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Substrate Specificity and Some Physicochemical Properties of Autolytic Enzymes of the Bacterium *Lysobacter* sp. XL 1

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Abstract—The substrate specificity of autolytic enzymes of the bacterium *Lysobacter* sp. XL 1 has been established. The periplasmic enzyme A_8, the cytosolic enzyme A_1, and the enzyme A_{10} solubilized from the cell walls and membranes with Triton X-100 exhibit glucosaminidase activity; the cytosolic enzyme A_4 and the enzyme A_9 solubilized from the cell walls and membranes with LiCl exhibit the muramidase activity. The cytosolic enzymes A_3 and A_6 have N-acetylmuramoyl-L-alanine amidase activity, and the enzyme A_5 exhibits the diaminopimelinoyl-alanine endopeptidase activity. Some physicochemical properties of the most active autolytic cytosolic enzymes of *Lysobacter* sp. XL 1 (endopeptidases A_5 and A_7 and N-acetylmuramoyl-L-alanine amidase A_6) were studied. The enzymes exhibit maximal activity over a wide range of buffer concentrations in weakly alkaline medium and moderate temperatures. The investigated enzymes are comparatively thermolabile proteins.

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Intracellular bacteriolytic (autolytic) enzymes destroying the covalent bonds in peptidoglycan (the main structural component of the bacterial cell wall) play an important role in the growth and division of the bacterial cell [1-3]. Some bacteria under certain conditions produce not only autolytic, but also extracellular bacteriolytic enzymes that are necessary to occupy an ecological niche and to provide the bacteria with food and energy [4, 5]. It is not known whether the extracellular enzymes are originally intracellular proteins leaving the cell under certain conditions, or they function independently. Presumably, some extracellular enzymes are modified intracellular enzymes, or the intracellular enzymes are precursors of the extracellular ones.

The gram-negative bacterium *Lysobacter* sp. XL 1, while growing under certain conditions, releases into the environment several extracellular bacteriolytic enzymes destroying peptidoglycan of different bacteria: endopeptidase L_1 [6], N-acetylmuramoyl-L-alanine amidase L_2 [7], muramidase L_3 [8], and endopeptidase L_4 [9]. These enzymes are components of the antibacterial medication lysoamidase obtained from the culture liquid of *Lysobacter* sp. XL 1 [10]. Their properties have been well studied. All these enzymes are thermolabile alkaline proteins exhibiting maximal activity at low ionic strength and rather high temperature [6-9].

While investigating the intracellular autolytic enzymes of *Lysobacter* sp. XL 1, 10 enzymes were found (A_1-A_{10}) exhibiting lytic activity towards their own cells. They have different intracellular localization: seven enzymes (A_1-A_7) were found in the cytosol, one in the periplasm (A_8), and two enzymes (A_9 and A_{10}) were revealed in the fraction of cell walls and membranes. Most of the autolytic enzymes are acidic proteins [11]. The enzyme A_1 was purified and characterized. This enzyme possesses glucosaminidase activity that is not characteristic for the extracellular bacteriolytic enzymes, and its physicochemical properties differ significantly from those of the extracellular enzymes of *Lysobacter* sp. XL 1 [12]. It was found that the cytosolic enzyme A_7 exhibits diaminopimelinoyl-alanine endopeptidase activity towards peptidoglycan of *Lysobacter* sp. XL 1 and the glycylglycine endopeptidase activity towards peptidoglycan of *S. aureus* [13]. However, the substrate specificity and properties of other autolytic enzymes of *Lysobacter* sp. XL 1 remained unknown.

The goal of the present work was investigation of the substrate specificity and properties of unstudied autolytic enzymes of the bacterium *Lysobacter* sp. XL 1.

Abbreviations: DNP) dinitrophenol; DTAC) 3,4-dinitrophenyl-tetra-N-acetyl-β-D-chitotetraoside; NADG) *p*-nitrophenyl-2-acetamido-2-deoxy-β-D-glucopyranoside.

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MATERIALS AND METHODS

In the present work, we used the strain *Lysobacter* sp. XL 1 from the All-Russian Collection of Microorganisms (Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences. The bacterial cells were grown in liquid medium 5/5 developed in our institute containing yeast and soybean extracts, casein hydrolyzate, and aminopeptide. The culture was grown in shaken flasks at 29°C. As a result, we obtained the cells of the logarithmic (9 h) or stationary (18 h) growth phases. The cells were removed from the culture liquid by centrifugation at 5000*g* for 20 min and then washed 3 times with 0.01 M Tris-HCl buffer, pH 8.0, centrifuging under the same conditions. The periplasmic and cytoplasmic fractions, as well as the fraction of the cell walls and membranes, were obtained by osmotic shock of the cells with their subsequent extrusion on a press under 9 atm [11, 14].

The preparations of autolytic enzymes were obtained by preparative electrophoresis in polyacrylamide gel under non-denaturing conditions. An anode system (pH 8.3) was employed for enzymes A_1, A_3-A_6, A_9, and A_{10}, and a cathode system (pH 4.3) for enzymes A_7 and A_8 [11, 15, 16]. Before electrophoresis, the enzymes bound to the cell walls and membranes were solubilized by sequential treatment with 5 M LiCl in 0.05 M Tris-HCl, pH 7.5 (A_9) and 1% Triton X-100 in the same buffer (A_{10}) [11]. After electrophoresis, the enzymes were eluted from the polyacrylamide gel with 0.1 M NaCl in 0.05 M Tris-HCl, pH 8.0. The areas exhibiting autocatalytic activity were cut out of the gel as 3-mm bands, minced, supplemented with 7 ml of the eluting solution, and incubated for 4 h at 37°C while stirring. The eluate was separated by centrifugation (18,000*g*, 20-30 min) and dialyzed against 0.05 M Tris-HCl buffer.

Peptidoglycan of *Lysobacter* sp. XL 1 was obtained in two steps. First, the peptidoglycan–lipopolysaccharide complex was isolated from the cells using 2.4% SDS, and then the lipopolysaccharide was removed by treatment with of 0.5 N TCA [17].

The autolytic activity of the cell fractions and enzyme preparations was determined by their ability to lyse autoclaved cells of *Lysobacter* sp. XL 1 (1 mg/ml) fused into 1% agarose gel in 0.05 M Tris-HCl buffer, pH 8.0, and also turbidimetrically by the reduction of the absorption of the autoclaved suspension of *Lysobacter* sp. XL 1 cells at 400 nm (A_{400}). The reaction mixture (0.1 ml of the enzyme preparation and 0.5 ml of the cell suspension of *Lysobacter* sp. XL 1 in a corresponding buffer (A_{400} = 0.3-0.4)) was incubated at 30°C during 1-2 h, and the absorbance of the mixture was measured at 400 nm. The unit of the activity was defined as the amount of the enzyme reducing the absorbance by 0.001 in 1 min [11].

The glucosaminidase activity was determined by cleaving *p*-nitrophenyl-2-acetamido-2-deoxy-β-D-glucopyranoside (NADG) [12, 18]. An enzyme preparation (0.1-0.2 ml) was added to 0.5 ml of NADG solution (0.3 mg/ml) in 0.05 M Tris-HCl buffer, pH 8.0, and incubated at 37°C. The activity was determined by the increase in the absorbance at 400 nm.

The muramidase activity was determined by the cleavage of 3,4-dinitrophenyl-tetra-N-acetyl-β-D-chitotetraoside (DTAC) [19]. An enzyme preparation (0.1-0.2 ml) was added to 0.5 ml of a DTAC solution (0.2 mg/ml) in 0.05 M Tris-HCl buffer, pH 8.0, and incubated at 37°C. The activity was determined by the increase in the absorbance at 400 nm.

The peptidase activity was determined by the ability of the enzymes to hydrolyze the peptide bonds of peptidoglycan of the cell walls of *Lysobacter* sp. XL 1. During the hydrolysis of peptidoglycan by a bacteriolytic enzyme, the number of released NH_2-groups was measured and the N-terminal amino acids in the resulting peptides were determined.

The NH_2-groups were determined by the method of Ghuysen and Strominger [20]. To 2 ml of a peptidoglycan suspension (1 mg/ml) in 50 mM Tris-HCl buffer, pH 8.0, 0.5 ml of an enzyme preparation in the same buffer was added, and the mixture was incubated at 30°C for 12-14 h. Then the soluble fraction of the resulting hydrolyzate (0.5 ml) was supplemented with 0.06 ml of 2,4-dinitrofluorobenzene solution (130 μl in 10 ml of absolute ethanol), and the solution was incubated in a thermostat at 60°C for 30 min. The samples were supplemented with 2.4 ml of 2 N HCl, and the absorbance was measured at 420 nm.

N-Terminal amino acids were determined by dinitrophenylation [20]. After hydrolysis of 3-5 mg of peptidoglycan by a bacteriolytic enzyme, 1 ml of the hydrolyzate was supplemented with 0.1 ml of dinitrophenol (DNP) solution (13 μl in 1 ml of absolute ethanol). The resulting solution was incubated in a thermostat at 60°C for 30 min and dried under vacuum. Then the samples were hydrolyzed in the presence of 6 M HCl at 100°C for 18 h. The DNP-derivatives of the amino acids were extracted with chloroform and analyzed using an LC 6000 E amino acid analyzer (Biotronic, Germany). The product of the acidic hydrolysis of peptidoglycan not treated with the enzyme was used as the control. The type of bonds cleaved by the enzyme was estimated from the difference in the amino acid composition. The root mean value (σ) was calculated from the data of six experiments. The results exceeding the root mean values were considered as reliable.

Optimal pH value for the activity of the autolytic enzymes was determined using 0.05 M sodium acetate buffer (pH 5.0, 6.0, and 6.5) and 0.05 M Tris-HCl buffer (pH 6.5, 7.0, 7.5, 8.0, and 9.0). The activity was measured the turbidimetric method.

Optimal concentration of the buffer for the enzymatic reaction was determined using Tris-HCl buffer solutions (0.01, 0.025, 0.05, 0.1, 0.2, 0.3, and 0.4 M) at the

optimal pH value. The activity was measured turbidimetrically.

Optimal temperature for the activity of the autolytic enzymes was determined by incubation the reaction mixture at 20, 29, 35, 40, or 45°C in Tris-HCl buffer of the optimal pH value and concentration. The activity was measured turbidimetrically.

Thermal stability of the enzyme preparations was determined as follows: 0.1 ml of the enzyme solution was incubated at 30, 40, 45, 50, 55, or 60°C for 15 min, cooled on ice, and supplemented with 0.5 ml of the cell suspension of *Lysobacter* sp. XL 1 in Tris-HCl buffer of the optimal pH value and concentration. The activity was measured turbidimetrically.

RESULTS AND DISCUSSION

Depending on the type of the cleaved bond in the peptidoglycan, autolytic enzymes are divided into three groups. The first group includes glycosidases destroying the glycan chains of the peptidoglycan. Glycosidases are subdivided into endo-N-acetylmuramidases hydrolyzing the bond between the N-acetylmuramic acid and the N-acetylglucosamine, yielding fragments of the peptidoglycan with the N-acetylmuramic acid on the reducing end, and endo-N-acetylglucosaminidases hydrolyzing the bond between the N-acetylglucosamine and the N-acetylmuramic acid, yielding the fragments of the peptidoglycan with the N-acetylglucosamine on the reducing end. The second group involves N-acetylmuramoyl-L-alanine amidases. They hydrolyze the amide bond between the lactyl group of N-acetylmuramic acid and α-amino group of the first amino acid residue of the peptide subunit (usually L-alanine). The third group, endopeptidases, includes the enzymes hydrolyzing inter-peptide bridges or peptide bonds formed by the neighbor peptide subunits of the peptidoglycan [1, 2].

In the cells of *Lysobacter* sp. XL 1, we found 10 autolytic enzymes of different intracellular localization [11]. To investigate the specificity and properties of the enzymes, the fractions of cytosol, periplasm, cell walls, and membranes were isolated. The enzymes A_5 and A_6 were isolated from cells of the logarithmic growth stage, and the other enzymes were obtained from cells of the stationary stage, since we demonstrated the dependence of the composition of the autolytic enzymes on the growth stage. Enzymes A_1 and A_3-A_7 were isolated from the cytosolic fraction and A_8 from the periplasmic fraction. The enzymes A_9 and A_{10} were obtained from the fraction of the cell walls and membranes after their extraction with LiCl and Triton X-100 solutions. The proteins were separated by preparative PAGE under nondenaturing conditions. The areas corresponding to the proteins A_1-A_{10} were revealed, and the enzymes were eluted from the gel with 0.1 M NaCl in 0.05 M Tris-HCl, pH 8.0. The resulting preparations of the autolytic enzymes were assayed for glucosaminidase and muramidase activities, and the content of the NH_2-groups released after the action of these enzymes on peptidoglycan of *Lysobacter* sp. XL 1 was determined (Table 1).

As seen from Table 1, enzymes A_1, A_8, and A_{10} exhibit glucosaminidase activity, and enzymes A_4 and A_9 possess muramidase activity. Enzymes A_3, A_5, and A_6 hydrolyze peptidoglycan releasing NH_2-groups, this indicating the peptidase activity of the enzymes.

To determine the peptide bond of the peptidoglycan cleaved by enzymes A_3, A_5, and A_6, the hydrolyzates of

Table 1. Substrate specificity of autolytic enzymes of *Lysobacter* sp. XL 1

Enzyme	Localization	Activity towards the substrate of glucosaminidases	Release of NH_2-groups from peptidoglycan of *Lysobacter* sp. XL 1	Activity towards the substrate of muramidases
A_1	cytosol	+	–	–
A_3	cytosol	–	+	–
A_4	cytosol	–	–	+
A_5	cytosol	–	+	–
A_6	cytosol	–	+	–
A_8	periplasm	+	–	–
A_9	membranes and cell walls (LiCl extraction)	–	–	+
A_{10}	membranes and cell walls (Triton X-100 extraction)	+	–	–

peptidoglycan of *Lysobacter* sp. XL 1 obtained after treatment with these enzymes were analyzed on an amino acid analyzer after reaction with 2,4-dinitrofluorobenzene, acidic hydrolysis, and chloroform extraction of the DNF-derivatives of the amino acids. The conclusion concerning the cleaved peptide bonds was made based the difference in the amino acid composition of the peptidoglycan before and after the enzymatic hydrolysis (Table 2). Peptidoglycan of *Lysobacter* sp. XL 1, as that of most gram-negative bacteria, has the A1γ structure [17]. The glycan chain is composed of alternating residues of N-acetylglucosamine and N-acetylmuramic acid bound to the tetrapeptide subunit (L-alanine–γ-D-glutamic acid–meso-diaminopimelic acid–D-alanine). The peptidoglycan of *Lysobacter* sp. XL 1 contains no inter-peptide bridges. The peptide subunits of the neighbor glycan chains are connected with the peptide bond formed by the NH_2-group of the diaminopimelic acid of one subunit and the COOH-group of the D-alanine of other subunit. As seen from Table 2, the action of enzymes A_3 and A_6 releases 0.34 and 0.35 mol alanine per mol glutamic acid, respectively, indicating the cleavage of the bond between the L-alanine and the N-acetylmuramic acid. Thus, these enzymes possess N-acetylmuramoyl-L-alanine amidase activity towards peptidoglycan of *Lysobacter* sp. XL 1. The action of enzyme A_5 results in the release of 0.29 mol diaminopimelic acid per mol glutamic acid, indicating the cleavage of the bond between the meso-diaminopimelic acid residue of one subunit and the D-alanine residue of other subunit. Thus, enzyme A_5 exhibits diaminopimelinoyl-alanine endopeptidase activity towards peptidoglycan of *Lysobacter* sp. XL 1. Since each of the isolated enzymes possessed only one certain substrate specificity, it can be concluded that the preparations of autolytic enzymes do not contain admixtures of other autolytic enzymes. However, we cannot exclude that several autolytic enzymes exhibiting the same substrate specificity can move in polyacrylamide gel as a single band.

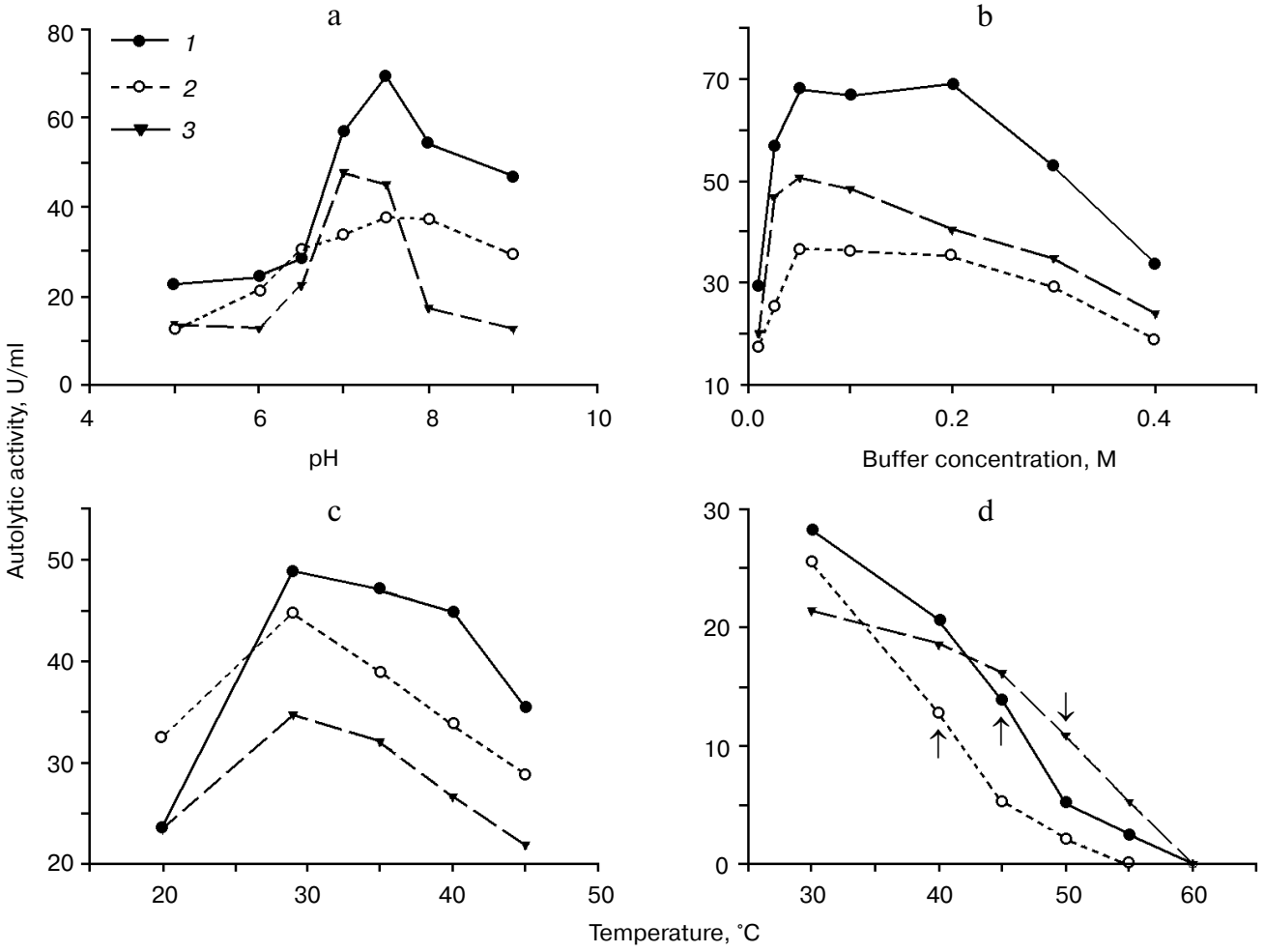
The specificity of the autolytic enzyme A_2 was not investigated since it exhibited low activity and was isolated in very small amount. The specificities of cytosolic autolytic enzymes A_1 and A_7 were determined previously. Enzyme A_1 is glucosaminidase [12], and enzyme A_7 exhibits diaminopimelinoyl-alanine endopeptidase activ-

Table 2. Amino acid composition of peptidoglycan of *Lysobacter* sp. XL 1 after treatment with enzymes

Component	Peptidoglycan, mol/mol Glu			
	without treatment	after 12 h hydrolysis by enzyme A_3	after 12 h hydrolysis by enzyme A_5	after 12 h hydrolysis by enzyme A_6
Glu	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
m-A_2pm	1.00 ± 0.05	0.95 ± 0.03	0.71 ± 0.16	0.96 ± 0.10
Ala	2.03 ± 0.07	1.69 ± 0.17	1.95 ± 0.09	1.68 ± 0.28

Table 3. Properties of extra- and intracellular bacteriolytic enzymes of *Lysobacter* sp. XL 1

Enzymes	Optimal conditions for activity			Half-inactivation temperature, °C
	pH	buffer concentration, mM	temperature, °C	
Intracellular (autolytic)				
endopeptidase A_5	7.5	50.0-200.0	29.0	45.0
amidase A_6	7.5-8.0	50.0-200.0	29.0	40.0
endopeptidase A_7	7.0	50.0-100.0	29.0	50.0
glucosaminidase A_1 [12]	8.0-8.5	10.0-500.0	37-40	47.0
Extracellular bacteriolytic				
endopeptidase L_1 [6]	7.0-11.0	50.0	70.0	50.0
amidase L_2 [7]	8.0	5.0	65.0	65.0
muramidase L_3 [8]	8.0	0.1	60.0	65.0
endopeptidase L_4 [9]	8.0	50.0	50-55	52.0



Physicochemical properties of autolytic enzymes A_5, A_6, and A_7 of *Lysobacter* sp. XL 1: a) pH dependence of activity; b) dependence of activity on concentration of working buffer; c) temperature dependence of activity; d) thermostability of the enzymes (temperature values corresponding to half-inactivation of the enzymes are designates by arrows). *1-3*) The activity of autolytic enzymes A_5-A_7, respectively

ity towards peptidoglycan of *Lysobacter* sp. XL 1 and glycylglycine endopeptidase activity towards peptidoglycan of *S. aureus* [13].

We have determined the substrate specificity of all found autolytic enzymes of *Lysobacter* sp. XL 1 except for A_2. The bacterium *Lysobacter* sp. XL 1, as other gram-negative bacteria [1, 2], produces autolytic enzymes of different substrate specificity and different intracellular localization. There are muramidases (A_4 and A_9), glucosaminidases (A_1, A_8, and A_{10}), N-acetylmuramoyl-L-alanine amidases (A_3 and A_6), and endopeptidases (A_5 and A_7). It should be noted that enzymes possessing the same substrate specificity were found in different cell compartments. For example, glucosaminidases are located both in the cytosol and in the periplasm and membranes, and the enzymes with muramidase activity are found in the cytosol and in the membranes.

The most active autolytic enzymes of *Lysobacter* sp. XL 1 are glucosaminidase A_1, amidase A_6, and endopeptidases A_5 and A_7. Glucosaminidase A_1 was purified and characterized earlier [12]. In the present work, we investigated biochemical properties of amidase A_6 and endopeptidases A_5 and A_7 obtained by elution from the polyacrylamide gel after the non-denaturing preparative electrophoresis (figure). The enzymes exhibit maximal activity in the range of pH 7.0-8.0 (figure, panel (a)). For endopeptidases A_5 and A_7, the dependence of the activity on pH is more pronounced than for amidase A_6. The optimal temperature for the activity of these enzymes is similar, about 29°C (figure, panel (c)), but the dependence of the activity on temperature is different. The activity of enzymes A_6 and A_7 decreases sharply at temperatures exceeding 29°C, and enzyme A_5 retains a high activity even at 40°C. The activity of the autolytic enzymes is maximal at buffer concentration 0.05-0.1 M (figure, panel (b)). Endopeptidase A_5 and amidase A_6 retain maximal activity at buffer concentration of 0.2 M, but the activity of endopeptidase A_7 significantly decreases with

increase in concentration of the buffer. Investigation of thermolability of the enzymes showed that they all, especially the amidase A_6, are sensitive to higher temperatures (figure, panel (d)). The properties of the investigated enzymes are similar, but there are some differences.

The properties of some intra- and extracellular peptidoglycan hydrolases of *Lysobacter* sp. XL 1 are presented in Table 3. As seen from the table, the extracellular enzymes are sensitive to ionic strength of the medium, exhibit maximal activity at higher pH values and temperature, and are half-inactivated at higher temperatures. All investigated extracellular bacteriolytic enzymes of *Lysobacter* sp. XL 1 are alkaline proteins with molecular weights of 21-29 kD. Among the intracellular enzymes, only endopeptidase A_7 is an alkaline protein, the other enzymes being acidic proteins. The molecular weights of the intracellular enzymes could not be determined since their content in the cell is very low.

Differences in the properties of the extracellular and intracellular bacteriolytic enzymes of *Lysobacter* sp. XL 1 indicate that the bacterium possesses two independent systems of bacteriolytic enzymes performing different physiological functions.

The results obtained in the present work demonstrating that extracellular and intracellular bacteriolytic enzymes differ in their properties (optimal values of pH, buffer concentration, and temperature) are confirmed by the literature data [1-3, 5, 21-23]. However, it should be noted that the data presented in the literature were obtained for either autolytic or extracellular enzymes. None of the bacteria was the subject of simultaneous investigation of the extra- and intracellular systems of bacteriolytic enzymes. To date, it has been shown that most of the extracellular enzymes are alkaline, thermostable proteins exhibiting maximal activity at low ionic strength and higher temperatures. In contrast, the intracellular enzymes are usually thermolabile proteins that are resistant to higher ionic strength of the reaction medium and exhibit maximal activity at moderate temperatures. Such differences in the properties of the intracellular and extracellular bacteriolytic enzymes suggest that, in most cases, there is no genetic connection between them. However, some autolytic enzymes can leave the bacterial cell and become extracellular enzymes. For example, a major autolysin of the bacterium *Pseudomonas aeruginosa* PAO1 is excreted into the environment as a component of membrane vesicles produced by the bacterium [24, 25].

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