PHYSICAL AND CHEMICAL PROPERTIES OF RECOMBINANT KPP10 PHAGE LYSINS AND THEIR ANTIMICROBIAL ACTIVITY AGAINST *PSEUDOMONAS AERUGINOSA*

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One of the most promising approaches to combatting multiple drug resistance is based on the use of recombinant endolysins. In this work we study physical, chemical and lytic properties of phage KPP10, a recombinant endolysin effective against *Pseudomonas aeruginosa* at concentrations of 3 μg/ml. Fused with a fragment of positively charged SMAP-29 peptide into one reading frame, artilysin KPP10 is effective against laboratory strains and clinical isolates of *Pseudomonas aeruginosa* in the absence of permeabilizers. Our findings encourage preclinical trials of this artilysin in infectious models.

Keywords: nosocomial infections, antimicrobial resistance, bacteriophages, lysins of bacteriophages, phage KPP10, artilysin KPP10

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ФИЗИКО-ХИМИЧЕСКИЕ СВОЙСТВА И ПРОТИВОМИКРОБНАЯ АКТИВНОСТЬ РЕКОМБИНАНТНОГО ФАГОЛИЗИНА БАКТЕРИОФАГА KPP10, ДЕЙСТВУЮЩЕГО НА *PSEUDOMONAS AERUGINOSA*

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Одним из наиболее перспективных современных подходов для преодоления множественной лекарственной устойчивости можно назвать применение рекомбинантных эндолизинов. Нами получены данные о физико-химических и литических свойствах бактериолизина фага KPP10 — рекомбинантного эндолизина, который эффективно действовал на штаммы *Pseudomonas aeruginosa* в концентрации от 3 мкг/мл. Слитый в одной рамке трансляции с фрагментом положительно заряженного пептида SMAP-29, артилизин KPP10 действует на лабораторные штаммы и клинические изоляты *Pseudomonas aeruginosa* без использования пермеабилизаторов. Полученные результаты позволяют рассчитывать на успешное проведение доклинических испытаний эффективности артилизина AL-KPP10 с использованием инфекционных моделей.

Ключевые слова: внутрибольничные инфекции, антибиотикорезистентность, бактериофаги, эндолизины бактериофагов, фаг KPP10, артилизин AL-KPP10

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The alarming rate of antibiotic resistance is one of the major concerns of contemporary healthcare. The number of bacterial strains with multiple or extensive resistance keeps growing. Some of them do not respond to treatment with otherwise highly effective medications, including drugs of last resort; some become totally resistant “superbugs” causing persistent infections to any used antibiotic. Current approaches to the therapy of bacterial infections cannot handle the spreading variety of rapidly emerging mechanisms of antibiotic resistance. A temporary solution lies in the use of improved approaches to

standard therapies based on known antibiotics. However, in the long run we need novel antibacterial agents with a reduced resistance potential.

A possible alternative to antibiotics and synthetic antibacterial agents comes in the form of bacteriophages that have been long used to combat infections [1, 2]. Bacteriophages offer a few advantages, including the ability to auto-dose depending on a bacterial titer, very little impact on the normal microbiota, low resistance potential, compatibility with antibiotics, the ability to destroy biofilms, rapid discovery, and low cost; a therapeutic effect of a bacteriophage can be achieved with a single or low dose, and phages are relatively environmentally friendly [3]. Still there are downsides to their clinical application. A pure phage isolate is difficult to obtain. Its pharmacokinetic properties need to be carefully controlled and normalized throughout the entire storage period. Besides, to produce a target phage host strains/species closely related to the pathogen of interest often have to be cultured in the absence of effective standard protocols under strict safety requirements. Also, bacteriophages can carry genes of bacterial toxins and harbor certain resistance potential. Lastly, phages are highly immunogenic, meaning that they will have a weaker therapeutic effect if used repeatedly [3–5].

Phage lysins (endolysins) constitute a new class of antibacterial agents free of the abovementioned limitations. Phage lysins are enzymes encoded by bacteriophages that are capable of degrading the peptidoglycan layer of the bacterial cell wall. Over the course of their lifecycle, phages secret these enzymes to facilitate phage DNA passage into the bacterial cell and to aid release of new virions from the infected bacterium into the surrounding environment. The lytic activity of their catalytic domains allows phage lysins to cleave peptidoglycan bonds in the bacterial cell wall, which eventually leads to hypotonic lysis of the infected bacterium caused by the difference of the osmotic pressures inside and outside the cell [6].

Phage lysins have a few indisputable advantages over other antimicrobial agents. First, they are selective and attack only certain bacterial species while sparing the normal microbiota. Second, phage lysins ensure rapid lysis as they do not rely on slow metabolic reactions. Therefore, phage lysin-based therapies may not take as much time as standard antibacterial treatments. Third, the risk of developing resistance to phage lysins is low. Phage lysins target specific molecules crucial for the normal life cycle of the host, rendering emergence of a resistant isolate highly improbable as it would have to be accompanied by the massive rearrangement of the bacterial cell wall. Fourth, phage lysins can kill antibiotic-resistant bacterial strains providing a solution to one of the most pressing problems of contemporary healthcare. Fifth, due to their capacity to destabilize the peptidoglycan layer of the cell wall, phage lysins can both kill metabolically active or latently rested cells and access bacterial cells hidden by biofilms [7].

One of the most dangerous causative agents of nosocomial infections is *Pseudomonas aeruginosa*. It causes a variety of different infections which are hard to cure, because it rapidly develops resistance to different antibiotics and is capable of forming biofilms [8]. Discovery of new drugs against *Pseudomonas aeruginosa* is a matter of a nearly paramount importance.

Bacteriophage KPP10 has a broad spectrum of activity directed against *Pseudomonas aeruginosa* strains [9]. A study of physical and chemical properties and the spectrum of lytic activity of its lysin could yield interesting results and reveal its potential as a basis for a novel antibacterial drug against *Pseudomonas aeruginosa*. Below we describe a synthetic recombinant artilysin of the KPP10 bacteriophage, its physical and chemical properties and bactericidal activity.

METHODS

Synthesis of proteins AL-KPP10 and L-KPP10

Sequences coding for endolysin L-KPP10 and artilysin AL-KPP10 were cloned into the commercial vector pAL2-T (Evrogen, Russia) and checked for errors using Sanger sequencing. The L-KPP10-encoding gene inserted in the pAL2-T vector was cleaved by restriction nucleases at sites BamH — SacI and BamHI — PstI. The same procedure was performed on the pQE-30 expression vector. The obtained restriction fragments were separated by agarose gel electrophoresis, viewed under UV light and extracted from the gel using the Silica Bead DNA Extraction Kit (Thermo, USA). The restriction fragments of the vector and the inserts were then ligated together in the standard T4 ligase reaction buffer (Thermo, USA).

For recombinant protein production, two obtained expression vectors pQE-30 carrying genes AL-KPP10 and L-KPP10, respectively, were introduced into the competent *Escherichia coli* cells (strain M15) using the heat shock transformation protocol. Briefly, the competent cells were added to the ligase mixtures, incubated on ice for 25 min and then kept at 42 °C for 45 s, chilled on ice for 2 min and combined with plain LB. The cells were then grown at 37 °C for 1 hour, seeded on Petri dishes and left there overnight at 37 °C. Upon the overnight incubation the colonies were picked and grown in LB containing appropriate antibiotics.

Once the cultures reached OD_{600} = 0.6, they were ready for induction (3 hours in 1 mM IPTG) followed by purification in the Ni-NTA agarose column. Briefly, the induced cells were lysed in a buffer containing 6 M guanidine hydrochloride, 10 mM Tris and 100 mM $NaH_2PO_4 \bullet H_2O$ (pH 8.0) for 1 hour. The obtained lysate containing the protein of interest was run through a gravity flow column. The protein was eluted from the sorbent by lowering the pH of the gradient buffer (8 M urea, 10 mM Tris, 01.M $NaH_2PO_4 \bullet H_2O$, pH range from 8.0 to 5.5).

The obtained fractions of proteins AL-KPP10 and L-KPP10 were purified by affinity chromatography in the HisTrap HP column (GE Healthcare, UK). Before the protein solution was applied to the column, the column was equilibrated with a buffer consisting of 8 M urea, 10 mM Tris, 0.1 M $NaH_2PO_4 \bullet H_2O$, and 10 mM imidazole. The elution buffer contained 8 M urea, 10 mM Tris, 100 mM Na_2HPO_4 and 500 mM imidazole (pH 5.5). The obtained protein fractions were dialyzed from the buffer containing 5 mM HEPES and 1 mM DTT (pH 5.5) at 4 °C. Protein concentrations were measured spectrophotometrically on HITACHI U-2900 (Hitachi, Japan) in cuvettes with a 1 cm long optical path. The optical density of the solutions was measured at 280 nm wavelength. Protein concentrations were calculated given that the molar extinction coefficient $E^{0.1\%}_{280}$ nm computed in ProtParam [10] was 1.455.

Physical and chemical measurements

1. Circular dichroism spectroscopy

Circular dichroism spectra of the recombinant proteins obtained under denaturing conditions and dialyzed in 0.1 mM phosphate buffer were measured on the modified Jobin-Yvon Mark V dichrograph (Horiba, Japan) at room temperature in cuvettes with a 1mm long optical path at protein concentrations of about 200 μg/ml. The spectra were measured over the range of 190 to 260 nm.

2. Dynamic laser light scattering

The hydrodynamic size of the particles in the solution was measured on ZetaSizer Nano-ZS (Malvern Instruments LTD, USA) at 25 °C in polystyrene cuvettes with an optical path of 1 cm. The laser wavelength was 633 nm. The dialyzed protein samples studied at this stage of the experiment were obtained under denaturing conditions using the technique described above. Protein concentrations were measured spectrophotometrically in advance. The data were processed in Dispersion Technology Software, version 5.10 (Malvern, USA).

3. Fluorescence spectrometry

Tryptophan fluorescence emission from 40 μg/ml protein samples obtained under denaturing conditions and purified chromatographically was recorded by FluoroMax-3 (HORIBA Jobin Yvon GmbH, Horiba, USA) at room temperature in cuvettes with an optical path of 1 cm at the excitation wavelength of 280 nm, at which tryptophan absorbs actively. The spectra were measured over the range of 300 to 400 nm.

Investigating antibacterial properties of the lysins

1. Bactericidal activity of L-KPP10 and AL-KPP10

The samples of L-KPP10 and AL-KPP10 preparations were purified in the chromatography column and dialyzed in the buffer containing 5 mM HEPES and 1 mM DTT (pH 5.5). Protein concentrations were adjusted using the same buffer.

Two ml of the bacterial culture left to sit in LB overnight were diluted and grown until the cells reached the optical density OD_{600} of 0.6. The resulting culture (2 ml) was pelleted by centrifugation at 3000 g for 10 min; then the cells were resuspended in 2ml of 5 mM HEPES (pH 5.5); turbidity of the obtained suspension was 0.5 McFarland standards. The culture was then diluted 100-fold using the same buffer (the final cell density was 10^6 cells per ml). The following mixtures were prepared in a 96-well plate:

1) 100 μl bacterial suspension, 50 μl protein preparation at the required concentration and 50 μl EDTA (the final concentration was 0.5 mM);

2) 100 μl bacterial suspension and 100 μl protein preparation at the required concentration;

3) 100 μl bacterial suspension bacterial suspension and 100 μl buffer (the control mixture);

4) 100 μl bacterial suspension, 50 μl buffer and 50 μl EDTA (the control mixture).

Those mixtures were incubated at room temperature for 30 min and diluted tenfold in the phosphate buffer (pH 7.2); 100 μl of each dilution were applied on Petri dishes containing agarised LB. The colonies were picked after the overnight incubation at 37 °C.

In our study we used the PA103 strain of *Pseudomonas aeruginosa* and clinical bacterial isolates Ts 38-16, Ts 43-16, Ts 44-16, Ts 47-16, Ts 48-16 and Ts 49-16 from the collection of Gamaleya Federal Research Center for Epidemiology and Microbiology.

2. Electron microscopy

AL-KPP10 at 50 μg/ml concentrations was added to 10^6 CFU of the sensitive Ts 43-16 isolate combined with the HEPES buffer and incubated for 10, 20 or 30 min at room temperature.

Ten μl of the mixture were applied on a grid mesh, dried at room temperature, stained with 1% uranyl acetate solution by submerging the mesh in a drop of the stain for 30 seconds. The residual uranyl acetate was removed by washing the mesh in a drop of water for 30 seconds, blotted with filter paper and dried at room temperature. The obtained protein preparations were inspected under the electron microscope JEOL JEM-101 (FEI Phenom World, Netherlands).

RESULTS

Synthesis of the recombinant endolysin and artilysin and their physical and chemical properties

To obtain recombinant endolysin L-KPP10 and its modified variant artilysin AL-KPP10 with a fragment of the myeloid antimicrobial peptide SMAP-29 ([K2,7,13]-SMAP-29(1–17)) at its C-terminus, we used custom-synthesized gene sequences. The genes were inserted into the vector pQE-30 for further expression. Both expression vectors carrying AL-KPP10- and L-KPP10-encoding genes, respectively, were introduced into the competent *Esherichia coli* cells (strain M15) using a heat shock transformation protocol. Protein production was induced in the cultured cells by isopropyl β-D-1-thiogalactopyranoside (IPTG), the synthetic lactose analog. Upon induction, the cells were lysed under denaturing conditions. The resulting fractions of AL-KPP10 and L-KPP10 proteins His-tagged at the N-terminus were purified and refolded by dialysis using affinity chromatography.

In the next step we assessed physical and chemical properties of the obtained proteins. Structures of L-KPP10 and its modified variant AL-KPP10 were studied under denaturing conditions and after renaturation. Among the methods used were circular dichroism spectroscopy (to study the secondary structure of the proteins), fluorescence spectrometry (to measure tryptophan spectra in order to assess the presence of a tertiary structure) and dynamic light scattering (to study the ability of the proteins to aggregate or oligomerize).

Circular dichroism spectroscopy revealed that AL-KPP10 and L-KPP10 had a typical αβ-protein spectrum, with a small contribution of disorganized structures (Fig. 1A). The presence of the peptide component [K2,7,13]-SMAP-29(1–17) did not affect the secondary structure of AL-KPP10 (protein and peptide spectra almost entirely overlapped).

Tryptophan fluorescence measurements were necessary to assess whether the proteins could fold into a tertiary structure (Fig.1B). Unlike denatured proteins, renatured proteins demonstrated a shift of emission peaks to shorter wavelengths. This indicates that tryptophan located inside the protein core started to lose its contacts with the solvent as a result of tertiary structure recovery, suggesting that renatured AL-KPP10 and L-KPP10 could retain their enzymic activity.

Stability of protein solutions was assessed by dynamic laser light scattering. This method is used to measure the hydrodynamic size of a protein complex in a solution and assess the ability of the molecule to aggregate. Small (3 to 7 nm) sizes of the complex suggest that the protein is monomeric, implying its stability in a solution. We discovered that the majority of native proteins were aggregates of 60 nm in size, which is normal for recombinant proteins obtained through renaturation (Fig. 1C, 1D).

Antimicrobial activity of the recombinant endolysin and artilysin against *Pseudomonas aeruginosa*

Antimicrobial activity of the obtained recombinant endolysin and artilysin was tested on the laboratory strains and clinical isolates from the collection of the Laboratory for Translational Biomedicine (Gamaleya Federal Research Center for Epidemiology and Microbiology). Prepared *Pseudomonas*

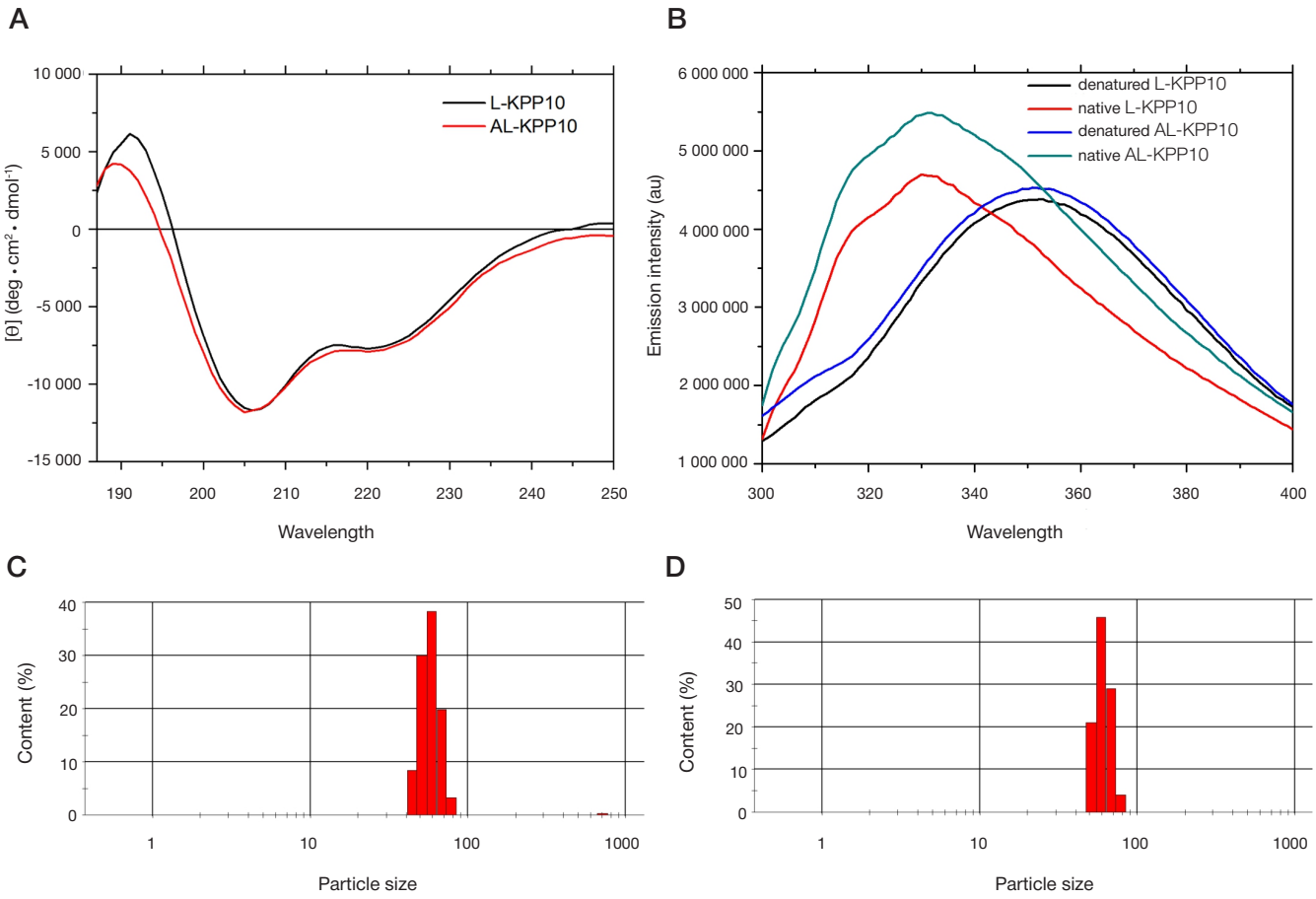

Fig. 1. Physical and chemical properties of endolysin L-KPP10 and artilysin AL-KPP10. Circular dichroism spectra of AL-KPP10 and L-KPP10 (**A**). Spectra of tryptophan fluorescence of proteins under denaturing and native conditions (**B**). Dynamic laser light scattering in the solutions of native AL-KPP10 (**C**) and L-KPP10 (**D**)

aeruginosa cells were treated with different concentrations of the recombinant endolysin and artilysin, and then seeded onto solid agarized LB.

The studied concentrations of endolysin L-KPP10 did not have a bactericidal effect on strain PA103 (Fig. 2A). This was not unexpected: the outer membrane protects the cell wall of gram-negative bacteria from the attacks of the protein. Treatment of bacterial cells with a combination of L-KPP10 and 0.5 mM EDTA increased membrane permeability and stimulated lysis at concentrations as low as 3 μg/ml (Fig. 2B).

In contrast, AL-KPP10 exhibited antimicrobial activity against PA103 and 4 of 6 clinical *Pseudomonas aeruginosa* isolates (Table 1 and Fig.3) due to the presence of a positively charged peptide component. Upon incubation of 10^6 bacterial cells with 25 μg/ml or 50 μg/ml AL-KPP10 for 30 min, the population of *Pseudomonas aeruginosa* CFU decreased by 10^5 cells (Fig. 3A).

Lysis was visualized by electron microscopy. Briefly, the clinical isolate Ts 43-16 of *Pseudomonas aeruginosa* sensitive to artilysin was treated with 50 μg/ml AL-KPP10 and incubated at room temperature for 10, 20 or 30 minutes. The cells were stained with uranyl acetate and inspected under the electron microscope (Fig. 3B). As shown in the picture, the phage lysin causes the cell wall of *Pseudomonas aeruginosa* to degrade (in the pictures the cell wall appears as an electron-dense colored flake-resembling mass).

To sum up, incubation of AL-KPP10-treated *Pseudomonas aeruginosa* strains sensitive to this protein results in the massive death of cultured bacteria, as exemplified by the clinical isolate Ts 49-16 (Fig. 3C).

DISCUSSION

There is an ongoing search for phage lysins that could be used to effectively combat gram-positive and gram-negative bacteria. Gram-negative pathogens are harder to kill because of their outer membrane that protects the peptidoglycan layer from phage attacks. One of the possible solutions to this problem lies in the use of permeabilizers, such as polymyxins or their derivatives, aminoglycosides, EDTA, citric acid, etc. [11]. For example, endolysin KZ144 obtained from the antipseudomonal phage phiKZ has been shown to exhibit bactericidal activity against Pseudomonas strains in the presence of permeabilizers [12]. Another example is endolysin OBPgpLYS. It has a broad spectrum of activity against gram-negative bacteria and can reduce their population by an order of magnitude. Its combinations with small quantities of EDTA have been shown to enhance the antimicrobial effect against multidrug-resistant *Pseudomonas aeruginosa* by 2 to 3 orders of magnitude [13].

However, it is possible to do without permeabilizing agents and use a new class of phage lysins called artilysin instead [14, 15, 16]. An artilysin consists of an endolysin and a positively charged peptide fused into one reading frame. The role of the peptide is to facilitate the passage of a protein through the outer membrane of gram-negative bacteria. One of the most promising peptides here is the sheep myeloid antimicrobial peptide SMAP-29. This amphiphilic molecule binds to a negatively charged membrane phospholipids incorporating its hydrophobic moiety into the membrane and thus creates pores [17]. One of the examples of SMAP-29-containing artilysin is called Art-175, a modified endolysin of phage KZ144. Unlike

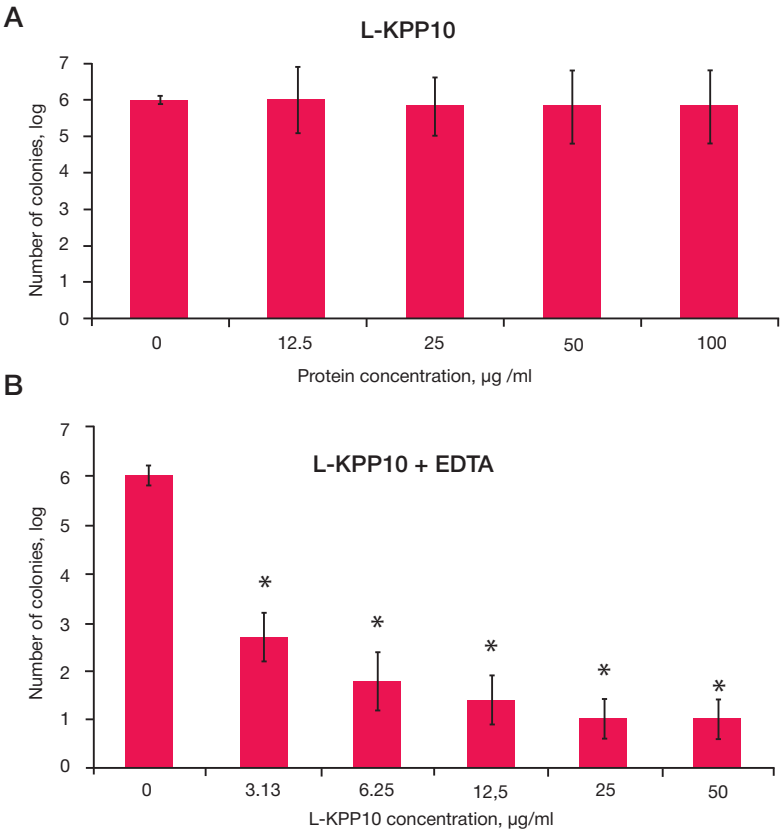

Fig. 2. Antimicrobial activity of the recombinant endolysin against strain PA103 of *Pseudomonas aeruginosa*. Treatment with different concentrations of the recombinant L-KPP10 in the absence of permeabilizing agents (**A**). 0.5 mM EDTA added to the bacteria combined with L-KPP10 (**B**). * — Difference is significant at p = 0.05 (Mann-Whitney U)

the original KZ144, Art-175 can pass through the outer membrane of *Pseudomonas aeruginosa* and kill multidrug resistant bacteria reducing their population by more than 10^4 [16]. The bactericidal effect of phage lysins is not limited to intraspecies attacks [18]. Thus, Art-175 has been shown to be effective against *Acinetobacter baumannii*, including persistent strains and strains with multiple drug resistance, [19].

Unfortunately, the original SMAP-29 is cytotoxic for human red cells. Its antibacterial and hemolytic activity has been shown to depend on its length and the original sequence. The non-toxic but still optimally bactericidal variant of SMAP-29 was obtained by removing and substituting some its original amino acids and had the following structure: [K2,7,13]-SMAP-29(1–17) [20, 21].

In the course of this work we obtained two recombinant phage lysins: endolysin L-KPP10 and its modified variant artilysin AL-KPP10. These proteins have a tertiary structure that can recover after renaturation, suggesting that they can retain their enzymic activity. Both proteins are highly stable in a solution. Importantly, the presence of the positively charged peptide SMAP-29(1–17) in KPP10 does not change the structure and properties of the protein. Those findings allowed us to continue our investigation and explore the antimicrobial activity of the obtained recombinant molecules.

We have established that the studied concentrations of endolysin L-KPP10 are effective against Pseudomonas only in the presence of EDTA. Being a permeabilizing agent, EDTA aided the passage of the endolysin into the cell through the outer membrane of the cell wall. To exert a bactericidal effect against a wide range of *Pseudomonas aeruginosa* strains, artilysin AL-KPP10 did not need permeabilizers, such as EDTA, since it contained a fragment of the myeloid peptide SMAP-29 ([K2,7,13]-SMAP-29(1–17)). Five of seven studied clinical isolates of *Pseudomonas aeruginosa* turned out to be sensitive to the recombinant artilysin. The best antimicrobial effect was seen at concentrations of 25 μg/ml and 50 μg/ml, when the bacterial populations shrunk by 10^5 cells within 30 min.

The closes functional analog of AL-KPP10 is artilysin Art-175. It consists of endolysin KZ144 linked to the original SMAP-29. Just like AL-KPP10, Art-175 can pass through the outer membrane of *Pseudomonas aeruginosa* and kill the cell. These two artilysins are different in their structure: AL-KPP10 retains the original KPP10 sequence and contains a modified shortened peptide SMAP-29 with three amino acid substitutions at positions K2,7,13, which makes the protein less toxic to human cells [20, 21]. We also expect AL-KPP10 to have a better antibacterial effect than Art-175, because the bactericidal activity of AL-KPP10 exhibited in our experiments was higher than that of Art-175 [16].

CONCLUSIONS

Our findings confirm that lytic properties of phage lysins targeting gram-negative bacteria from both inside and outside the cell can be modified and enhanced by the use of

Table 1. Antimicrobial activity of artilysin AL-KPP10 against clinical isolates of *Pseudomonas aeruginosa*

Strain/Clinical isolate of *P. aeruginosa*	Effect
PA103	+
Ts 38-16	–
Ts 43-16	+
Ts 44-16	+
Ts 47-16	–
Ts 48-16	+
Ts 49-16	+

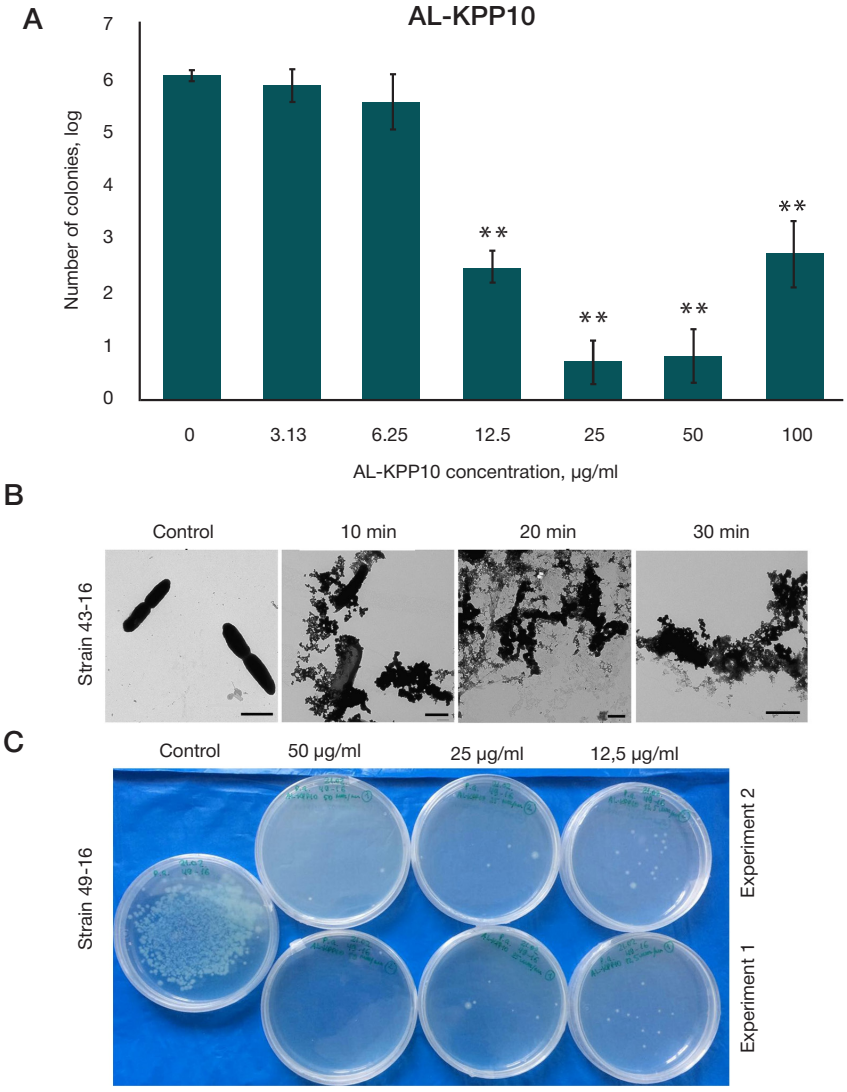

Fig. 3. Antimicrobial activity of the recombinant artilysin AL-KPP10 against sensitive strains of *Pseudomonas aeruginosa*. Treatment of *Pseudomonas aeruginosa* sensitive strains with different concentrations of recombinant AL-KPP10 in the absence of permeabilizing agents (**A**), $p < 0.01$ (Mann-Whitney U). Electron microscopy of Ts 43-16 (clinical isolate of *Pseudomonas aeruginosa*) following incubation with 50 μg/ml AL-KPP10 for 10 min, 20 min and 30 min (**B**). A Petri dish with artilysin and clinical isolate Ts 49-16 of *Pseudomonas aeruginosa* upon incubation for 30 min (**C**)

permeabilizing agents and positively charged peptides. The results of our work are consistent with previously published data on endolysin KZ144 isolated from antipseudomonal bacteriophage phiKZ and endolysin OBPgpLYS that exhibited antimicrobial activity in the presence of permeabilizers. This gives hope for the discovery of therapeutic agents based on recombinant phage lysins that could become a real alternative to antibiotics.

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