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INFLUENCE OF LIPOPOLYSACCHARIDES OF *ESCHERICHIA COLI* ON THE PROTEASE ACTIVITY OF SEVERAL *BACILLUS* STRAINS

L. D. VARBANETS[1], O. S. BROVARSKA[1], O. V. GUDZENKO[1]✉, K. G. GARKAVA[2], A. R. MAKARENKO[2]

[1]Institute of Microbiology and Virology named after D. K. Zabolotny, National Academy of Sciences of Ukraine, Kyiv, Ukraine;
[2]National Technical University of Ukraine "Igor Sykorsky Kyiv Polytechnic Institute", Kyiv, Ukraine;
✉e-mail: alena.gudzenko81@gmail.com

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We have previously shown that lipopolysaccharides (LPS) of a number of strains of the phytopathogenic species Pantoea agglomerans are capable of increasing the activity of Bacillus peptidases with fibrinolytic, elastase and collagenase activities by 2-4 times. The aim of this work was to investigate the effect of isolated intracellular LPS1 and extracellular LPS2 of Escherichia coli on the activity of purified bacilli proteases with elastase and fibrinogenolytic activity. It was shown that both LPS2 and LPS1 of E. coli 23 can increase the elastase activity of Bacillus sp. IMV B-7883 by 600 and 416% respectively. Both LPS are able to increase fibrinogenolytic activity in all studied Bacillus strains, but its greatest stimulation (200%) was observed under the action of LPS2 of Bacillus sp. L9.

K e y w o r d s: Escherichia coli, Bacillus, lipopolysaccharides, proteases with fibrinogenolytic and elastase activity, effect of lipopolysaccharides on protease activity.

Many aspects of the biological activity of bacteria of the genus *Escherichia*, as well as other gram-negative bacteria, are determined by the specific structure of the components of their cell membrane, among which an important role belongs to lipopolysaccharides (LPS). They are characterized by a wide range of physiological effects on the body of warm-blooded animals, showing both positive and negative effects [1]. Despite the fact that the study of LPS was started in the 40s of the XX century, this problem has not lost its relevance to this day. Interest in the study of LPS is due to the fact that the molecular and cellular mechanisms of their biological activity remain insufficiently studied and in many cases their elucidation is associated with the resolution of fundamental issues of immunogenesis and cellular reception. The decisive moment in clarifying these questions was the research of Bruce Butler, who in 1998, while studying the effect of LPS on mice, discovered the Toll-like gene responsible for the receptor for LPS [2]. This receptor is similar to the protein encoded by the Toll gene discovered in 1995 in Drosophila [3]. The identification of the receptor for LPS was a significant contribution to the understanding of the problem of innate immunity. For this discovery, the Nobel Prize in Medicine and Physiology in Immunology was awarded in 2011 to three scientists, including Bruce Butler and Jules Hoffmann for establishing one of the first molecular mechanisms for triggering innate immunity. The discovery of LPS receptors on the surface of a number of cells has contributed to large-scale studies to establish the mechanisms of their biological action. Today, the question of the interaction of LPS bacteria with proteins of various systems of the human and animal body is well studied in the literature [4]. They are able to stimulate or modulate the activity of a number of enzymes that break down proteins, especially in conditions of sepsis, endotoxemia or systemic inflammatory response. Thus, LPS can trigger or enhance the action of serine proteases, such as neutrophil elastase, cathepsin G, which are able to destroy pathogen proteins, but can also damage their own tissues. Under the influence of LPS, the permeability of lysosomes increases and cathepsins (B, D, L) are released, which are involved in intracellular

proteolysis. Some proteases activated by LPS cleave membrane proteins or receptors, changing the cellular response to inflammation or leading to apoptosis. LPS causes a decrease in the levels of α1-antitrypsin and other inhibitors, which allows excessive protease activity, which can result in destructive changes in the lungs, kidneys, and blood vessels. Thus, in the human body, LPS are able to enhance the activity of proteolytic enzymes as part of the protective mechanism, but their excessive activation can cause pathological changes and tissue damage. As for studies on the interaction of LPS with proteins of microorganisms, they are currently extremely limited. Thus, Kramer R.A. et al. [5] first established that the activation of the integral protease OmpT of the outer membrane of *Escherichia coli* requires its interaction with lipopolysaccharide. Earlier [6] we showed that LPS of a number of strains of the phytopathogenic species *Pantoea agglomerans* are able to increase the activity of *Bacillus* peptidases with fibrinolytic, elastase and collagenase activities by 2-4 times. In recent years, we have not found a single new report in the available literature. Since bacterial proteases are of interest for use in practical medicine in connection with the development of effective means of combating human heart diseases, studies on the influence of *Escherichia coli* LPS, which is a constant component of the gastrointestinal tract of humans and animals, on the activity of proteases of a number of *Bacillus* strains, some representatives of which may be part of probiotic preparations, are relevant. Therefore, the aim of this work was to investigate the influence of *Escherichia coli* lipopolysaccharides on the activity of proteases (with elastase and fibrinogenolytic activity) of a number of *Bacillus* strains.

Materials and Methods

The objects of research were: 1) lipopolysaccharides of *Escherichia coli* 23, which were isolated from a chicory plant collected in the area near the Zhulyany airfield [7]; 2) proteases with fibrinogenolytic and elastase activity, obtained from cultures of *Bacillus* sp. IMV B-7883, *Bacillus* sp. L 9; *Bacillus atrophaeus* 08. Bacillus cultures were isolated from different ecological niches: soil (*Bacillus* sp. IMV B-7883) [8], from plants of the coastal zone of the Kinburn Spit (*Bacillus* sp. L 9) [9] and bottom sediments of the Black Sea (*Bacillus atrophaeus* 08) [10].

To accumulate bacterial mass, the *E. coli* 23 culture was grown on meat peptone agar (MPA) mattresses for 24 h at a temperature of 28°C. The cells were washed with 0.9% NaCl solution and centrifuged at 5000 g, 20 min. The supernatant was evaporated to 400 ml and ammonium sulfate was added to 90% saturation, the mixture was kept at 4°C for a day, centrifuged, the supernatant was discarded, and the precipitate was dissolved in 20 ml of distilled water and dialyzed against distilled water, evaporated to 10 ml and lyophilized. The resulting preparation was extracellular lipopolysaccharide.

Lipopolysaccharide (LPS) from *E. coli* cells was extracted using the classical water-phenol method of O. Westphal and K. Jann [11]. Isolation of LPS was performed by adding equal volumes of hot (65-70°C) 90% phenol and water and incubating the mixture in a water bath at 70°C for 30 min with vigorous stirring. The mixture was cooled and centrifuged at 5000 rpm for 30 min to separate the aqueous phase from the phenolic phase. The phenolic phase was re-extracted by adding 300 ml of distilled water. The aqueous phases were combined, dialyzed against tap and distilled water to neutral pH, concentrated by evaporation, then lyophilized.

Purification of LPS from nucleic acids was carried out by precipitation with trichloroacetic acid for 24 h in refrigerator conditions, after which the mixture was centrifuged. The supernatant was dialyzed against distilled water, concentrated by evaporation and lyophilized.

Chemical characterization of LPS was carried out by standard methods: the presence of 2-keto-3-deoxyoctanoic acid (KDO) was determined by reaction with thiobarbituric acid (Chaby et. al., 1993) [12], heptose (Dische, 1953) [13]. The total carbohydrate content was determined by Dubois (Dubois, 1956) [14], proteins by Lowry (Lowry, 1951) [15], and nucleic acids by Spirin (Spirin, 1958) [16].

The monosaccharide composition (Albersheim et al., 1967) [17] was analyzed as polyol acetates on an Agilent 6890N/5973 inert chromatography–mass spectrometry system equipped with a DB 225mS column (30 m × 0.25 mm × 0.25 μm); the carrier gas was helium at a flow rate of 1 ml/min. The identification of monosaccharides was performed by comparison of the retention times with the authentic samples

The quantitative content of individual monosaccharides was expressed as % to the total sum of peak square.

O-antiserum to a heated (2.5 h, 100°C) culture of *E. coli* 23 grown on MPA was obtained by four intravenous injections of increasing doses of a sus-

pension of microbial bodies (from 500 thousand to 2 billion cells/ml at 0.5 ml per rabbit) with the interval between injections is 5 days. On the 5th day after the last injection, a blood sample (20-30 ml) was taken from the vein of the ear to obtain O-antisera. The titer of sera was determined by the ring precipitation reaction. The antigenic activity of LPS was studied by the method of double immunodiffusion in agar according to Ouchterlony (Ouchterlony, 1962) [18].

For the synthesis of extracellular proteases, the strains of bacillus were cultivated on a liquid nutrient medium of the following composition (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 – 6.5-6.7. The culture was grown for 24 h in Erlenmeyer flasks on shakers at 250 rpm, at 28°C. The inoculum was grown on the appropriate medium for 24 h, then inoculated into flasks at a concentration of 105-106 CFU/ml.

Proteases were isolated by precipitation with ammonium sulfate 90% saturation (*Bacillus* sp. IMV B-7883 and *Bacillus atrophaeus* 08) and 60% saturation (*Bacillus* sp. L 9) the supernatant of the culture liquid obtained by centrifugation at 5000 g for 30 min. The precipitate was collected by centrifugation at 5000 g for 30 min, dissolved in 0.01 M Tris-HCl buffer (pH 7.5) and applied to a column (2.5×40 cm) with anion exchanger TSK DEAE 650 (M) ("Toyosoda", Japan). Elution was carried out with 0.01 M Tris-HCl buffer (pH 7.5) in a sodium chloride gradient from 0 to 1 M, at a rate of 0.5 ml/min. Protein fractions that exhibited fibrinogenolytic and elastase activity were selected, combined and applied to a column (1.8×40 cm) with neutral TSK gel – Toyopearl HW-55 ("Toyosoda", Japan). Elution was performed with the same buffer with a flow rate of 0.85 ml/min. The degree of purification of enzyme preparations was characterized by the measures of specific elastolytic and fibrinogenolytic activity (U/mg of protein).

At all stages of the study, the protein content was recorded at 280 nm, and its amount was determined by the method of Lowry et al. [15]. The method for deciding elastolytic activity is based on colorimetric measurement of the intensity of the color of a solution containing Congo-red elastin as an enzyme substrate [19]. 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 solution. The mixture was incubated for 3 h at 37°C. The reaction was stopped by keeping the tubes with the reaction mixture in an ice bath for 30 min. Unhydrolyzed elastin was separated by centrifugation at 10,000 g for 5 min. The color intensity was measured at 515 nm. The amount of enzyme that catalyzes the hydrolysis of 1 mg of elastin in 1 min was taken as a unit of activity.

Fibrinogenolytic activity was measured by the Masada method [20], and fibrinogen obtained from human blood plasma was used as a substrate. The reaction mixture contained 1 mg of fibrinogen, 1.8 ml of 0.01 M Tris-HCl buffer (pH 7.5) with the addition of 0.005 M $CaCl_2$ and 0.2 ml of the solution of the test enzyme. The incubation mixture was kept for 30 min at 37°C. The formation of cleavage products was determined at 275 nm.

The protein concentration was 1 mg/ml.

The effect of *Escherichia coli* LPS 23 on the protease activity of *Bacillus* strains was studied at a final concentration of 0.01%, with an incubation time of 60 min at room temperature.

All experiments were performed in no less than 5–7 replications. Statistical processing of the results of the experimental series was carried out by standard methods using Student's *t*-test. The value of the null hypothesis, $P < 0.05$, was taken as the critical level of reliability.

Results and Discussion

Since the interaction between different microorganisms occurs constantly, their structural components can also affect each other. It is known that LPS of gram-negative bacteria is one of the important factors of bacterial pathogenicity, therefore, the study of their influence on various types of biological activity of polymers is necessary to understand the mechanisms of interference in these activities. Enzymes with proteolytic activity are very important for the life of both pro- and eukaryotes. In eukaryotes, they participate in the processes of transition of enzymes from an inactive state (zymogen) to an active enzyme, while the main function of extracellular proteases of prokaryotes is the cleavage of proteins contained in the environment and their conversion into a form that can easily penetrate the cell. Among the extracellular proteases of microorganisms, a special place is occupied by enzymes that are able to cleave insoluble or poorly soluble proteins, such as collagen and elastin. Since both LPS and proteases interact with each other in the human body and in environmental objects, it is relevant to study the results of such interaction.

The data available in the literature are mainly devoted to the study of the effect of LPS on the activation of enzymes in the human body. As for prokaryotes, the literature only reports about the production of enzymatically active protease *E. coli*, in particular Omp T proteins with the participation of LPS [5], as well as the effect of LPS of three strains of phytopathogenic bacteria *Pantoea agglomerans* on the activity of proteases with fibrinolytic, elastase and collagenase activity of five strains of *Bacillus* [6]. We found that the effect of LPS on them was very diverse. The largest increase in fibrinolytic activity was 4 times, elastase – 2 times, collagenase – 1.5 times. Since LPS were characterized by different composition, and proteases were isolated from 5 strains, we were unable to establish certain correlations between the composition of LPS or the strain from which they were isolated, and the nature of their influence on the proteolytic activities of bacilli. Therefore, in this work, we decided to investigate the influence of LPS of only one strain of *E. coli* 23 on the activity of proteases with fibrinogenolytic and elastase activity. It is known that cells of gram-negative bacteria are characterized by heterogeneity of lipopolysaccharides, i.e. the presence in one cell of several lipopolysaccharides that differ in both composition and biological activity. Therefore, we isolated 2 types of LPS from cells of one strain of *E. coli* 23: LPS1, isolated from whole cells (classical LPS), as well as extracellular LPS2. LPS are the main components of the outer membrane, in which they are anchored by lipid A, which is part of LPS. However, the strength of this interaction varies, so LPS molecules that are weakly bound to the outer membrane can pass into the saline solution with which cells are washed away after growth on the agar medium is complete (LPS2).

Studies of the total content of protein, carbohydrates and nucleic acids in LPS1, which we chose as a standard for comparison (Table 1), indicate that it is almost devoid of protein, but a fairly high content of carbohydrates and nucleic acids was found (Table 1). The same percentage of carbohydrates was found in LPS2 as in LPS1, but in contrast to it, it has a high protein content and a low content of nucleic acids. This may be due to different methods of obtaining LPS1 and LPS2. The distribution of ketodeoxyoctonic acid (KDO) acid and heptose in the studied LPS is of interest (Table 2). Since KDO is an essential component of LPS of most gram-negative bacteria, which binds lipid A to the polysaccharide part of the molecule, its presence can serve as a kind of marker indicating the lipopolysaccharide nature of the isolated substance. The content of KDO in LPS2 even exceeds that in LPS1, which confirms the lipopolysaccharide nature of the extracellular glycopolymer easily associated with the outer membrane. The studied LPS differ slightly in the content of heptose, characteristic components of the LPS molecule (Table 1).

The studied LPS also differ in qualitative and quantitative monosaccharide composition, namely the absence of rhamnose and mannose in LPS2, but a larger amount of ribose (almost 2 times) and glucose (1.5 times). The presence of ribose in both LPS indicates that it is a component of LPS, and not contamination from nucleic acids, which are obtained by the same method as lipopolysaccharides, which pass into the aqueous fraction during extraction by the aqueous-phenol method, and nucleic acids are in the phenolic fraction.

Significant differences in the fatty acid composition of LPS1 and LPS2 were found, which may indicate the presence of structural variants of lipid A (Table 3). Thus, in the composition of LPS1, the main fatty acid was 3-hydroxytetradecanoic acid (3-OH-$C_{14:0}$), the content of which was 73.37%. At the same time, its content in the composition of LPS2 was only 1.32%. Palmitic acid ($C_{16:0}$, 62.09%) and its monounsaturated analogue 9-$C_{18:0}$ (12.41%) were dominant. The results obtained indicate the structural diversity of LPS, which can potentially affect their biological activity.

It is known that LPS are the main antigens of the bacterial cell, which determine its serological specificity. Today, based on the structural features of O-specific polysaccharides of lipopolysaccharides, intraspecies serological groups have been created. More than 180 different serogroups have been established for representatives of *E. coli*. The agar immunodiffusion method (Fig. 1) showed that LPS1 and LPS2 react with antiserum obtained from a heated culture of *E. coli* 23, i.e. they belong to the same serogroup. It can be indirectly assumed that the immunodominant monosaccharides in the structure of their O-specific polysaccharides are the same. The presence of antigenic activity in LPS2 also confirms its lipopolysaccharide nature.

The absence of cross-serological reaction between antiserum to treated cultures of *E. coli* 23 with LPS isolated from other strains of *E. coli* indicates their belonging to other serogroups.

Table 1. Composition of the main components of the studied LPS (% to dry weight of LPS)

Component	LPS1	LPS2
Carbohydrates	35.23	35.21
Proteins	0.96	10.48
Nucleic acids	18.43	2.22

Table 2. Monosaccharide composition of E. coli 23 LPS

Monosaccharide	LPS1	LPS2
% to total sum of peaks square		
Rhamnose	9.39	–
Ribose	13.85	25.85
X_1	3.41	–
X_2	4.28	–
Mannose	1.25	–
Galactose	25.36	26.09
Glucose	29.58	44.21
X_3	5.02	5.85
X_4	4.63	–
X_5	3.23	–
% to dry weight of LPS		
KDO	0.05	0.11
Heptose	0.49	3.9

Table 3. Fatty acid composition of E. coli 23 LPS

Fatty acids	LPS1	LPS2
	% to total sum of peaks square	
$C_{12:0}$	11.73	–
$C_{14:0}$	4.47	1.19
3-OH-$C_{14:0}$	73.37	1.32
9-$C_{16:0}$		12.74
$C_{16:0}$	11.43	62.09
$C_{18:0}$	–	10.25
9-$C_{18:0}$	–	12.41

In further studies, it was shown how lipopolysaccharides, heterogeneous in composition, isolated from one culture of *E. coli* 23, affect the activity of proteases of a number of *Bacillus* strains with elastase and fibrinogenolytic action. When studying the effect of LPS1 and LPS2 of *E. coli* 23 on the fibrinogenolytic activity of proteases of the bacilli, it was found (Fig. 2) that they activated them to different degrees. Thus, the fibrinogenolytic activity of *Bacillus* sp. IMV B-7883 increased by 70% under the action of LPS1 and by 60% under the action of LPS2. A somewhat different picture was observed under the action of LPS on the fibrinogenolytic activity of *Bacillus* sp. L 9 (Fig. 2). The greatest activation (by 200%) was observed under the influence of LPS2, while under the influence of LPS1 only by 20%. As for fibrinogenase *B. atrophaeus* 08 (Fig. 2), the greatest activation (by 60%) was carried out by LPS2 and only at 32% by LPS1.

When studying the effect on the elastase activity of *Bacillus* sp. IMV B-7883 (Fig. 3), the greatest activation (almost 6 times) was observed under the action of LPS2, and somewhat less (4.16 times) by LPS1. Whereas the effect of LPS on the elastase activity of the other two *Bacillus* strains was insignificant and ranged from 12 to 30%.

Thus, it was shown that the effect of LPS1 and LPS2 on the activity of proteases with elastase and fibrinogenolytic activity obtained from different strains of *Bacillus* was very diverse. The most promising for increasing the elastase activity of *Bacillus* sp. IMV B-7883 may be both LPS2 and, to a

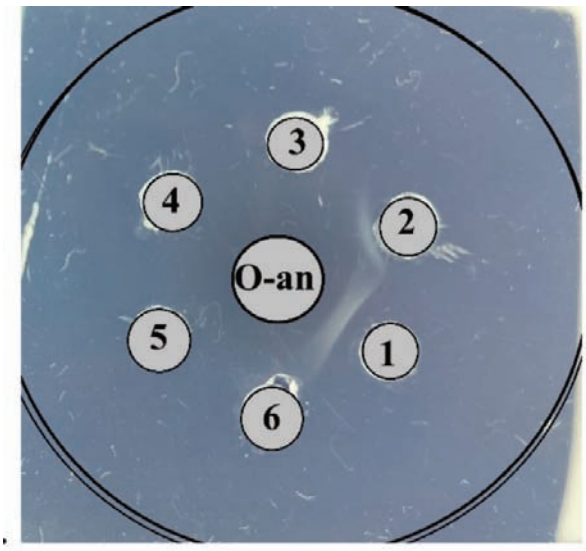

Fig. 1 Double immunodiffusion reaction in Ouchterloni agar of E. coli 23 O-antiserum (central well) with: 1 – LPS1 E. coli 23; 2 – LPS2 E. coli 23; 3 – LPS E. coli 43; 4 – LPS E.coli K; 5 – LPS E. coli O111; 6 – LPS E. coli F-50

lesser extent, LPS1 of *E. coli* 23. As for fibrinogenolytic activity, both LPS are able to increase it in all studied *Bacillus* strains, but its greatest stimulation (200%) was observed when LPS2 acted on the proteolytic activity of *Bacillus* sp. L9. Therefore, today we, as before [6], cannot establish certain correlations between the composition of LPS1 and LPS2 and the nature of their influence on the activity of the studied proteases. Analysis of the results of the obtained experimental material allows us to assume that the decisive role in the interaction of LPS *E. coli* 23 with proteases of the studied strains of *Bacillus* is played not by the peculiarities of the composition of LPS, but by the peculiarities of the structure of the active centers of proteases of different strains of *Bacillus.* It is known that minor changes in the structure of the active center of enzymes affect their stability and functional activity. It can be assumed that the binding of LPS to the protein leads to its conformational changes, which are necessary for obtaining a more active form of the protease. But in order to approach the study of the mechanism of interaction of LPS molecules with enzymes, it is necessary to conduct further research to study the interaction with them not only of the native LPS molecule, but also of its individual structural components: O-specific polysaccharide (OPS), core-oligosaccharide and lipid A, which differ in composition. When isolat-

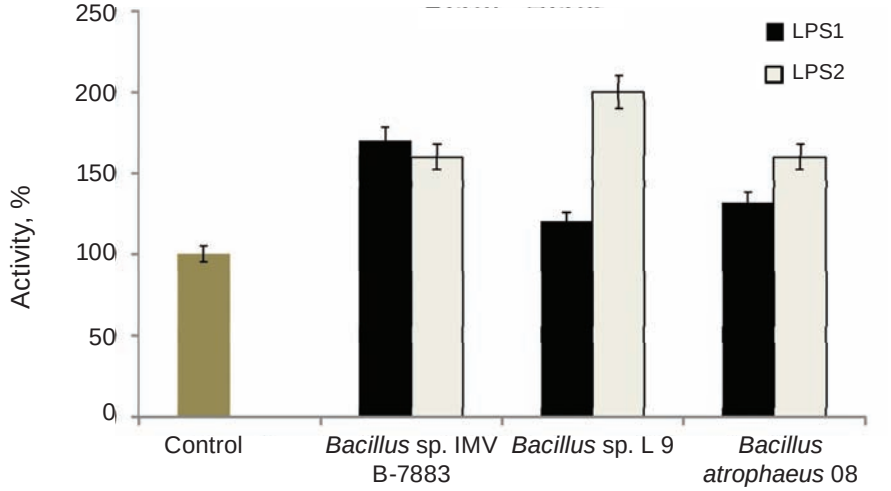

Fig. 2. Effect of LPS1 and LPS2 of E. coli 23 on the fibrinogenolytic activity of a number of Bacillus strains

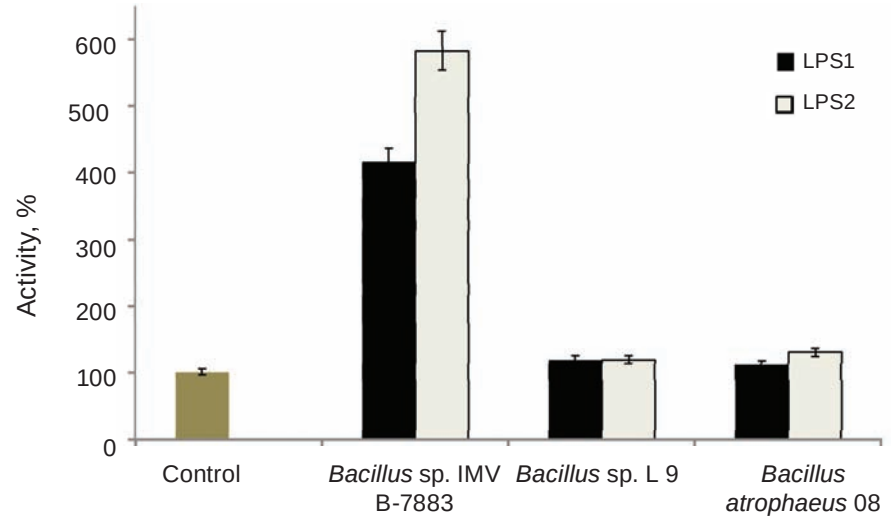

Fig. 3. Effect of LPS1 and LPS2 E. coli 23 on the elastase activity of strains: Bacillus sp. IMV B-7883; Bacillus sp. L 9; Bacillus atrophaeus 08

ing individual LPS fractions, it is possible that some other parts of the LPS molecule become available for interaction with certain groups of enzyme binding centers. Therefore, further studies will be aimed at elucidating some mechanisms of action of LPS and their structural components on the protease activity of bacilli, since these data are absent in the literature. But the results of this study indicate that both cellular and extracellular LPS *E. coli* 23 can be activators of the elastase activity of proteases *Bacillus* sp. IMV B-7883 (6 times), as well as the fibrinogenolytic activity of all three *Bacillus* strains studied.

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

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ВПЛИВ ЛІПОПОЛІСАХАРИДІВ *ESCHERICHIA COLI* НА АКТИВНІСТЬ ПРОТЕАЗ РЯДУ ШТАМІВ *BACILLUS*

Л. Д. Варбанець[1], *О. С. Броварська*[1], *О. В. Гудзенко*[1]✉, *К. Г. Гаркава*[2], *А. Р. Макаренко*[2]

[1]Інститут мікробіології і вірусології ім. Д.К. Заболотного Національної Академії наук України, Київ
[2]Національний технічний університет України «Київський політехнічний інститут ім. Ігоря Сикорського», Київ;
✉e-mail: alena.gudzenko81@gmail.com

Раніше нами було показано, що ліпополісахариди (ЛПС) ряду штамів фітопатогенного виду *Pantoea agglomerans* здатні в 2-4 рази підвищувати активність пептидаз *Bacillus* з фібринолітичною, еластазною та колагеназною активностями. Метою даної роботи було дослідити вплив внутрішньоклітинного ЛПС1 та позаклітинного ЛПС2 *Escherichia coli* на активність очищених протеаз бацил з еластазною та фібриногенолітичною активністю. Показано, що як ЛПС2, так і ЛПС1 *E. coli* 23 можуть підвищувати еластазну активність *Bacillus* sp. IMV B-7883 на 600 та 416% відповідно. Обидва ЛПС здатні підвищувати фібриногенолітичну активність всіх досліджених штамах *Bacillus*, але найбільша її стимуляція (200%) спостерігалася під дією ЛПС2 *Bacillus* sp. L9.

Ключові слова: ліпополісахариди *Escherichia coli*, протеази з фібриногенолітичною і еластазною активністю *Bacillus*, вплив ліпополісахаридів на активність протеаз.

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