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Construction, expression, functional characterization and practical application of fusion protein SPA-BAPmut

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Aim. *The creation of genetically engineered fusion protein SPA-BAPmut and its application as a secondary immunoreagent in immunoassays.* ***Methods***. *Gene cloning, PCR, electrophoresis, DNA sequencing, bacteria cells culturing, protein expression and purification, ELISA, Western-blotting were used.* ***Results***. *The DNA sequences encoding Staphylococcus aureus protein A (SPA) and bacterial alkaline phosphatase with enhanced catalytic activity (BAPmut) were used for construction of gene encoding fusion protein SPA-BAPmut that was expressed in the high-productive Escherichia coli system and obtained in a soluble form. Cultivation conditions to provide a high-level expression of SPA-BAPmut (> 1 g/l) were determined. The target protein was obtained with purity more than 95 % using IMAX method. SPA-BAPmut is thermostable, and both parts of fusion protein (SPA and BAPmut) retain their IgG binding and alkaline phosphatase activity for a long time. SPA-BAPmut was used as a substitute of secondary an- tibodies in immunoassays. As little as 5 ng of the antigen could be detected in Western blotting and 1 μg/ml of IgG in ELISA.* ***Conclusions***. *The possibility of using SPA-BAPmut as universal secondary immunoreagent for different types of immunoassays was shown.*

Keywords: protein A, bacterial alkaline phosphatase, fusion protein, immunoassays.

Introduction. *Staphylococcus aureus* protein A (SPA) consists of five immunoglobulin-binding domains (E, D, A, B, C), each of them being capable of specific interaction with Fc-fragments of IgG of different animal species and humans [1]. Therefore, SPA is widely used in affinity chromatography to create sorbents for purification of antibodies or separation of blood of patients with autoimmune diseases from autoantibodies and circulating immune complexes [2, 3]. Another promising approach in SPA application is diagnostics. The SPA conjugates with alkaline phosphatase, horseradish peroxidase and stained iodine are used in immunological tests. This conjugation is usually conducted chemically. However chemical conjugation has a number of disadvantages: 1) a great number of required purified components; 2) high heterogeneity of the final product; 3) necessity of separating full-size conjugates from non-conjugated components. In turn, the recombinant DNA technology assures the elaboration of genetic constructions, which can be used to obtain chimeric bifunctional proteins in heterological systems, including *Escherichia coli*.

The bacterial alkaline phosphatase with enhanced catalytic properties (BAPmut) was selected for fusion with SPA. An increase in the BAPmut catalytic activity was achieved via two aminoacid substitutes D153G and D330N (substitution of aspartic acid for asparagine and glycine in positions 153 and 330 respectively) [4].

Materials and Methods. *Construction and expression of SPA-BAPmut.* DNA-sequence of SPA was obtained via PCR-amplification from chromosomal DNA of *S. aureus*, isolated using Genomic DNA Purification Kit (*Fermentas*, Lithuania). A pair of specific primers, introducing restriction sites *NdeI* and *NotI*, was used to conduct PCR. The purified PCR product (~880 b.p.) was hydrolyzed by respective restrictases and cloned into plasmid vector *pET-24a* (resulting plasmid – *pETSPA*) [5].

PCR was conducted using the following pair of primers: sense SPA – *NdeI*:
5'-ATCATATGGCGCAACACGATGAAGCTCAAC-3';
antisense SPA – *NotI*:
5'-ATGCGGC CGCTTCCT CTTTTGGTGC-3'.

Plasmid *pCANTAB-ScFv-BAPmut* and a pair of primers, introducing restriction sites *NotI* and *XhoI*, were used to obtain DNA sequences, encoding BAPmut. The purified PCR product (~1360 b.p.) was hydrolyzed by respective restrictases and subcloned into plasmid vector *pET-SPA*. PCR was conducted using the following pair of primers: sense BAPmut – *NotI*:
5'-ACGGGCGGCCGCTACACCAGAA-3';
antisense BAPmut – *XhoI*:
5'CGGCGAGACCCCGACTTTGAGCTCACTA-3'.

For the expression of SPA-BAPmut, *E. coli* BL21 (DE3) cells were transformed by obtained plasmid *pET24-SPA-BAPmut.* The Protein expression was induced by the autoinduction protocol [6]. The localization and content of the target protein in the total lysate of cells-producents were determined by electrophoretic separation of the soluble and insoluble fractions of cell cytoplasm proteins.

The purification of SPA-BAPmut was performed by Immobilized-metal affinity chromatography (IMAC) in native conditions. 1 ml HiTrap chelating column balanced with Ni^{2+} ions was connected to an FPLC system (Pharmacia) , equilibrated with the buffer (100 mM tris-HCl (pH 8.0), 300 mM NaCl, 10 mM imidazole) with the flow rate of 0.5 ml/min and a fraction of soluble proteins of cytoplasm of *E.coli* cells previously filtered through 0.45 μm- membrane PVDF filter (*Millipore*, USA) was added . The column was washed from non-specifically bound proteins by the mentioned buffer until the recording pen got to the baseline. SPA-BAPmut was eluted by the buffer – 100 mM tris-HCl (pH 8.0), 300 mM NaCl, 300 mM imidazole. The purified protein was dialyzed against the buffer, containing 100 mM tris-HCl (pH 9.5), 140 mM NaCl, 15 mM $MgSO_4$. The homogeneity of the purified protein was analyzed in 12% SDS-PAAG [7], SPA-BAPmut concentration was determined using the known value of A_{280} adsorption, calculated from this sequence using Vector NTI software.

The application of SPA-BAPmut as a secondary immunoreagent. Antibody-dependent ELISA. rhIFNα2b (*PharmBiotec* Scientific Production Company, Ukraine) in 50 mM Na-carbonate buffer (pH 9.5) in the concentration of 10 μg/ml was added to the wells of polystyrene plate for ELISA (*Nunc,* Denmark) and incubated for 1 h at 37°C. After washing the wells with PBST buffer, the affinity purified rabbit polyclonal anti-rhIFNϭ2b antibodies in the concentration range of 100–0.1 μg/ml and SPA-BAPmut, 0.5 μg/ml, were added. The obtained immune complexes were visualized using *p*-nitrophenylphosphate, (pNPP, *Sigma*, USA). After staining the reaction was terminated by the introduction of 1 M NaOH and A_{405} adsorption value was measured using multichannel photometer Multiscan MCC/340 (*Titertek*, USA).

Antigen-dependent ELISA. rhIFNϭ2b in 50 mM Na-carbonate buffer (pH 9.5) in the concentration of 1–500 ng per well was added to the wells of polystyrene plate for ELISA (*Nunc*) and incubated for 1 h at 37°C. After washing the wells with PBS buffer which contains 0.1% twin-20 (PBST), the affinity purified rabbit polyclonal anti-rhIFNϭ2b antibodies [8] in the concentration 1 μg/ml and SPA-BAPmut in the concentration 0.5 μg/ml, were added.

Dot-blot analysis. Antigen rhIFNϭ2b in the amount of 5–20 ng was applied to the nitrocellulose membrane Hybond-C Extra (*Amersham Biosciences*, UK). After blocking the sites of non-specific binding by buffer PBS + 3 % milk (PBSM), the membrane with immobilized proteins was incubated for 1 h with purified rabbit polyclonal anti-rhIFNϭ2b antibodies in the concentration of 1 μg/ml. Antigen rhExCD34 was applied onto the membrane as a negative control [9]. The target protein was detected using SPA-BAPmut in the concentration of 0.5 μg/ml. For visualization of immune complexes Alkaline Phosphatase chromogen substrate BCIP-T/NBT (*Sigma*) was used.

The determination of the thermostability of the fusion protein SPA-BAPmut. The aliquotes of the purified SPA-BAPmut (1 mg/ml in the buffer of 0.1 M tris-HCl (pH 9.5), 0.14 M NaCl, 15 mM $MgSO_4$) were incubated at the following temperatures: +4, +50, +70, +85, +95, –20, –70°C for 10 min. The enzymatic activity of the alkaline phosphatase and IgG-binding activity of protein A were determined in ELISA.

Results and Discussion. In addition to affinity chromatography, the application of SPA, fused with the marker molecule, is promising for the determination of antibodies and specific antigens via specific antibodies. While selecting the marker molecule , the following parameters were taken into consideration: high enzymatic activity, a wide spectrum of commercially available substrates, considerable thermostability, small size, possibility of the obtaining in bacteria, and stability after conjugation with other proteins. The selection was ended with alkaline phosphatase (AP), widely used to elaborate immunoconjugates for immunochemical methods. Mammalian alkaline phosphatase (MAP) is remarkable for its high k_{cat} value (about 2000 s^{-1}) and thermolability ($T_m \sim 65°C$) [4]. The immunoconjugates with MAP are usually obtained via chemical conjugation [10]. Bacterial alkaline phosphatase (BAP) is less active compared to MAP, its k_{cat} value being 65–80 s^{-1}, but it is more thermostable – T_m is ~ 95°C. The immunoconjugates with BAP can be obtained by traditional chemical conjugation and in a more convenient way, i.e. by gene engineering combination of BAP sequence and the sequence of the marker molecule. High thermostability, the capability to forming gene-engineered conjugation as well as the possibility of efficient obtaining such conjugates in the active form via bacterial expression make BAP a promising alternative to MAP. Therefore, we preferred BAP as a marker molecule with the increased catalytic activity (BAPmut).

The comparative analysis of the structure of active sites of MAP and BAP demonstrated aminoacid substitutes, highly improving the catalytic activity of the latter. For instance, the aminoacid substitutes K328H/D330N increase the enzymatic activity of BAP three-fold, D153H/K328H, D153H/K328H/D330N – eight-fold, D153H/D330N – up to 17-fold. However, along with the enzymatic activity majority of the

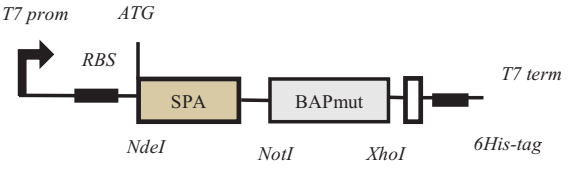

Fig. 1. The scheme of location of the elements of the expression cassette of SPA-BAPmut in vector *pET-24a(+)*

mentioned mutations lead to considerable decrease in the thermostability of the enzyme, which decreases the value of the latter as a component of immunoreagents. Mutations D330N/D153G were found, which increase the catalytic activity of BAP by about 17–40 times (depending on the incubation medium content) and do not impact the enzyme thermostability [4]. BAP with the abovementioned properties (aminoacid substitutes D330N/D153G) was used to obtain SPA-BAPmut.

The interactive design of the chimeric protein was conducted considering structural specificities of both moieties of SPA-BAPmut and the topology of their active sites. It was determined that optimal design of the chimeric protein presupposes fusion of BAPmut with C-terminus of SPA. This location secures the functional properties of phosphatase, as C- terminus, participating in the formation of the molecule active site, is not involved. The abovementioned location of SPA relative to the enzyme does not hinder the dimerization of a phosphatase molecule, required for its functioning, and increases the avidity of the formed immunoconjugate, thus increasing its sensitivity (Fig. 1). At the same time the location of the phosphatase on the N-terminus of the fused partner is a reason of of the loss of functional activity of the latter [11].

For large-scale production of SPA-BAPmut *E. coli* cells, strain BL21 (DE3), were transformed by the plasmid vector *pET-24-SPA-BAPmut* (Materials and Methods). Expression of SPA-BAPmut was induced by the autoinduction protocol [6]. The electrophoretic analysis of the lysates of bacterial cells demonstrated the band of expected molecular mass (~78 kDa) (Fig. 2). The electrophoretic separation of soluble and insoluble fractions of cytoplasm proteins showed that SPA-BAPmut is synthesized in the soluble form, the level of its accumulation is 37% of the total proteins of *E. coli* cells (~ 0.96 g/l of the bacterial culture) at the optic density $A_{600} = 16.3$. This value is almost 45 times

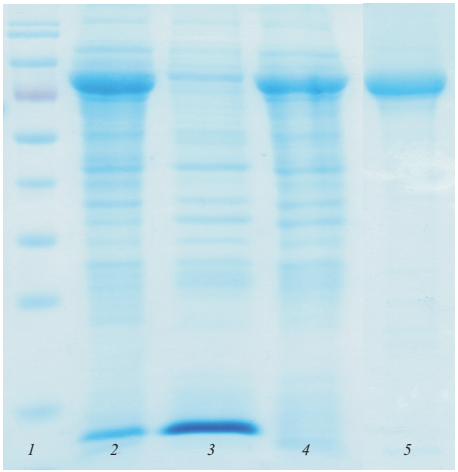

Fig. 2. The expression of SPA-BAPmut in *E. coli* cells: *1* – molecular weight markers (130, 100, 70, 55, 40, 35, 25, 15 and 10 kDa); *2* – total lysate of producent cells with induced production of SPA-BAPmut; *3* – fraction of insoluble proteins of the cell; *4* – fraction of soluble proteins of the cell; *5* – SPA-BAPmut, isolated on metal affinity sorbent and dialyzed against the buffer of 0.1 M tris-HCl, 0.14 M NaCl, 15 mM $MgSO_4$, pH 9.5

higher than the yield of similar fusion protein SPA APK328A, the obtaining of which is described [12].

The isolation of such conjugates requires combinations of the following methods: cells disruption by sonication, centrifugation, ion-exchange chromatography, gel-filtration, chromatography of hydrophobic interactions, precipitation, affinity chromatography, which, it should be noted, is the reason of the decrease in the functional activity of the enzyme due to strict elution conditions. As SPA-BAPmut contains a genetically introduced His-tag sequence, it was purified using IMAC under native conditions. To decrease non-specific adsorption of contaminant proteins, which may have significant impact on the purity of the final product, the column was washed with the buffer, containing 300 mM NaCl and 10 mM imidazole.

The elution of SPA-BAPmut was conducted with the buffer, which contained 300 mM imidazole. The mentioned conditions ensure obtaining SPA-BAPmut with purity more than ~95% (Fig. 2). After the purification SPA-BAPmut was dialyzed against the buffer, optimal for maintenance of the enzymatic activity of alkaline phosphatase (Materials and Methods).

The data, summarized according to the results of purification of SPA-BAPmut with using IMAC are presented in the Table. It was demonstrated that this method ensures obtaining SPA-BAPmut without considerable loss of the product (yield ~91.6%) and its functional activity.

Purification of SPA-BAPmut on Ni-NTA sepharose

Fraction	Volume, ml	Total amount of protein, mg	Functional activity BAPmut, %
Soluble cytoplasm proteins	10	6	100
After purification of SPA-BAPmut on Ni-NTA sepharose	5,7	5,6	Not determined
After the replacement of the buffer for elution with the buffer optimal for BAPmut	5,0	5,0	87

The purified SPA-BAPmut was used as a secondary antibody for the detection of antibodies (ELISA) and antigenes (ELISA, dot-blotting). It was determined that SPA-BAPmut reliably detects IgG in the concentration of ~500 ng/ml and less than 15 ng/ml of antigen (rhIFNб2b) (Fig. 3, 4). Besides, contrary to most of the immunoenzymatic systems of detection with specific secondary antibodies conjugated with a marker molecule, the application of SPA-BAPmut allows extending the range of primary antibodies (SPA recognizes Fc-fragments of IgG of humans, mice,rabbits, guinea pigs and bovine).

The thermostability of the fusion protein SPA-BAPmut was also determined. For this reason the aliquotes of SPA-BAPmut with the same concentration were incubated at different temperatures (Materials and Methods). SPA-BAPmut, isolated right prior to the experiment, was used as control. As seen from the diagram, presented in Fig. 5, SPA-BAPmut does not lose its immunoglobulin-binding and phosphatase activity during incubation at the temperature up to 70°C; it also endures the impact of low temperatures (Fig. 5). High thermostability of SPA-BAPmut is of relevant diagnostic significance as usually commercially available immunoconjugates are rather thermolabile. It was shown that SPA-BAPmut preserves its functional activity for at least 6 months if stored in 50 % glycerol at 4°C in the corresponding buffer.

Compared to the analogues, the suggested immunoconjugates has higher sensitivity due to an

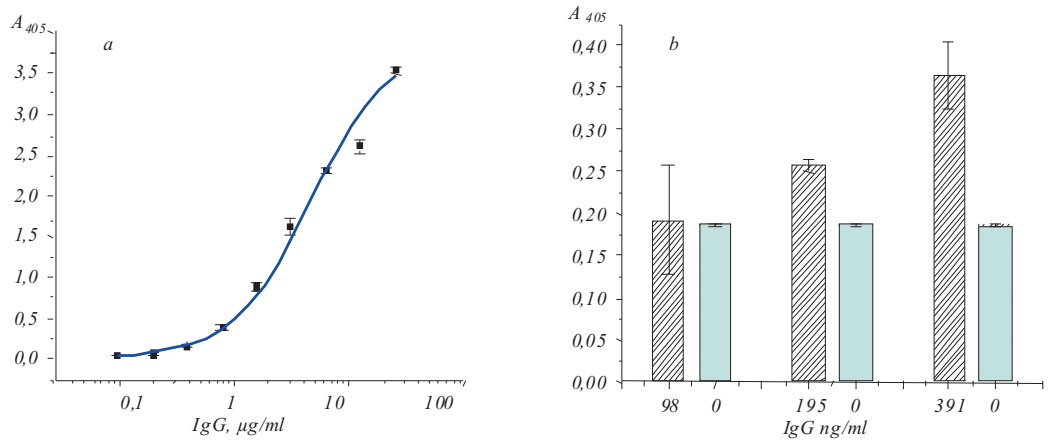

Fig. 3. The determination of the minimal concentration of IgG, detected using SPA-BAPmut in ELISA

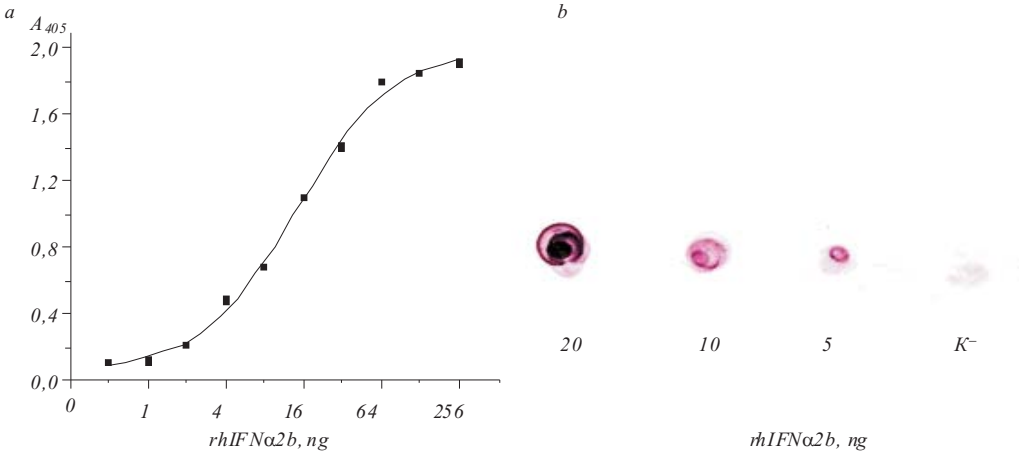

Fig. 4. The determination of antigen concentration (rhIFNб2b), detected using SPA-BAPmut in ELISA (*a*) dot-blotting (*b*)

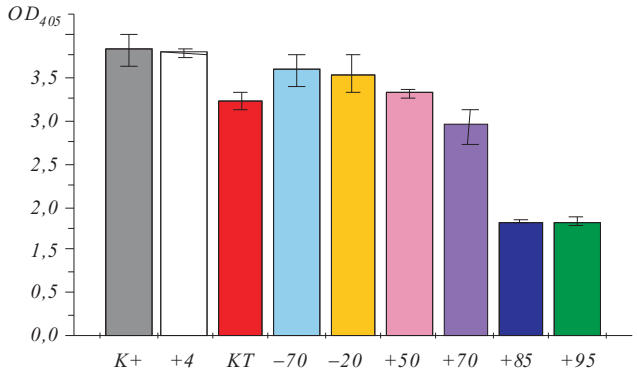

Fig. 5. The impact of different temperatures on functional activity of the fusion protein SPA-BAPmut: *K*+ – SPA-BAPmut immediately after the isolation from *E. coli* cells; *RT* – room temperature (24 h); +4, +50, +70, +85, +95, –70, –20°C (10 min)

increase in the enzymatic activity of BAP. Besides, contrary to traditional immunoreagents, created by chemical conjugation, the fusion protein SPA-BAPmut, obtained in preparative amounts by high productive and simple bacterial expression, allows for considerable simplification of the laboratory and industrial production of similar immunoreagents and reduction in the cost of immunoconjugates.

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Конструювання, синтез, функціональна характеристика і практичне застосування злитого білка SPA-BAPmut

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Резюме

Мета. *Створення генно-інженерного злитого білка SPA-BAPmut та його застосування як вторинного імунореагенту в імунологічних тестах.* ***Методи***. *Клонування генів, ПЛР, секвенування ДНК, культивування бактерій, електрофорез, синтез і очищення білків, ELISA, вестерн-блот.* ***Результати***. *З використанням*

послідовновностей ДНК, що кодують білок А Staphylococcus aureus (SPA) і бактерійну лужну фосфатазу з покращеними каталітичними властивостями (BAPmut), сконструйовано ген злитого білка SPA-BAPmut та забезпечено його препаративне отримання у розчинній формі внаслідок синтезу в клітинах Escherichia coli. Визначено умови ферментації, за яких вихід SPA-BAPmut становить приблизно 1 г/л культури E. coli. Із застосуванням методу металоафінної хроматографії одержано цільовий білок з чистотою понад 95 %. SPA-BAPmut термостабільний, а обидва його компоненти (SPA і BAPmut) зберігають імуноглобулінзв'язувальну і фосфатазну активність тривалий час. SPA-BAPmut дозволяє виявляти щонайменше 5 нг антигену та 1 мкг/мл антитіл. ***Висновки****. Показано можливість використання SPA-BAPmut як універсального вторинного імунореагенту в імунохімічних тестах.*

Ключові слова: білок А, бактерійна лужна фосфатаза, злитий білок, імунодіагностика.

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Конструирование, синтез, функциональная характеристика и практическое применение слитого белка SPA-BAPmut

Резюме

Цель*. Создание генно-инженерного слитого белка SPA-BAPmut и его использование как вторичного иммунореагента в иммунологических тестах.* ***Методы****. Клонирование генов, ПЦР, секвенирование ДНК, культивирование бактерий, электрофорез, биосинтез и очистка белков, ELISA, вестерн-блот.* ***Результаты****. С использованием последовательностей ДНК, кодирующих белок А Staphylococcus aureus (SPA) и бактериальную щелочную фосфатазу с улучшенными каталитическими свойствами (BAPmut), сконструирован ген слитого белка SPA-BAPmut и обеспечено его препаративное получение в растворимой форме вследствие синтеза в клетках Escherichia coli. Определены условия ферментации, при которых выход SPA-BAPmut составляет около 1 г/л культуры E. coli. С применением метода металлоаффинной хроматографии целевой белок получен с чистотой более 95 %. SPA-BAPmut термостабилен, а оба его компонента (SPA и BAPmut) сохраняют иммуноглобулинсвязывающую и фосфатазную активность на протяжении длительного времени. SPA-BAPmut позволяет детектировать 5 нг антигена и 1 мкг/мл антител.* ***Выводы****. Показана возможность применения SPA-BAPmut как универсального вторичного иммунореагента в иммунохимических тестах.*

Ключевые слова: белок А, бактериальная щелочная фосфатаза, слитый белок, иммунодиагностика.

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