

INCREASING OF THE EXPRESSION OF RECOMBINANT scFv-ANTIBODIES EFFICIENCY

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Obtaining single-chain variable fragments (scFv) of recombinant antibodies in *E. coli* cells is often associated with numerous problems causing low yields or inactive conformation of the product.

The aim of this work was to study the influence of staphylococcal protein A fragment fused with scFv antibodies (SpA-tag) on the efficiency of expression of final product. Examination of scFv antibodies of different origin and specificity has shown that in similar expression systems fused scFv is synthesized in much higher quantities than free scFv. Furthermore, the scFv antibodies in fused form retained their antigen-binding properties and the SpA fragment the ability to bind other immunoglobulins.

Thus, the proposed strategy can be considered effective in improving the efficiency of scFv-antibodies production in *E. coli* cells.

Key words: scFv-antibodies, protein A *Staphylococcus aureus*, chimeric proteins, *E. coli*.

ScFv — single-chain fragment variable — is a widely used type of recombinant antibodies. They are obtained by combined translating of genes in variable domains of the heavy and light immunoglobulin chains. Though scFv antibodies lack constant domains, they are specific and bind antigens similarly to natural antibodies. ScFvs are widely used in practice as highly specific immunological reagents to isolate and establish the characteristics of biomolecules, for quantitatively identification of relevant biomarkers in biological fluids, to sort and label cells, to determine the immune status of the organism, to locate sites of tumor growth, to neutralize toxins, etc. [1, 2].

In the beginning, the technology for obtaining scFv antibodies was focused on using the *Escherichia coli* cells as a producer. This expression system is still the most convenient for routine studies [3]. The sequence of scFv antibodies is unique for each clone, which determines the peculiarities of their expression. Quite often, expression in *E. coli* cells provides less than enough scFv antibodies. Also, they can be synthesized in a functionally

inactive form and accumulated in the inclusion bodies. Refolding of recombinant antibodies *in vitro* is one of the solutions to this problem, but usually, the output of renatured protein does not exceed 25% of the amount before refolding [4]. Moreover, the process of allocation is greatly complicated by this procedure and it requires additional funds and time.

Using co-expression or hybridization with chaperons and chaperone-like proteins such as isomerases of the disulphide bonds of DsbABCD, thioredoxin (Trx1) etc., is another set of approaches to improve expression of recombinant antibodies in *E. coli* cells [5, 6].

Previously we obtained a series of scFvs against the B subunit of diphtheria toxin, which contained the functional fragment of D-E-A-A* staphylococcal protein A (SpA) as a C-terminal tag. Since the D-E-A-A* fragment, like the natural SpA, specifically interacts with immunoglobulins of different mammalian species, the tagged immunoglobulins of varying specificity can be used to detect scFvs combined with the D-E-A-A* fragment. Our results also indicated that the production of

recombinant antibodies can be improved by combining them with the D-E-A-A* fragment of SpA [7]. Therefore, the purpose of the present work was to construct a vector for scFv antibodies, and to test the effectiveness of using the SpA-scFv complex to improve the recombinant antibody fragments production on the example of scFv antibodies of different origin and specificity.

Notably, SpA can be used as a tag to detect, isolate, and purify recombinant antibodies. Moreover, it was shown that scFv fused with SpA has a significantly longer half-life time in blood plasma compared to free scFv [8].

Materials and Methods

Manufacturing the pET-28c-SpA modified vector for expression of SpA-scFv hybrid protein in E. coli. The SpA gene fragment sequence was cloned from the previously obtained construct based on the pET-28c vector and DNA sequence of the D-E-A-A* fragment [9]. For amplification, sense primer complementary to the sequence of pET-28c vector and antisense primer complementary to the sequence of SpA were used; the latter also contained a site for SfiI restriction endonuclease (emphasized).

Sense primer:

5'-GCTAGTTATTGCTCAGCGGTG-3'

Antisense primer:

5'-GTGTGGCCGGCTGGGCCTGTTGTT-TTGGTGCTTGAGCATCA-3'

The conditions of PCR amplification were: denaturation at 95 °C for 1 min, 25 cycles according to the scheme 94 °C for 30 s, 50 °C for 30 sec, 72 °C for 40 s and the extension at 72 °C for 7 min at the end. 10 µl of 10x polymerase buffer, a mixture of deoxyribonucleotides to a final concentration of 0.2 mM (Amersham, USA), sense and antisense primers of 20 pmol each, 4.5 Taq DNA polymerase activity units (Fermentas, Lithuania), an aliquot of the plasmid used as a matrix, and nuclease-free water to a final volume of 100 µl were used per 100 µl of reaction.

The resulting PCR product was treated with DNA polynucleotide kinase and T4 DNA polymerase for extension of protruding ends.

The pET-28c was hydrolyzed with BamHI restriction endonuclease and then treated with T4 DNA polymerase to complete the protruding ends. Then the restriction with NdeI endonuclease (Fermentas, Lithuania) was performed. A fragment of SpA gene was treated with the same endonuclease. The reaction was carried out in accordance with the

recommendations of the manufacturer. The resulting fragments of pET-28c and SpA gene were ligated and the *E. coli* DH10B cells were transformed with a ligase mixture.

Subcloning the scFv sequences into pET-28c-SpA vector was performed according to sites for the *NotI* and *SfiI* endonucleases. *E. coli* Rosetta strain was used as a producer.

Subcloning the sequences of scFv in the pET-28a vector. Sequences of scFv were amplified from the construct based on pCANTAB 5E vector. The primer completing *EcoRI* site (ATATGAATTCGGCCAGCCGCC) was used as sense, the sequencing primer S6 for the pCANTAB 5E plasmid was used as antisense. The scFv sequences were embedded in pET-28a vector based on the sites for *EcoRI* and *NotI* endonucleases. As a producer, *E. coli* Rosetta strain was used.

Analysis of target proteins expression.

The cell propagation of respective producer clones took place at 37 °C with active aeration on the LB medium (Sigma, USA) with kanamycin (50 µg/ml) and 2% glucose to a density corresponding $A_{600} = 0.7$. After this, cells were precipitated and resuspended in LB medium with kanamycin (50 µg/ml) and an inducer of expression of IPTG (isopropyl-β-D-1-thiogalactopyranoside) in a concentration of 1 mM. Then they were kept for 3.5 hours at 30 °C with active aeration, and then precipitated by centrifugation. The precipitation was resuspended in a buffer containing 10 mM of Tris-HCl (pH 7.5), 2.5 mM of MgCl₂, 0.1 mM of CaCl₂, 10 U/ml of DNAase. Then the samples were treated by the Labsonic ultrasonic homogenizer (Sartorius, Germany). The fraction of soluble proteins was separated from insoluble fraction in centrifugal separation at 12.000 rpm. The fractions were analyzed by SDS electrophoresis in polyacrylamide gel [10]. The results of electrophoresis were analyzed with the TotalLab TL120 v 2009 program.

Immunoblotting. After the electrophoresis, proteins were transferred to nitrocellulose membrane by electromigration at 38 mA for 75 min. After the transfer, the nitrocellulose membrane was incubated for 60 min at 37 °C in blocking solution (5% skimmed milk in PBS (physiologic buffered saline (PBS): 0.8% NaCl, 0.02% KCl, 0.144% Na₂HPO₄, 0.024% KH₂PO₄; pH 7.4)). After blocking, the membrane was washed with PBS and transferred to a solution of antibodies to the marker sequence (his-tag) conjugated to horseradish peroxidase in PBS with the addition of 0.04% Tween-20. After incubation

at 37 °C for 60 min, the membrane was washed with PBS and transferred for developing to a solution (0.06% diaminobenzidine (Sigma, USA) and 0.001% hydrogen peroxide in PBS).

Cleaning of scFv-SpA fusion proteins. The purification was carried out in column of Ni-NTA agarose sorbent with soluble and insoluble fractions. Conversion of proteins to the active form in the latter case was done by refolding in accordance with the method described above [11].

Enzyme immunoassay. It was conducted as described above [12]. The recombinant subunit B of diphtheria toxin, HB-EGF and the recombinant fragment of $\alpha 7$ subunit of the nicotinic acetylcholine receptor were used as antigens, and the bovine serum albumin was used as negative control.

The SpA-scFv fusion protein was used as antigen to determine the necessary dilution of antibodies conjugated to horseradish peroxidase.

Results and Discussion

Production of the pET-28c-SpA modified vector. SpA or several its domains can improve the production of recombinant proteins with which they are fused, for instance, protecting them from N-terminal degradation and increasing solubility [13]. To verify whether the fusion with D-E-A-A* fragment of SpA affects the level of scFv antibodies production, a vector was constructed for obtaining different scFvs fused with the D-E-A-A* fragment. This vector contained DNA sequence of the D-E-A-A* fragment and sites for the restriction endonuclease to embed scFv (Fig. 1).

In construction of the scFv antibodies libraries, the scFv sequences are generally

flanked by sites for the *SfiI* and *NotI* restriction endonuclease [14]. The multicloning site pET28 has only a site for *NotI* endonuclease. Hence, during the amplification of the D-E-A-A* DNA sequence of SpA fragment, in the antisense primer we added the site for *SfiI* enzyme and the additional nucleotide, required for the SpA gene fragment and scFv to be in the same reading frame.

The resulting PCR fragment was embedded in a pET-28c vector and transformed *E. coli* DH10B cell with ligase mixture (Fig. 2, 3).

Thus, a genetic construct based on the pET-28c vector was obtained to produce the scFv antibodies combined with D-E-A-A* fragment of SpA.

Obtaining the SpA-scFv-1E, SpA-scFv-7E and SpA-scFv-II-15 fusion proteins. The sequence of SpA D-E-A-A* fragment was combined with the sequences of scFv antibodies of different origin and specificity, derived from the library of human scFv antibodies: scFv-1E specific to the extracellular domain of the $\alpha 7$ subunit of nicotinic acetylcholine receptor; scFv-7E to the growth factor HB-EGF from libraries of mouse antibodies [15] and scFv-II-15 to the diphtheria toxin B subunit [16]. The scFv sequences were isolated from the phagemid vector by hydrolysis with *SfiI* and *NotI* restriction endonucleases, and then they were embedded in the pET-28c-SpA resulting construct. The cells of *E. coli* DH10B were transformed with resulting ligase mixtures and clones with the target construct were selected.

The SpA-scFv-1E, SpA-scFv-7E and SpA-scFv-II-15 clones were tested for the ability to produce the target protein. The cells were propagated in a liquid medium and the expression of target protein was induced. The

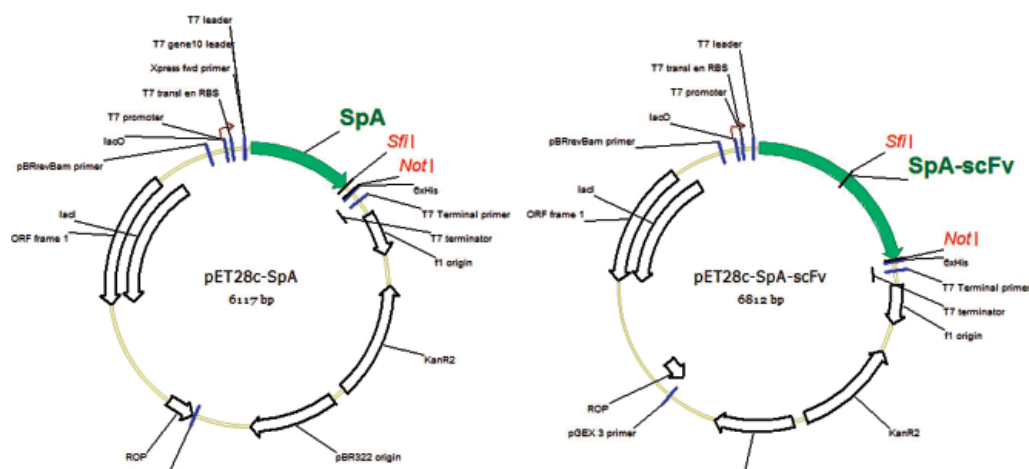


Fig. 1. The pET-28c-SpA and pET-28c-SpA-scFv constructions

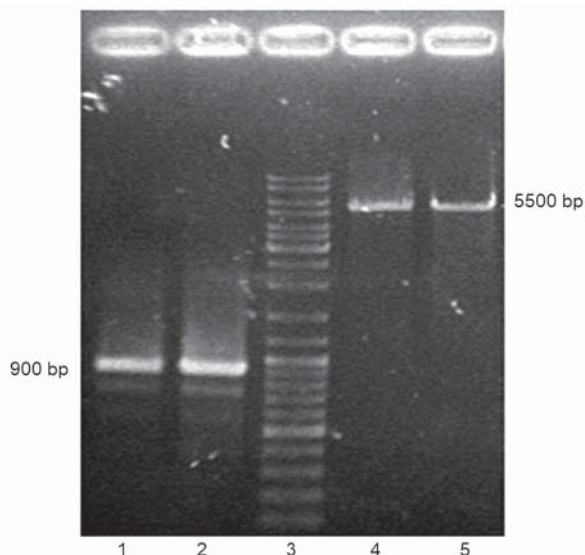


Fig. 2. The procedure of pET-28c-SpA vector manufacturing:

1, 2 — SpA gene fragment, treated with T4 DNA polymerase and then with NdeI restriction endonuclease; 3 — markers; 4, 5 — pET-28c plasmid, hydrolyzed by BamHI restriction endonuclease succeeded by extension of protruding ends with T4 DNA polymerase, and treated with NdeI restriction endonuclease

soluble and insoluble fractions were isolated from the obtained cells and analyzed by electrophoresis in polyacrylamide gel.

Cell lysates contained a protein with size corresponding to the expected size of scFv fused with SpA (63 kDa approximately). Target proteins were produced in insoluble form predominantly (Fig. 4, a). Presence of both soluble and insoluble target recombinant protein in cell lysates was confirmed by immunoblotting with monoclonal antibodies against histidine tag (Fig. 4, b).

As calculated in the TotalLab program, the total output of the target protein in soluble form was 20% for the scFv-1E-SpA producer, for the scFv-7E-SpA producer it was 13.6%, and for the producer scFv-II-15-SpA it was 10%.

It was important to confirm that even as components of scFv-SpA complex, scFv retain the ability to bind to the corresponding antigen, and the fragment of SpA can bind to the Fc fragments of immunoglobulins. In enzyme immunoassay it was shown that obtained fusion proteins are recognized by the peroxidase-conjugated polyclonal rabbit antibodies specific to mouse immunoglobulins (Fig. 5, a). Subsequently, these labeled antibodies were used to detect scFv-SpA complexes with antigen. Fig. 5, b, c, d shows

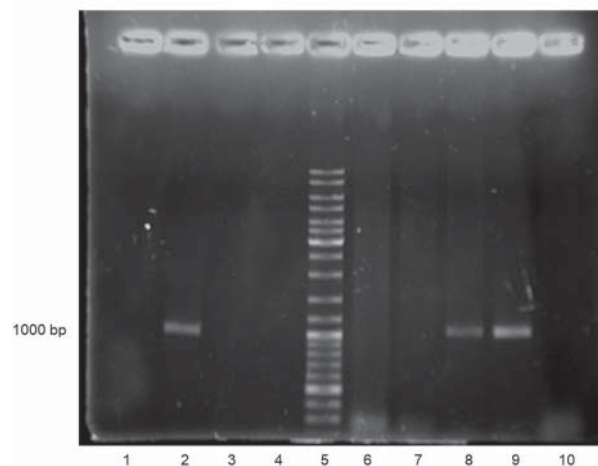


Fig. 3. Analysis of transformants containing the structure based on pET-28c vector with integral SpA gene fragment sequence and site for SfiI restriction endonuclease:

2, 8, 9 — positive clones, transformed by pET-28c-SpA; 5 — markers

the results of enzyme immunoassay, in which the recombinant B subunit of diphtheria toxin, HB-EGF and a recombinant $\alpha 7$ subunit of the nicotinic acetylcholine receptor were used as antigens to which scFv-II-15, scFv-7E and scFv-1E are the specific respective recombinant antibodies. All scFv-SpA fusion proteins effectively detected the target antigen and did not recognize the bovine serum albumin. This indicated the retention of the antigen-binding function in scFv after binding with the SpA fragment.

Obtaining the scFv antibodies in pET-28a vector. The nucleotide sequences of the scFv-1E, scFv-7E and scFv-II-15 recombinant antibodies were subcloned in pET-28a vector. For this, scFv sequences were amplified from pCANTAB 5E vector construct. The resulting PCR products were embedded in pET-28a vector following *EcoRI* and *NotI* sites (Fig. 6). Transformants were obtained *E. coli* cells Rosetta strain as in the case of scFv fused to the SpA gene fragment. Thus, the producers of the corresponding scFv antibodies were obtained in an expression system similar to that for obtaining scFv fused to SpA.

Comparison of the output level of SpA-scFv-1E, SpA-scFv-7E and SpA-scFv-II-15 fusion proteins with the analogues not fused with SpA. The cell lysates of clones producing SpA-scFv-1E, SpA-scFv-7E and SpA-scFv-II-15 fusion proteins were obtained and compared with the lysates of the producer

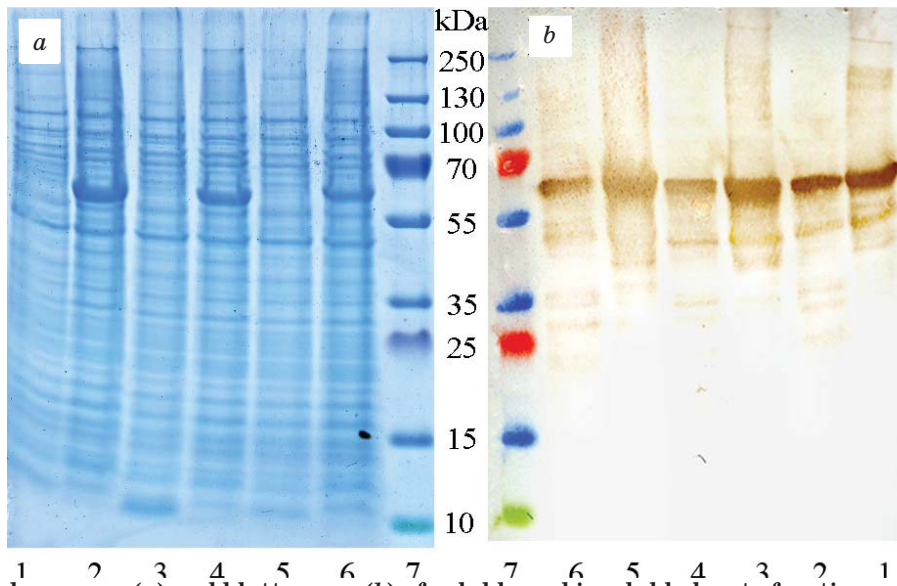


Fig. 4. Electrophoregram (a) and blottogram (b) of soluble and insoluble lysate fractions of clone cells producing scFv antibodies fused to SpA:

1 and 2 — soluble and insoluble fractions of cells of scFv-1E-SpA clone; 3 and 4 — soluble and insoluble fractions of cells of scFv-7E-SpA clone; 5 and 6 — soluble and insoluble fractions of cells of scFv-II-15-SpA clone; 7 — markers

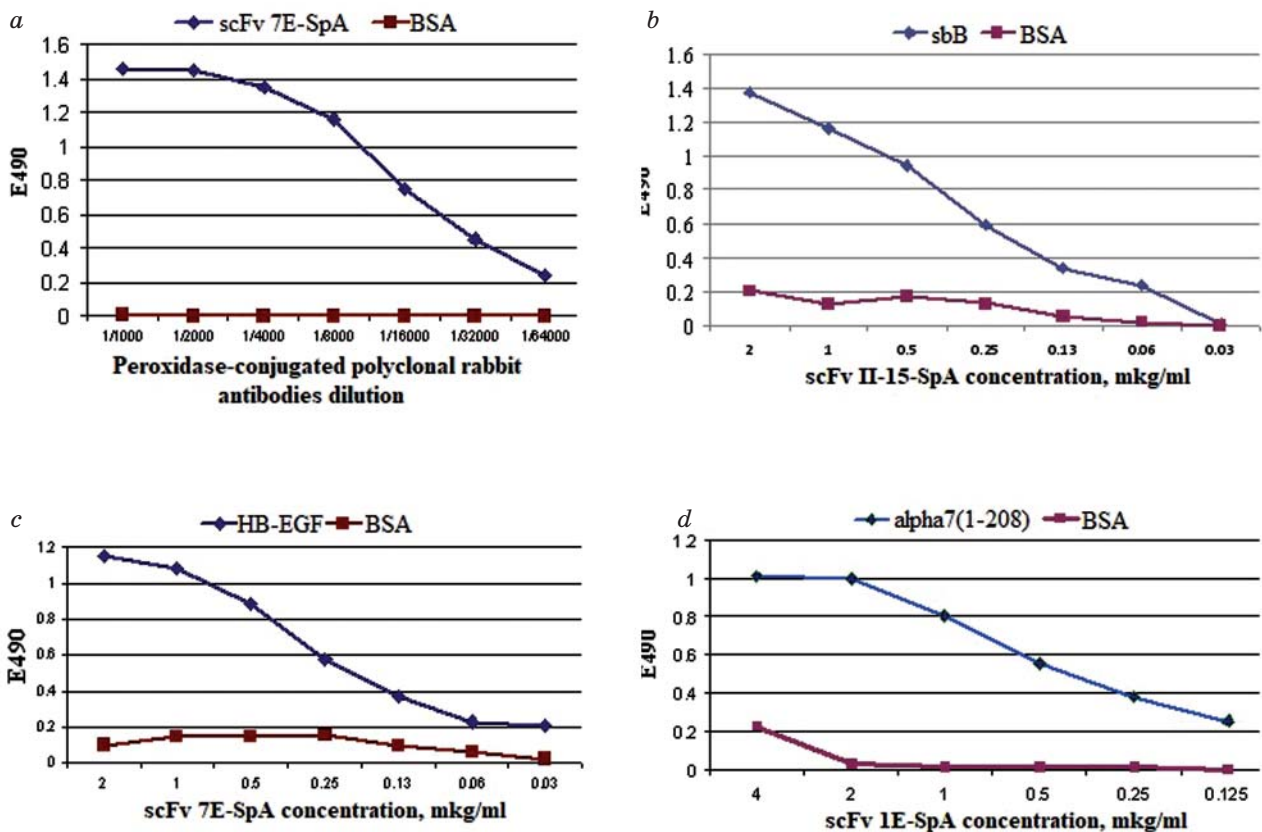


Fig. 5. Enzyme immunoassay of scFv antibodies fused to protein A:

a — binding of rabbit antibodies conjugated to horseradish peroxidase with the scFv antibodies fused to SpA; b — binding of scFv-antibodies II-15 to recombinant B subunit of diphtheria toxin (sbB); c — binding of scFv-antibodies 7E to HB-EGF; d — binding of scFv-antibodies 1E to the extracellular domain of $\alpha 7$ subunit of the nicotinic acetylcholine receptor (alpha7 (1-208); BSA — bovine serum albumin. The shown results of one of series of experiments are significant compared to BSA control ($P < 0.05$)

clones of the not fused with SpA analogues. The obtained fractions were analyzed by electrophoresis in polyacrylamide gel, as well as by immunoblotting method.

Fig. 7, *a* of electrophoregram shows the impossibility to detect the recombinant protein bands in the lysates of strains producing free scFv in the region of about

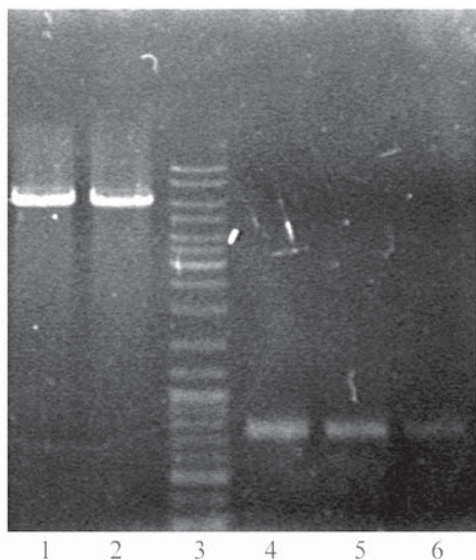


Fig. 6. Subcloning the scFv-1E, scFv-7E, and scFv-II-15 DNA sequences into the pET-28a vector: 1, 2 — DNA of pET-28a vector, treated with EcoRI and NotI restriction endonucleases; M — markers (top down 10; 8; 6; 5; 4; 3.5; 2; 1.5; 1.2; 1; 0.9; 0.8; 0.7; 0.6; 0.5; 0.4; 0.3; 0.2; 0.1 bp); 3, 4, 5 — scFv-1E, scFv-7E, and scFv-II-15 sequences treated with EcoRI and NotI restriction endonucleases

33 kDa, which corresponds to the expected sizes of free scFv. However, the target product is identified during immunoblotting with antibodies against the histidine tag (Fig. 7, *b*). This indicates low level of expression of free scFv. Instead, the output of recombinant SpA-scFv proteins in the lysates of the producer clones was from 10% to 20% of total bacterial protein. The sample of scFv fused with SpA was 50 times smaller than the sample of free scFv for the immunoblotting method for comparing lysates. Thus, the cytoplasmic production of scFv antibodies of different origins and specificity was substantially increased due to the fusion with the D-E-A-A* fragment of SpA.

Obtaining the modified pET-22b-SpA vector

The pET-22b plasmid containing PelB transport signal was used to obtain a modified vector containing a protein transport signal for periplasm, where stable disulfide bonds can form (Fig. 8). For this purpose, a fragment of the previously obtained pET-28c-SpA construct containing the D-E-A-A* fragment sequence and the site for SfiI restriction endonuclease was incorporated into the pET-22b vector by the EcoI and NotI restriction endonucleases sites. E. coli DH10B cells were transformed with the resulting ligase mixture and clone with the target construct was selected (Fig. 9).

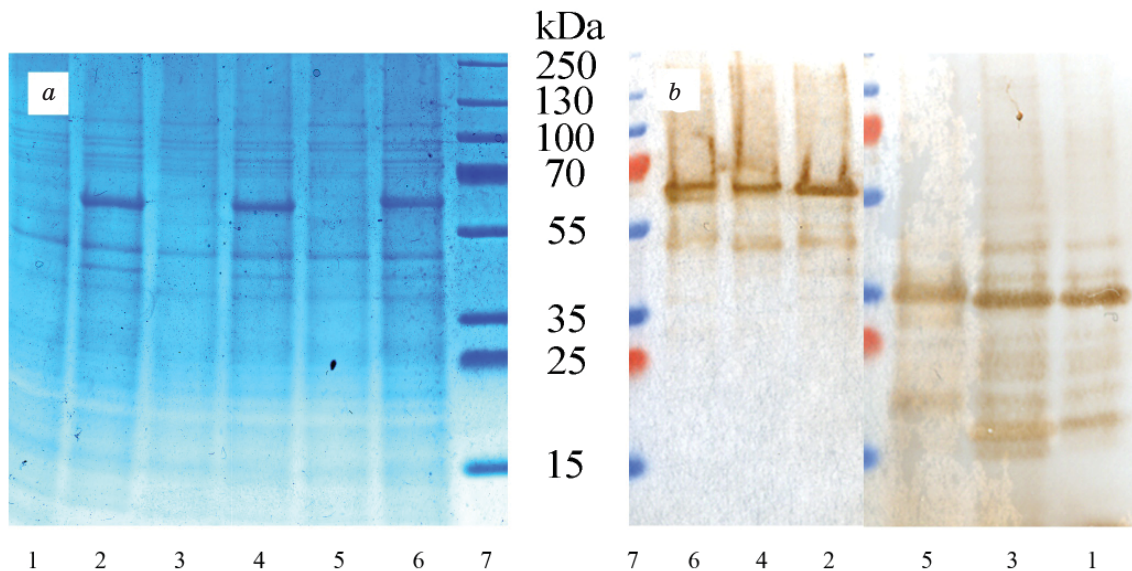


Fig. 7. Electrophoregram (a) and blotogram (b) of the cell lysates of the producer clones of free scFv antibodies and scFv antibodies fused to the protein A fragment:

1 — scFv 1E; 2 — scFv-1E-SpA; 3 — scFv 7E; 4 — scFv-7E-SpA; 5 — scFv -II-15; 6 — scFv-II-15-SpA; 7 — markers

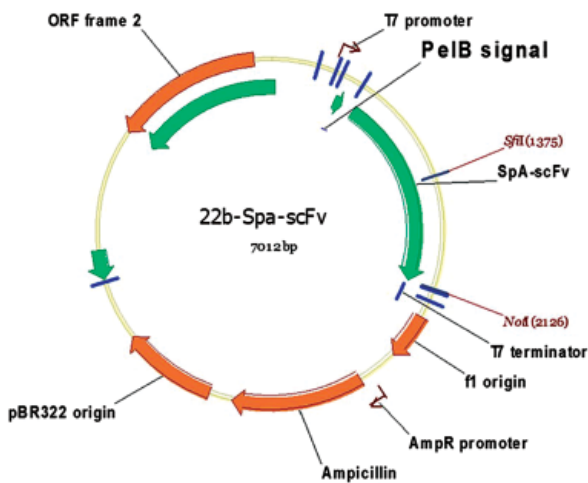


Fig. 8. The design of pET-22b-SpA-scFv construct

As a result, a universal genetic construct based on the pET-22b vector was obtained to produce scFv antibodies, fused with SpA.

Manufacturing SpA-scFv-1E, SpA-scFv-7E and SpA-scFv-II-15 fusion proteins in pET-22b vector

The DNA sequences of scFv antibodies of different origin and specificity (scFv-1E, scFv-7E and scFv-II-15) were combined with the SpA, located in the pET-22b vector. The scFv sequences were hydrolyzed from phagemid vector by *Sfi*I and *Not*I restriction endonucleases, than they were embedded in the pET-22b-SpA resulting construct. *E. coli* DH10B cells were transformed by the obtained ligase mixtures and the clones with the target construct were selected (Fig. 10).

For comparison, the producer clones of the corresponding scFv antibodies were used. In those clones, the scFv sequences were embedded in the pET-22b vector, where scFv-1E, scFv-7E and scFv-II-15 fused with SpA fragment were subcloned.

The resulting producer clones were analyzed electrophoretically in polyacrylamide gel for the ability to produce the target protein. Soluble and insoluble fractions isolated from cells were studied.

The appropriate target proteins with an expected molecular weight of about 30 kDa for free scFv antibodies and about 63 kDa for scFv fused with protein A can be identified in the producer lysates on the electrophoregram (Fig. 11, a). The presence of the target recombinant protein in cell lysates, in both

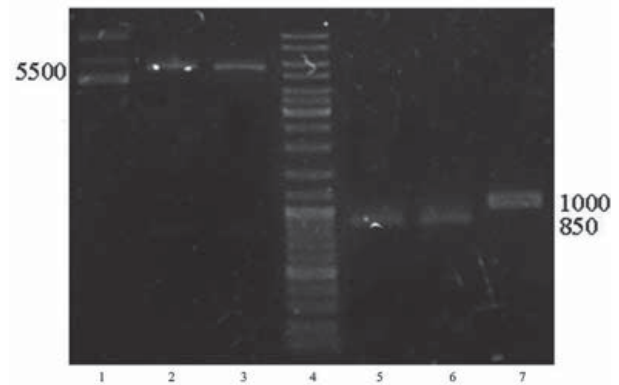


Fig. 9. Production of the pET-22c-SpA vector: 1 — isolated pET-22v vector, 2, 3 — pET-22v vector, hydrolyzed by EcoI and NotI restriction endonucleases; 4 — markers; 5 — SpA gene fragment, treated with EcoI and NotI restriction endonucleases; 6 — fragment SpA gene, amplified from the pET-22c-SpA construct.

soluble and insoluble fractions, was confirmed by immunoblotting with monoclonal antibodies against histidine tag (Fig. 11, b).

According to calculations made in the TotalLab program, the ratio of target protein produced in a soluble form was 38% for the scFv-1E-SpA producer, 36.5% for the scFv-7E-SpA producer, and 40% for the scFv-II-15 producer.

An enzyme immunoassay was carried out to confirm the uninterrupted functions of scFv-SpA fusion, and its results showed that all fusion proteins effectively detected the target antigen and did not recognize the bovine serum albumin (Fig. 12). This indicated the maintenance of the antigen-binding function of scFv, and the Fc-fragment-binding function for *Staphylococcus aureus* protein A.

Comparison of the level of production of SpA-scFv-1E, SpA-scFv-7E and SpA-scFv-II-15 fusion proteins in the pET-22v vector with that of the not fused to SpA analogues

To compare the output of free and fused with the protein A fragment antibodies, the soluble and insoluble fractions of producers were obtained. For SpA-scFv producers, target proteins were produced in both soluble and insoluble forms (Fig. 13). The immunoblotting method has confirmed that the bands identified by electrophoresis in a polyacrylamide gel as SpA-scFv actually correspond to the target proteins and are synthesized both in insoluble and soluble form. Instead, in the lysates of free scFv producers, the target protein was found only in insoluble fraction (Fig. 14).

Developing inexpensive and efficient production systems and introducing

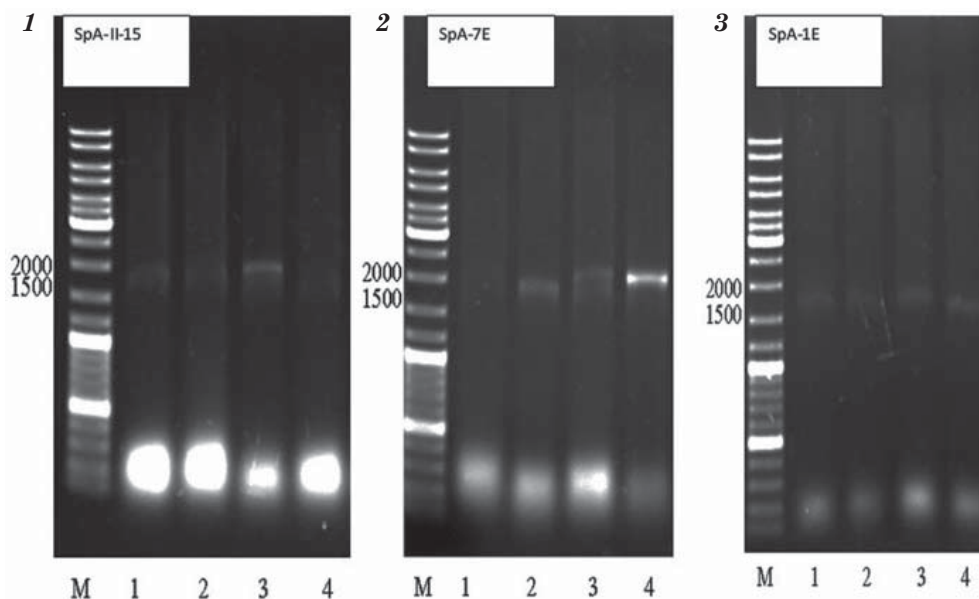


Fig. 10. PCR analysis of clones transformed by pET-22b-SpA with genes sequences of corresponding scFv antibodies:

1-4 — clones transformed by pFET-22b-SpA sequences of scFv gene; M — markers

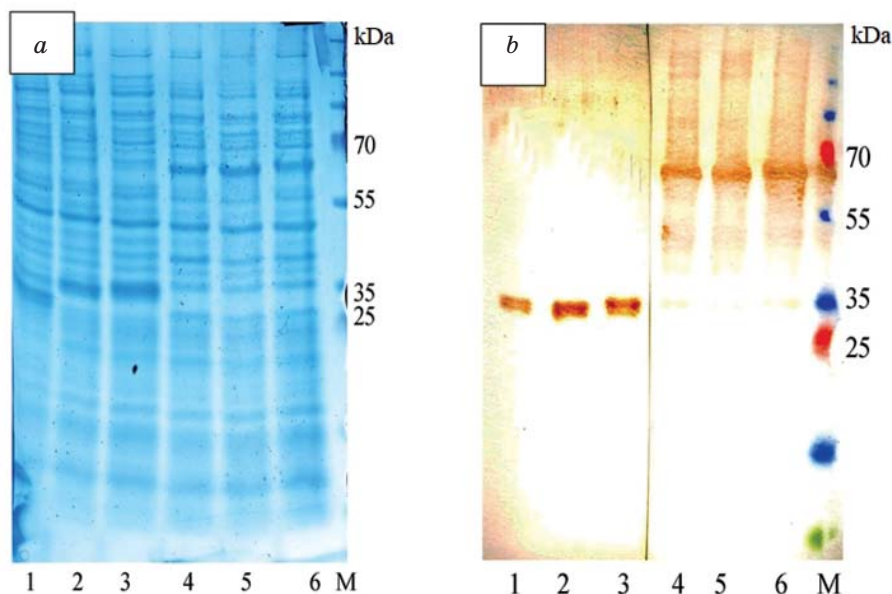


Fig. 11. Electrophoregram (a) and blotogram (b) of lysates of cells producing scFv antibodies either free or fused with the fragments of SpA, subcloned in the pET-22b vector:

1, 2, 3 — free scFv II-15, 7E, 1E, respectively; 4, 5, 6 — SpA-scFv II-15, 7E, 1E, respectively; M — markers. Immunoblotting was conducted with antibodies to his-tag, conjugated to horseradish peroxidase.

technological methods for the extraction of the purified and biologically active target product are two of the most pressing challenges of modern biotechnological production of recombinant antibodies. Fusion of the scFv gene sequence to the SpA gene sequence makes it possible to increase the efficiency of scFv application in enzyme immunoassay and can be used to

create test systems. Presence of the SpA gene sequence in the complex greatly enhances the production of target protein in a functionally active form, as shown in this experiment.

Thus, the strategy proposed in this paper can be used as a universal and effective approach to increase the efficiency of production of scFv antibodies in *E. coli* cells.

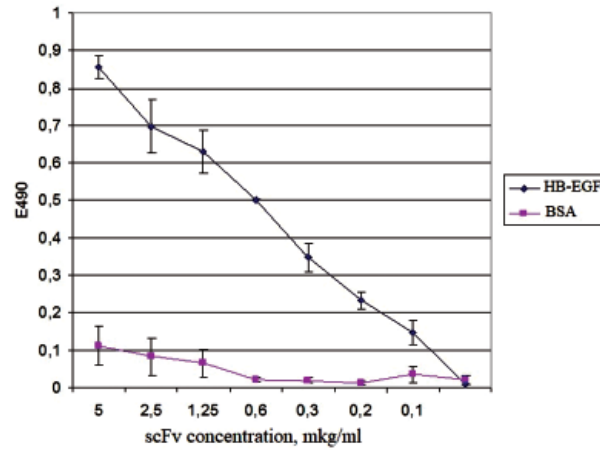


Fig. 12. Enzyme immunoassay analysis of 7E scFv antibodies fused with SpA:
BSA — bovine serum albumin. The error bars represent a standard deviation. Significant compared to control results with BSA ($P \leq 0.05$)

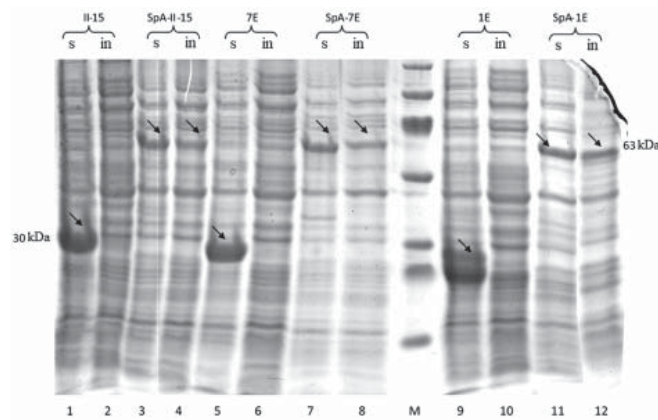


Fig. 13. Electrophoregram of soluble and insoluble fractions of cell lysates of clones producing free scFv antibodies and scFv antibodies fused to a protein A fragment:
s — soluble fraction; in — insoluble fraction. The clone names are indicated on top

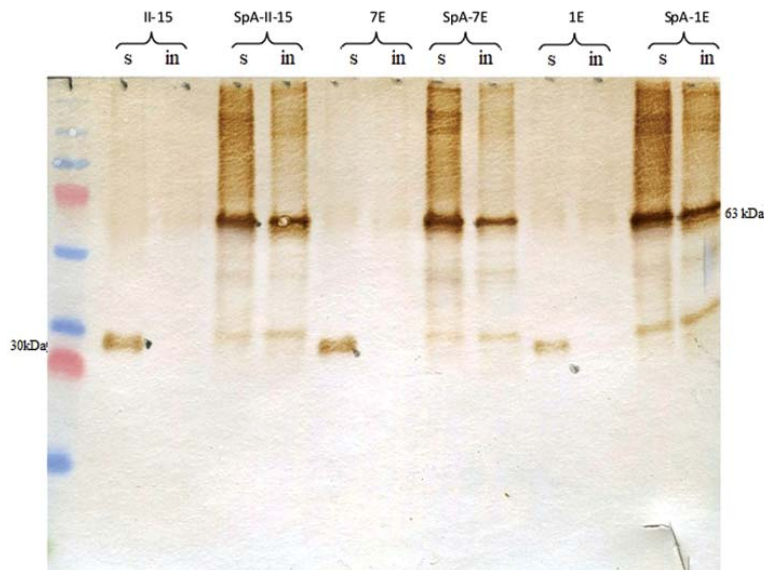


Fig. 14. Blotogram of soluble and insoluble fractions of cell lysates of clones producing free scFv antibodies and scFv-antibodies fused to protein A fragment:
s — soluble fraction; in — insoluble fraction. The clone names are indicated on top

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**ПІДВИЩЕННЯ ЕФЕКТИВНОСТІ
ЕКСПРЕСІЇ РЕКОМБІНАНТНИХ
scFv-АНТИТІЛ**

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Одержання рекомбінантних одноланцюгових варіабельних фрагментів антитіл scFv (single-chain fragment variable) в клітинах *E. coli* часто пов'язане з низкою проблем, які зумовлюють низький рівень виходу та неактивну форму продукту.

Метою роботи було дослідити вплив фрагмента стафілококового протеїну А (SpA) у складі злитого протеїну із scFv-антитілами на ефективність експресії кінцевого продукту. На прикладі scFv-антитіл різного походження і специфічності показано, що рівень їх експресії у злитій із D-E-A-A* фрагментом SpA формі значно перевищував рівень експресії вільних scFv у аналогічній експресійній системі. При цьому у складі злитих протеїнів scFv-антитіла зберігали свої антигензв'язувальні властивості, а фрагмент протеїну А — здатність зв'язувати інші імуноглобуліни.

Таким чином, запропоновану стратегію можна використовувати як ефективний підхід для підвищення ефективності продукції scFv-антитіл у клітинах *E. coli*.

Ключові слова: scFv-антитіла, протеїн А *Staphylococcus aureus*, химерні протеїни, *E. coli*.

**ПОВЫШЕНИЕ ЭФФЕКТИВНОСТИ
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Получение рекомбинантных одноцепочечных вариабельных фрагментов антител scFv (single-chain fragment variable) в клетках *E. coli* часто связано с рядом проблем, которые обуславливают низкий уровень выхода и неактивную форму продукта.

Целью этой работы было исследовать влияние фрагмента стафилококкового протеина А (SpA) в составе слитого протеина с scFv-антителами на эффективность экспрессии конечного продукта. На примере scFv-антител различного происхождения и специфичности показано, что уровень их экспрессии в слитой с D-E-A-A* фрагментом SpA форме значительно превышал уровень экспрессии свободных scFv в аналогичной экспрессионной системе. При этом в составе слитых протеинов scFv-антитела сохраняли свои антигенсвязывающие свойства, а фрагмент протеина А — способность связывать другие иммуноглобулины.

Таким образом, предложенная стратегия может использоваться как эффективный подход для повышения эффективности продукции scFv-антител в клетках *E. coli*.

Ключевые слова: scFv-антитела, протеин А *Staphylococcus aureus*, химерные белки, *E. coli*.