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Destabilase-Lysozyme of Medicinal Leech. Multifunctionality of Recombinant Protein

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Abstract—Preparation and purification of a recombinant protein are described along with characteristics of its specific (for ε-(γ-Glu)-Lys and D-dimer substrates) and nonspecific (for L-γ-Glu-pNA) isopeptidase activities; the absence of peptidase function for α-(α-Glu)-Lys substrate is noted. It is shown that the protein exhibits muramidase (cell walls of *Micrococcus lysodeikticus*) and specific glycosidase activities. The latter was determined towards the fluorogenic substrate 4-methylumbelliferyl-tetra-N-acetyl-β-chitotetraoxide. Antimicrobial activity of recombinant destabilase-lysozyme protein (recDest-Lys) and its 11-membered amphipathic peptide was revealed towards cells of the strict anaerobic Archaean *Methanosarcina barkeri*, whose cell walls contain no murein. Possible mechanisms of the effect of recDest-Lys on these cells are discussed.

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Key words: endo-ε-(γ-Glu)-Lys- and exo-ε-(γ-Glu)-Lys-isopeptidolysis, specific glycosidase activity, 4-methylumbelliferyl-N-acetyl-β-chitotetraoxide, muramidase activity, antibacterial activity, *Methanosarcina barkeri*

Destabilase-lysozyme of the medicinal leech is the first among lysozyme members of invertebrates which combines properties of endo-ε-(γ-Glu)-Lys isopeptidase (destabilase) and lysozyme (Dest-Lys). It was identified for the first time as destabilase within the salivary gland secretion and isolated as a total preparation [1]. Its action dissolves stabilized fibrin [1, 2] and cleaves D-dimer (a fragment of stabilized fibrin degradation) to monomers [3, 4] not by proteolysis but by isopeptidolysis of ε-(γ-Glu)-Lys bonds joining the monomers. This mechanism was demonstrated during observation of retention of integrity of D-dimer chains after incubation with Dest-Lys. Analysis of the N-terminal amino acid sequences upon conversion of D-dimer γ-γ-chains to monomers showed their complete identity [5]. It was shown in experiments on animals that such mechanism results in slow destruction of old, preliminarily formed thrombi [2], and therefore destabilase seems to not be an urgent thrombolytic enzyme. We showed in 2000 that a fragment of destabilase primary structure is homologous to a site of the polypeptide chain of one invertebrate lysozyme [6], which made it possible to reveal the lysozyme (muramidase) activity of destabilase upon destruction of *Micrococcus lysodeikticus* cell walls. However, the enzyme deprived of muramidase activity by long-term heating at 90°C blocks microbial growth, and this ability exceeds the activities of many known antibiotics [7]. Besides, lysozyme activity is revealed as specific glycosidase relative to N-acetyl-glucosamine hexamer [8].

The functions of Dest-Lys found by us show that the enzyme can be of practical clinical importance. Therefore, in recent years our efforts have been aimed at obtaining the recombinant form of multifunctional enzyme while accounting for results of previous investigations on detection and cloning of three genes encoding different destabilase forms – *Ds1*, *Ds2*, and *Ds3* [9]. In

Abbreviations: CM, cytoplasmic membrane; CW, cell wall; $(GlcNAc)_4$-MeU, 4-methylumbelliferryl-tetra-N-acetyl-β-chitotetraoxide; recDest-Lys, recombinant destabilase-lysozyme; SEM, scanning electron microscopy; TEM, transmission electron microscopy.

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this work a plasmid carrying the *Ds2* gene was constructed to obtain a Dest-Lys producing strain of *Escherichia coli*. Its expression with subsequent purification and renaturing of the recombinant protein from inclusion bodies yielded soluble electrophoretically homogenous recDest-Lys enzyme and allowed analysis of its biological functions – such as lysozyme (both muramidase and specific glycosidase), isopeptidase, peptidase, and antimicrobial. The fluorogenic substrate 4-methylumbelliferryl-tetra-N-acetyl-β-chitotetraoxide ($(GlcNAc)_4$-MeU) was used for determination with high sensitivity of the specific glycosidase activity of the enzyme [10]. Antimicrobial activity of recDest-Lys and its polypeptide fragments was determined towards the anaerobic archae *Methanosarcina barkeri* whose cells are covered by unique walls, largely of protein nature and free of murein, while the cytoplasmic membrane has unique structure characteristic only of Archaeans [11].

MATERIALS AND METHODS

Preparation of recDest-Lys. Plasmid (courtesy of E. V. Snezhkov, Institute of Bioorganic Chemistry, Russian Academy of Sciences (IBCh, RAS)) containing the Dest-Lys *Ds2* gene was used to transform *E. coli* strain JM-110. The transformation mixture was plated on solid agar-containing medium with ampicillin (30 μg/μl). After 16-18 h, 10-12 colonies grown on selective medium were placed into 300-1000 ml nutrient medium TB heated in advance to 37°C and cultivated in an orbital thermostatically controlled shaker at 37°C and 200 rpm to A_{600} = 0.8-1.0. Then isopropyl-β,D-thiogalactopyranoside was added to 0.1 mM concentration and cultivation was continued at 30°C for 2-4 h.

Escherichia coli cells containing already produced chimeric recombinant protein were sedimented by centrifugation at 3000*g* for 20 min. The pellet was washed with 1/3 volume of physiological solution, centrifuged for 20 min at 3000*g*, and resuspended in 10-30 ml buffer (25 mM Na phosphate, pH 7.4, 400 mM NaCl). The cells were broken by sonication (22 kHz, four times, 20 sec each), insoluble fraction was removed by centrifugation at 50,000*g* for 45 min, and the cell extract underwent further purification.

Amphipathic Dest-Lys fragments. Amphipathic Dest-Lys peptide fragments corresponding to residues 67-77 (fragment 1), 85-92 (fragment 2), and 105-112 (fragment 3) of the protein primary structure [10] were revealed by analysis of its secondary structure [12]. Then these fragments were synthesized (99% purity) in the Laboratory of Peptides, IBCh, RAS.

Endo-isopeptidase activity. The recDest-Lys monomerizing (endo-isopeptidase) activity towards D-dimer was determined electrophoretically by appearance of D-monomer after incubation with standard D-dimer preparation from bovine blood (HyTest, Finland) as described earlier [3]. Preparations of BSA (Sigma, Germany) and bovine fibrinogen (Bacpreparations Enterprise, Lithuania) were used as controls.

Exo-isopeptidase and peptidase activities. The recDest-Lys exo-isopeptidase and peptidase activities towards di-isopeptide ε-(γ-Glu)-Lys and dipeptide α-(α-Glu)-Lys were determined by TLC. Samples of 30 μl containing enzyme (0.18 mg/ml), 0.05 M Tris-HCl, pH 7.4, 0.01 M $CaCl_2$, 0.15 M NaCl, and ε-(γ-Glu)-Lys (Sigma) or α-(α-Glu)-Lys (Bachem AG, Switzerland) (1 mg/ml) were prepared. Similarly prepared samples but containing Glu or Lys (Sigma) (1 mg/ml) instead of substrates were used as controls. Samples were incubated at 22°C. Before beginning incubation, 7 μl aliquots were taken from each sample and then after 24, 72, and 192 h of incubation. Then 0.7 μl 50% TCA (final concentration 5%) was added to each sample, and they were left on ice for 30 min and centrifuged for 10 min at 10,000*g*. Supernatants were taken and applied onto Fixion plates (Reanal, Hungary) onto a point in a volume of 4 μl. Thin layer chromatography was carried out in 0.4 M Na citrate buffer, pH 3.3. Reaction products were developed with 1% ninhydrin solution in acetone.

Amidolytic activity. Amidolytic activity of recDest-Lys towards L-γ-Glu-pNA was determined spectrophotometrically at 405 nm by cleavage of *p*-nitroaniline after incubation with enzyme for 20 h at 22°C as described earlier [13]. Incubation samples of 250 μl contained enzyme (0.056 mg/ml), 0.03 M Tris-HCl, pH 8.0, 6 mM $CaCl_2$, and L-γ-Glu-pNA (Bachem AG) from 0.04 to 0.2 mM. No enzyme was present in control samples. Kinetics of enzymic hydrolysis of L-γ-Glu-pNA was analyzed from double reciprocal values.

Lysozyme (muramidase) activity. It was determined nephelometrically by clarification of bacterial (*M. lysodeikticus*) cell wall suspension (MP Biomedicals, LLC, France) after incubation for 20 min at 37°C by the earlier described method [6]. The recDest-Lys concentration in the incubation mixture was 6 μg/ml.

Specific hydrolase activity. Specific glycosidase activity of recDest-Lys and egg lysozyme towards fluorogenic substrate was determined according to Yang and Hamaguchi [10]. A 0.1-ml sample of recDest-Lys (0.1-0.5 mg/ml) or egg lysozyme (4-6 mg/ml) (Fluka, Switzerland) was added to 0.05 ml $(GlcNAc)_4$-MeU (Toronto Research Chemical Inc., Canada) solution in 20 mM Na-acetate buffer, pH 5.2, and incubated for 1 h at 37°C. The reaction was stopped by addition of 1 ml glycine-carbonate buffer, pH 10.4-11.5. Fluorescence (*F*) was measured on a Perkin Elmer LS 55 spectrofluorimeter (Great Britain) at 365 nm (excitation) and 450 nm (emission), slit width 4.5 nm. The amount of released 4-methyl-umbelliferone (MeU) was determined using a calibration curve. Kinetic constants were determined from double reciprocal values.

Antimicrobial activity. Antimicrobial activity of recDest-Lys and its peptide fragments towards Archaean cells was determined by inhibition of *M. barkeri* growth in liquid medium in the presence of the protein and peptides [14]. One of best studied Archaeans, the strictly anaerobic *M. barkeri* DSMZ 804 was used as the standard culture. The culture was grown using Hangate's technique in medium of the following composition (g/liter): KH_2PO_4, 0.53; $MgSO_4 \cdot 7H_2O$, 0.43; NaCl, 0.43; NH_4Cl, 0.4; $CaCl_2 \cdot 2H_2O$, 0.053; $FeSO_4 \cdot 7H_2O$, 0.002; yeast extract, 1; Na acetate, 1; Na formiate, 2; $NaHCO_3$, 4; cysteine-$HCl \cdot H_2O$, 0.5; $Na_2S \cdot 9H_2O$, 0.5; mixture of fatty acids (g/liter): valeric acid, 0.5; isovaleric acid, 0.5; 2-methylbutyric acid, 0.5; isobutyric acid, 0.5; distilled water, 20 ml, pH 6.7-7.0. Antibiotic effect of recDest-Lys and its fragments was judged by inhibition of growth of the Archaean (by measuring optical absorbance of cell suspension at 600 nm) one day later at 37°C in liquid medium containing (10% by volume) protein and peptide solutions in the following buffer: 0.1 M Tris-acetate, pH 8.1, 10 mM GSH, 1 mM GSSG. Buffer was used as control.

To obtain an electron-microscopic pattern of the effect of recDest-Lys on *M. barkeri* cells, a 1-day-old cell culture was incubated in advance at 37°C with enzyme solution (35 μg/ml) for 15, 30, and 60 min, and then fixed in 2.5% glutaraldehyde for 60 min. After 60 min incubation with the protein, the cells were prepared for examination in a scanning electron microscope after dehydration of the cells in a series of solutions of increasing alcohol concentrations, finally in absolute acetone, and then critical point drying [15]. The sample was shadowed with platinum in a LKB (Sweden) device. The shape and surface of the sample were studied using a CamScan (Great Britain) microscope at accelerating voltage 20 kV.

For analysis of cell slices in a transmission electron microscope (TEM) after cell fixation by glutaraldehyde and additional fixation by 2% aqueous solution of OsO_4, sections were dehydrated in a series of increasing alcohol concentration and impregnated with resins using a known method [15]. Contrasting of obtained sections was carried out by 1% uranyl acetate and Reynolds reagent. Sections were examined in a TEM (Geol, Japan) at 80 kV accelerating voltage.

RESULTS

Purification of recombinant protein. Cell extract containing recombinant protein was centrifuged and for subsequent denaturing metal-affinity chromatography the pellet was resuspended in buffer (25 mM Na-phosphate buffer, pH 7.4, 400 mM NaCl, 20 mM imidazole, 8 M urea, 5 mM β-mercaptoethanol). For complete dissolution of inclusion bodies, the resuspended pellet was incubated in the same buffer for 1 h with vigorous mixing. Then the insoluble fraction was separated by centrifugation at 50,000*g* for 45 min. The supernatant was filtered through a nylon filter (pore diameter 0.45 μm) and treated by metal-affinity chromatography under denaturing conditions.

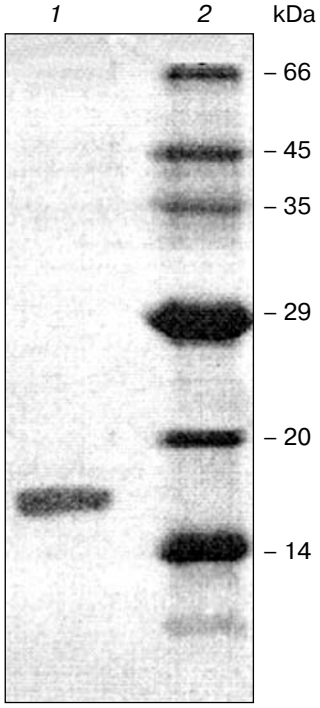

Fig. 1. Electrophoregram of purified renatured protein preparation recDest-Lys: *1*) 25 μl of the preparation; *2*) molecular mass markers.

The sample was fractionated using an AKTA FPLC chromatographic device (Amersham Biosciences, USA) using a Tricorn 10/50 column (Amersham Biosciences) containing 3 ml Ni-Sepharose FastFlow (Amersham Biosciences). The above-mentioned buffer was used initially, and then the sample was eluted in a step-wise imidazole concentration gradient (20, 100, 200, 300 mM) in the same buffer. Eluate fractions with $A_{280} > 0.05$ were collected and analyzed by PAGE under denaturing conditions to reveal fractions with the least amount of contaminants. The Dest-Lys was renatured by rapid 20-fold dilution with buffer (0.1 M Tris-acetate, pH 8.1, 10 mM GSH, 1 mM GSSG) with intensive mixing. The resulting solution was centrifuged at 5000*g* for 10 min to remove un-renatured protein. The supernatant containing renatured Dest-Lys preparation was concentrated 4-6-fold using a Microcone microconcentrator (Millipore, USA). The resulting preparation was analyzed by denaturing PAGE (Fig. 1).

Isopeptidase function of recDest-Lys. *D-dimer monomerizing activity*. Figure 2 (lanes *1* and *2*) shows D-dimer monomerization. For fibrinogen and BSA, SDS-

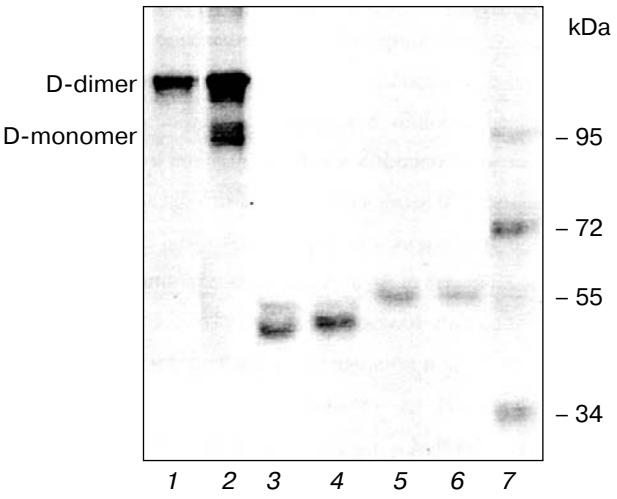

Fig. 2. Effect of recDest-Lys on protein substrates during incubation for 20 h at 22°C. Original preparations: D-dimer from bovine blood (*1*), bovine fibrinogen (*3*), BSA (*5*); the same preparations after incubation with recDest-Lys (*2*, *4*, *6*, respectively); *7*) molecular mass markers.

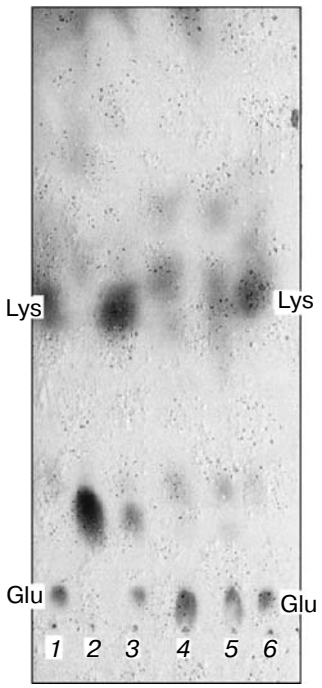

Fig. 3. TLC of products of 24 h incubation of recDest-Lys (at 22°C) with ε-(γ-Glu)-Lys (*3*) and with α-(α-Glu)-Lys (*5*) compared to original components (*2* and *4*, respectively); *1* and *6*) mixture of glutamic acid with lysine.

electrophoresis did not reveal any changes. This points to selectivity of the effect of recDest-Lys on hydrolysis of isopeptide bonds joining monomers in D-dimer as we showed previously.

Exo-isopeptidase and *peptidase activities*. The existence of exo-isopeptidolysis is supported by the results shown in Fig. 3. Isopeptide ε-(γ-Glu)-Lys (lane *2*) is hydrolyzed to appropriate amino acids (lane *3*), whereas α-(α-Glu)-Lys is unchanged during its incubation with recDest-Lys (lanes *4* and *5*). In this case contaminants in the dipeptide preparation also remain unchanged.

Amidolytic activity. An indirect indication of isopeptidolysis is also the amidolytic activity of recDest-Lys towards L-γ-Glu-pNA. The pH dependence of this activity shown in Fig. 4 indicates that maximal amidolytic activity of the enzyme is revealed at pH 6.2.

Kinetics of L-γ-Glu-pNA hydrolysis by recDest-Lys was measured at optimal pH value 6.2 and temperature 22°C (Table 1).

Lysozyme activity of recDest-Lys. *Specific glycosidase activity towards fluorogenic substrate (GlcNAc)$_4$-MeU.* Kinetic constants characterizing both enzyme functions of recDest-Lys, specific lysozyme activity towards (GlcNAc)$_4$-MeU and amidolytic activity towards L-γ-Glu-pNA, are shown in Table 1. Egg lysozyme was used as a positive control in determination of lysozyme activity. Comparison of K_m values shows that in the case of (GlcNAc)$_4$-MeU hydrolysis by recDest-Lys the latter exhibits 7.5-fold lower affinity to this substrate compared to egg lysozyme. Comparison of k_{cat} values is indicative of low efficiency of the hydrolysis of this substrate by both enzymes. It is also seen that amidolytic and specific glycosidase substrates are characterized by similar K_m values.

Muramidase activity towards M. lysodeikticus cell walls. The revealed dependence of the muramidase activity of recDest-Lys on cell wall concentration is shown in Fig. 5. Linear dependence exists practically to the concentration of 4 mg/ml.

Antimicrobial activity of recDest-Lys. *Nephelometric analysis of effects of recDest-Lys and its amphipathic fragments on growth of M. barkeri cells.* Antibiotic effect of the recombinant protein and its amphipathic fragments on

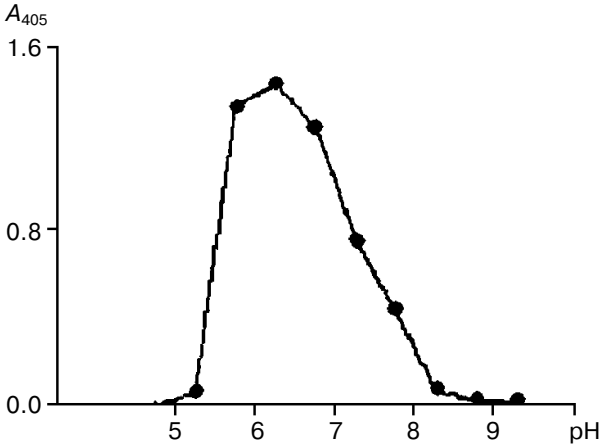

Fig. 4. pH-dependence of recDest-Lys amidolytic activity.

Table 1. Kinetic characteristics of enzymes

Enzyme	Substrate	$K_m \times 10^{-4}$, M	$k_{cat} \times 10^{-4}$, sec^{-1}	Activity type
RecDest-Lys	$(GlcNAc)_4$-MeU	0.6	2	specific glycosidase
Egg lysozyme	$(GlcNAc)_4$-MeU	0.08	0.21	–"–
RecDest-Lys	L-γ-Glu-pNA	0.5	9	amidolytic

growth of *M. barkeri* cells was judged by the decrease in optical absorption of a cell suspension at 600 nm after 1-day-long cultivation at 37°C. These results are shown in Table 2.

RecDest-Lys causes practically complete cell lysis. In this case formation of a characteristic pellet (flocculation) is indicative of disturbance of normal physiological state of the culture, most cells of which underwent lysis. Amphipathic peptide fragments of Dest-Lys appeared to be significantly less efficient even at concentrations tenfold exceeding the concentration of recDest-Lys: fragment 1 two times decreased optical absorption of the Archaean cell suspension compared to control, fragment 3 decreased it by less than one third, while fragment 2 had practically no antimicrobial effect.

Electron microscopy. Peculiarities of Archaean cell destruction by recDest-Lys were studied in scanning (SEM) and transmission (TEM) electron microscopes. Figure 6 shows the Archaean cell SEM images before and after incubation with recDest-Lys (35 μg/ml) at 37°C for 1 h.

Analysis using TEM (Fig. 7) revealed the state of cells at different stages of lysis. An electron-transparent layer of cell walls (Fig. 7, control) has low, approximately even thickness. The cytoplasm is almost homogeneous. The nucleoid and conglomerates of ribosomes are partially seen. It is important that due to dissociation characteristic of populations of various prokaryote species, these conglomerates always contain cells with different wall thickness and different sensitivity to antimicrobial activity of analyzed compounds [16] including recDest-Lys. Because of this, the pattern of cell population lysis is not homogeneous, and cells at different stages of lysis are seen in Fig. 7. Gradually the amount of completely lysed cells increases, and clumps of cells most resistant to antimicrobial activity are lysed last. Since cells are accumulated in conglomerates, destruction of their cell walls (CW) and cytoplasmic membranes (CM) is more intensive in the outer regions of the conglomerates.

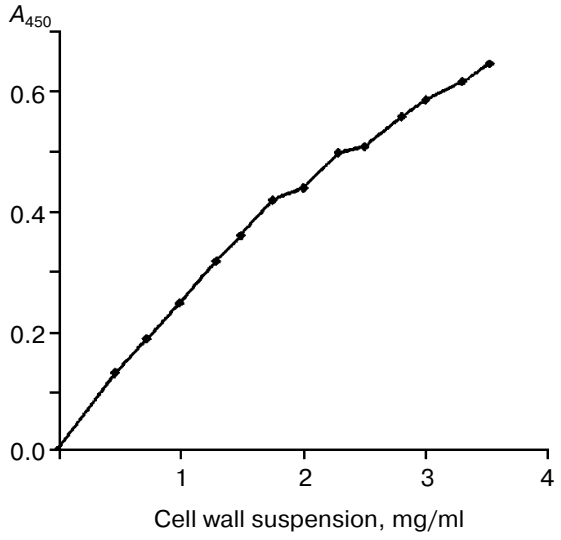

Fig. 5. Dependence of recDest-Lys muramidase activity on concentration of *M. lysodeikticus* cell walls.

Table 2. Inhibition of *M. barkeri* growth by recDest-Lys and its synthetic fragments

Protein or fragment solution added to the medium (to final concentration, μg/ml)	Archaean growth 1 day later, A_{600}
Control	0.73 ± 0.1 (n =3)
RecDest-Lys (10)	0.11 ± 0.05 (n =3)
Fragment 1 (100)	0.38 ± 0.05 (n =3)
Fragment 2 (100)	0.71 ± 0.1 (n =3)
Fragment 3 (100)	0.61 ± 0.09 (n =3)

Patterns of sections of cells lysed by recDest-Lys are described below. Figure 7 (*1*) shows the beginning of cytoplasmic granularity and cell wall loosening. The section of a cell conglomerate (Fig. 7, *2*) clearly shows cell wall loosening, especially intensive at the outer side of the conglomerate, enhancement of cytoplasmic granularity, and occurrence of invaginations in the CM.

A section of a large cell conglomerate (Fig. 7, *3*) shows different stages of CW loosening (significant outside the conglomerate and almost unnoticeable inside it), and in some cells vacuoles of different sizes emerge in a relatively homogeneous cytoplasmic layer. The cytoplasmic granularity and CM invaginations are seen on sections of

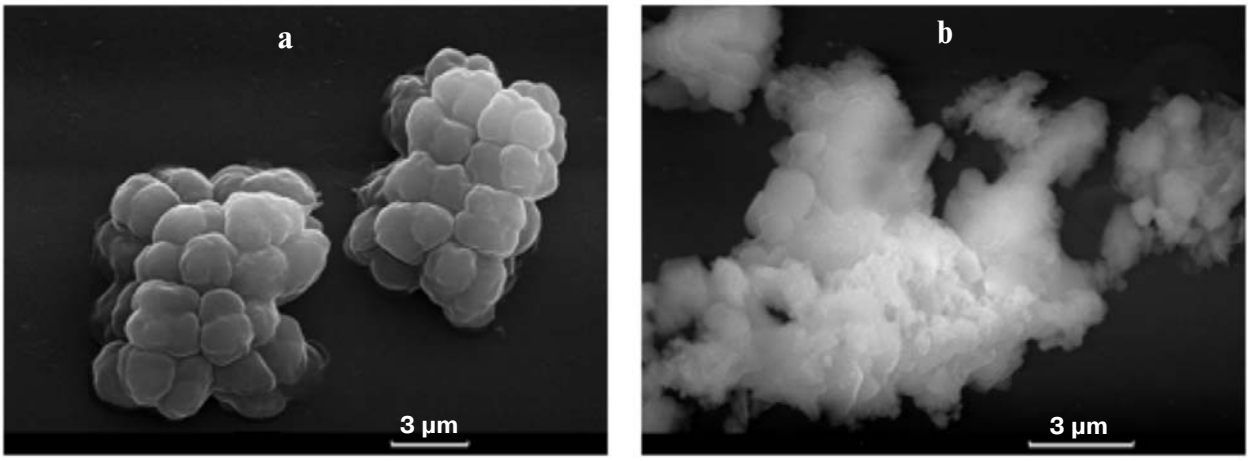

Fig. 6. SEM of *M. barkeri* cell conglomerates before (a) and after (b) incubation with recDest-Lys. Lysis of most of the cells in the conglomerates is observed.

cells arranged outside a conglomerate. Cell wall around some outer cells, localized on the right in the figure, underwent lysis, and around other cells the CW was loosened to different extents. It is seen in the section shown in Fig. 7 (*4*), where just shadows remain of practically fully lysed CW. There is significant cytoplasm clarification, which can be due to distortion of CM permeability. In the upper right segment of the cell section there are regions of CM destruction, while on other sections formation of circular membrane structures in the cytoplasm is seen. Shadows of lysed CW are seen only around the cell section on the left (Fig. 7, *5*). The right section shows significant breaks in the CM, and the cytoplasm has significant clarified regions and pronounced granularity in other places, probably due to local conglomerates of ribosomes. Some such conglomerates are restricted by CM invaginations. Divergence of the cell conglomerate was found.

Figure 7 (*6*) shows fragmentation of remaining regions of lysed cells with practically complete absence of CW around them. Residues of granularity are retained. The CM is broken in many places, and cell regions are fragmented, which is seen on the lower left; within such fragments there are sometimes circular CM structures. Figure 7 (*7*) shows lysis of a great number of conglomerated cells, residues of CW and CM shadows; there are cells containing numerous small circular CM structures. Cytoplasmic clarification is observed, but in some regions granularity is still retained. The enhanced blur of the cell section contours due to their lysis is seen, but most cell conglomerates by that time are already completely lysed.

DISCUSSION

Destabilase-lysozyme of the medicinal leech salivary gland secretion (SGS) was for a long time the only known example of a multifunctional enzyme exhibiting isopeptidase, lysozyme, and antimicrobial functions. In 2003 there appeared data on structure and properties of a lysozyme isolated from the marine bivalve mollusk *T. japonica* [17] that revealed its endo-isopeptidase activity [18]. It was shown in our early experiments [2] that isopeptidolysis is a manifestation of the SGS thrombolytic activity that can be realized by the leech not so much during sucking up the blood, but rather during storage of the blood in the intestine due to destruction of developing blood clots. From the very beginning of our work with destabilase, our goal (that remains the priority even now) was production of a thrombolytic drug on the basis of recombinant Dest-Lys enzyme. Similarly to us, Japanese authors suggest the use of recombinant protein as a thrombolytic drug [19].

Results of this work document a homogeneous recombinant protein on the basis of one gene (*Ds2*) of the destabilase-lysozyme family [9] (Fig. 1) exhibiting isopeptidase activity (Figs. 2-4). It is important that the enzyme first of all breaks endo-isopeptide bonds in D-dimer and catalyzes hydrolysis of ε-(γ-Glu)-Lys bond in di-isopeptide (Fig. 3) without causing degradation of fibrinogen and serum albumin (Fig. 2) and cleavage of dipeptide α-(α-Glu)-Lys (Fig. 3). Indirect evidence in favor of isopeptidase activity is the ability of the recombinant enzyme to catalyze hydrolysis of chromogenic substrate L-γ-Glu-pNA with $K_m = 0.05$ mM. This K_m value is close to that obtained upon analysis of amidolytic activity of native destabilase preparation (0.22 mM [3]) (Table 1). For L-γ-Glu-pNA, $k_{cat} = 0.9 \cdot 10^{-3}$ sec^{-1} and the ratio $k_{cat}/K_m = 18$ M^{-1}·sec^{-1}, which is indicative of low efficiency of the hydrolysis of this substrate by Dest-Lys. We determined earlier that the active enzyme content in electrophoretically homogeneous preparation was 77.4% of enzyme content calculated spectrophotometrically [13].

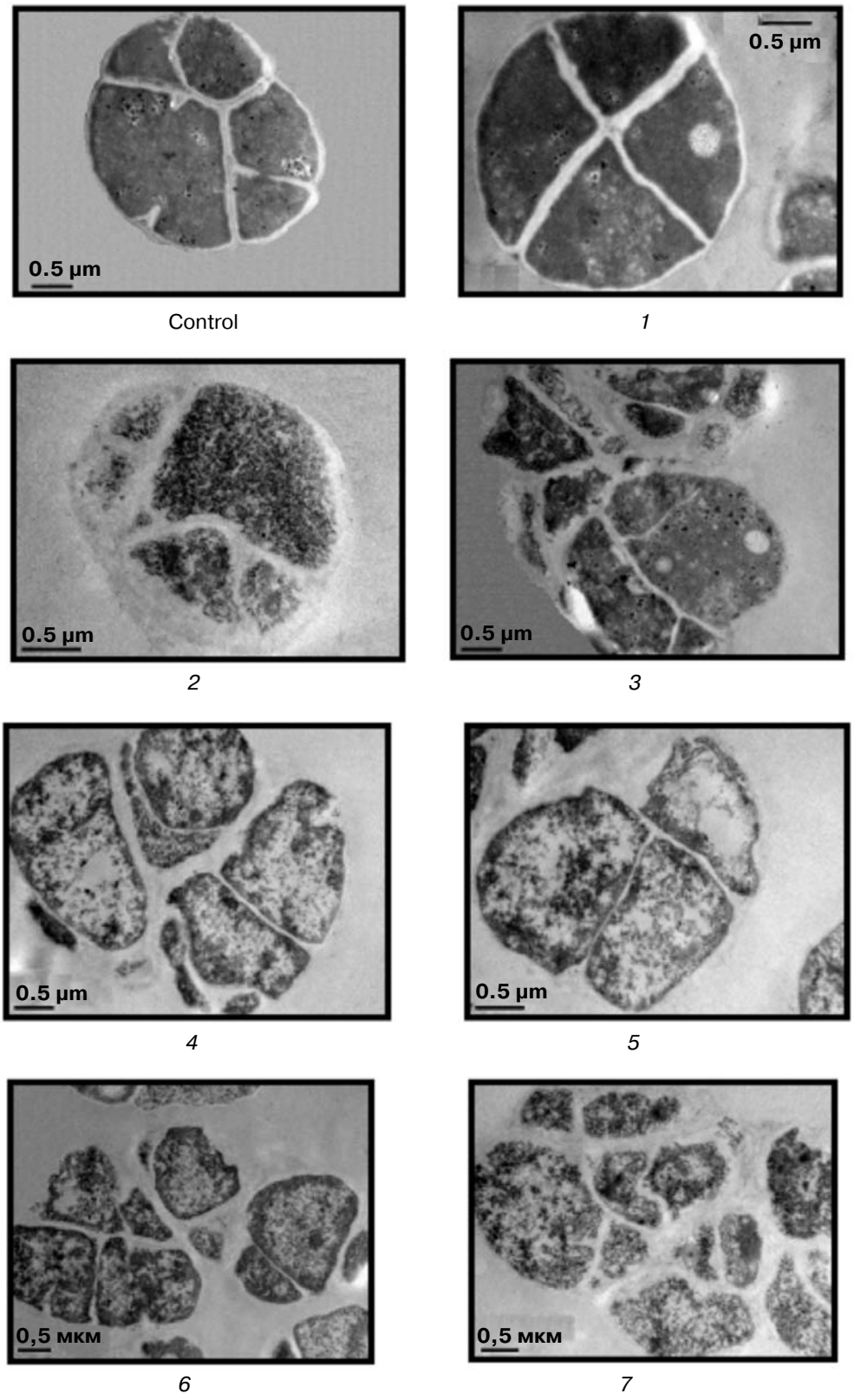

Fig. 7. TEM of sections of *M. barkeri* cell suspension after incubation at 37°C with recDest-Lys. Control, section of cell a conglomerate; *1-3*) lysed cells after incubation with recDest-Lys for 15 min; *4-6*) cells lysed by recDest-Lys after incubation for 30 min; *7*) debris of the lysed cell population after incubation with recDest-Lys for 1 h.

These data suggest that low efficiency of L-γ-Glu-pNA hydrolysis by Dest-Lys is due to the specificity of the enzyme.

We have studied lysozyme activity of recDest-Lys as a specific glycosidase towards fluorogenic substrate $(GlcNAc)_4$-MeU described in 1980 [20], but this substrate was not widely used in estimation of the glycosidase activity of lysozymes. The K_m value of recDest-Lys towards this substrate (0.06 mM) is almost one order of magnitude lower than that for egg lysozyme (0.008 mM), which is indicative of some structural difference between the active centers of these enzymes. At the same time, the similarity of received data to K_m values in single-substrate enzymic reactions, unlike more highly specific constants in bi-substrate reactions, might point to a mechanism of independent functioning of the Dest-Lys lysozyme and amidolytic active centers. Both enzymes exhibit extreme inefficiency towards $(GlcNAc)_4$-MeU hydrolysis, but our data correlate with those in the literature where the following parameters were determined with this substrate for egg lysozyme: $k_{cat} = 1.13 \cdot 10^{-4}$ sec^{-1} and $K_m = 0.0026$ mM [10]. Some difference in k_{cat} values can be explained by a slight modification of the method of the egg lysozyme activity determination (temperature and pH) optimized in our works for the recDest-Lys activity. It is necessary to take into account that it is impossible to evaluate the actual amount of active recombinant enzyme in the case of comparative estimation of rates of such glycosidase reaction of recombinant enzyme and egg lysozyme. The same should also be considered upon estimation of the recDest-Lys lysozyme muramidase activity towards *M. lysodeikticus* cell walls. Our data (Fig. 5) show that the rate of cell wall hydrolysis at the recDest-Lys concentration 6 μg/ml is comparable with that of egg lysozyme concentration 0.8 μg/ml. It should be noted that recombinant Dest-Lys, earlier obtained by us from cell extracts of *Spodoptera frugiperda* infected with baculovirus carrying the *Ds2* cDNA, exhibited both glycosidase and chitinase activities. This was revealed in the hydrolysis of the N-acetyl-glucosamine hexamer not only to di- and tetramers, but formation of a trimer characteristic of chitinase action was registered as well [7].

Earlier we demonstrated antimicrobial effect of native enzyme deprived of muramidase activity by prolonged heating at 90°C [7]. It was expressed in the ability of Dest-Lys to inhibit growth of *E. coli* and *Micrococcus luteus*. Since the primary structure of Dest-Lys does not contain the HLH (helix–loop–helix) fragments that destroy cell membranes by penetrating them similarly to a hairpin [21], which provides for the antimicrobial activity of human and chicken egg lysozyme, we supposed that the antimicrobial effect of Dest-Lys can be provided by α-helical amphipathic peptides within the primary structure of the enzyme. It has been shown that such peptides, due to multistep interaction with the cell wall regions of cytoplasmic membrane of Gram-negative bacteria, cause perforation of the membrane accompanied by antimicrobial effect. At the same time their effect on membrane potential of Gram-positive bacteria is rather pronounced [22, 23]. Antimicrobial activity of lysozymes, independent of their muramidase activity, is reported in a number of recent publications [24-26]. For analysis of the antimicrobial effect of recDest-Lys, in this work we have used *M. barkeri* cells (a traditional model object for studying Archaeans whose CW consist mainly of proteins and of heteropolysaccharides and are free of both murein and pseudomurein). As known, Archaean cells differ from cells of the procaryotic and eukaryotic domains first of all by the chemical composition and structure of their CM [11]. Lipids of Archaean CM are formed by glycerol residues in L-form (sometimes by residues of a different alcohol such as nonitol) and joined by ether bonds to branched isoprene chains. Such ether bonds are more resistant to effects of oxygen, high temperatures, and some other factors compared to ester bonds in the CM lipids of other living beings. The Archaean CM can consist of mono- and/or bilayer formed by unique lipids. Structural variability of Archaean membranes is also provided by the possibility of different joining the isoprene molecules as well as by their ability for tetraether formation and various cyclizations [11, 27]. Unique lipids structurally similar to lipids of eukaryotic membranes also appear in Archaean CM. Thus, in *M. barkeri* one of main CM lipids, glucosaminylarchetidyl inositol, is similar to glucosaminphosphatidyl inositol that is important for eukaryotic cells [28]. Methanochondroitin was found among the heteropolysaccharides of *M. barkeri* cell wall [29].

Results presented in Figs. 6 and 7 show that recDest-Lys causes rapid lysis of *M. barkeri* cell conglomerates without involvement of enzymic (glycosidase) recDest-Lys activity whose substrate is murein. Note that the synthetic 11-membered peptide (fragment 1, Table 2), an amphipathic fragment of Dest-Lys, exhibits more than one order of magnitude lower lytic activity. Thus, antimicrobial (muramidase-independent) effect of Dest-Lys and its amphipathic fragments on Gram-positive and Gram-negative bacteria (*M. luteus* and *E. coli*) [7] is observed along with effect on the member of a unique Archaean domain, the strict anaerobe *M. barkeri*.

It can be supposed that the effect of recDest-Lys on *M. barkeri* cells is due to total effect dependent on both properties and characteristics of the enzyme itself and of chosen conditions of recombinant enzyme interaction with cells:

– first, immediate effect of the amphipathic fragments of the protein on the cell wall and perforation of *M. barkeri* CM via interaction of its hydrophilic positively charged fragments with negatively charged regions of the lipid layer;

– second, enzymatic degradation of the *M. barkeri* cell wall proteins by recombinant enzyme, transformed to

serine protease as occurs with native Dest-Lys [30] in the multicomponent medium used for *M. barkeri* growth;

– third, it is a possibility that fragments of *M. barkeri* cell wall proteins contain isopeptide bonds of endo-ε-(γ-Glu)-Lys hydrolyzed by recDest-Lys;

– fourth, another possible mechanism resulting in lysis of *M. barkeri* cells is that interaction of the amphipathic fragment of the enzyme with the cell wall can alter the rest of the recDest-Lys molecule, which will result in alteration of its properties and, due to cooperativity, will enhance perforation of the cell wall (including the above-described mechanisms).

Pore formation in bacterial membrane is a multistep process including joining to the membrane, incorporation into the membrane, and oligomerization. This is supported by the ability of lysozyme fragments relieving membrane potential and ion gradients of Gram-negative bacteria, although the effect on membrane potential of Gram-positive bacteria is more pronounced [23]. Unlike the Dest-Lys protein, its 11-membered peptide fragment (fragment 1, Table 2) is not able to exert such concentrated effect on cell wall, which is why its effect is not very marked. The cell wall destruction and CM perforation upon lysis of *M. barkeri* by recDest-Lys are shown adequately in Figs. 6 and 7.

So, the ability of recombinant Dest-Lys to exhibit not only isopeptidase and lysozyme activities similarly to native enzyme, but also to exert antimicrobial effect by destroying cells of the strict anaerobic Archaean *M. barkeri* was demonstrated.

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