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Isolation and Characterization of a New Extracellular Bacteriolytic Endopeptidase of *Lysobacter* sp. XL1

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Abstract—The previously unstudied bacteriolytic enzyme L_4 was isolated from the culture liquid of the bacterium *Lysobacter* sp. XL1 in electrophoretically homogeneous state. The enzyme L_4 is a diaminopimelinoyl-alanine endopeptidase relative to peptidoglycan of *Lysobacter* sp. XL1. The enzyme is an alkaline protein of ~21 kD. The N-terminal amino acid sequence of the enzyme has been determined — A V V N G V N Y V Gx T T A ... The maximal activity of the enzyme was observed in 0.05 M Tris-HCl at pH 8.0 and 50-55°C. The half-inactivation temperature of the enzyme is 52°C. The endopeptidase L_4 is not a metalloenzyme since it is not affected by EDTA. The enzyme is inhibited by *p*-chloromercuribenzoic acid by 72% and by phenylmethylsulfonyl fluoride by 43%, which indicates the involvement of serine and thiol groups in its functioning.

Key words: bacteriolytic enzyme, *Lysobacter* sp., lysoamidase, purification, properties

Investigations of bacteriolytic enzymes destroying bacterial cell walls are important since these enzymes are necessary for the vital functions of the bacterial cell itself and for the interaction of the organisms in natural ecosystems. They are also used in contemporary cell and molecular biology. In addition, bacteriolytic enzymes can be used as medications against pathogenic bacteria that cause different infectious diseases [1].

Extracellular bacteriolytic enzymes of *Lysobacter* sp. XL1 are the main components of the antibacterial preparation lysoamidase [2]. So far, three bacteriolytic enzymes of lysoamidase have been isolated, purified, and characterized: muramidase [3] and two bacteriolytic proteases. One of the proteases (L_1), being an endopeptidase according to the classification of the bacteriolytic enzymes [4], also exhibits N-acetyl muramoyl-L-alanine amidase activity [5, 6], and the other (L_2) is N-acetyl muramoyl-L-alanine amidase [6-8]. The present work is devoted to isolation, purification, and characterization of another bacteriolytic enzyme of *Lysobacter* sp. XL1.

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MATERIALS AND METHODS

In the present work we used strain *Lysobacter* sp. XL1 from the All-Russian Collection of Microorganisms (Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, Pushchino). The cells of *Lysobacter* sp. XL1 were grown in liquid medium containing glucose, peptone, yeast extract, $Na_2HPO_4 \cdot 12H_2O$, KH_2PO_4, KCl, $MgSO_4 \cdot 7H_2O$, and $FeSO_4 \cdot 7H_2O$ in shaking flasks at pH 7.0 and 29°C for 18 h. Then the cells were separated by centrifugation at 5000 rpm for 30 min using a K-70 D centrifuge. The culture liquid was used for isolation of the bacteriolytic enzymes. The bacteriolytic enzyme L_1 was isolated from lysoamidase using the previously described procedure [5].

Protein composition of fractions was analyzed by SDS-PAGE as described by Laemmli [9] after the precipitation of the proteins with 10% trichloroacetic acid. We used 12% polyacrylamide gel; the ratio of acrylamide to methylene-bis-acrylamide was 29.2 : 0.8. Protein concentration was determined by Bradford's method [10] or by measuring the absorption at 280 nm.

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The bacteriolytic activity of the enzyme preparations was determined using autoclaved cells of *Staphylococcus aureus* 209-P as the substrate. The enzyme preparation (100 μl) was added to 2 ml of the suspension of the staphylococcus cells in 0.01 M Tris-HCl, pH 8.0 (A_{540} = 0.6). The mixture was incubated for 10-30 min at 37°C. The reaction was stopped by placing the tubes into ice, and then the absorption of the mixture was measured at 540 nm using a Shimadzu UV-160 A spectrophotometer (Japan). The unit of the bacteriolytic activity was determined as the amount of the enzyme decreasing the absorbance of the bacterial suspension by 0.01 in 1 min.

The peptidase activity was determined as the ability of the enzyme to hydrolyze the peptide bonds of peptidoglycan of the cell walls of *Lysobacter* sp. XL1. Enzyme L_4 (1 ml, 1.23 U/ml) in the working buffer was added to 1 ml of peptidoglycan of *Lysobacter* sp. XL1 (5 mg/ml) in 0.05 M Tris-HCl at pH 8.0. The reaction was allowed to proceed at 37°C for 12 h. During the hydrolysis of the peptidoglycan by the bacteriolytic enzyme, the content of the released NH_2-groups was measured and the N-terminal amino acids in the resulting peptides were determined.

The amount of free NH_2-groups released during the lysis of the cell walls was determined by the method of Ghuysen and Strominger [11]. To 0.6 ml of the peptidoglycan hydrolyzate, 0.06 ml of ethanol solution of 2,4-dinitrofluorobenzene (DNFB) (13 μl of DNFB in 1 ml of anhydrous ethanol) was added. The mixture was incubated at 60°C for 30 min. Then 2.4 ml of 2 M HCl was added to the samples and the absorption at 420 nm was measured on a Shimadzu UV-160 A spectrophotometer. The content of the free NH_2-groups was determined from a standard curve plotted with the use of glutamic acid. The peptidoglycan not treated with the enzyme was used as the control.

The N-terminal amino acids were determined by the dinitrophenylation method [11]. To 1 ml of the hydrolyzate obtained after the hydrolysis of 3-5 mg of peptidoglycan in the presence of the bacteriolytic enzyme, 0.1 ml of the DNFB solution (13 μl of DNFB in 1 ml of anhydrous ethanol) was added. The mixture was thermostatted at 60°C for 30 min and then dried under vacuum. Then the samples were hydrolyzed in the presence of 6 M HCl for 18 h at 100°C. The DNFB amino acid derivatives were extracted with chloroform. The resulting samples were analyzed using an LC 6000 E amino acid analyzer (Biotronic, Germany). The control samples were acid hydrolyzates of the peptidoglycan not treated with the enzyme. The type of the bonds cleaved by the enzyme was estimated from the difference in the amino acid composition. The results of six experiments were used to calculate the standard deviation (σ). The values that exceeded the value of the standard deviation were considered as significant values.

To determine the optimal pH value for the lysis of the *S. aureus* cells, we used the following buffer solutions: 0.05 M sodium acetate buffer, pH 5.0, 5.5, 6.0, and 6.5; 0.05 M Tris-HCl, pH 6.5, 7.0, 7.5, 8.0, and 8.5; and 0.05 M sodium carbonate buffer, pH 9.0, 9.5, and 10.0. The reaction mixture contained 0.1 ml of the enzyme preparation and 2 ml of the suspension of *S. aureus* in the corresponding buffer.

Optimal concentration of the buffer solution was determined using different concentrations of Tris-HCl buffer, pH 8.0: 0.01, 0.05, 0.1, 0.15, 0.20, 0.25, 0.30, and 0.35 M. The reaction mixture contained 0.1 ml of the enzyme preparation and 2 ml of the suspension of *S. aureus* in the corresponding buffer.

To study the temperature dependence of the bacteriolytic activity of the enzyme, the reaction mixture (0.1 ml of the enzyme preparation and 2 ml of the suspension of *S. aureus* in 0.05 M Tris-HCl, pH 8.0) was incubated at 30, 35, 40, 45, 47, 49, 50, 51, 53, 55, 60, 65, 70, and 75°C.

To study the thermal stability of the enzyme, samples containing 0.1 ml of the enzyme preparation were incubated at different temperatures (30, 40, 45, 50, 55, 60, and 70°C) for 15 min. Then the samples were cooled in ice, supplemented with 2 ml of the suspension of *S. aureus* in 0.05 M Tris-HCl, pH 8.0, and the bacteriolytic activity was determined at 37°C.

The effect of inhibitors on the bacteriolytic activity of the enzyme was investigated using the following reagents: ethylene diamine tetraacetic acid (EDTA), the inhibitor of metalloenzymes (final concentrations, 10^{-3} and 10^{-2} M); phenylmethylsulfonyl fluoride (PMSF), the inhibitor of serine proteinases (10^{-3} and $2.5 \cdot 10^{-3}$ M); *p*-chloromercuribenzoic acid (*p*-CMB), the inhibitor of the thiol proteinases (10^{-4} and 10^{-3} M). The reaction mixture contained 0.1 ml of the enzyme preparation and the inhibitor in 0.05 M Tris-HCl, pH 8.0 (final volume, 0.5 ml). After 15 min of incubation at room temperature, the mixture was supplemented with 0.5 ml of the suspension of *S. aureus* in 0.05 M Tris-HCl, pH 8.0, and the bacteriolytic activity was determined.

Proteins were separated by electrophoresis following Laemmli [9] and then transferred to an Immobilon membrane in 0.05 M borate buffer, pH 8.0, without methanol or β-mercaptoethanol (36 V, 0.2 A) for 3 h. The proteins were stained with solution containing 0.1% Coomassie R-250, 50% methanol, and 10% acetic acid for 10 min.

The N-terminal amino acid sequences were determined by automatic Edman degradation using a 477A protein sequencer (Applied Biosystems, USA) with automatic identification of the phenylthiohydantoin amino acid derivatives on a 120 A amino acid analyzer (Applied Biosystems).

The following chemicals were used: CM-Sephadex C-50, SP-Sephadex C-50, and Sephacryl S-200 from Pharmacia (Sweden); protein standards from Bio-Rad (USA); Coomassie Brilliant Blue R-250, *p*-CMB, and

chemicals for electrophoresis from Serva (Germany); EDTA and PMSF from Sigma (USA). Other chemicals were of domestic production.

RESULTS AND DISCUSSION

Recently, the main subject of our investigation had been the extracellular bacteriolytic endopeptidase L_1 of *Lysobacter* sp. XL1, a component of the antimicrobial preparation lysoamidase. Investigation of the enzyme structure required large amounts of the protein. Previously, the enzyme was isolated from lysoamidase according to a procedure including the S-200 Sephacryl chromatography in 0.05 M glycine-NaOH buffer, pH 12.0, CM-Sephadex ion-exchange chromatography, and MonoS FPLC in 0.05 M Tris-HCl buffer, pH 8.0 [5].

To obtain lysoamidase, the enzymes of the culture liquid of *Lysobacter* sp. XL1, including those having the bacteriolytic activity, were precipitated with acetone or alcohol and then with ammonium sulfate. This resulted in a significant loss of the bacteriolytic activity. To avoid such a loss, the step of precipitation with solvents was excluded from the procedure. The enzymes were precipitated from the culture liquid directly with ammonium sulfate (80% saturation). After this procedure, the precipitate retained 100% of the original bacteriolytic activity. Then the bacteriolytic endopeptidase was isolated according to the previously described procedure [5]. The obtained preparation of endopeptidase L_1 was analyzed by SDS-PAGE under slightly modified conditions compared to those used in the previous works (the ratio acrylamide/methylene-bis-acrylamide was 29.2 : 0.8 instead of 30 : 0.8). The electrophoresis revealed the presence of an unknown protein with molecular weight close to that of the endopeptidase (Fig. 1a, lane *3*).

As seen from Fig. 1a, the new protein exhibits a higher electrophoretic mobility than the endopeptidase L_1 (lane *2*), corresponding to the molecular weight of ~21 kD. Accordingly, the molecular weight of endopeptidase L_1 is ~22 kD (in the previous works on purification and characterization of endopeptidase L_1 we indicated that the molecular weight of the enzyme was ~21 kD). Thus, it was necessary to separate this protein from the endopeptidase. For this purpose, different variants of ion-exchange chromatography were tested and the procedure of purification was changed. The proteins of the culture liquid of *Lysobacter* sp. XL1 were precipitated with ammonium sulfate (80% saturation), the pellet was dissolved in 0.05 M Tris-HCl, pH 8.0, dialyzed against the same buffer, and applied to a CM-Sephadex column under the same conditions. All the proteins exhibiting the bacteriolytic activity were adsorbed to the CM-Sephadex and then eluted with 0-0.3 M gradient of NaCl in 0.05 M Tris-HCl buffer, pH 8.0. The proteins exhibiting the bacteriolytic activity were eluted from the column as a single symmetric peak. The active fractions were pooled, dialyzed against 0.05 M Na-acetate buffer, pH 5.0, and

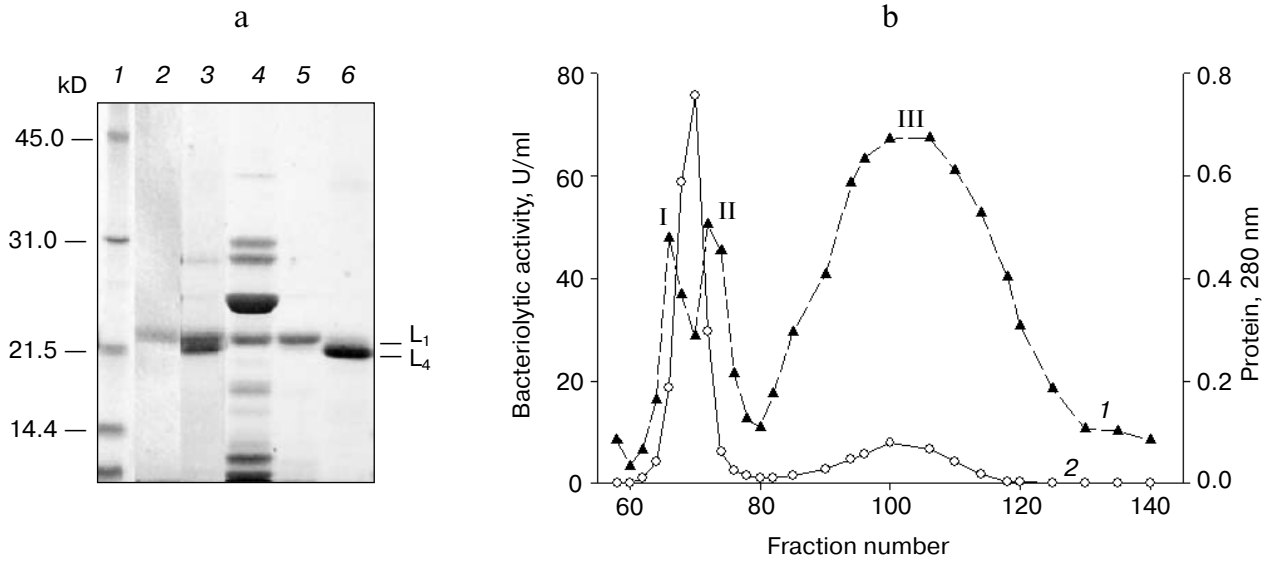

Fig. 1. Purification of bacteriolytic enzymes from the culture liquid of *Lysobacter* sp. XL1. a) Electrophoregram of the bacteriolytic enzymes of *Lysobacter* sp. XL1 (SDS-PAGE by the Laemmli method in 12% polyacrylamide gel, staining with Coomassie R-250): *1*) protein standards: ovalbumin (45 kD), carboanhydrase (31 kD), trypsin inhibitor (21.5 kD), lysozyme (14.4 kD); *2*) preparation of endopeptidase L_1 obtained by the previously described procedure with the use of solvents [5]; *3*) preparation of endopeptidase L_1 obtained by the new procedure without the use of solvents; *4-6*) protein composition of the peaks I, II, and III after SP-Sephadex chromatography, respectively. b) Elution of the bacteriolytic activity (*1*) and protein (*2*) from an SP-Sephadex column (5 × 15 cm) with 0-0.5 M gradient of NaCl in 0.05 M Na-acetate buffer, pH 5.0. I, II, and III are the peaks of the bacteriolytic activity.

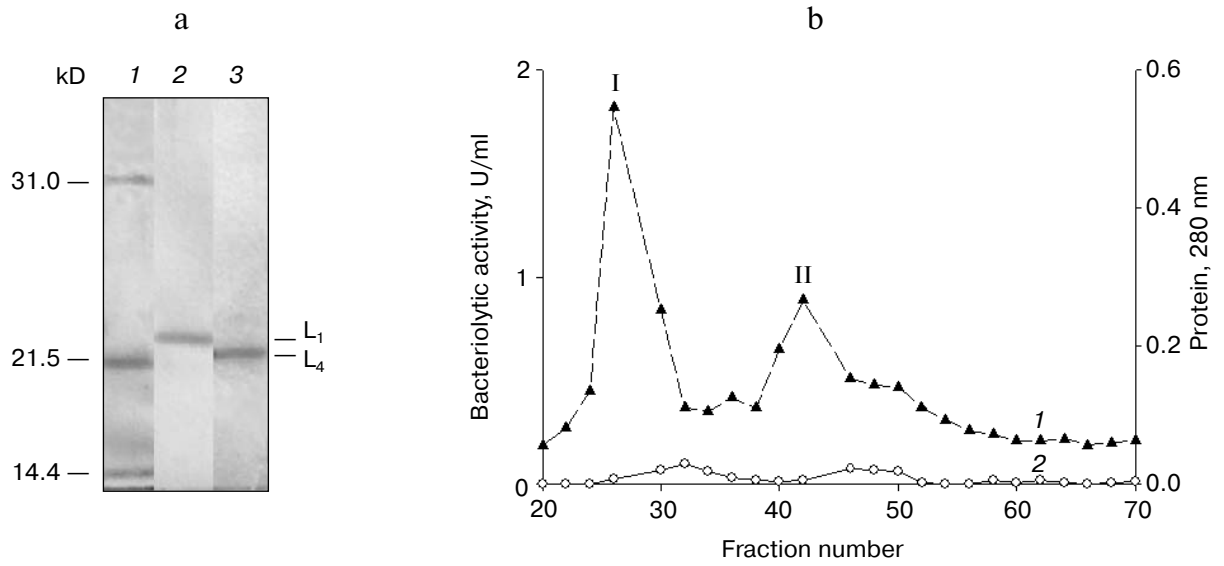

Fig. 2. Purification of the new bacteriolytic enzyme L_4 of *Lysobacter* sp. XL1 by means of Sephacryl S-200 gel chromatography. a) Electrophoregram of the active peaks I and II (SDS-PAGE by the Laemmli method, 12% polyacrylamide gel, staining with Coomassie R-250): *1*) protein standards: carboanhydrase (31 kD), trypsin inhibitor (21.5 kD), lysozyme (14.4 kD); *2*) peak I; *3*) peak II. b) Elution of the bacteriolytic activity (*1*) and protein (*2*) (1 × 60-cm column, 0.01 M citrate buffer, pH 6.0, containing 0.4 M NaCl). I and II are the peaks of the bacteriolytic activity.

applied to an SP-Sephadex column equilibrated with the same buffer. Under the conditions employed, all the bacteriolytic proteins bound to the column. After washing the column with the starting buffer, the proteins were eluted with 0-0.5 M gradient of NaCl in the same buffer (Fig. 1b). The bacteriolytic activity was eluted in three peaks that did not coincide with the protein peak. The results of the electrophoresis (Fig. 1a, lanes *4*-*6*) showed that the bacteriolytic activity of the first peak (lane *4*) was due to the presence of N-acetyl-muramoyl-L-alanine amidase L_2 (~25 kD) and endopeptidase L_1 (~22 kD), and the second peak (lane *5*) was due to the endopeptidase L_1 (~22 kD). The third peak (lane *6*) contained a new unknown protein (~21 kD) and some admixtures of endopeptidase L_1. This fraction exhibited high bacteriolytic activity. Based on these results, it concluded that the discovered protein of ~21 kD is a new bacteriolytic enzyme of *Lysobacter* sp. XL1. Previously, three bacteriolytic enzymes of *Lysobacter* sp. XL1 were isolated and characterized, so the new enzyme was arbitrarily named L_4 [5]. We assume that enzyme L_4 was not revealed previously because it denatured irreversibly in the presence of acetone, alcohol, or at high pH values. After SDS-PAGE, the proteins of fractions II and III were transferred to an Immobilon-P membrane (Millipore, USA) by electroblotting. The N-terminal amino acid sequences of the proteins of 21 and 22 kD were analyzed by automatic Edman degradation using a protein sequencer. The protein of 22 kD was found to be the previously described protein L_1 [12]. Analysis of the 21-kD protein allowed determination of the amino acid sequence A V V N G V N Y V Gx T T A... Comparing the given sequence with the protein sequences presented in the data bank of PIR and Swiss-Prot [http://pir.georgetown.edu], no homology with other proteins was revealed. This indicates that the bacteriolytic enzyme L_4 of *Lysobacter* sp. XL1 is a new unstudied protein.

The new bacteriolytic protein was admixed with a small amount of endopeptidase L_1. Thus, it was necessary to find methods for purification of enzyme L_4 from the admixtures of endopeptidase L_1. It is known from the literature that some bacteriolytic enzymes exhibit a nonspecific affinity to dextrans, similarly to lipophilic compounds [13]. Therefore, the preparation containing endopeptidase L_1 and the bacteriolytic enzyme L_4 (Fig. 1a, lane *3*) was applied to a Sephacryl S-200 column. The proteins were eluted with 0.01 M citrate buffer, pH 6.0, containing 0.4 M NaCl. The elution profile is presented

Table 1. Amino acid composition of peptidoglycan of *Lysobacter* sp. XL1 (mol/mol Glu) after treatment with enzyme L_4

Component	Untreated peptidoglycan	Peptidoglycan after 12 h of hydrolysis in the presence of L_4
Glu	1.00 ± 0.00	1.00 ± 0.00
m-A^2pm	1.10 ± 0.19	0.74 ± 0.12
Ala	2.02 ± 0.09	2.03 ± 0.10

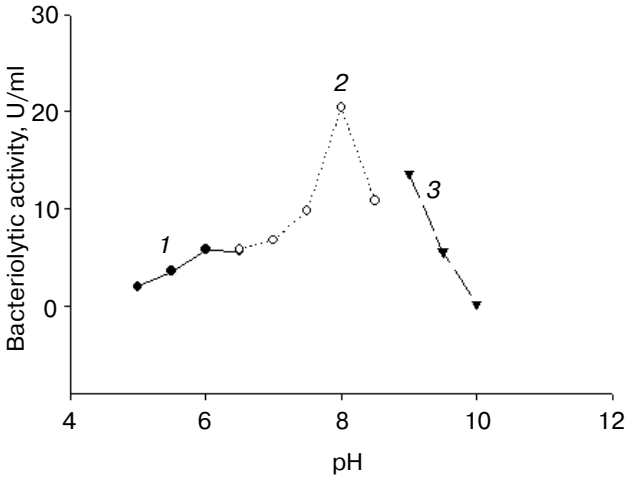

Fig. 3. Dependence of the activity of enzyme L_4 on pH: *1-3*) 0.05 M Na-acetate, 0.05 M Tris-HCl, and 0.05 M carbonate buffer, respectively.

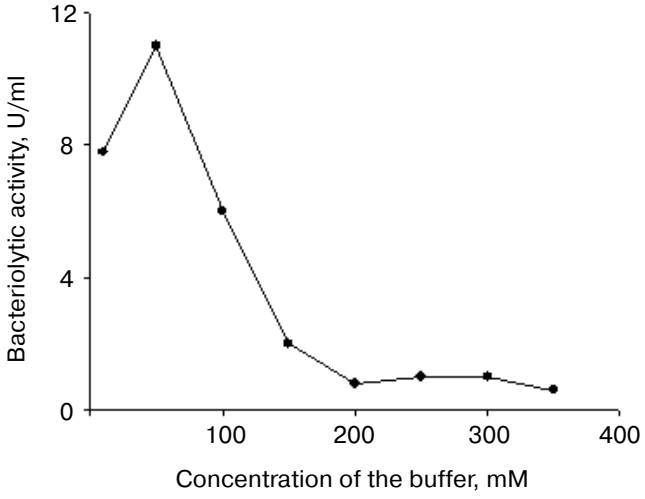

Fig. 4. Dependence of the activity of enzyme L_4 on buffer concentration (0.01, 0.05, 0.1, 0.15, 0.20, 0.25, 0.30, 0.35 M Tris-HCl buffer, pH 8.0).

in Fig. 2b. As seen from the figure, the procedure separated the new bacteriolytic protein L_4 from endopeptidase L_1 (Fig. 2a).

The substrate specificity of enzyme L_4 to peptidoglycan of *Lysobacter* sp. XL1 was determined. The structure of peptidoglycan of *Lysobacter* sp. XL1 is known [14]. According to the classification of Schleifer and Kandler [15], peptidoglycan of *Lysobacter* sp. XL1 is A1γ peptidoglycan. The glycan chain of the peptidoglycan of this type is composed of alternating residues of N-acetylglucosamine and N-acetylmuramic acid, each muramic acid residue carrying a tetrapeptide of the following amino acid sequence: L-alanine–γ-D-glutamic acid–mesodiaminopimelic acid–D-alanine. There are no interpeptide bridges in A1γ peptidoglycan.

Peptidoglycan of *Lysobacter* sp. XL1 was hydrolyzed by enzyme L_4. It was found that NH_2-groups are released during the hydrolysis. To determine the peptide bond hydrolyzed by enzyme L_4, the hydrolyzate was treated with DNFB. After acid hydrolysis of the preparations and the subsequent extraction of the dinitrophenyl derivatives of the amino acids with chloroform, they were analyzed on an amino acid analyzer. The control sample was the preparation of the peptidoglycan not treated with enzyme L_4. The hydrolyzed peptide bond was estimated from the difference in the amino acid composition (Table 1). As seen from the table, the action of enzyme L_4 on peptidoglycan of *Lysobacter* sp. XL1 resulted in the release and extraction of 0.36 mol diaminopimelic acid per mol of glutamic acid, this indicating the diaminopimelinoyl-alanine endopeptidase activity of the enzyme toward peptidoglycan of *Lysobacter* sp. XL1.

Endopeptidase L_1, similarly to endopeptidase L_4, hydrolyzes the bond between the diaminopimelic acid and alanine in the peptidoglycan of *Lysobacter* sp. XL1.

The pH optimum of the enzyme is 8.0 (Fig. 3). As seen from the figure, the bacteriolytic activity strongly depends on pH, change in the pH value by 0.5 resulting in a sharp decrease in the enzyme activity.

The dependence of the activity of enzyme L_4 on ionic strength of the solution is presented in Fig. 4. According to the data, the activity depends significantly on the ionic strength, exhibiting maximal value in 50 mM Tris-HCl.

The optimal temperature for the lysis of the *S. aureus* cell walls by enzyme L_4 is 50-55°C (Fig. 5). The half-inactivation temperature of enzyme L_4 is 52°C (Fig. 6).

To study the effect of inhibitors on the bacteriolytic activity of enzyme L_4, we used EDTA (an inhibitor of

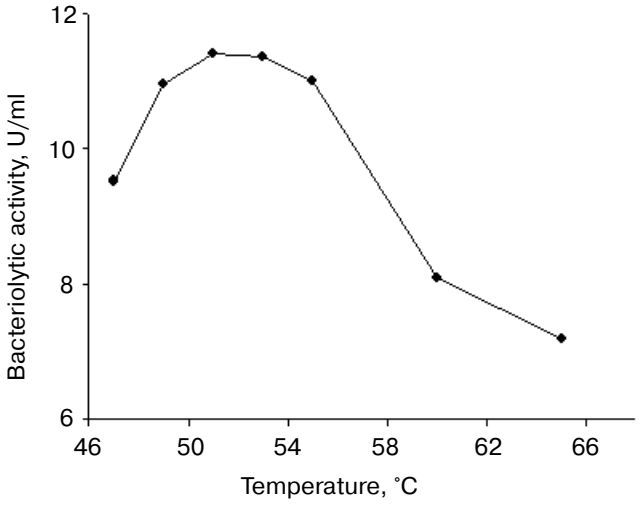

Fig. 5. Temperature dependence of the activity of enzyme L_4. The reaction was performed in 0.05 M Tris-HCl, pH 8.0, at different temperatures.

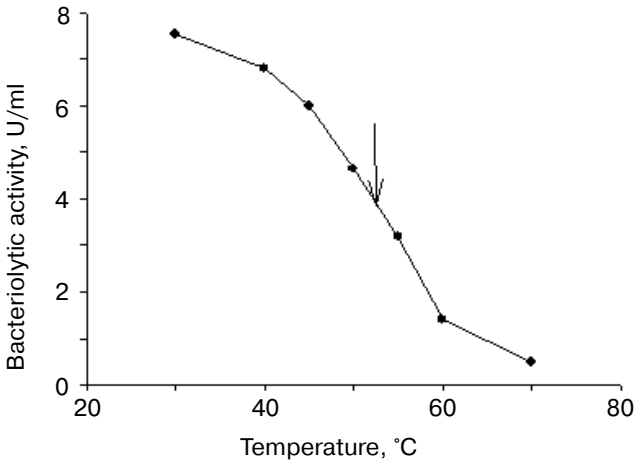

Fig. 6. Thermal stability of enzyme L_4. Samples of the enzyme in 0.05 M Tris-HCl buffer, pH 8.0 (0.1 ml) were incubated at different temperatures for 15 min, then cooled in ice, and the bacteriolytic activity of the samples was determined.

metalloenzymes), PMSF (an inhibitor of serine proteinases), and *p*-CMB (an inhibitor of thiol proteinases). To compare the properties of the enzymes, the effect of inhibitors on enzyme L_1 was also studied. The results are presented in Table 2. It is seen from the table that endopeptidases L_1 and L_4 of *Lysobacter* sp. XL1 are sensitive to the action of PMSF and *p*-CMB. The effect of PMSF on the activity of enzyme L_4 (40% inhibition at the maximal concentration of the inhibitor) exceeds the effect on enzyme L_1 (30% inhibition). The sensitivity of enzyme L_4 to *p*-CMB (70% inhibition) is also much higher than that of enzyme L_1 (20% inhibition at the maximal concentration of the inhibitor). Thus, endopeptidases L_1 and L_4 are serine proteinases containing thiol groups in the active sites. The enzymes L_1 and L_4 are EDTA-insensitive, this indicating that they are not metalloenzymes.

Thus, a new previously unstudied bacteriolytic enzyme L_4 was isolated from the culture liquid of *Lysobacter* sp. XL1, and some of its physical and chemical properties were investigated.

Comparison of the properties of enzyme L_4 with the properties of other extracellular bacteriolytic enzymes of *Lysobacter* sp. XL1 showed that each of the enzymes possesses a unique combination of properties, but these properties are very close in general. The differences concern the optimal concentration of the buffer, optimal temperature, and effect of the inhibitors. Unlike the activity of endopeptidases L_1 and L_4, the activity of amidase L_2 is inhibited by EDTA (the inhibitor of metalloenzymes) by ~40% [7, 8]. Endopeptidase L_1, like endopeptidase L_4, exhibits the highest activity in 50 mM Tris-HCl, but the shape of the curves of the dependence of the activity on buffer concentration differs significantly: a sharp peak in the case of endopeptidase L_4 and an extended peak in the case of endopeptidase L_1 [5]. The bacteriolytic amidase L_2 exhibits the highest activity at 5 mM concentration of the buffer [3, 7]. The optimal concentration of the working buffer for muramidase is very low: the enzyme exhibits the highest bacteriolytic activity at 0.1 mM concentration of the buffer, the activity being maximal in distilled water [3].

It is interesting to note a high optimal temperature for the lysis of *S. aureus* cells by the extracellular enzymes of *Lysobacter* sp. XL1. The optimal temperature is 50-55°C for endopeptidase L_4, 70°C for endopeptidase L_1 [3, 5], 65°C for N-acetyl muramoyl-L-alanine amidase L_2 [3, 7], and 60°C for muramidase [3]. This might be due to a protective action of the high-molecular-weight substrate, the cell walls of *S. aureus*.

Table 2. Effect of inhibitors on the activity of bacteriolytic enzymes L_1 and L_4 of *Lysobacter* sp. XL1

Inhibitor	Inhibitor concentration, M	L_1			L_4		
		activity		inhibition, %	activity		inhibition, %
		U/ml	%		U/ml	%	
EDTA	$1 \cdot 10^{-3}$	2.9	100	0	19.4	100	0
	$1 \cdot 10^{-2}$	2.8	100	0	19.1	100	0
PMSF	$1 \cdot 10^{-3}$	2.5	89	11	14.5	75	25
	$2.5 \cdot 10^{-3}$	2.0	71	29	11.0	57	43
p-CMB	$1 \cdot 10^{-4}$	2.9	100	0	10.6	55	45
	$1 \cdot 10^{-3}$	2.2	79	21	5.4	28	72
No	—	2.8	100	0	19.3	100	0

The half-inactivation temperature of the enzymes is rather high, 52°C for endopeptidase L_4, 55°C for endopeptidase L_1 [3, 5], 65°C for amidase L_2 [3, 7], and 62°C for muramidase [3].

The molecular weights of the extracellular lytic enzymes of *Lysobacter* sp. XL1 are very similar: 21 kD for L_4, 22 kD for L_1, 25 kD for L_2 [3], and 22.4 kD for muramidase [3]. The enzymes also exhibit their maximal activity at the same pH value [3, 5, 7].

Thus, it should be noted that although each of the bacteriolytic enzymes of lysoamidase possess its individual properties, they do not differ generally from the properties of most of bacteriolytic enzymes described in the literature: a low molecular weight (18-25 kD), basic pH optima, low optimal values of the ionic strength of the reaction mixture [16].

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