

TECHNOLOGICAL OPTIONS FOR THE PRODUCTION OF LACTOFERRIN

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Abstract: Lactoferrin (LTF) is a multifunctional protein of the transferrin family present in human and other mammals' milk. Global demand for originated protein is currently much higher than the supply, and lactoferrin remains the one of the most expensive proteins. Therefore, the development of cost efficient methods to produce lactoferrin is extremely important. In this study, human lactoferrin is obtained in bacterial system. *lrf* gene, coding sequence of protein - human lactoferrin (hLTF), was cloned into the expression vector pET28a+, modified signal sequence IMKKTAIAlAVALAGFATVAQA AS. *E.coli* BL21DE3 strain was selected for expression. Productivity of the producing strain in regard to recombinant human lactoferrin was 30–35% of the cellular protein. The strain produced the recombinant lactoferrin in the form of inclusion bodies. The maximum yield of the target protein (including in soluble form) was achieved by culturing of the recombinant strain at 25°C, induction by 1.1 mM concentration of IPTG and 8-h period after induction. Optimization of solubilization and renaturation conditions allowed once to recover lactoferrin in a concentration of 2.9 mg/ml by the pulsed system. The protein of high purity at least 90% was obtained by means of affinity chromatography on Ni⁺-agarose.

Keywords: lactoferrin, milk protein, gene, *E.coli*

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INTRODUCTION

Milk can be considered the first functional food in life as it contains, not only nutrients for the newborn, but also essential components for organ development and normal physiology. An overarching goal is to identify and produce beneficial components of milk for improving nutrition and for specific therapeutic interventions. While some components are already being isolated from bovine milk to meet this goal, others, especially components of human milk cannot be easily obtained from the natural source. This is illustrated by lactoferrin [1, 2].

Lactoferrin is a polyfunctional protein of the transferring family, which has anti-bacterial, anti-viral, anti-cancer, antifungal, antiparasitic, antioxidant and regenerative properties. Lactoferrin can be found in the human and other mammal's milk [3]. On the basis of the many biological activities of hLF, researchers have considered a wide variety of possible applications in human health care, such as prophylaxis and treatment of infectious and inflammatory diseases [4].

Until recently, the therapeutic use of lactoferrin has been limited by the lack of an efficient and cost-effective method for the production of the human protein in large quantities. The limited availability of human milk and purified hLF has been a major hurdle for (clinical) studies on potential nutraceutical and pharmaceutical applications of hLF. To overcome this limitation, the feasibility of large-scale production of functional recombinant human lactoferrin (rhLF) was studied in a variety of expression systems [5]. The small quantities of lactoferrin have been expressed in eukaryotic systems, including baby hamster cells and human 293 cells. While the recombinant lactoferrin produced was biologically active, these systems are not readily amenable for scale production. The lactoferrin has been expressed in yeast expression system: *Saccharomyces cerevisiae*, *Pichia pastors* [6]. The secretion of recombinant lactoferrin in these systems was inefficient, with less than 10% of the lactoferrin produced being secreted into the growth medium and this host cell itself is found to be highly susceptible for

the lactoferrin produced. The ability to get lactoferrin by means of fungal expression system (*Aspergillus* [7]) was demonstrated. The bioavailability of the lactoferrin, obtained by means of this system, was less than 0.5%. Also, the using of *Aspergillus* for the lactoferrin production was associated with a high risk of contamination with aflatoxins, which are potential carcinogens. *Ltf* has been expressed in plant culture: potato plants [8], tobacco plants [9], ginseng cell culture [10], rice cell culture [11]. However, such problems as low level of lactoferrin expression, even when using a strong promoters; congestion with carbohydrate component; risk of the transgene leakage into the environment (out of control) make these approaches not suitable for large scale production.

Ltf has been expressed in the mammalian expression system: mice, rabbits [12], transgenic cows [13], transgenic goats [14]. However, the expression level was unstable and not suitable for fast expression of the proteins. Also, the success of this approach, including the ability to purify the recombinant protein in a viral- and toxin- free form and the cost-effectiveness of large transgenic animal program, remains to be established.

The advantages making microbial synthesis of the lactoferrin promising are metabolic flexibility and high ability of the microorganisms to adapt, high growth rate, ease of cultivation, the study of genetics and othe [15].

Expression of recombinant proteins in *E.coli* cytoplasm is widely used. However, improper folding of many target proteins may occur during this process. Improper folding often results in the formation of

inclusion bodies despite attempts to optimize growth conditions. One possible approach to obtaining a correctly folded recombinant protein is to export the protein into the *E. coli* periplasm [16, 17].

The purpose of this study is to develop a technology of obtaining human lactoferrin from *E. coli* cells.

OBJECTS AND METHODS OF STUDY

Reagents. The following reagents were used in the work: isopropyl-β-thiogalactopyranoside (IPTG) ("ALMABION", Russia), lactose ("ProfiPark", Russia), T4 DNA ligase, *Taq*- and *pfu* DNA polymerase, buffer for *taq*-polymerase, buffer for *pfu*-polymerase, DNA markers, deoxyribonucleases, mineral oil, the restriction enzyme XhoI and HindIII, buffer XhoI, buffer HindIII ("Sibensim", Russia), ethylenediaminetetraacetic acid (EDTA), sodium dodecyl sulphate (SDS), magnesium chloride, boric acid, sodium hydroxide, acrylamide, N,N,N',N'-tetramethylethylenediamine (TIMED), persulfate ammonium, glycine, Coomassie R-250, sodium perchlorate, bromophenol blue, Tris, hydrochloric acid, 0,1% DS-Na, glucose, PEG, the solution of TE, phenol and chloroform, potassium acetate, sodium chloride ("Invitrogen", USA), suspension of silica, carbod ("Helicon", Russia), LB-medium ("Gibco BRL", US), ribonuclease, kit "DNA isolation from agarose gels", ("Sileks", Russia), ampicillin, agarose, ethidium bromide, 2-mercaptoethanol, lactoferrin ("Sigma", USA), kit "Lactoferrin-IFA-best" ("Vector-best", Russia).

Plasmids.

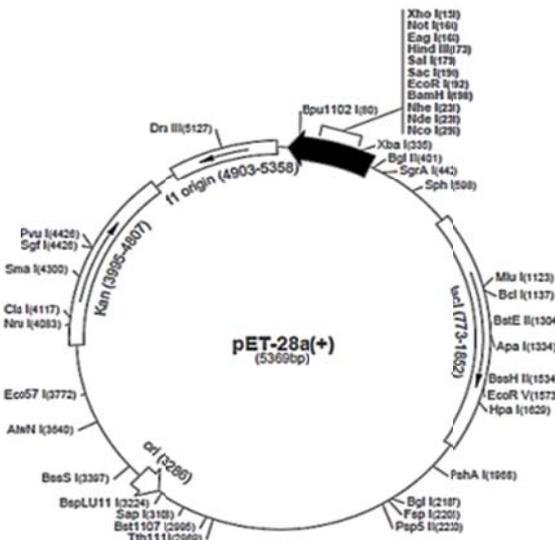


Fig. 1. pET28a+ plasmid which was used in the work

Construction (Fig. 1) was modified by signal sequence of OmpA, providing transport of proteins across the inner membrane into the periplasm - IMKKTAIAIAVALAGFATVAQA AS on sites (BspH I)OmpA (HindIII):

atcatgaagaaaaccgcatcgccatcgccgtggcgcctggcaggtttccaccctggca
 I M K K T A I A I A V A L A G F A T V A

cagccgctag
 Q A A S
 Strain – *E.coli* BL21 DE3

mRNA synthesis. mRNA of human lactoferrin (LTF laciotransferrin [Homo sapiens]) based on the sequence of GeneBank database (GenBak: 23268458), in which were held insignificant nucleotide substitutions, was synthesized by the Evrogen (Russia).

mRNA of lactoferrin gene was synthesized so that directly behind the coding sequence was located the site corresponding to hexahistidine sequence. As a result the induced expression of the cloned gene will be synthesized lactoferrin, containing additional hexahistidine sequence in the C-terminal region of the polypeptide chain, allowing to carry out subsequent affine cleaning of the protein on Ni-NTA-agarose.

Hydrolysis of the DNA fragments of XhoI u HindIII by means of restriction enzymes was performed in 100 µl of special buffers at optimal temperature to each enzyme – 37°C. Completeness of the hydrolysis was monitored by means of gel – electrophoresis on agarose gel.

Agarose gel electrophoresis is carried out according to the standard procedure described in [18].

Isolation of DNA fragments from the gel. The DNA samples were separated by means of electrophoresis in TBE buffer in 0.8–1% of a gel block containing 0.5 µg/ml ethidium bromide, and were analyzed in the UV. Pieces of the gel containing the fragment of interest was excised and transferred to microcentrifuge tubes. Then elution of fragments was performed using the kit “Isolation of DNA fragments from agarose gels”.

Ligation. Electrophoretically purified HindIII-ltf-His-Tag-XhoI fragment and fragment of the mpET28a+ vector were ligated in ligase buffer with T4 DNA ligase for 12 hours at 10°C. Thereafter ligase was inactivated by heating of the mixture for 5 min at 70°C. Purification from unreacted PCR fragments was performed by excising from the gel.

Preparation and transformation of competent cells with the obtained genetic structure were performed by means of heat shock. To this, 1 ml of overnight culture was grown at 30°C with intensive aeration in medium, supplemented with 0.9% glycine, 0.02 M MgCl₂ and ampicillin, in a shaker. The cells were pelleted by centrifugation after cooling on ice for 10 minutes. The pellet was resuspended in 1 ml of cooled TB1 buffer, further tubes were incubated on ice for 10 minutes and placed for 30 sec. in a water bath preheated to 42°C.

2M glucose was added in the tube after the thermal shock and the mix was incubated for 1 hour at 37°C (or 1.5 h at 30°C). Then mix was plated on Petri dishes with LB medium, containing 30 µg/ml ampicillin. The dishes were incubated in a thermostat at 37°C for 16 hours.

Isolation of plasmid DNA was carried out according to the method of Maniatis.

Lactoferrin heterologous expression. The plasmid was transformed into BL21(DE3)/mpET28a competent cells, then the cells were spread on LB-agar plates, followed by overnight culture at 37°C. The colonies from LB-agar plates were selected and cultured in 5 ml of LB liquid medium, plus 50 µl of 50% glucose, overnight at 37°C with shaking. The next morning, 1

ml of overnight culture was inoculated in 100 ml of fresh LB liquid medium, plus 1 ml of 50% glucose, and cell culture was continued at 37°C with shaking while monitoring growth of the culture by measuring the optical density at 600 nm (OD₆₀₀). At OD₆₀₀ of 0.6–0.8, 100 ml of culture was inoculated into 4 L LB, plus 30 ml of 50% glucose. The cell culture was continued again at 37°C with shaking while monitoring growth of the culture. Once OD₆₀₀ reached 0.6 again, the temperature was decreased to 23°C, the inducer was added (1 mM IPTG). All plates and LB liquid media used here contained 25 µg/ml of kanamycin.

Screening of cultivation conditions. The experiments were performed in 250 ml of LB medium containing 25 µg/ml kanamycin. 1 ml of culture was diluted 50 times in fresh LB nutrient medium and then cultivated (at 37°C, 250 rpm) until OD₆₀₀ nm of 0.4–0.8 of the cell density.

Response surface methodology (RSM) of statistic. RSM was used for optimize the induction conditions selected at univariate experiments, and for increase the production of recombinant lactoferrin. Composite design was used for optimization of three significant factors: temperature after induction, concentration of inducer and period after induction. The following polynomial second order equation explains the relationship between dependent and independent variables:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2, \quad (1)$$

where Y is the dependent variable; X₁, X₂ and X₃ are the independent variables (temperature after induction, concentration of inducer and time after induction); β₀ is the time of intersection; β₁, β₂, β₃ the linear correction factors; β₁₂, β₁₃ and β₂₃ the interaction coefficients and β₁₁, β₂₂ and β₃₃ the quadratic coefficients. The approximating polynomial equation in the form of contour and surface response plots was used for image the relationship between responses and experimental levels of each of the variables.

Determination of the lactoferrin concentration in accordance with the protocol to “Lactoferrin-ELISA-BEST” (“Vector-best”, Russia).

Protein analysis. SDS-PAGE was carried out using a 10% gradient gel according to Laemmli [19]. TotalLab program was used for the processing of electrophoregram results.

Isolation, solubilization and renaturation. After fermentation the culture medium was separately by centrifugation. The cells were harvested by centrifugation and suspended in 50 mM phosphate buffered saline (PBS, pH 8.0) buffer containing 200 mM NaCl. Protease inhibitors were added to the suspension. The cells were destroyed by ultrasound homogenizer Scienta-IID (China) by 4 times in the mode: total time – 30 seconds (2 sec – voicing, 2 sec – break), cooled in an ice bath for 1 minute. After sonification, the suspension was centrifuged at 5800g for 30 min at 4°C. The pellet was suspended several times in 50 mM PBS (pH 8.0) buffer containing 10% Triton X-100 and 200 mM NaCl to remove

nonspecifically adsorbed proteins, and the solution was centrifuged again at 5800 g for 30 min at 4°C. The pellet was used for optimization of solubilization with different concentration of Gn-HCl at pH 12 in 2 M Tris-HCl buffer. Optimization of renaturation was carried out in standart buffer: 0.5 M/L Gn-HCl, 50 mM/L Tris-HCl (pH 8.5), 0.75 M/L arginine, 1 M/L NaCl, 5 mM/L EDTA and 3 mM of glutathione in a ratio of 10 : 1 GSH : GSSG.

Purification on a Ni²⁺-NTA agarose. The sample was loaded onto a column of Ni²⁺-NTA agarose equilibrated with 150 mM PBS (pH 8.0) comprising 10 mM imidazole. The column was washed of unbound material with the source buffer, then with the 150 mM phosphate buffer (pH 8.0), comprising 20 mM imidazole. Then it was eluted with 150 mM phosphate buffer (pH 8.0), comprising 250 mM imidazole.

RESULTS AND DISCUSSION

The *ltf* gene expression in *E.coli* BL21DE3/mpET28a+*-ltf*

The cells received after the transformation were cultivated at 37°C for 24 h until optical density 0.6–0.8 in conditions when transcription of lactoferrin cloned gene was repressed. Then effective transcription was induced by the addition of the inducer. This approach allows to grow cells containing the mRNA of human lactoferrin gene to a high titer and only after to trigger the production of protein.

The target protein with a molecular mass of 78–80 kDa was accumulated in conditions of induction in *E. coli* BL21DE3/mpET28a+*-ltf* (Fig. 2).

The level of target protein expression (~78–80 kDa) was from 30 to 33% of the total cellular protein, as shown by electrophoresis in 10% polyacrylamide gel in the presence of sodium dodecyl sulfate (Fig. 2).

The level of the lactoferrin in the samples washed precipitations (inclusion bodies) was 1–3% less than the total expressed protein. Thus, the strain producing the recombinant lactoferrin mainly in the form of inclusion bodies (IB) (Fig. 2) in standart condition was obtained.

Selection of cultivation conditions

The expression of heterologous genes leads to metabolic depletion of cells, which is expressed in the inhibition of cell growth, decrease of the biomass yield and the target protein. Several one-factor experiments for study the impact of various factors on the production of target protein were carried out.

The following parameters presented in Table 1 were selected for testing. The experiments were conducted in three replicates.

Cell concentration

The cell density before induction is important factor for the expression of recombinant proteins.

Testing was performed at different cell concentrations from 0.4 to 1.0 at OD 540 nm. The results are presented in Fig. 3.

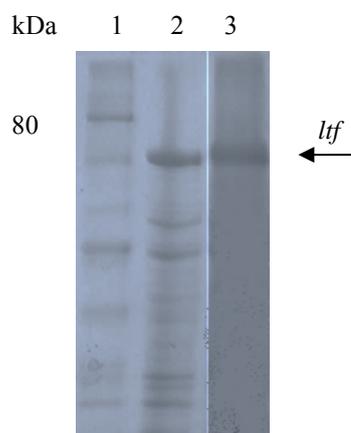


Fig. 2. The *ltf* gene expression in *E. coli* BL21DE3/mpET28a+*-ltf*. Lane: 1 – markers; 2 – lysate of the strain, transformed with the genetic construct containing *ltf* gene; 3 – after solubilisation of IB.

Table 1. Induction parameters selected for the study of lactoferrin production

| Induction condition | Range |
|---|-------------------|
| Cell concentration | OD 540 nm 0.4–1.0 |
| Type of inductor | IPTG / lactose |
| Concentration of inductor | 0.2–2.0 mM |
| Temperature cultivation after induction | 15–37°C |
| Time after induction | 2–24 h |

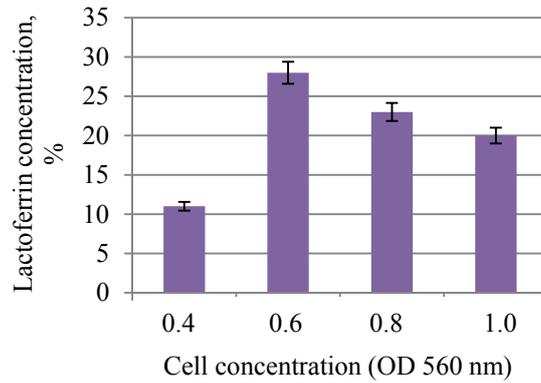


Fig. 3. The effect of cell concentration on the lactoferrin synthesis by cultivation with 1 mM IPTG, 37°C and 24 h after induction of strain.

The concentration of lactoferrin varies over wide range. The most amount of lactoferrin (28% of total cellular protein) was obtained during early induction (the cell concentration 0.6)

The effect of concentration and type of inducer

The used expression system of lactoferrin requires the addition of inducer (IPTG) for ensure of the cloned gene transcription in the plasmid and initiation of lactoferrin translation. This leads to

some changes in the metabolism of the host cell. The concentration of the inducer depends on how expressed product is toxic to the strain and used vector. In order to reduce the cost of induction, the effect of lactose concentration on the efficiency of expression and the ability to effectively replace IPTG were studied. This strain was grown at 37°C. The inducer was added in concentrations from 0.2 to 2 mM. The results are presented in Fig. 4.

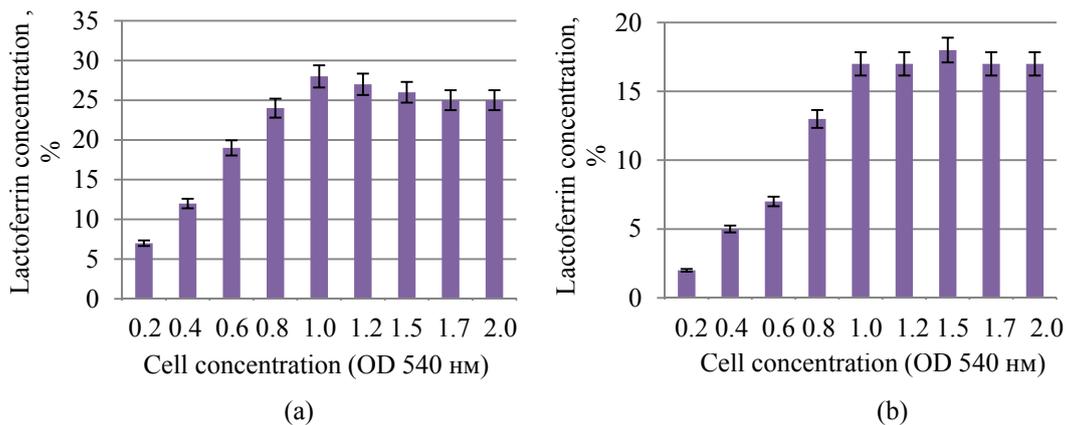


Fig. 4. The effect of IPTG (a) and lactose (b) concentrations on the lactoferrin synthesis (percentage of the total cellular protein) by cultivation at 37°C and 24 h after induction.

As Fig. 4a shows, the concentration of inducer (IPTG) affects on the lactoferrin synthesis. The increase of the lactoferrin concentration was observed by increasing of the IPTG concentration from 0.2 to 1.0 mM. The maximum percentage (28%) of lactoferrin was reached at a concentration of 1.0 mM. From 1.2 mM of IPTG the decrease of lactoferrin synthesis was observed due to the slower growth of bacteria at high concentrations of IPTG. High concentrations of IPTG in this case can lead to ribosomal degradation and the production of heat shock proteins and eventually to cell death.

As Fig. 4b shows, the concentration of the inducer (lactose) also affects on the lactoferrin synthesis. The increase of the lactoferrin concentration was observed by increasing of IPTG concentration from 0.2 to 1.5 mM. The maximum percentage was reached at a concentration of 1.5 mM.

Temperature of cultivation after induction

Lactoferrin expression in cells, transformed with pET28a+ plasmid, was studied at different temperatures. That is how the selection of temperature for cultivation of bacteria aimed at the maximum yield of the protein in a soluble form, was carried out. After induction, the recombinant strains were cultivated at different temperatures (37°C, 23°C, 15°C). The concentration of lactoferrin was measured in the culture medium after the destruction and dissolution of the inclusion bodies (IB).

As SDS-analysis shows (Fig 5), the expression of lactoferrin was observed at all investigated temperatures. Maximum protein synthesis was observed at 23°C. The maximum yields (62%) of target protein in the soluble form were achieved by culturing the recombinant strain also at 23°C. This is because

slower rates of production give sufficient time to the system to fold a protein that decreases the rate of aggregation and the possibility of random formation of intermolecular hydrophobic interactions. Almost all lactoferrin is produced in the form of inclusion bodies at 37°C. Lowering of temperature to 15°C also was not lead to greater accumulation of lactoferrin than at 23°C. Thus, cultivation of the strain at 23°C is the most appropriate.

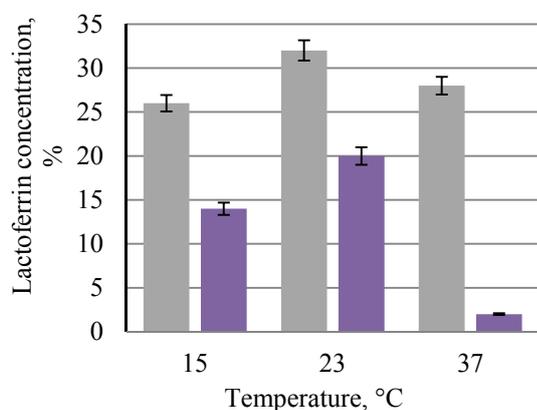


Fig. 5. The effect of temperature after induction on the lactoferrin synthesis (percentage of the total cellular protein) by cultivation at 37°C and 24 h after induction. The first column is total lactoferrin, second column – soluble lactoferrin.

Period after induction

The optimal time of expression was determined by analysis of samples taken after 2, 4, 6, 8, 10, 12 and 24 hours after induction. The total product (soluble and insoluble forms) of the total cellular protein was analyzed.

The effect of the duration of period after induction on the lactoferrin production is shown in Fig. 6.

As Fig. 6 shows, the maximum percentage of product to regard to total protein was reached in 8 h after induction.

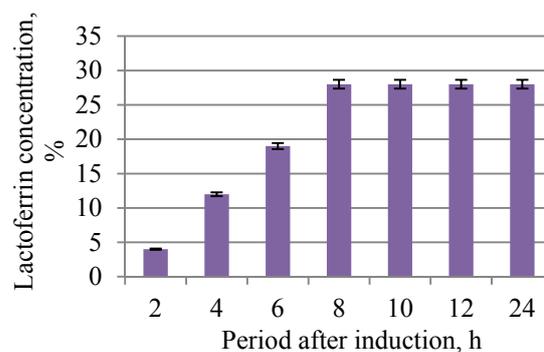


Fig. 6. The effect of duration of period after induction on the lactoferrin synthesis (percentage of total cellular protein) at cultivation at 37°C and 24 h period after induction.

Analysis of the effect of variable parameters on the lactoferrin synthesis by RSM

RSM (Response surface methodology) method of statistics was used for further optimization of the conditions. The use of statistically based experimental design is an important tool in optimizing of induction conditions. The analysis gives several important advantages, such as the effect of the studied factors, the determination of the optimal values, etc. Multifactorial experiment with 3 variable parameters selected in previous research: temperature after induction – X_1 , the concentration of inducer – X_2 , and period after induction – X_3 was conducted for optimization of induction conditions. Each parameter was varied on three levels (Table 3): low (-1) middle (0) and high (+1) (Table 2).

Composite matrix of multi-factorial experiment and obtained results of protein yield are presented in Table 3.

The mathematical processing of data presented in Table 3 was carried out for impact assess of variable induction parameters on the lactoferrin concentration. The results of the statistical analysis of effects of variable factors are showed in Fig 7.

Table 2. Levels of variation of independent parameters

| Parameter | Variable | Range | | |
|--------------------------------|----------|-------|----|-----|
| | | -1 | 0 | 1 |
| Temperature after induction, C | X_1 | 15 | 23 | 37 |
| Concentration of inducer, mM | X_2 | 0.6 | 1 | 1.7 |
| Period after induction, h | X_3 | 6 | 8 | 12 |

Table 3. Matrix multi-factor experiment for optimize the conditions (a key parameter is the concentration of lactoferrin)

| № | Levels of factors | | | Lactoferrin concentration, mg/L |
|---|-------------------|-------|-------|---------------------------------|
| | X_1 | X_2 | X_3 | |
| 1 | 1 | 1 | 1 | 20,2 |
| 2 | +1 | -1 | -1 | 22 |
| 3 | -1 | +1 | -1 | 46,4 |
| 4 | -1 | -1 | +1 | 36,8 |
| 5 | +1 | +1 | -1 | 27,8 |
| 6 | +1 | -1 | +1 | 42,1 |
| 7 | -1 | +1 | +1 | 65,7 |
| 8 | +1 | +1 | +1 | 80,9 |
| 9 | 1 | -1 | +1 | 63,8 |

Table 3. Ending. Matrix multi-factor experiment for optimize the conditions (a key parameter is the concentration of lactoferrin)

| № | Levels of factors | | | Lactoferrin concentration, mg/L |
|----|-------------------|----------------|----------------|---------------------------------|
| | X ₁ | X ₂ | X ₃ | |
| 10 | -1 | 1 | -1 | 59.1 |
| 11 | -1 | -1 | 1 | 29.6 |
| 12 | 1 | 1 | -1 | 70.4 |
| 13 | 1 | -1 | 1 | 57.4 |
| 14 | -1 | 1 | 1 | 75.2 |
| 15 | 1 | 1 | 1 | 96.9 |
| 16 | +1 | +1 | 1 | 84.6 |
| 17 | +1 | 1 | +1 | 93.1 |
| 18 | 1 | +1 | +1 | 87.5 |
| 19 | 1 | 1 | +1 | 102.0 |
| 20 | 1 | +1 | 1 | 76.7 |
| 21 | +1 | 1 | 1 | 93.5 |
| 22 | -1 | 1 | +1 | 83.5 |
| 23 | -1 | +1 | 1 | 64.5 |
| 24 | +1 | 1 | -1 | 50.7 |
| 25 | 1 | -1 | -1 | 39.9 |
| 26 | 1 | +1 | -1 | 56.5 |
| 27 | +1 | -1 | 1 | 32.1 |

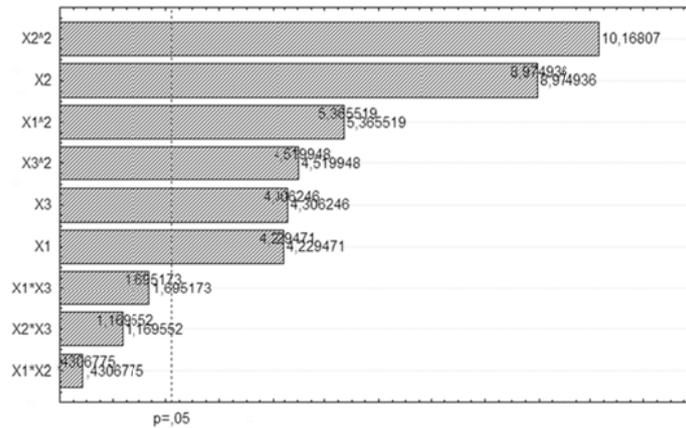


Fig. 7. Pareto diagram for assess of effects significance of variable factors on the lactoferrin concentration.

As the data shows, the concentration of lactoferrin, produced by *E. coli* BL21DE3/mpET28a+*-lrf* strain, is determined by all three considered parameters (X₁, X₂, X₃). Linear and quadratic factors of inductor concentration have most pronounced effect on protein yield. The results of the analysis show no significant interaction effect between variable parameters.

Multiple regression analysis of the experimental data provided parameters of equation. The polynomial equation of the second order (equation 1) was used for express the empirical relationship between the response and significant variables:

$$Y = -324.6 + 7.02X_1 - 0.15X_1^2 + 273.242X_2 - 115.4X_2^2 + 32.9X_3 - 1.81X_3^2, \quad (2)$$

where Y is the concentration of lactoferrin, mg/L; X₁, X₂, X₃ – temperature after induction, concentration of inducer and period after induction respectively.

The three surface are showed (Fig. 8), considering all possible combinations. The quadratic dependence of the lactoferrin concentration on the temperature after induction, inducer concentration and period after induction was showed.

The profiles of desirability are presented in Fig. 9. Profiles of desirability function also show a quadratic dependence between variable parameters and the lactoferrin concentration. Optimal levels for variable parameters according to the profile of the desirability are following: induction by 1.1 mM of IPTG, followed by 8.6 hour period after induction at 25°C. The change of desirability function for variation of the relevant independent variables was presented in the lower series of graphs.

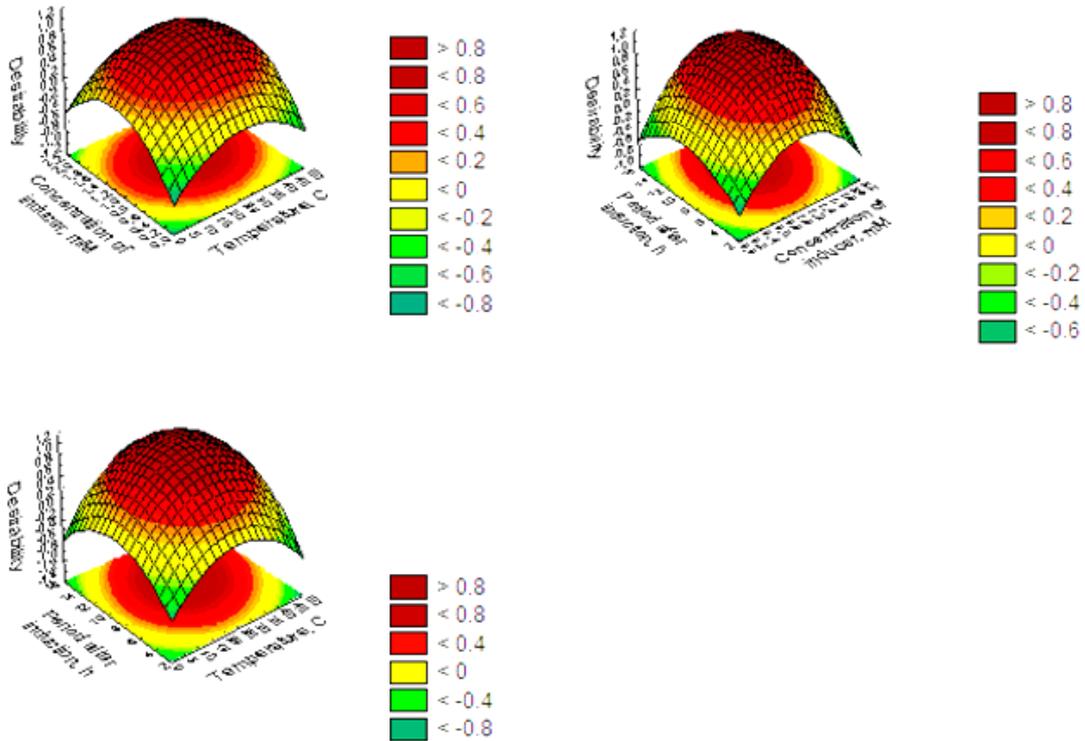


Fig. 8. Response surface showing the dependence of lactoferrin yield on variable parameters of induction.

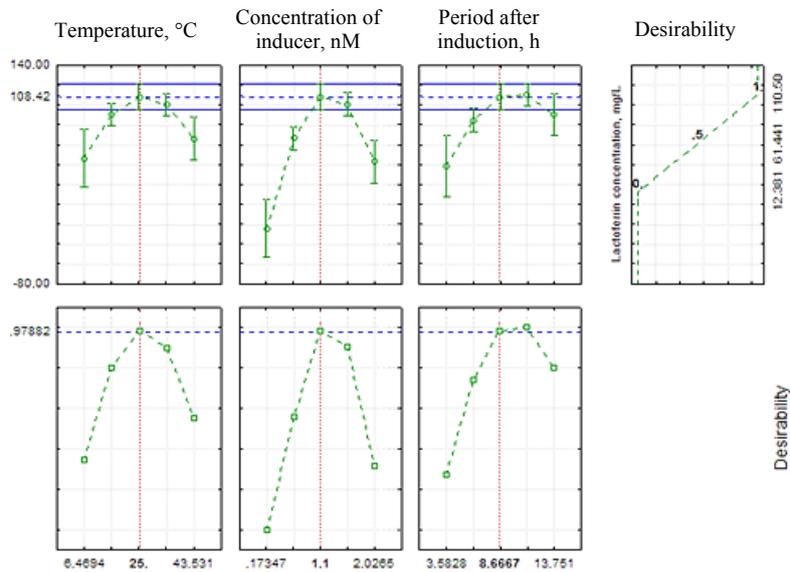


Fig. 9. Desirability profiles of lactoferrin release by culturing BL21DE3/mpET28a+-lrf strain.

Optimization of solubilization, renaturation and purification of lactoferrin

Optimization of solubilization

Portion of lactoferrin was synthesized in the form of inclusion bodies by cultivation of strain under optimal conditions. Additional stages of isolation, solubilization and renaturation were conducted for translate the protein in a soluble form.

Before solubilization inclusion bodies were washed with 20 mM/L Tris-HCl (pH 8.5), 0.5 mM/L EDTA and 2% Triton X-100.

For solubilization of protein from inclusion bodies,

the experiments were carried out with different concentrations of IB both in the presence and in the absence of Gn-HCl. Fig. 10 shows the results obtained by dissolving various concentrations of inclusion bodies at pH 12 in 2 M Tris-HCl buffer with various Gn-HCl concentration.

With increasing of protein concentration from 0.05 to 5 mg/ml the solubility of inclusion bodies was decreased from 68% to 20% due to the alkaline pH (without adding of Gn-HCl). The addition of Gn-HCl in the buffer improve the solubility of protein inclusion bodies in higher concentrations.

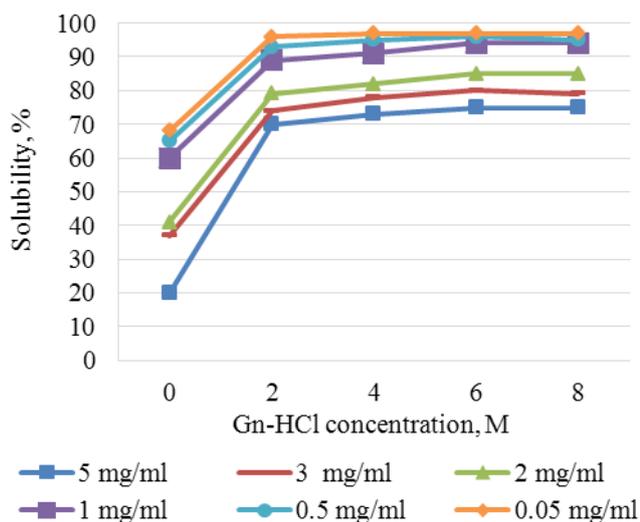


Fig. 10. The effect of Gn-HCl concentration on the solubility of inclusion bodies at different concentrations of inclusion bodies. Protein concentration varied from 0.05 to 5 mg/ml.

Solubilization more than 89% of the total protein was achieved by addition of 2M Gn-HCl to 1 mg/ml of IB. Approximately 70% of the total protein was received in soluble form at solubilization 5 mg/ml of inclusion bodies. Further adding of large amounts of Gn-HCl increased 1–4% more the yield of soluble protein. The maximum soluble protein was achieved at 6M Gn-HCl (from 75% at solubilization of 5 mg/ml protein to 97% at 0.05 mg/ml).

The increasing of Gn-HCl concentration to 8 M or more did not lead to greater yield of soluble lactoferrin. At low concentrations of protein 2 M Gn-HCl was sufficient for maximum efficiency of solubilization. Subsequent increase of Gn-HCl concentration did not lead to a significant increase of protein yield. The optimal mode is solubilization 1 mg/ml of protein by 6 M Gn-HCl, allowing to solubilise 96% of protein.

Optimization of refolding

Refolding of solubilized protein is initiated by the removal of the denaturing agent.

The following factors: composition of the buffer, protein concentration, temperature, reagents suppressing aggregation and redox conditions were considered for optimizing of refolding.

Effect of pH

Experiments were performed at different pH to create the optimal conditions for refolding (Fig. 11). 0.5 M/L Gn-HCl, 50 mM/L Tris-HCl (pH 8.5), 0.75 M/L arginine, 1 M/L NaCl, 5 mM/L EDTA and 3 mM of glutathione in a ratio of 10 : 1 GSH : GSSG solution was used as standart buffer.

As Fig. 11 shows, at pH below 8 the renaturation was not observed, the pH prevents the formation of disulfide bonds. In more alkaline conditions, the renaturation of lactoferrin is also unfavorable, probably due to the instability of the protein.

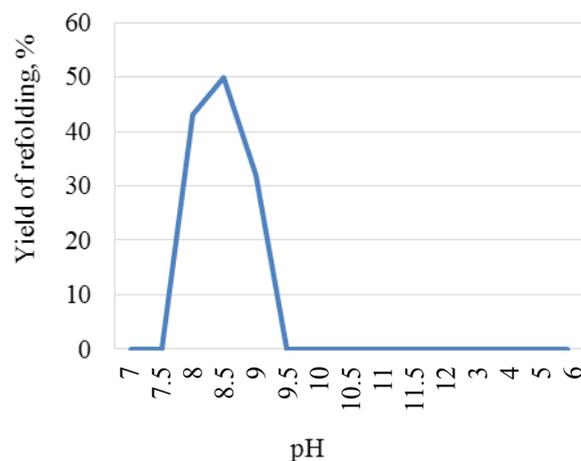


Fig. 11. Influence of pH conditions on lactoferrin renaturation was carried out at 20°C and a total protein concentration of 0.1 mg/ml in standard renaturation buffer with 3 mM/L of glutathione in a ratio of 10 : 1 (GSH : GSSG).

The influence of redox conditions

Since lactoferrin is a protein containing disulfide bonds, the refolding was carried out using redox systems. Adding in a mixture of oxidized and reduced forms of thiol reagent of low molecular weight usually provides the appropriate redox potential, which promotes the formation and rearrangement of disulfide bonds. In our research both oxidized and reduced glutathione (GSH : GSSG) were used.

In contrast to the relatively narrow range of renaturation pH, the constant of lactoferrin renaturation yield was obtained in a wide range of redox conditions (Fig. 12a).

Only strong oxidizing conditions did not lead to the lactoferrin renaturation. The maximum yield of renaturation was received in excess of reduced glutathione (2 : 1), in which case the yield of renaturation was 51%. Only 2 and 6% less the yield of renaturation was obtained at a ratio of 10 : 1 and 1 : 1.

Thus, in the case of lactoferrin, pH and the ratio of GSH : GSSG is critical variable for optimization of renaturation.

The influence of protein concentration and temperature on the lactoferrin renaturation

Protein aggregation is one of the major side reactions at the renaturation high concentrations of protein.

Several strategies, including renaturation at very low protein concentrations, low temperature, and/or addition of aggregation suppressors prevents and reduces aggregation. However, low protein concentrations slow down the process of renaturation, which lead to a decrease of the renaturation yield.

For identify the best conditions for the renaturation, the experiments were carried out in standard solutions of renaturation at temperature in the range of 10–30°C and the lactoferrin concentration 1.0 mg/ml. Severe aggregation was observed at all temperatures with the renaturation 1.0 mg/ml of protein.

At lower concentration of lactoferrin and temperature aggregation was decreased (Fig. 12b). For example, by reducing the temperature from 30 to 10°C the refolding yield was increased from 36 to 57% (on 21%). The best conditions for the renaturation are 10°C and 0.2 mg/ml of lactoferrin.

As the results show, the concentrations used in this refolding were not approached for application. In this regard, more research was investigated for increase of the protein concentration. Denatured protein can be added in pulses mode, avoiding aggregation.

In the experiments used 10 hour pulse system. With each impulse (total 10 pulses) the protein concentration was increased on 0.3 mg/ml to a final concentration of 2.9 mg/ml protein, followed by 48 hour incubation. This approach allowed to increase the concentration of protein to 2.4 mg/ml and to achieve a yield of 55%.

Optimization of solubilization and renaturation conditions to regard to pH, redox conditions, temperature, protein concentration, aggregation

suppressors allowed to recover lactoferrin in a concentration of 0.2 mg/ml, resulting the renaturation yield was 58%. The increase of the lactoferrin concentration to 2.9 mg/ml was achieved by a pulsed system. Yield of renaturation was 55%.

The replacement of buffer at 4°C by dialysis of received solution against 5 volumes of buffer (with 0.2 mM of Gn-HCl, 10 mM Na₂HPO₄/NaH₂PO₄, 1 mM EDTA) was carried out for further purification.

Purification of lactoferrin

For purification of lactoferrin, mRNA of lactoferrin gene was synthesized so that immediately after the coding sequence was located the plot corresponding hexahistidine sequence (Xho I - gene - His-Tag - Eco I). As a result of induced expression of the cloned gene lactoferrin containing additional hexahistidine sequence in the C-terminal region of the polypeptide chain, allowing subsequent affine cleaning protein on Ni-NTA-agarose, is synthesized.

Purification of lactoferrin was based on affinity chromatography.

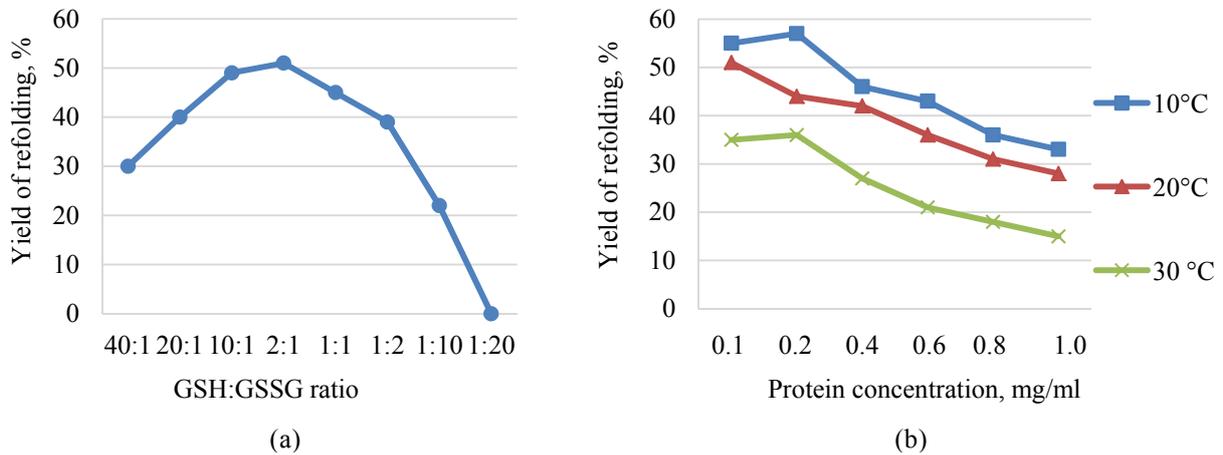


Fig. 12. The effect of redox conditions (a) and temperature and protein concentration (b) on lactoferrin renaturation.

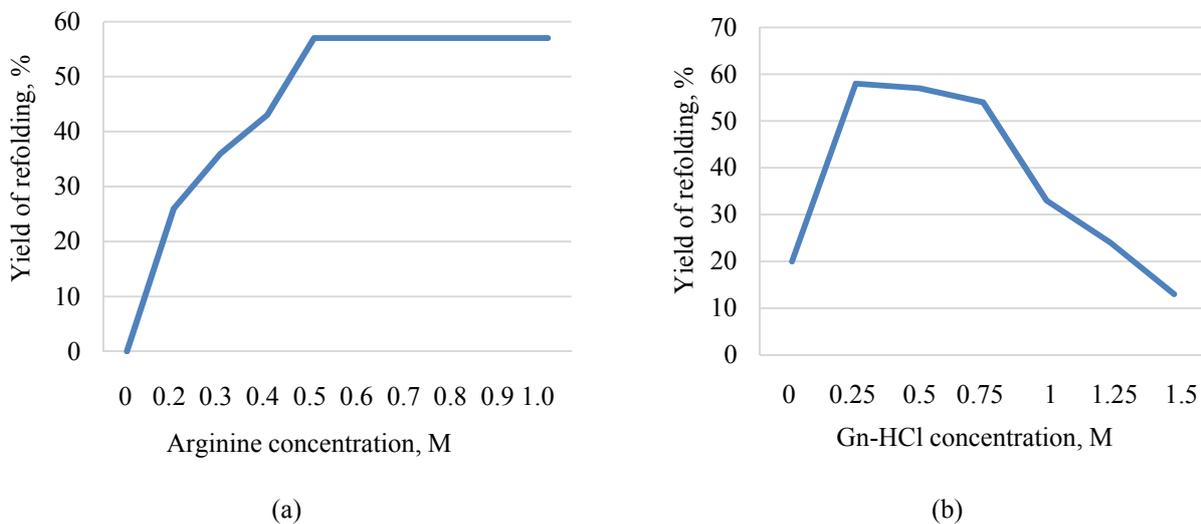


Fig. 13. Effect of arginine concentration (a) and Gn-HCl concentration (b) on the lactoferrin renaturation.

Data analysis of the protein electrophoresis, that is shown in Fig. 14a, presents that the fractions giving the highest efficiency bands near 76–80 kDa are fractions of lactoferrin.

The chromatogram in Fig. 14b shows the results obtained after the purification. The fraction of lactoferrin was from 10 to 12 minutes. The purity of the isolated protein was not less than 90%.

The yields of total protein and lactoferrin at different stages of isolation, solubilization, renaturation and purification were analyzed for determine the effectiveness of optimized methods. The results are presented in Table 4.

The maximum yield of lactoferrin (97.2 mg) was obtained by cultivation of *E. coli* BL21DE3/mpET28a+*-lrf* strain, 58 mg of which was

in the form of a dissolved protein and 38.8 in the form of inclusion bodies. Such stages as: washing of inclusion bodies, renaturation and solubilization was required for 38.8 mg of protein. Total of 11 mg of lactoferrin from inclusion bodies, representing 11% of the primary was obtained after washing of inclusion bodies, renaturation, solubilization and purification. Most of the protein was lost at the stage of renaturation, which is mainly connected with the formation of insoluble aggregates.

Thus, 61 mg of lactoferrin, 11 mg of which was obtained during the renaturation of solubilised inclusion bodies, and 50 mg in the form of dissolved protein was obtained at full processing of biomass obtained from 1 L.

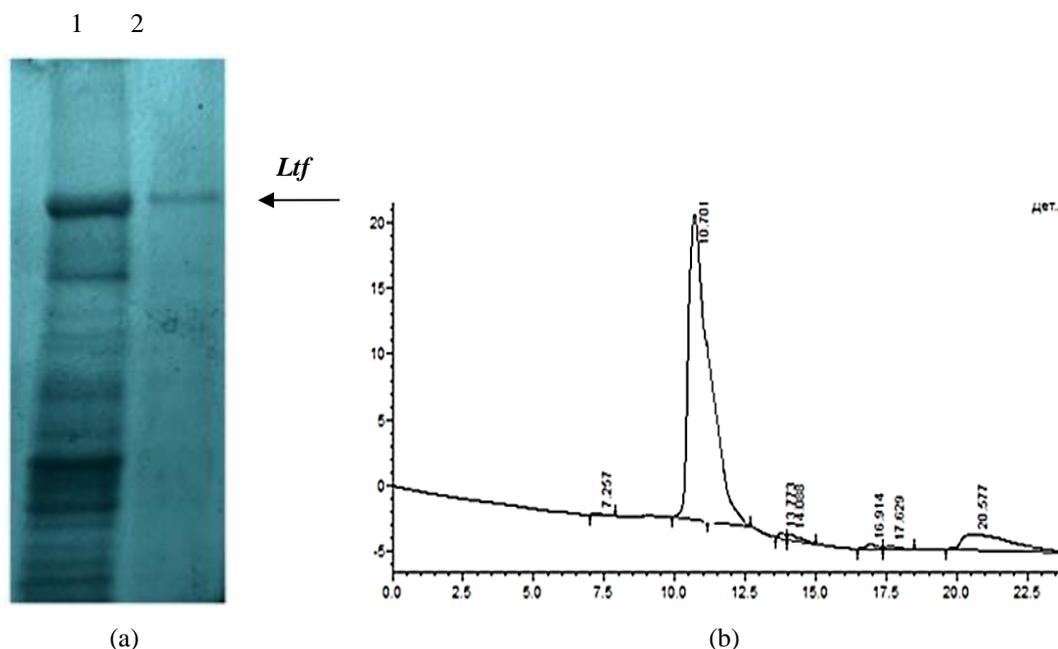


Fig. 14. Purification of lactoferrin received from *E. coli* BL21DE3 / mpET28a + cells: (a) electrophoregram: 1 – the culture medium after sonication; 2 – purified lactoferrin obtained after optimization of purification conditions; (b) the chromatogram of the purified lactoferrin.

Table 4. The parameters of the lactoferrin production with optimised methods of solubilization, renaturation and purification

| Stage | M_{protein} , mg | M_{lf} , mg | | Yield of LF, % | |
|-----------------------------|---------------------------|----------------------|----|----------------|----|
| | | S | IB | S | IB |
| Biomass after separation | 270 | 102 | | 100 | |
| After sonication | 230 | 97.2 | | 96 | |
| Separated fractions | 116 | 58 | 39 | 56 | 38 |
| Washing of inclusion bodies | 116 | – | 38 | – | 36 |
| Solubilization | 113 | – | 35 | – | 34 |
| Renaturation | 72 | – | 19 | – | 18 |
| Purification | 63 | 50 | 11 | 49 | 11 |
| Total yield of IB and S | 63 | 61 | | 60 | |

Discussion

Thus, the cloning of *ltf* gene encoding the human lactoferrin was described in this paper. Evidence of the created recombinant construct functionality was submitted. Ability to produce lactoferrin at level of lactoferrin synthesis – 30–35% of the total protein content was shown. The maximum yield of the target protein (including in soluble form) was achieved by culturing of the recombinant strain at 25°C, induction by 1.1 mM concentration of IPTG and 8-h period after induction. Optimization of solubilization and renaturation conditions to regard to pH, redox conditions, protein concentration, reagents suppressing aggregation and temperature allowed once to recover lactoferrin in a concentration of 2.9 mg/ml by the pulsed system, the income of which renaturation was

55%. The protein of high purity at least 90% was obtained by means of affinity chromatography. 61 mg of lactoferrin, 11 mg of which was obtained during the renaturation of solubilised inclusion bodies, and 50 mg in the form of dissolved protein was obtained at full processing of biomass obtained from 1 L.

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List of abbreviations

LTF – lactoferrin hltf- human lactoferrin; *rhlf* – recombinant human lactoferrin; IPTG – isopropyl-β-thiogalactopyranoside; SDS – sodium dodecyl sulphate; IB – inclusion bodies; SDS-PAGE – polyacrylamide gel electrophoresis; EDTA – ethylenediaminetetraacetic acid; PBS – Phosphate buffered saline.

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