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Staphylococcus simulans Recombinant Lysostaphin: Production, Purification, and Determination of Antistaphylococcal Activity

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Abstract—*Staphylococcus simulans* lysostaphin is an endopeptidase lysing staphylococcus cell walls by cleaving pentaglycine cross-bridges in their peptidoglycan. A synthetic gene encoding *S. simulans* lysostaphin was cloned in *Escherichia coli* cells, and producer strains were designed. The level of produced biologically active lysostaphin comprised 6-30% of total *E. coli* cell protein (depending on *E. coli* M15 or BL21 producer) under batch cultivation conditions. New methods were developed for purification of lysostaphin without affinity domains and for testing its enzymatic activity. As judged by PAGE, the purified recombinant lysostaphin is of >97% purity. The produced lysostaphin lysed cells of *Staphylococcus aureus* and *Staphylococcus haemolyticus* clinical isolates. *In vitro* activity and general biochemical properties of purified recombinant lysostaphin produced by M15 or BL21 *E. coli* strains were identical to those of recombinant lysostaphin supplied by Sigma-Aldrich (USA) and used as reference in other known studies. The prepared recombinant lysostaphin represents a potential product for development of enzymatic preparation for medicine and veterinary due to the simple purification scheme enabling production of the enzyme of high purity and antistaphylococcal activity.

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Key words: *Staphylococcus simulans*, *Escherichia coli*, recombinant lysostaphin, heterologous expression, liquid chromatography, lytic activity, *Staphylococcus* sp. clinical isolates

Staphylococcus simulans lysostaphin (EC 3.4.24.75) is a glycylglycine endopeptidase possessing bacteriolytic activity against various species of staphylococci, such as *S. epidermidis*, *S. haemolyticus*, *S. saprophyticus*, *S. carnosus*, *S. hominis*, *S. simulans*, *S. xylosus*, *S. hyicus*, and *S. intermedius*, containing pentaglycine cross-links in their cell wall [1].

The possibility of using lysostaphin as an anti-staphylococcus therapeutic agent has been discussed in the literature, particularly in recent publications [2-4], and lysostaphin has been regarded as a prospective biotechnological object. Successful application of lysostaphin preparations in veterinary and medicine has been described in the literature [1, 5, 6]. However, currently available commercial lysostaphin preparations are very expensive and are designed for research (not clinical) use only. Therefore, the development of enzymatic lysostaphin preparation for clinical applications is still relevant.

Many articles have been published since 1990 describing recombinant lysostaphin production in *E. coli* [3, 7-12], *Bacillus* spp. (*B. subtilis* and *B. sphaericus*) [13], as well as in eukaryotic cells [4, 14]. Unfortunately, in most of these works the level of target protein production was rather low. *Escherichia coli* lysostaphin producing strains seem to be the most promising, but in the majority of cases the variants of produced recombinant lysostaphin contain $(His)_6$ tag introduced for target protein purification by metal affinity chromatography [9-12]. However, metal affinity chromatography is associated not only with high cost of target product, but also with target protein pollution with metal, particularly cobalt, ions immobilized on the stationary phase [9]. This fact forced the authors of other works to use zinc affinity chromatography, which yielded more active (and safe for clinical application) lysostaphin [15, 16].

Abbreviations: IPTG, isopropyl-1-thio-β-D-galactopyranoside; PIZ, partial inhibition zone; PMSF, phenylmethylsulfonyl fluoride; TIZ, total inhibition zone.

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Besides, Szweda and coworkers [9] obtained the active lysostaphin only from unfrozen cell biomass. When attempting lysostaphin isolation and purification from frozen biomass, they observed precipitate formation and inactivation of the enzyme. Based on these observations, they indicated the necessity to fulfill all protein purification stages immediately after biomass growth and pelleting of cells by centrifugation [9], which is not reasonable from the technological point of view.

Since further evaluation of sensitivity to lysostaphin in clinical staphylococcal isolates required large amounts of highly purified lysostaphin with high specific activity, our study aimed to increase production level of lysostaphin in cytoplasm of *E. coli* cells transformed to contain the recombinant plasmid. This work describes the construction of *E. coli* producer strains and a method for purification of soluble recombinant lysostaphin free of affinity (tag) domains. The idea of lysostaphin expression in *E. coli* as a cytoplasmic mature enzyme without affinity tag domains belongs to Khatri and Sharma [17], although rather low yield was achieved in their work. Therefore, the present work particularly aimed to enhance the yield of the target protein.

MATERIALS AND METHODS

Cloning of gene encoding *S. simulans* lysostaphin. *Escherichia coli* M15 NaI^S, Str^S, Rif^S, Thi^-, Lac^-, Ara^+, Gal^+, Mtl^-, F^-, $RecA^+$, Uvr^+, Lon^+, Km^r (Rep4) genotype and expressing vector pQE6 (Qiagen, USA) were used for the cloning procedure. Also, *E. coli* BL21(DE3) cells, *fhuA2 [lon] ompT gal (λ DE3) [dcm] ΔhsdSλ DE3 = λ sBamHIo ΔEcoRI-B int::(lacI::PlacUV5::T7 gene1) i21 Δnin5)* genotype (New England Biolabs, USA) were employed for vector construction, gene expression, and protein production using pET-45b(+) expression vector (Novagen, Merck KgaA Biosciences, Germany).

The sequence of the synthetic gene encoding *S. simulans* lysostaphin was designed considering both *E. coli* codon composition and lack of RNA secondary structure constraints. The amino acid sequence encoded by the synthetic gene corresponds to 207-452 a.a. of the C-terminal part of *S. simulans* bv. *staphylolyticus* lysostaphin (NCBI Reference Sequence: YP_003505772.1). The oligonucleotide synthesis and gene assembly were performed by Eurogen (Russia). The nucleotide sequence used for further cloning (767 bp, GenBank deposited, KP025769) was flanked with NcoI and Kpn2I restriction sites for cloning in pQE6 vector in the case of *E. coli* M15 strain, or NcoI and HindIII in the case of pET45b(+) vector for *E. coli* BL21 strain. Chemicals, T4 ligase, and other enzymes used for cloning were purchased from Fermentas (Thermo Fisher Scientific, USA).

Escherichia coli M15 or BL21(DE3) cells were transformed with the obtained plasmids by electroporation using MicroPulser equipment from Bio-Rad (USA). Clones obtained after transformation were screened by selection on ampicillin. To confirm correct cloning result, the gene construction was subjected to restriction analysis and sequencing. The plasmid DNA was isolated by the alkaline lysis-mini-prep procedure (in accordance with the procedure described in the manual of Sambrook et al. [18]) and sequenced (to verify and confirm the sequence) in the N. F. Gamaleya Federal Research Centre for Epidemiology and Microbiology, Ministry of Health of the Russian Federation.

Biomass production of producer stains. Overnight culture of *E. coli* M15 [Rep4, pLys] or *E. coli* BL21 was inoculated into 500-ml flasks in 200 ml of LB liquid medium with kanamycin (25 μg/ml) and ampicillin (150 μg/ml) for *E. coli* M15 strain, and ampicillin (150 μg/ml) for *E. coli* BL21 strain. The cultures were grown on a rotary shaker in a thermostat (170 rpm, 37°C). Isopropyl-1-thio-β-D-galactopyranoside (IPTG) (0.2 mM) was added to the cultures after their turbidity reached 1.0-1.5 OD units at 600 nm, and the cultures were grown for additional 3-4 h. Biomass was collected by centrifugation at 6000*g* for 15 min. The biomass yield comprised approximately 5 g/liter.

Lysostaphin purification. In the case of *E. coli* M15 producer, 50 g of the produced biomass was resuspended in 25 mM Tris-HCl buffer, pH 7.5, containing 50 mM NaCl and 1 mM phenylmethylsulfonyl fluoride (PMSF) (biomass to buffer ratio (w/v, g/ml) was 1 : 9) using a homogenizer. Lysozyme (100 μg/ml) was added to the suspension and incubated for 30 min at room temperature. Then the cells were disrupted by sonication on ice for 2 min (5 s pulses with 3 s intervals, amplitude 60%; Bandelin Sonopuls HD3200, Germany). The lysate was cleared by centrifugation (10,000*g* for 35 min at 4°C), and the supernatant containing the recombinant lysostaphin was subjected to fractional precipitation with $(NH_4)_2SO_4$. Maximum output of lysostaphin was achieved in the range of 15-40% $(NH_4)_2SO_4$ saturated solution. This step allowed the isolation, as a pellet, of almost all of the soluble lysostaphin synthesized by *E. coli* M15 producer. After centrifugation (10,000*g* for 35 min at 4°C), the pellet was dissolved in uQ water, and conductivity of the protein solution was adjusted to 30 mS/cm.

The protein solution was applied to the column with cation exchanger Unosphere S (Bio-Rad) equilibrated with 25 mM Tris-HCl, pH 7.5, 50 mM NaCl, and 1 mM PMSF, at 1 ml/min flow rate. The column was washed with 15 volumes of the same buffer. Lysostaphin was eluted with 0-0.5 M NaCl gradient buffered with 100 mM Tris-HCl, pH 7.5, in three column volumes. All chromatographic operations were conducted on BioLogic LP equipment (Bio-Rad). UV absorption (at 280 nm) and conductivity data were monitored and collected using LP Data View software (Bio-Rad).

In the case of *E. coli* BL21 producer, 2.9 g of produced biomass was treated as described above, with the

exception of fractional precipitation with $(NH_4)_2SO_4$, and the chromatography was carried out under the same conditions. All the purification procedures, except treatment with lysozyme, were carried out at 4°C or on ice. An equal volume of glycerol was added to the solutions of purified lysostaphin and the material was stored at –20°C.

Protein concentration was determined spectrophotometrically by the Lowry method using Bio-Rad DC Protein Assay kit according to the protocol and using bovine serum albumin (Sigma-Aldrich, USA) as a protein standard for calibration, or by the BCA Protein Assay (AppliChem GmbH, Germany). Protein electrophoresis was carried out in 12 or 15% polyacrylamide gel using chemicals from Bio-Rad according to the protocol recommended for denaturing conditions. Target protein yield at various purification stages and molecular weights were estimated using Gel-Pro Analyzer 3.1 software (Media Cybernetics, USA) or Quantity One (Bio-Rad) equipment and software, and determination error was below 0.5 kDa. Recombinant lysostaphin obtained from Sigma-Aldrich was used as a reference in comparative experiments on effects of temperature and pH on lysostaphin activity.

Determining susceptibility of *S. aureus* and *S. haemolyticus* clinical isolates to lysostaphin. *Staphylococcus aureus* and *S. haemolyticus* strains were isolated from clinical sources in the Laboratory of Biologically Active Nanostructures at the N. F. Gamaleya Federal Research Centre for Epidemiology and Microbiology. The strains were identified using a STAPHYtest 16 kit (ErbaLachema 10003378). Lysostaphin activity against clinical strains of staphylococci was determined by the agar well diffusion method. Cultures of strains stored in cryoprotectant Cryoinstant (Deltalab, Spain) were inoculated on tryptone soya agar (HiMedia Laboratories Pvt. Ltd., India) and incubated for 24 h at 37°C. Then suspensions of bacterial cells in phosphate buffer solution were prepared from the grown bacterial cultures, wherein their turbidity corresponded to 0.5 McFarland ($1.5 \cdot 10^8$ CFU/ml).

Petri dishes were filled with Mueller–Hinton Agar (Conda, Spain) to agar layer thickness of 4 mm in all dishes. Bacterial suspensions were inoculated to the Petri dishes by even distribution over the agar surface. Wells of 6 mm diameter were made in the agar gel. Then the tested lysostaphin solution (30 μl with the protein concentration of 0.5 mg/ml) was dropped into the wells (i.e. 15 μg per well). The dishes were incubated for 24 h at 37°C. Diameters of clear growth inhibition zones around the wells were determined after the incubation. Diameters of zones with total and partial inhibition of staphylococcus cell growth (TIZ and PIZ, respectively) were measured. Tests and measurements were performed in triplicate, and results were expressed as mean value ± standard error of the mean (SEM) of growth inhibition zone diameters obtained with lysostaphin.

Evaluation of lysostaphin activity by turbidity decrease of inactivated staphylococcus cell suspensions. In this work, clinical isolate *S. aureus* R 116-14 was used as a substrate for testing lysostaphin lytic activity. Two methods were used for complete inactivating the staphylococci: the bacterial cells were (i) autoclaved at 132°C under 2.0 atm pressure for 20 min or (ii) incubated in 1% SDS (Serva, Germany) solution in 100 mM Na-acetate buffer, pH 4.5. In the second case, 24-h culture grown on BHI agar medium was washed with 1% SDS solution in 100 mM Na-acetate buffer, pH 4.5, and the suspension was incubated under shaking at 24°C for 3 h. Then the cells were washed free from SDS twice with 20 mM Tris-HCl containing 50 mM NaCl, pH 7.5. The final cell suspensions in buffer were inoculated on growth medium to control their sterilization. In both inactivation techniques, a suspension of inactivated cells was prepared in sterile isotonic NaCl solution containing 50 mM Tris-HCl buffer, pH 7.5 (or 100 mM Tris-HCl with other pH values in experiments on determination of pH dependence of lysostaphin activity), wherein OD at 600 nm (A_{600}) was adjusted at 0.25 ± 0.01 (mean ± absolute deviation from mean) in a 1-cm photometric cell. Then aliquots of lysostaphin solution were added to aliquots of cell suspension, wherein the volume of lysostaphin aliquots did not exceed 0.2% of the total volume of the mixture (6 ml), and incubated for 10 min at 37°C (or other temperature, when studying temperature dependence of lysostaphin activity). The initial light absorption of the suspensions (A_{600}) was measured, and then the decline of light absorption was determined (ΔA_{600}, i.e. turbidity clearance of cell suspensions in 10 min), and the resulting value was expressed in percentages of the initial value (mean ± absolute deviation from the mean), wherein the measurements were done in triplicate.

The sterile staphylococcus cell suspension could be stored for two weeks at 4°C unless frozen (being frozen, it lost its validity probably because of peptidoglycan layer destruction and cell deformation resulting from the freezing–thawing cycle).

RESULTS AND DISCUSSION

Gene cloning, protein purification, and testing for the presence of lytic activity in recombinant lysostaphin preparations against two species of live staphylococci. Recombinant *S. simulans* lysostaphin was produced in the present work by cloning of the synthetic gene using pQE6 vector with subsequent production in *E. coli* M15 cells, or pET-45b(+) vector followed by protein production in *E. coli* BL21. Induction with IPTG resulted in production of the recombinant protein achieving 6 and 30% of total cell proteins in *E. coli* M15 and BL21, respectively (Fig. 1a, lane *1* and Fig. 1b, lane *2*), that shows the higher efficacy of the *E. coli* BL21 producer. The target protein was very

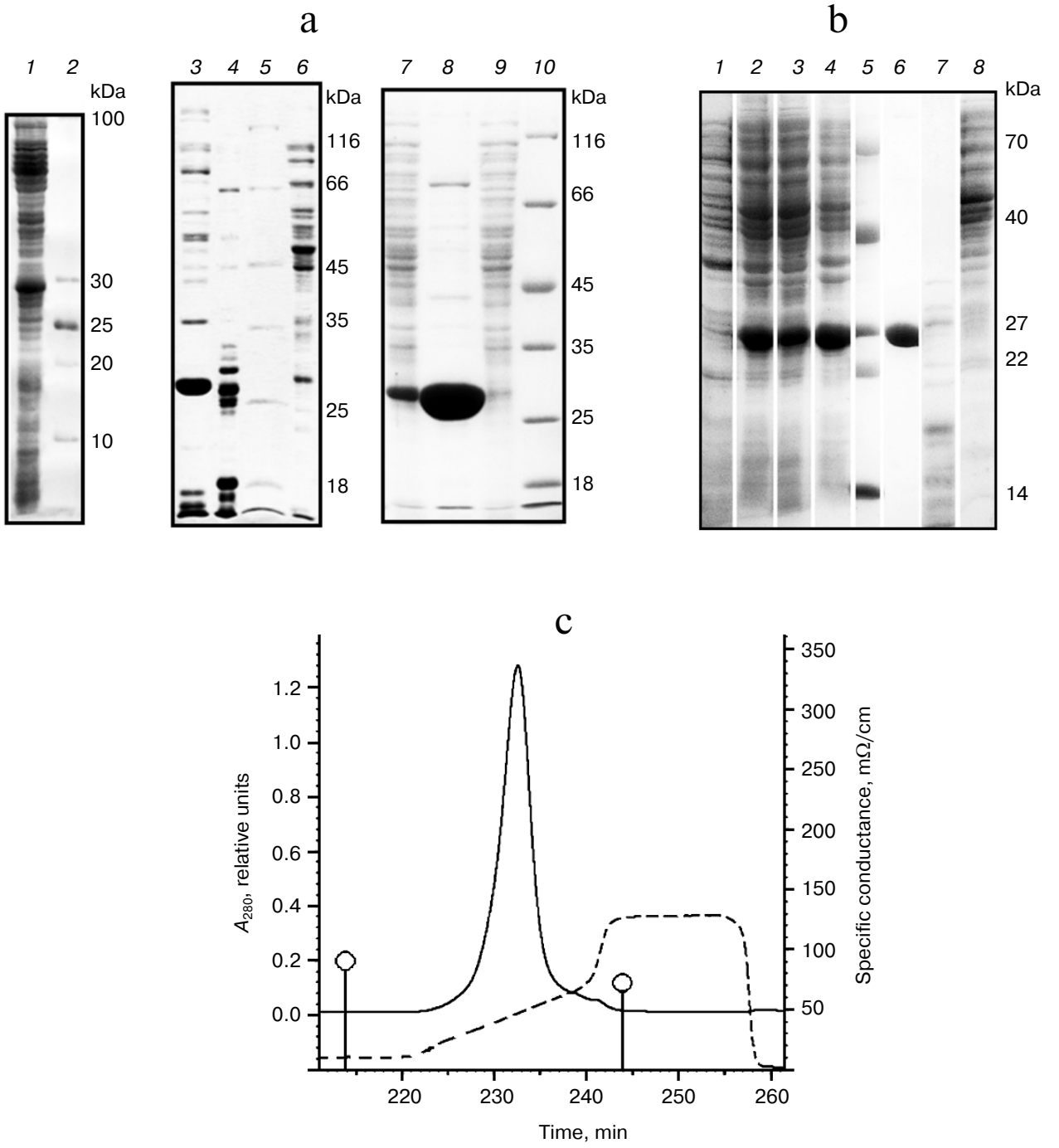

Fig. 1. a) Patterns of 12 and 15% polyacrylamide gels after Laemmli electrophoresis of protein samples obtained from *E. coli* M15 producer (stained by Coomassie BB R250). *1*) Extract of total cell proteins after induction with IPTG, 15 μg; *2*, *5*, *10*) protein markers of molecular weight; *3*, *8*) protein "peaks" containing lysostaphin after cation-exchange chromatography, 10 and 3.5 μg, (*3* – without fractional precipitation with $(NH_4)_2SO_4$, *8* – with the fractional precipitation); *4*) protein eluted from Unosphere S column with 1 M NaCl, 10 μg; *7*) lysostaphin-containing fraction after fractionation with $(NH_4)_2SO_4$, before loading on the Unosphere S column, 5.2 μg; *6*, *9*) flow-through from Unosphere S, 10 and 14.2 μg. b) Pattern of 12% polyacrylamide gel after Laemmli electrophoresis of protein samples obtained from *E. coli* BL21 producer (stained by Coomassie BB R250). *1*) Cell protein extract before induction with IPTG, 15 μg; *2*) cell protein extract after the induction with IPTG, 15 μg; *3*) readily soluble protein extract (cytoplasmic cell proteins), 15 μg; *4*) proteins from pellet after separation of readily soluble protein fraction, 15 μg; *5*) protein markers of molecular weight; *6*) lysostaphin "peak" after cation-exchange chromatography, 2.1 μg; *7*) flow-through from Unosphere S, 2.1 μg; *8*) proteins eluted from Unosphere S with 1 M NaCl, 6.2 μg. c) Lysostaphin elution profile from cation exchange Unosphere S column of 25 ml volume (an example for the protein from *E. coli* M15 producer, including fractionation with $(NH_4)_2SO_4$). The left circle – start of NaCl gradient formation protocol, the right circle – the end of the protocol.

soluble in *E. coli* M15 producer cells, whereas a readily soluble (cytoplasmic) and insoluble (deposited in inclusion bodies and forming a pellet under cell disruption) forms of lysostaphin were synthesized in approximately equal amounts in *E. coli* BL21 producer cells.

When attempting further increase of lysostaphin production and yield (in the case of *E. coli* BL21 producer) by the addition of 0.5% glucose to the growth medium, the greater portion (up to 50% of total protein based on PAGE) of the target protein is synthesized as insoluble form and is deposited in inclusion bodies. In this case, attempts to solubilize and refold the lysostaphin from its insoluble form led to substantial loss in specific lysostaphin activity, and the target-purified lysostaphin had lower specific activity in comparison with the protein isolated from cells grown without glucose addition (data not shown).

Images of gels obtained after electrophoresis of protein fractions containing lysostaphin at its various purification steps are given in Fig. 1. Lysostaphins isolated and purified from *E. coli* M15 or BL21 cell biomasses had virtually identical properties (as described further), but the portion of the target protein in its insoluble form was greater in BL21 cells and comprised almost 50% (Fig. 1b), whereas it was negligible in M15 (Fig. 1a).

Previously, Khatri and Sharma [17] reported lysostaphin production comprising approximately 20% of the total proteins of the induced cell extract using *E. coli* BL21(DE3) strain. In the present work, the production of target protein in *E. coli* BL21 has been increased 1.5 times, probably due to the codon composition optimization of the cloned gene sequence.

Since the amount of lysostaphin synthesized in *E. coli* M15 cells (in percentages) was smaller than in BL21 strain, purification of the target protein from M15 cells included an additional stage, such as precipitation with $(NH_4)_2SO_4$, and the cation-exchange chromatography step was applied in both cases. Fractional precipitation with $(NH_4)_2SO_4$ for lysostaphin purification was initially proposed by Schindler with coworkers [19, 20]. In the present work, this purification step has removed the major portion of admixtures and enhanced purity of the target protein from 58 to >97% (Fig. 1a, lanes *3* and *8*).

Since calculated lysostaphin polypeptide average charge is +11.5 in Tris-HCl buffer, pH 7.5, it predictably binds to cation exchanger Unosphere S (Bio-Rad) and can be eluted at elevated ionic strength. Elution from the column with gradient concentration of NaCl in 100 mM Tris-HCl, pH 7.5, resulted in a single protein peak (Fig. 1c) possessing the specific enzymatic activity of lysostaphin. Further elevation of ionic strength results in elution of protein admixtures, and their amount is much greater in the case of M15 producer (Fig. 1a, lane *4*) than for BL21 (Fig. 1b, lane *8*). Earlier Khatri and Sharma [17] introduced an additional purification step, such as anion-exchange chromatography at pH 8.5. In the present work, higher expression level achieved in BL21 producer cells and initially higher portion of the target protein in the

Table 1. Lysostaphin isolation and purification from 5 g biomass of *E. coli* M15 producer

Fractionation step	Total protein amount, mg	Lysostaphin purity, % of total protein	Yield, %	Lysostaphin amount, mg
Ultrasound treatment	362	6.0	100.0	21.7
Soluble protein extract	224	9.4	97	21.1
20-40% $(NH_4)_2SO_4$ fraction	40.4	49.3	92	19.9
Cation-exchange chromatography	16.9	97.6	76	16.5

Table 2. Lysostaphin isolation and purification from 3 g biomass of *E. coli* BL21 producer

Fractionation step	Total protein amount, mg	Lysostaphin purity, % of total protein	Yield, %	Lysostaphin amount, mg
Ultrasound treatment	420	29	100.0	121
Soluble protein extract	338	24	97	80
Inclusion bodies protein extract	72.3	52		38
Cation-exchange chromatography of soluble proteins	18.3	97	31	18
Cation-exchange chromatography of proteins from inclusion bodies	20.7	97		20

fraction precipitated with $(NH_4)_2SO_4$ in the case of M15 producer provided lysostaphin apparently homogeneous on electrophoresis while omitting the additional chromatography step.

For both producers, the resulting protein was >97% pure by Laemmli electrophoresis, and its apparent molecular mass of 27.5 kDa estimated from molecular weight protein standards (Fig. 1a) was close to the value calculated from the amino acid sequence (27.517 kDa).

For the M15 producer, the lysostaphin yield comprised about 4 mg per 1 g wet biomass (the mean of three repeated purification runs). For BL21 producer, the protein yield comprised about 13 mg of the target protein per 1 g wet biomass (the mean of three purification runs), about half of this protein (46%) was synthesized in completely soluble cytoplasmic form, and half (54%) as inclusion bodies. The specific enzymatic activities of lysostaphins purified from cytoplasmic fraction and inclusion bodies – according with the same purification scheme – were identical (data not shown). Tables 1 and 2 demonstrate examples of recombinant lysostaphin yields in the course of its purification run from biomass of producer strains – *E. coli* M15 and BL21, respectively.

Since about 4-5 g biomass can be obtained from 1 liter of a producer culture when it is grown in flasks, use of BL21 producer (in combination with the developed protein purification scheme) yields up to 55-70 mg of purified lysostaphin from 1 liter of flask culture. Khatri and Sharma [17] earlier achieved the yield of 8.9 mg.

Unlike recombinant lysostaphin described in earlier cited work [9], which became inactivated upon biomass freezing, the recombinant lysostaphin produced by *E. coli* M15 and BL21 cells was active after freezing and thawing of the biomass, this fact allowing time separation between the stages of biomass production and protein purification, which is technologically preferable. The method of lysostaphin purification proposed in the present work does not require affinity or other expensive sorbents and enables production of recombinant lysostaphin of high purity (>97%).

Enzymatic properties of purified recombinant lysostaphin. In most works known from the literature, turbidimetric assay of lysostaphin activity is based on decrease in absorption of suspensions of *S. aureus* cells after addition of lysostaphin [9, 11, 19]. Turbidity is measured by spectrophotometry at 600 nm (or 620 nm for viable cells) in a 1-cm spectrophotometric cell. The bacteriolytic activity of lysostaphin is expressed in relative (arbitrary) units as a proportion (%) in turbidity reduction (ΔA_{600}) of a standard bacterial suspension (either living or autoclaved cells), per definite time range (usually 10 min) at 37°C [19-21]. Sigma-Aldrich also recommends a protocol assuming spectrophotometric estimation of absorption clearance in suspensions of live *S. aureus* cells for determination of enzymatic activity in recombinant lysostaphin preparations offered by this company (L9043).

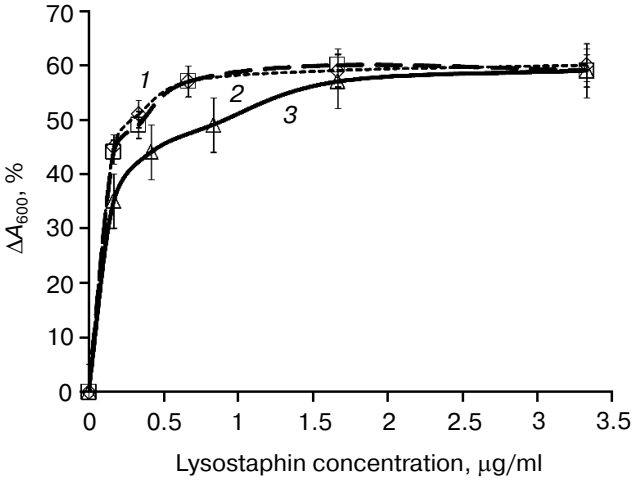

Fig. 2. Dependence of absorbance decrease at 600 nm (ΔA_{600}, %) in suspension of inactivated *S. aureus* cells in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl on lysostaphin concentration (μg/ml) in reaction medium after incubation for 10 min at 37°C. Initial absorbance $A_{600} = 0.25 \pm 0.01$. *1*, *2*) Lysostaphins obtained in the present work (M15 and Bl21, respectively); *3*) lysostaphin from Sigma-Aldrich.

Since use of live *S. aureus* cells cannot be recommended as a routine protocol for determination of lysostaphin activity in laboratory practice, the present work proposes a method for determination of lysostaphin activity using inactivated staphylococci and compares various preparations of lysostaphin under identical conditions. This semiquantitative method for determination of lysostaphin activity *in vitro* assumes the use of inactivated *S. aureus* cells (autoclaved or treated with SDS at pH 4.5, as described in "Materials and Methods"). The use of this method as a routine laboratory technique is safe and does not require any safety control when working with pathogens. Dependence of ΔA_{600} in a cell suspension on lysostaphin concentration in reaction medium is shown in Fig. 2. The dependencies obtained for autoclaved or SDS-inactivated cells were identical (data not shown). Maximum decrease in absorption (ΔA_{600}) comprised ~60% after 1.7 μg/ml lysostaphin addition and 10 min incubation.

In the present comparative study, preparations of purified recombinant lysostaphin produced by *E. coli* M15 or BL21 producers displayed virtually identical specific activities comparable with that in lysostaphin supplied by Sigma-Aldrich (Fig. 2).

It is difficult to compare specific activity between lysostaphin obtained in the present work and that of lysostaphin obtained by Khatri and Sharma [17] because specific activity units proposed by the authors differ from those proposed by Sigma-Aldrich: one unit of lysostaphin was defined as the amount of the enzyme required to decrease optical density (A_{600}) by 0.001 in 3 ml suspension of live *S. aureus* 237 cells after 5 min incubation at 37°C.

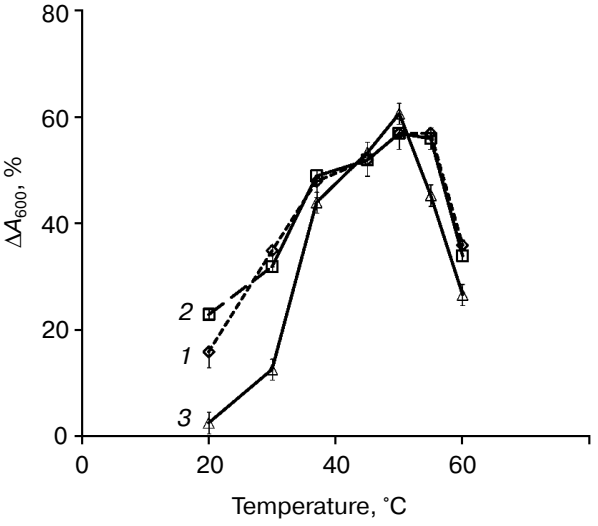

Fig. 3. Dependence of lysostaphin activity on temperature. Decrease in absorbance at 600 nm (ΔA_{600}, %) of autoclaved *S. aureus* cell suspension in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl solution after incubation for 10 min in the presence of 0.8 μg/ml lysostaphin in reaction mixture. Initial absorbance $A_{600} = 0.25 \pm 0.01$. *1*, *2*) Lysostaphins obtained in the present work from *E. coli* BL21 and M15, respectively; *3*) lysostaphin from Sigma-Aldrich.

However, the authors state that the "specific activity of 12,000 U/mg in purified recombinant lysostaphin was comparable with that of lysostaphin from Sigma-Aldrich" [17].

Huang with coauthors [22] determined lysostaphin activity using colored peptidoglycan KNR-PG as a substrate [23] and reported specific activity of their product equal to 1100 U/mg. They stated also that secretory expression of recombinant lysostaphin (containing a signal peptide determining the product secretion) produced by *E. coli* cells and purified in their work and the product from Sigma-Aldrich had "comparable specific activities". Thus, recombinant lysostaphins without additional tag domains of various origin and the secreted lysostaphin possess comparable specific activities.

The literature reports several attempts to develop methods for lysostaphin activity assay using synthetic substrates containing pentaglycine sequences either releasing colored products [23] or based on FRET analysis or designing polypeptide FRET substrate for lysostaphin [15, 16]. The dye-release assay described by Zhou et al. [23] required 3 μg/ml lysostaphin protein concentration. Its sensitivity was higher than the FRET assay of Warfield and coworkers [16] (14 μg/ml) and Bardelang and coworkers [15] (5.5-27 μg/ml). The concentration of lysostaphin protein produced in the present work and required for the assay varied within the range 0.15-2.5 μg/ml, whereas the dependencies of ΔA_{600} on protein concentration achieve a plateau (saturation) at ~1.7 μg/ml for lysostaphins produced in *E. coli* M15 or BL21 cells (Fig. 2). One can say that both preparations possess activity slightly higher than that described in literature, and the method adapted in the present work is sensitive enough for adequate comparison between various lysostaphin preparations.

Influence of reaction medium temperature on lysostaphin activity. Dependencies of lysostaphin activities on temperature of the reaction mixture for the lysostaphin preparations obtained in the present work are given in Fig. 3. Maximum activity corresponds to 50°C. The curves for both lysostaphins obtained in the present work are similar and close to that for lysostaphin from Sigma-Aldrich, as well as for $(His)_6$-lysostaphins described by Szweda and coworkers [9] and Sharma and coworkers [11], and close to that described by Khatri and Sharma [17] (with maximum about 47°C).

Since the pH dependence of enzymatic activity is significant for biotechnological and medical applications,

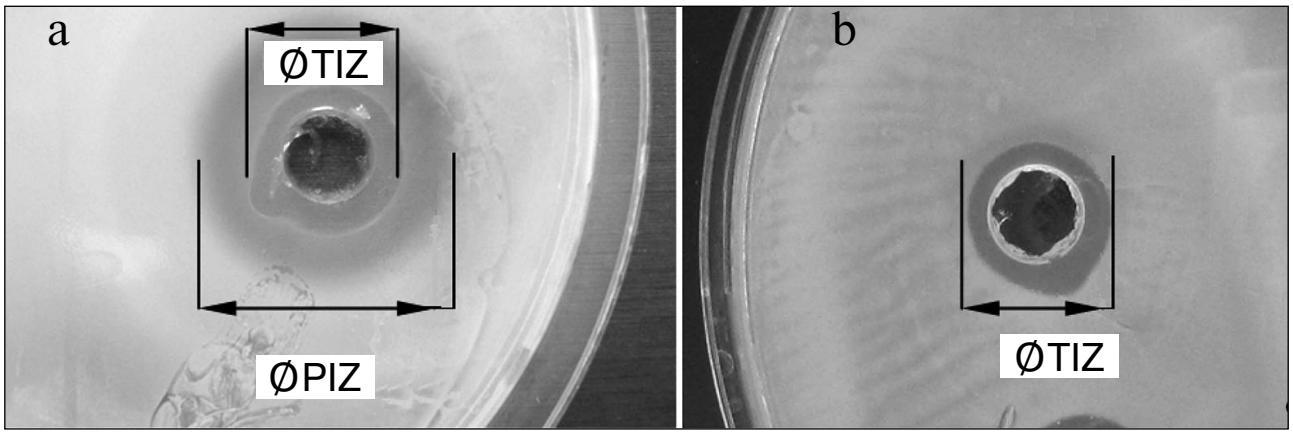

Fig. 4. Antistaphylococcal activity of lysostaphin *in vitro*. a) Total and partial growth inhibition zones (TIZ and PIZ, respectively) for *S. aureus* around a well with lysostaphin. b) Total growth inhibition zone (TIZ) for *S. haemolyticus* around a well with lysostaphin.

lysostaphin activity was measured under varying pH. We did not observe any significant influence of pH on lysostaphin activity within the studied pH range (6.5-8.8). Thus, activity of recombinant lysostaphins obtained in present work is virtually independent of pH in 100 mM Tris-HCl buffer. It is this property that distinguishes lysostaphin obtained in our work from the recombinant $(His)_6$-lysostaphins described in the literature and purified via metal-affinity chromatography: those lysostaphins displayed prominent – more than 2 times – alteration in their activity on varying pH from 6.5 to 8.8 [9, 11].

On the other hand, lysostaphin preparations obtained in the present work are similar in their stability under varying pH to the preparation of lysostaphin from Sigma-Aldrich. The same was noted by Sabala and coworkers [24] when they studied recombinant lysostaphin produced in their work.

Staphylococcus growth inhibition by lysostaphin. In experiments determining susceptibility to lysostaphin of staphylococcus cells grown on agar medium, *S. aureus* strain displayed TIZ (9.0 ± 0.9 mm), as well as PIZ (18 ± 1.5 mm), and the culture resembled a thin translucent film within PIZ (Fig. 4a). Gram-negative staining was observed on microscopy of cell preparations taken from PIZ (surprisingly for Gram-positive staphylococcus), suggesting substantial structural alterations of cell wall. This may be associated with thinning of the peptidoglycan layer due to the disruption of cross bridges with lysostaphin.

As for *S. haemolyticus*, it was also sensitive to lysostaphin displaying formation of a TIZ with similar diameter of 9.0 ± 0.9 mm (Fig. 4b). Several orders of magnitude difference in antistaphylococcal activity of lysostaphin is known from the literature for different staphylococcus species [25]. However, the example with two strains tested in present work demonstrates a more complex effect. In the case of *S. aureus*, the effect of lysostaphin at lower concentrations corresponding to PIZ is different from that of its higher concentrations: the cells have apparently damaged peptidoglycan layer resulting in change in their Gram-staining, but they maintain their viability, although their growth rate is decreased. It is hard to evaluate lysostaphin concentration at the outer border of the PIZ, but probably it corresponds to minimum inhibiting concentration of lysostaphin for *S. aureus* strains, in accordance with literature data comprising 2 μg/ml [26].

Thus, recombinant lysostaphin obtained in this work displays significant lytic activity *in vitro* against clinical isolates of *S. aureus* and *S. haemolyticus*. Of two strains used as producers for recombinant lysostaphin, the *E. coli* BL21 strain is clearly better than M15 in terms of further biotechnological application for the expression of the *S. simulans* bv. *staphylolyticus* lysostaphin gene. The study of general properties of the produced recombinant lysostaphin has demonstrated its similarity with the preparation from Sigma-Aldrich and several advantages over lysostaphin preparations described in the literature with $(His)_6$ tag. The effective producer of heterologous protein (*E. coli*) and simple technology for production of highly purified protein preparation possessing high lytic activity against tested staphylococci isolated from clinical sources are prerequisites for further use of recombinant *S. simulans* bv. *staphylolyticus* lysostaphin as a basis for pharmaceutical preparation for clinical applications.

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