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# A Chimeric LysK-Lysostaphin Fusion Enzyme Lysing *Staphylococcus aureus* Cells: a Study of Both Kinetics of Inactivation and Specifics of Interaction with Anionic Polymers

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**Abstract** A staphylolytic fusion protein (chimeric enzyme K-L) was created, harboring three unique lytic activities composed of the LysK CHAP endopeptidase, and amidase domains, and the lysostaphin glycyl-glycine endopeptidase domain. To assess the potential of possible therapeutic applications, the kinetic behavior of chimeric enzyme K-L was investigated. As a protein antimicrobial, with potential antigenic properties, the biophysical effect of including chimeric enzyme K-L in anionic polymer matrices that might help reduce the immunogenicity of the enzyme was tested. Chimeric enzyme K-L reveals a high lytic activity under the following optimal ($_{opt}$) conditions: $pH_{opt}$ 6.0–10.0, $t_{opt}$ 20–30 °C, $NaCl_{opt}$ 400–800 mM. At the working temperature of 37 °C, chimeric enzyme K-L is inactivated by a monomolecular

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mechanism and possesses a high half-inactivation time of 12.7 ± 3.0 h. At storage temperatures of 22 and 4 °C, a complex mechanism (combination of monomolecular and bimolecular mechanisms) is involved in the chimeric enzyme K-L inactivation. The optimal storage conditions under which the enzyme retains 100 % activity after 140 days of incubation (4 °C, the enzyme concentration of 0.8 mg/mL, pH 6.0 or 7.5) were established. Chimeric enzyme K-L is included in complexes with block-copolymers of poly-L-glutamic acid and polyethylene glycol, while the enzyme activity and stability are retained, thus suggesting methods to improve the application of this fusion as an effective antimicrobial agent.

**Keywords** Fusion enzyme · Staphylolytic activity · Enzyme kinetics · Applied biocatalysis

## Introduction

The resistance of bacteria to antibiotics is a global problem for both human and animal healthcare and agriculture. The use of bacteriophage lytic enzymes (cell wall degrading proteins that are important to both the initial and terminal stages of the phage infection and lytic cycle) as antimicrobial agents is a promising alternative to conventional antibiotic therapy. Such peptidoglycan hydrolases exhibit antimicrobial activity due to hydrolysis of chemical bonds in peptidoglycan, a complex structural molecule of bacterial cell walls [1, 2]. Phage endolysins reveal high efficacy in destroying antibiotic-resistant pathogens with a milligram quantities able to be produced in a recombinant form [3].

LysK (staphylococcal phage K endolysin) and lysostaphin (a bacteriocin secreted by *Staphylococcus simulans*) are capable of lysing antibiotic-resistant strains of *Staphylococcus aureus* [4–8]. LysK and lysostaphin also show synergy in lysing *S. aureus* cells in the checkerboard assay [4]. Phage endolysins are known to be highly refractory to resistance development (reviewed in [9]). In order to further reduce the risk of resistance development, it was decided to create fusion proteins with three, unique, simultaneous lytic activities, one of which was reported previously [10]. The fusion protein K-L was created with the LysK CHAP endopeptidase and amidase domains, and the entire mature lysostaphin protein (glycyl-glycine endopeptidase and SH3b cell wall binding domains). Chimeric enzyme K-L consists of 646 amino acids (71.6 kDa) and contains 390 amino acids from a molecule of LysK and 246 amino acids of mature lysostaphin (Fig. 1). It should be pointed out that this type of chimeric enzymes (K-L) shows all three activities (amidase, alanyl-glycyl endopeptidase, glycyl-glycyl endopeptidase) in the destruction of chemical bonds in the synthetic peptide substrate [10]. Triple-acting fusion antimicrobial proteins reduce the probability of resistant pathogen developments [11, 12].

Despite their many beneficial aspects, phage lytic enzymes have a few disadvantages such as high molecular weight, in vitro and in vivo instability, short half-lives, and immunogenicity [13]. The stability of enzymes is an important parameter when considering the possibility of using these active catalysts in both medicine and industrial processes. To ensure a long life in storage (for 1 year or more), protein products are stored at temperatures from −80 to −20 °C, mostly in a lyophilized form or in a medium with high concentrations of glycerol [14]. For convenience of storage (while used in practice), it is very important to study the stability of lytic enzymes at much higher temperatures (from +2–8 to +20–25 °C). When a temperature is selected the chosen parameters of pH, medium composition, the concentration of enzyme should be relevant to the maximum stability of the enzyme. To assess the possibility of

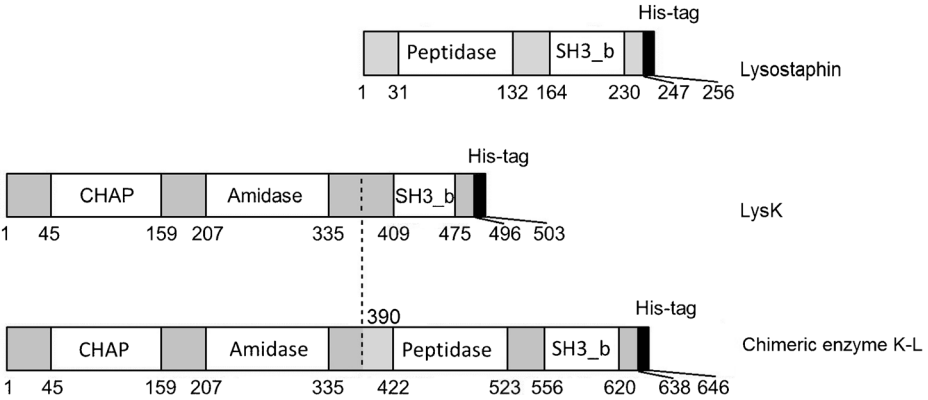


**Fig. 1** A schematic representation of the modular organization of the enzymes obtained using BLAST analysis of their amino acid sequences. The *gray color* indicates the areas of amino acid sequences that have no homologous structures; the *black color* indicates C-terminal 6xHis-tag sequences; catalytic and adsorption domains are marked with the *white color*. The *scales* refer to the positions of amino acids in the primary sequences

practical use, it is essential to study the activity and stability of chimeric enzyme K-L under different conditions and to establish potential causes of enzyme inactivation.

Various methods are applied to make better lytic enzymes of bacteriophages, such as genetic engineering techniques, covalent immobilization, stabilizing with low-molecular-weight additives and polymers [15–18]. When particles containing both enzyme and polymer molecules are formed, immunogenicity of the enzyme is decreased while its stability and solubility increase [19]. Pegylation (a covalent attachment of polyethylene glycol (PEG) to a protein molecule) is one of the most common ways to decrease the immunogenicity of proteins and to increase their stability [20]. However, pegylation results in a significant inactivation of the enzymes [21]; that is why we chose a method of forming particles containing enzyme molecules and block-copolymer molecules. Block-copolymers of the polycation-PEG-type are quite toxic; therefore, negatively charged PGLU-PEG block-copolymers were chosen. PGLU-PEG block-copolymers are composed of polyethylene glycol (PEG, nonionic block) linked to nontoxic and biodegradable fragment of poly-L-glutamic acid (PGLU, negatively charged ionic block). Oppositely charged fragments of molecules of both the enzyme and the polymer are attracted to each other, forming the core of a particle, with PEG chains facing the solvent. This is a so-called mild pegylation, which ensures maintaining of the activity with enzymes. Influence of PGLU-PEG block-copolymers on chimeric enzyme K-L activity was compared with effect of other polyanions (polyacrylic acids, dextran sulfates). In this work, we approached the incorporation of the chimeric enzyme K-L into nanomatrices of anionic polymers to reduce the immunogenicity of the enzyme.

## Reagents and Methods

### Reagents

Recombinant chimeric enzyme K-L (C-terminally 6xHis tagged) was prepared according to the procedure described previously [10]. Purified triple fusion protein (11–15 mg/mL) was

stored at −20 °C in nickel chromatography elution buffer (50 mM $NaH_2PO_4$, 300 mM NaCl, 250 mM imidazole, 30 % glycerol, pH 8.0).

Preparation of autoclaved *S. aureus* cells was produced as described previously [22]. The autoclaving of *S. aureus* cells was optimized subject to two requirements: (i) autoclaved bacteria should lose the ability to grow and reproduce, but the cell wall peptidoglycan should not be destroyed; (ii) the method of heat treatment of *S. aureus* cells should not cause a change in the chimeric enzyme K-L activity.

The following was used as additives to the enzyme: block-copolymers of poly-L-glutamic acid and polyethylene glycol $PGLU_{10}$-$PEG_{114}$, $PGLU_{50}$-$PEG_{114}$, and $PGLU_{100}$-$PEG_{114}$ with the molecular weights of 6.5, 13.5, and 20.0 kDa, respectively (Alamanda Polymers); polyacrylic acids with molecular weights of 5.1, 240.0, and 4000.0 kDa (Sigma-Aldrich); dextran sulfates with molecular weights of 8.0, 100.0, and 500.0 kDa (Alfa Aesar).

The following equipment was used: spectrophotometer Jen-way 6405 UV/Vis (Akvilon, Russia), Mini-PROTEAN Tetra System (Bio-Rad, Austria); BioSpectrum imaging system (Ultra-Uiolet Products Ltd, USA), chromatographic system Biologic LP (Knauer, Germany), Zetasizer Nano ZS (Malvern, UK).

## Measuring the Activity of Chimeric Enzyme K-L (Standard Enzyme Assay)

The activity of the enzyme was measured by turbidity reduction assay at the physiologically relevant conditions (*S. aureus* cell suspension, $OD_{600\ nm} = 0.6$*, 20 mM $KH_2PO_4$, pH 7.5, 37 °C). From 10 to 20 μL of the enzyme solution (0.2–0.8 mg/mL in 20 mM $KH_2PO_4$, pH 7.5) were added to 0.5 mL of *S. aureus* cell suspension, and the decrease in $OD_{600\ nm}$ was monitored every second for 15 min. For control purposes, the incubation of cell suspension was performed under the same conditions without enzyme.

The reaction rate was determined as a $\Delta OD_{600\ nm}$ per second (after subtraction of $\Delta OD_{600\ nm}$/s of control) per milligram of the enzyme (specific activity, $\Delta OD_{600\ nm}$/s/mg).

*The value of optical density is linearly dependent on the concentration of *S. aureus* cells in the range from 0 to 0.7 [18]. The value of the enzyme activity was measured at the optical density value equal to 0.6 (then changes in the optical density adequately reflect changes in the concentration of the substrate).

## Influence of pH on Chimeric Enzyme K-L Activity

*S. aureus* cells were suspended in a universal buffer (20 mM Tris, 20 mM potassium citrate, 20 mM potassium phosphate, pH values were varied from 6.0 to 10.0), and the $OD_{600\ nm}$ value was maintained at 0.6. 10 μL of the enzyme solution (0.4 mg/mL in universal buffer) were added to 0.5 mL of the cell suspension with the desired pH, and the decrease in $OD_{600nm}$ was monitored at 37 °C. Activity of the enzyme was calculated as shown above.

## Influence of Temperature on Chimeric Enzyme K-L Activity

*S. aureus* cells were suspended in 20 mM potassium phosphate buffer (pH 7.5), and the $OD_{600\ nm}$ value was maintained at 0.6. The resulting suspension was heated up to the temperatures from 20 to 50 °C. Ten microliters of the enzyme solution (0.4 mg/mL in 20 mM $KH_2PO_4$, pH 7.5) were then added to 0.5 mL of the cell suspension, and the decrease in $OD_{600\ nm}$ was monitored at the desired temperature. Activity of the enzyme was determined as shown above.

### Influence of NaCl Concentration on Chimeric Enzyme K-L Activity

*S. aureus* cells were suspended in 20 mM potassium phosphate buffer (pH 7.5) containing NaCl (salt concentration varied from 0.2 to 1000 mM), and the $OD_{600\ nm}$ value was maintained at 0.6. Ten microliters of the enzyme solution (0.4 mg/mL in 20 mM $KH_2PO_4$, pH 7.5) were added to 0.5 mL of the cell suspension, and the decrease in $OD_{600\ nm}$ was monitored in the presence of the desired NaCl concentration at 37 °C for 15 min. Activity of the enzyme was determined as shown above.

### Chimeric Enzyme K-L Stability

For the selection of the optimum storage conditions, the enzyme solution (12.0 mg/mL of chimeric enzyme K-L, 300 mM NaCl, 250 mM imidazole, 30 % glycerol, 50 mM phosphate, pH 8.0) was diluted with universal buffer (20 mM Tris, 20 mM potassium citrate, 20 mM potassium phosphate, pH values were varied from 6.0 to 9.0) to achieve an enzyme concentration of 0.2–0.8 mg/mL. Enzyme solutions were kept at 4 and 22 °C (storage temperatures) for different time intervals (days, weeks, months), and aliquots were taken to measure the activity under standard conditions (as shown above). The order of the inactivation reaction was determined by the equation of Ostwald-Noyes [23].

### Electrophoresis

Electrophoresis of chimeric enzyme K-L samples was performed as reported in [24]. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) electrophoregrams (gels) were analyzed using BioSpectrum imaging system.

### Preparation of Chimeric Enzyme K-L Complexes with Polymers

The enzyme solution (12.0 mg/mL of chimeric enzyme K-L, 300 mM NaCl, 250 mM imidazole, 30 % glycerol, 50 mM phosphate, pH 8.0) was diluted with a polymer solution (20 mM $KH_2PO_4$, pH 7.5) to achieve an enzyme concentration of 2.0 mg/mL. The resulting solutions were incubated 30 min at 22 °C to allow the formation of complexes; afterward, they were diluted with 20 mM $KH_2PO_4$ (pH 7.5) to an enzyme concentration of 0.4 mg/mL to study the activity and stability.

### Characterization of Protein-Polymer Interaction by Dynamic Light Scattering

The particle sizes were measured using a Zetasizer Nano ZS system (He-Ne laser, 5 mW, 633 nm) at 22 °C and enzyme concentration of 0.4 mg/mL. Solutions of chimeric enzyme K-L and its complexes were filtered with a 0.8-μm pore size filter (Millipore).

### Activity and Stability of Chimeric Enzyme K-L-Polymer Complexes

The activity of chimeric enzyme K-L-polymer complexes was measured under standard conditions (as shown above). To study the stability, activity measurements were made with the enzyme-polymer complexes (the enzyme concentration was 0.4 mg/mL in 20 mM $KH_2PO_4$, pH 7.5) at fixed time intervals of incubation at the required temperature (37 and 22 °C).

### HPLC

The molecular weight of the inactivated enzyme was determined using a chromatographic system (Biologic LP) equipped with a UV detector, a column (BioFox 17Sec), and software (LP Data View). During the mobile phase, the PBS buffer (137 mM NaCl, 3 mM KCl, 10 mM $Na_2HPO_4$, 2 mM $KH_2PO_4$, pH 7.4) was used and the flow rate was 0.5 mL/min.

### Data Analysis

All obtained results are expressed as a mean ± SD from three independent experiments ($n = 3$). Both the mean values and the standard deviations were calculated by the Microsoft Office Excel program.

## Results and Discussion

### Influence of Salt Content, pH, and Temperature on Chimeric Enzyme K-L Activity

Using a turbidity reduction assay, the influence of salt content, pH, and temperature on chimeric enzyme K-L activity was tested (Fig. 2a–c). Chimeric enzyme K-L is active across wide range of pH from 6.0 to 10.0 (Fig. 2a). Chimeric enzyme K-L shows the bell-shaped dependence of the activity on NaCl concentration, with an optimum at 400–800 mM NaCl (Fig. 2b). The activity of chimeric enzyme K-L decreases as temperature increases from 20 to 50 °C (Fig. 2c).

Activity of the LysK-lysostaphin fusion molecule shows no clear dependence on pH probably due to the presence of several amino acid residues in the active sites with a variety of pH sensitivities. There are at least four pH-sensitive groups (two from LysK and two from lysostaphin) in the chimeric enzyme K-L molecule. The LysK activity at different pH levels is controlled by groups with pK 6.0 and 9.5 [17], while the lysostaphin activity is controlled by glutamic acid and histidine residues with pK 5.9 and 9.2, respectively [25]. The dependence on the NaCl concentration shown by the enzyme activity may be due to increased hydrophobic interactions, hence compaction and added stability of the protein globule. The temperature increase apparently has a denaturing effect on chimeric enzyme K-L.

These correlations of the activity of chimeric enzyme K-L and the pH, salt concentration, and temperature cannot be interpreted only in terms of their effect on the enzyme. As a rule, a change in substrate/assay solution may also affect the state of the cell wall substrate. This, in turn, might also affect the level of the activity of the enzyme.

### Kinetic Features of Chimeric Enzyme K-L Inactivation

To study the stability of chimeric enzyme K-L in working conditions, the enzyme (0.2–0.8 mg/mL) was placed in the media with physiological parameters (20 mM K-phosphate buffer with 150 mM NaCl, pH 7.5, 37 °C). When analyzing the inactivation curves for chimeric enzyme K-L in working conditions, it was discovered that the inactivation of the enzyme occurs irreversibly with first order ($n = 1.0 \pm 0.1$) kinetics. First-order kinetic (monomolecular mechanism of inactivation) corresponds to enzyme

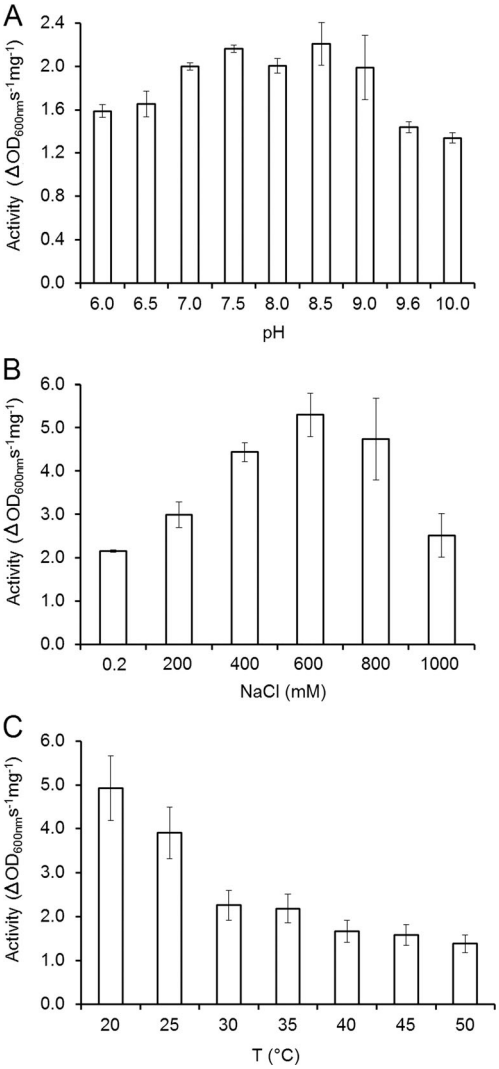


**Fig. 2** Influence of pH, NaCl, and temperature on chimeric enzyme K-L activity. **a** Dependence of chimeric enzyme K-L activity on pH. Experimental conditions: 37 °C, universal buffer (20 mM Tris, 20 mM potassium phosphate, 20 mM potassium citrate). **b** Dependence of chimeric enzyme K-L activity on NaCl concentration. Experimental conditions: 37 °C, 20 mM potassium phosphate, pH 7.5, 0.2–1000 mM NaCl. **c** Dependence of chimeric enzyme K-L activity on temperature. Experimental conditions: 20 mM potassium phosphate, pH 7.5, $OD_{600\ nm} = 0.6$, chimeric enzyme K-L concentration of $8 \times 10^{-3}$ mg/mL

unfolding (denaturation). The process of the enzyme inactivation is described by the equation below (Fig. 3):

$$\ln(A/A_0) = k_{in}t \tag{1}$$

where $A/A_0$ is the residual activity of the enzyme ($A$ is the activity of the enzyme at a particular time, $A_0$ is the activity of the enzyme at zero time), $k_{in}$ is the constant of the first-order inactivation, and $t$ is the time of the enzyme to achieve the residual activity $A/A_0$. When the inactivation has a monomolecular mechanism, the limiting step is the $N \rightarrow D$ transition, where N is a native form of the enzyme and D is a denatured form of the enzyme.

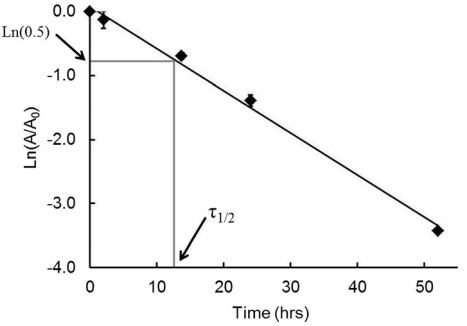


**Fig. 3** Dependence of chimeric enzyme K-L residual activity on time (first-order coordinates). Experimental conditions: 37 °C, 20 mM K-phosphate buffer with pH 7.5 and 150 mM NaCl, chimeric enzyme K-L concentration of 0.2–0.8 mg/mL

The calculation of the half-inactivation time (12.7 ± 3.0 h) by plotting the enzyme residual activity versus time is a suitable estimation of chimeric enzyme K-L stability (Fig. 3). It can be concluded that chimeric enzyme K-L is stable in working conditions.

To find optimal storage conditions, the influence of the following factors on chimeric enzyme K-L stability was investigated: the effect of pH (6.0–9.0), temperature (4 and 22 °C), and concentration of the biocatalyst (0.2–0.8 mg/mL). The maximum stability (maintaining of the 100 % activity during 140 days) is observed at 4 °C, pH 6.0/7.5 (universal buffer), chimeric enzyme K-L concentration of 0.8 mg/mL (Table 1). Of note, chimeric enzyme K-L is more stable compared with LysK and lysostaphin; the maximum half-inactivation time of LysK and lysostaphin at 4 °C equals to ~60 days [17, 26].

The enzyme inactivation under storage conditions is a complex process corresponding to the fractional orders (Table 1). The minimum value of the inactivation order ($n$) for chimeric enzyme K-L under storage conditions is equal to 1.0 ± 0.4, while the maximum value is 2.3 ± 0.1 (Table 1). The unfolding of the enzyme molecule is inherent to the monomolecular mechanism (first-order inactivation, $n = 1$); the enzyme inactivation by bimolecular mechanism (second order inactivation, $n = 2$) is provided by the collision of two molecules followed by the formation of dimers and, possibly, of higher order aggregates. If the enzyme is inactivated according to fractional orders ($1 \leq n \leq 2$), two different processes occur: the inactivation by denaturation (Fig. 4a, N → D) and the inactivation by collisions with the molecules (Fig. 4a,

**Table 1** Inactivation parameters of chimeric enzyme K-L (inactivation order, half-inactivation time) under storage conditions

| T (°C) | pH | Chimeric enzyme K-L (mg/mL) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0.2 | | 0.4 | | 0.8 | |
| | | $n$ | $\tau_{1/2}$ (days) | $n$ | $\tau_{1/2}$ (days) | $n$ | $\tau_{1/2}$ (days) |
| 22 | 6.0 | 1.4 ± 0.4 | 6 ± 2 | 1.8 ± 0.4 | 7 ± 3 | 1.8 ± 0.2 | 15 ± 0 |
| | 7.5 | 1.5 ± 0.1 | 3 ± 2 | 1.3 ± 0.5 | 8 ± 1 | 2.0 ± 0.5 | 12 ± 3 |
| | 9.0 | 1.4 ± 0.2 | 3 ± 0 | 1.5 ± 0.6 | 3 ± 1 | 1.9 ± 0.1 | 8 ± 3 |
| 4 | 6.0 | 1.1 ± 0.1 | 48 ± 4 | 1.4 ± 0.0 | 90 ± 14 | 2.0 ± 0.4 | >140 |
| | 7.5 | 1.2 ± 0.6 | 18 ± 7 | 1.1 ± 0.5 | 60 ± 0 | 2.3 ± 0.1 | >140 |
| | 9.0 | 1.0 ± 0.4 | 20 ± 4 | 1.3 ± 0.4 | 40 ± 10 | 2.1 ± 0.3 | 70 ± 20 |

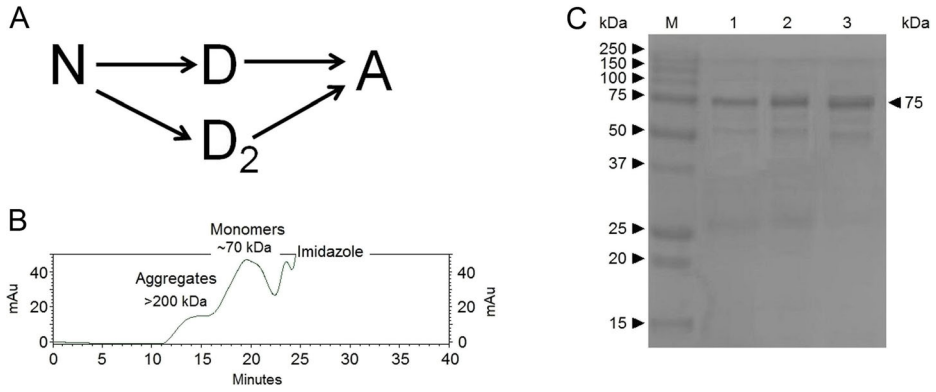


**Fig. 4** Analysis of the inactivation process of chimeric enzyme K-L under storage conditions. **a** A scheme of chimeric enzyme K-L inactivation under storage conditions. *N* native, *D*, $D_2$ inactivated, *A* aggregated forms of the enzyme. **b** A chromatogram of the products of chimeric enzyme K-L inactivation. Experimental conditions: BioFox 17Sec column, flow rate of mobile phase is 0.5 mL/min, sample volume is 0.2 mL, $\lambda = 240$ nm. **c** An electrophoregram of the products of chimeric enzyme K-L inactivation. *M* molecular weight protein markers (10–250 kDa); *1*, an active enzyme (control); *2*, *3*, the enzyme after inactivation (4 °C, 22 °C)

$N \rightarrow D_2$) with formation of inactive dimers. Irreversible process of inactivation was confirmed by measurement of activity of chimeric enzyme K-L cooled solutions.

The increase of half-inactivation time of chimeric enzyme K-L was observed as the enzyme concentration increased and pH decreased (Table 1). Dependence of chimeric enzyme K-L inactivation on its concentration is likely due to the change of the inactivation mechanism from a monomolecular to an aggregative (bimolecular) one. Different periods of the half-inactivation time of the enzyme correspond to different mechanisms of inactivation. At low enzyme concentrations (0.2 and 0.4 mg/mL), the inactivation reaction order is ~1, which means that the limiting step for the inactivation process is denaturation. When the enzyme concentration is increased to 0.8 mg/mL, the inactivation reaction order is ~2, which suggests that processes of intermolecular interactions are likely prevalent.

During the enzyme inactivation under storage conditions, the precipitation was visual indicator of aggregates being formed. Value of effective hydrodynamic radius of active chimeric enzyme K-L is equal to 3 nm, and particle size of inactive enzyme is higher than 1 μm (Fig. S1). It can be concluded that solution of chimeric enzyme K-L contains both water soluble and insoluble forms of the enzyme, and the inactive enzyme precipitates ($D \rightarrow A$, $D_2 \rightarrow A$, Fig. 4a).

To characterize the water-soluble forms of chimeric enzyme K-L, the precipitate centrifugation was produced and the supernatant was analyzed by HPLC method. It was shown that there is a monomer form (~70 kDa) and aggregated forms (>200 kDa) in chimeric enzyme K-L solutions (Fig. 4b). Fractions relevant to both monomeric and aggregated forms were collected, and their activity was further measured. No lytic activity was detected in aggregated forms (>200 kDa). The monomeric form (~70 kDa) shows an activity that decreases to zero with time. This peak most likely corresponds to the mixture of active form, partially denatured form and fully denatured form of the enzyme. The ratio between these forms varies at different points of the enzyme inactivation process. At the initial moment of inactivation, the peak

corresponds to the 100 % content of the active form of the enzyme, while at the final point of inactivation, this peak corresponds to the 100 % content of the enzyme denatured form.

Formation of K-L aggregates during the inactivation process is due to noncovalent interactions, as demonstrated, by identical bands of both the active enzyme and inactivated K-L in SDS-PAGE (Fig. 4c). We hypothesize that the inactivated form of the enzyme (D, $D_2$) form large aggregates due to the increased potential for protein-protein interactions in the unfolded state (Fig. 4a, $N \rightarrow D \rightarrow A$, $N \rightarrow D_2 \rightarrow A$).

## Interaction of Chimeric Enzyme K-L with Polyanions

The main disadvantage of lytic enzymes as candidate therapeutics is their immunogenicity. This disadvantage can be reduced or eliminated by incorporating the enzymes into matrixes of anionic polymers (polyacrylic acids, dextran sulfates, block-copolymers of poly-L-glutamic acid, and polyethylene glycol), thus blocking their recognition as foreign antigen by the immune system [21].

The interaction between the lysin molecules and polymers results in the formation of protein-polyelectrolyte complexes. In solutions of individual polymers or chimeric enzyme K-L, there are no major associates: the values of effective hydrodynamic radii ($R_h$) of the particles are in the range of 1–10 nm for all the concentrations considered in this work (Table 2, Fig. 5). When the enzyme is mixed with polymers, there is an increase in values of the effective hydrodynamic radius of the particles from 1–10 to 50–200 nm (Table 2). The polydispersity index values range from 0.1 to 0.2, indicating the formation of particles with a narrow size distribution. In cases where the particle radius is small (50–100 nm, Fig. 5), the enzyme-polymer interaction was additionally controlled by measuring the zeta potential of the particles. The enzyme molecule is positively charged (+5 ± 1 mV) during the formation of complexes; particles with a negative zeta potential (−4 to −1 mV) are formed when polymers are added (unpublished data).

When chimeric enzyme K-L is incorporated into complexes with polyanions, there is a change in the enzyme activity (Table 3). The way in which polymers affect the lysin activity

**Table 2** Influence of polymers on chimeric enzyme K-L particle size (the number particle size distribution)

| Polymer | M (kDa) | $R_h$ (nm) | | |
|---|---|---|---|---|
| | | Z[a] = 1:1 | Z = 10:1 | Z = 0 (no enzyme) |
| No polymer | – | 3 ± 1 | | |
| Polyacrylic acid | 5.1 | 150 ± 50 | 150 ± 10 | 10 ± 5 |
| | 240.0 | 150 ± 50 | 160 ± 50 | 5 ± 3 |
| | 4000.0 | 150 ± 50 | 150 ± 10 | 10 ± 4 |
| Dextran sulfate | 8.0 | 70 ± 10 | 80 ± 30 | 5 ± 1 |
| | 100.0 | 50 ± 10 | 70 ± 10 | 5 ± 1 |
| | 500.0 | 80 ± 10 | 100 ± 20 | 10 ± 1 |
| $PGLU_{10}$-$PEG_{114}$ | 6.5 | 70 ± 5 | 65 ± 5 | 1 ± 0 |
| $PGLU_{50}$-$PEG_{114}$ | 13.0 | 80 ± 10 | 90 ± 10 | 2 ± 1 |
| $PGLU_{100}$-$PEG_{114}$ | 20.0 | 50 ± 10 | 50 ± 10 | 3 ± 1 |

[a] Z is polymer/enzyme molar charge ratio (-/+ molar charge ratio)

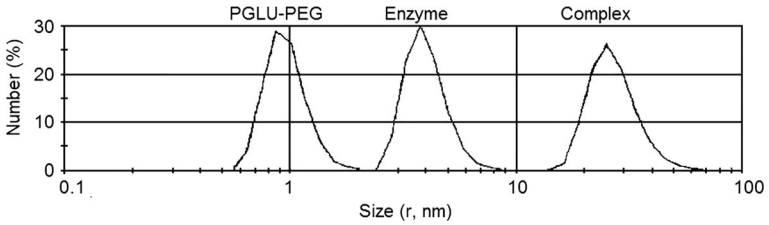


**Fig. 5** Comparison of particle sizes of chimeric enzyme K-L before and after the interaction with PGLU-PEG. Experimental conditions: 22 °C, 20 mM potassium phosphate, pH 7.5, chimeric enzyme K-L concentration of 0.4 mg/mL, Z = 10:1

depends on the nature of their structural units and molecular weight while being independent of the polymer/enzyme molar charge ratio (Z, -/+ molar charge ratio). Formation of complexes with polyacrylic acids and dextran sulfates (8.0 and 100.0 kDa) causes a complete inactivation of the enzyme. Interaction with PGLU-PEG block-copolymers and dextran sulfate with molecular weight of 500.0 kDa allows retention of chimeric enzyme K-L activity at 80–100 %.

Polyacrylic acids and dextran sulfates are the most extensively studied polymers used in the formation of protein-polymer complexes [27, 28]. It can be concluded that polyacrylic acids (5.1–4000.0 kDa) and dextran sulfates with low molecular weights (8.0–100.0 kDa) are not suitable reagents for chimeric enzyme K-L encapsulation, and biomedical applications of enzyme-polymer complexes are restricted. The deactivating effect of polyacrylic acids and dextran sulfates (8.0–100.0 kDa) may be due to their denaturing effect on the enzyme molecules.

Different impact of dextran sulfates on the chimeric enzyme K-L activity is likely due to differences in the structures of protein-polymer complexes. Complexes of the enzyme with 8.0 kDa dextran sulfate contain 2 (Z = 1) or 20 (Z = 10) molecules of polymer per molecule of protein; complexes of chimeric enzyme K-L with 500.0 kDa dextran sulfate contain 4 (Z = 1) or 40 (Z = 10) molecules of the enzyme per molecule of polymer. This can cause a significant difference in the properties of the enzyme-polymer complexes (activity, stability).

**Table 3** Influence of polymers on chimeric enzyme K-L activity

| Polymer | M (kDa) | Activity of the enzyme[a] (%) | |
|---|---|---|---|
| | | Z[b] = 1:1 | Z = 10:1 |
| – | – | 100 ± 0 | |
| Polyacrylic acid | 5.1 | 0 | |
| | 240.0 | 0 | |
| | 4000.0 | 0 | |
| Dextran sulfate | 8.0 | 0 | |
| | 100.0 | 0 | |
| | 500.0 | 100 ± 0 | |
| $PGLU_{10}$-$PEG_{114}$ | 6.5 | 120 ± 10 | 85 ± 5 |
| $PGLU_{50}$-$PEG_{114}$ | 13.0 | 95 ± 10 | 95 ± 10 |
| $PGLU_{100}$-$PEG_{114}$ | 20.0 | 100 ± 10 | 100 ± 10 |

[a] Activity of the enzyme alone is equal to 100 %

[b] Z is polymer/enzyme molar charge ratio (-/+ molar charge ratio)

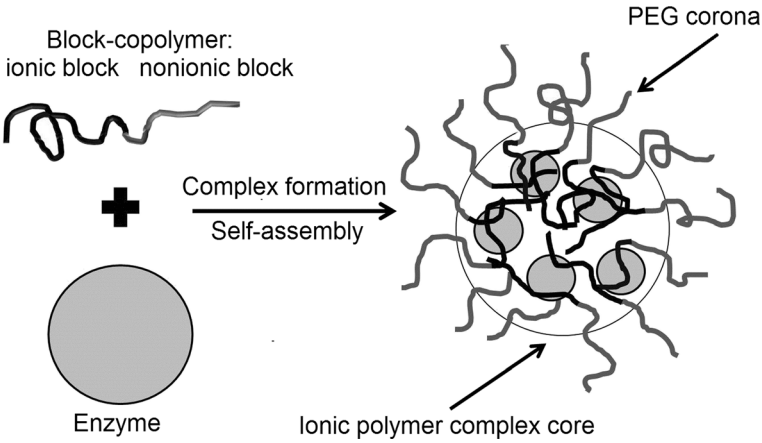


**Fig. 6** A schematic representation of the formation of a complex consisting of block-copolymer and enzyme molecules

$COO^-$ side groups in the block-copolymer molecules are linked to the main polymer chain by means of spacers (composed of two methylene groups $–CH_2–CH_2–$). During the interaction of chimeric enzyme K-L and block-copolymers, the spacers prevent or reduce direct contact between the enzyme and the polymer chain, thus protecting from chimeric enzyme K-L deactivation.

Lysin molecules when reacting with block-copolymers are incorporated in the particle cores which are similar to micelles ([29], Fig. 6). This process occurs through the formation of electrostatic contacts between positively charged sites of the enzyme molecules and negatively charged fragments of molecules of block-copolymers. Polyethylene glycol chains of the molecules of block-copolymers are probably interacting with the aqueous phase (a PEG-corona is shaped around the particle [29]). The formation of such particles takes place while maintaining both the activity and stability of the biocatalyst; $PGLU_{10}$-$PEG_{114}$ has little stabilization effect on the enzyme (Table 4). Moreover, this can potentially reduce immuno-reactivity of chimeric enzyme K-L, wherein the enzyme retains high stability and lytic activity.

**Table 4** Influence of block-copolymers on chimeric enzyme K-L stability

| Polymer | Z[a] | $\tau_{1/2c}/\tau_{1/2}$[b] | |
|---|---|---|---|
| | | 22 °C | 37 °C |
| $PGLU_{100}$-$PEG_{114}$ | 1:1, 10:1 | 1.0 ± 0.1 | |
| $PGLU_{50}$-$PEG_{114}$ | 1:1, 10:1 | 1.0 ± 0.1 | |
| $PGLU_{10}$-$PEG_{114}$ | 1:1 | 1.0 ± 0.1 | |
| | 10:1 | 1.5–2.0 | 1.0 ± 0.1 |

[a] Z is polymer/enzyme molar charge ratio (-/+ molar charge ratio)

[b] $\tau_{1/2c}$ is half-inactivation time of the enzyme complexed with polymer; $\tau_{1/2}$ is half-inactivation time of the enzyme alone

## Conclusion

At 37 °C, chimeric enzyme K-L is inactivated following a monomolecular mechanism. Under storage conditions, chimeric enzyme K-L is inactivated with a mixed mechanism that is a combination of monomolecular and bimolecular mechanisms. No additional stabilization of the enzyme is required to ensure its long-term storage at 4 °C. Incorporation of chimeric enzyme K-L into complexes with the PGLU-PEG block-copolymers occurs with retention of both enzyme activity and stability. The inclusion in a polymer matrix may result in a reduced immunogenicity of chimeric enzyme K-L which is a prerequisite for commercialization of a proteinaceous antimicrobial agent.

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