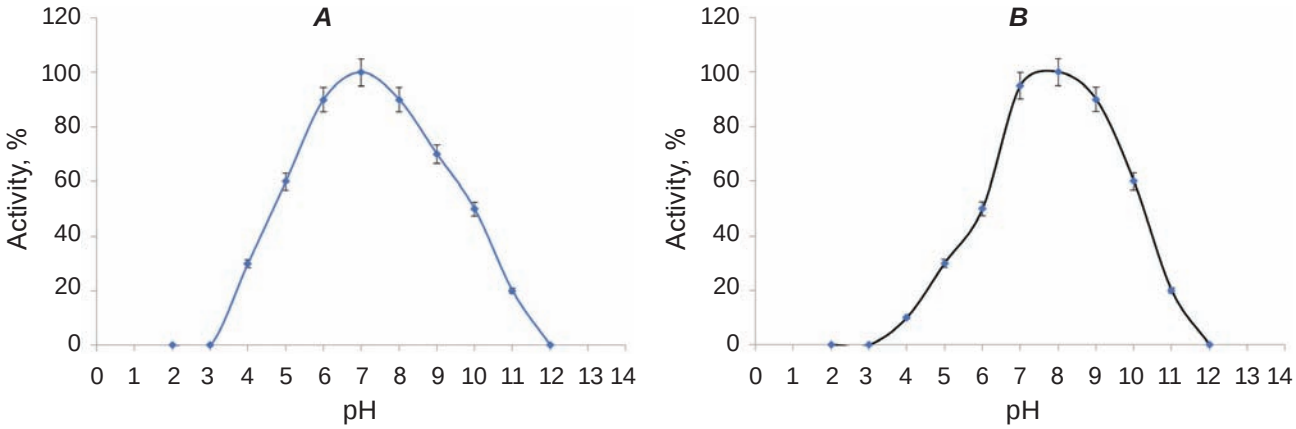

*Fig. 5. Effects of pH on elastase (**A**) and fibrinogenolytic (**B**) activity of Bacillus sp. IMV B-7883 (40°C)*

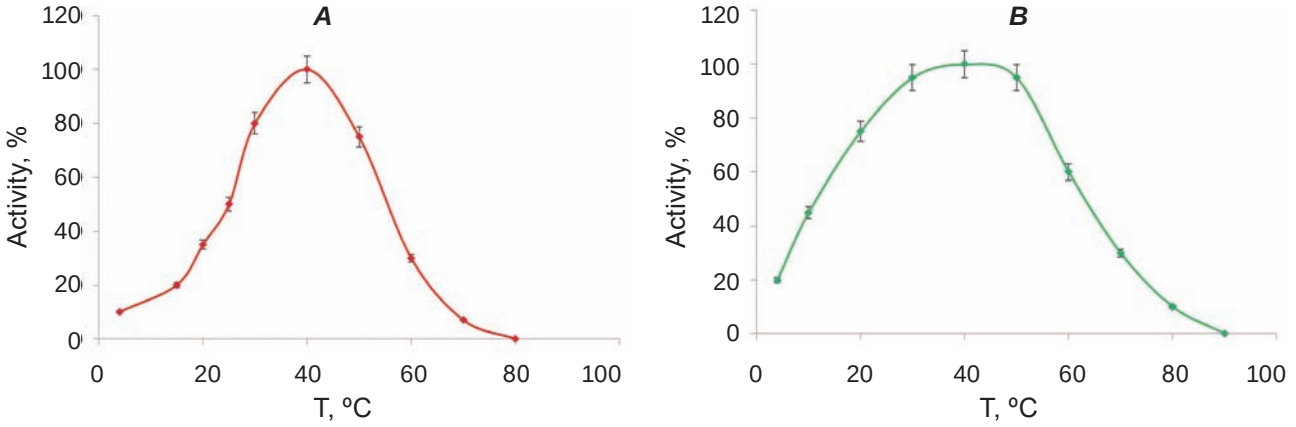

*Fig. 6. Effects of temperature on the Bacillus sp. IMV B-7883 enzyme with elastase (pH 7.0) (**A**) and fibrinogenolytic (pH 8.0) (**B**) activity*

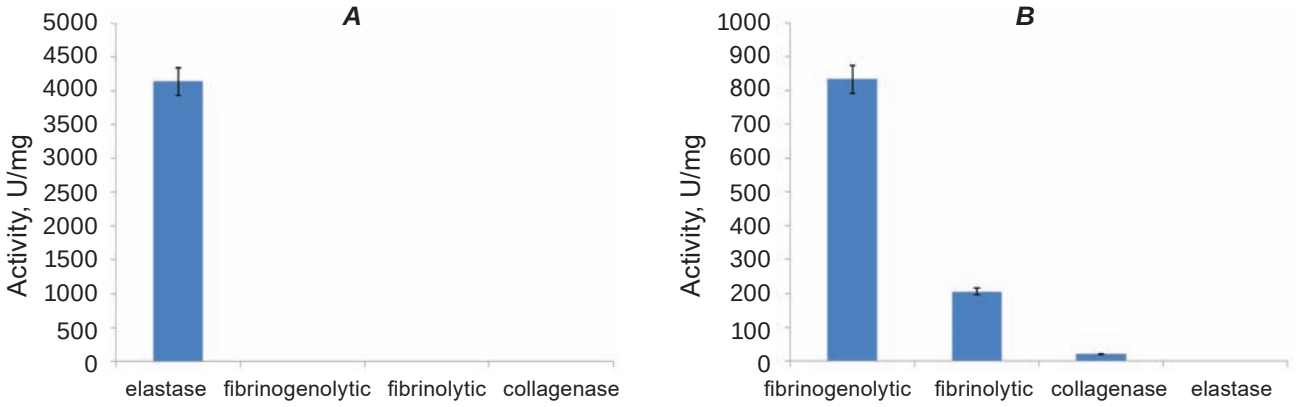

*Fig. 7. Substrate specificity of purified enzyme preparations: **A** – fraction with elastase activity, **B** – fraction with fibrinogenolytic activity*

tivity, from *Bacillus* sp. IMV B-7883 will not only make a certain contribution to the currently existing data on the biology of this type of microorganisms, but will join the range of industrially promising producers with the property of hydrolyzing elastin, fibrinogen and fibrin. The activity of the enzyme with elastolytic activity (4138 U/mg of protein) exceeds not only the described elastase producers, such as *Priestia megaterium* 273 and *Bacillus aryabhattai* BCN4-1 (the activity of which was 771.3 U/ml and 723.1 U/ml, respectively) [3], but it is almost two orders of magnitude higher than the activity of one of the commercially available pancreatic elastase.

Conflict of interest. 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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ВИДІЛЕННЯ ТА ХАРАКТЕРИСТИКА ПРОТЕАЗ *BACILLUS* SP. IMB B-7883

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Представники *Bacillus* є одними з найкращих продуцентів протеаз, вивчених досі, оскільки вони виявляють широку субстратну специфічність, значну активність, стабільність, короткий період ферментації та низьку вартість. Раніше ми показали, що штам *Bacillus* sp. IMV B-7883 синтезує позаклітинні протеази, які виявляють еластолітичну та фібриногенолітичну активність. Метою роботи було виділення та очищення цих ензимів із культуральної рідини штаму-продуцента *Bacillus* sp. IMB B-7883, а також вивчити їх властивості. Виділення та очищення протеаз проводили осадженням культуральної рідини сульфатом амонію, гельпроникною та іонообмінною хроматографією та рехроматографією на Sepharose 6B. У результаті виділено протеази з еластолітичною та фібриногенолітичною активністю з молекулярною масою 23 та 20 кДа, відповідно, активність еластази зросла у 63,6 раза та фібриногенолітична активність – у 44,1 раза. Ензим з еластазною активністю мав pH-оптимум 7,0 і гідролізував лише еластин, тоді як ензим із фібриногенолітичною активністю був лужною протеазою з pH-оптимумом 8,0 і на додаток до фібриногену виявляв специфічність до фібрину та, у слідових кількостях, до колагену.

Ключові слова: *Bacillus* sp. IMV B-7883, еластаза, фібриногеназа, pH оптимум, субстратна специфічність.

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