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RECOMBINANT SINGLE CHAIN VARIABLE FRAGMENT ANTIBODIES (scFv) AGAINST Pro^{144}-Leu^{155} FRAGMENT OF HUMAN PROTEIN C

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The aim of this work was to obtain the recombinant single chain variable fragments of antibodies (scFv) against human protein C, the key component of blood anticoagulation system. For this purpose a peptide that mimics a Pro^{144}-Leu^{155} sequence of protein C was synthesized and the murine immune scFv library against this peptide was constructed. The protein C specific scFv 9E were selected from the constructed library by the phage-display method. The scFv 9E dissociation constant was found to be $2 \cdot 10^{-9}$ M. It was shown that scFv 9E were suitable for protein C detection by ELISA and Western blotting. Selected scFv could be further used for protein C investigation and for the development of quantitative methods for protein C detection in human blood.

K e y w o r d s: protein C, scFv antibodies, phage display.

Protein C (blood coagulation factor XIV, autoprothrombin IIa, EC 3.4.21.69) is vitamin K-dependent serine proteinase which plays a key role in the anti-coagulation system. The activated form of protein C has powerful anticoagulant and profibrinolytic properties due to its ability to inactivate Va and VIIIa factors. The decrease of protein C plasma level correlates with the risk of thrombogenesis and has an important diagnostic value [1]. Moreover protein C demonstrates cytoprotective properties such as increasing of cell viability, anti-apoptotic and anti-inflammatory effects. The investigation of protein C regulating properties is essential for development of new therapeutic approaches for correction of numerous pathological states [2].

Antibodies are an important tool both for studying of protein C biological activity and developing of test systems for quantitative measurement of its blood concentration.

The aim of this study was to obtain the recombinant murine single-chain variable fragment antibody (scFv) to carboxyterminal end of human protein C light chain. ScFv-antibodies could be easily modified and fused with enzymatic and fluorescent labels [3, 4]. This could be farther used for development of the system for quantitative determination of protein C, as well as for investigation its biological properties. Also the recombinant antibodies may be used for the affine purification of protein C [5].

Materials and Methods

Conjugation of synthetic peptide with carrier proteins. The synthetic peptide, which imitates Pro^{144}-Leu^{155} amino acid sequence of human protein C, was conjugated to hemocyanine of mollusk *Megathura crenulata* (KLH) or bovine serum albumin (BSA) in accordance with the methods described in [6, 7]. Conjugation was performed in two steps: first carrier protein was modified by maleimidopropionic acid and then peptide was added to maleimidopropionyl-KLH or maleimidopropionyl-BSA [8].

Immunization of experimental mice. BALB/c mice (4-month old females) immunized with conjugate of synthetic peptide with KLH were used in the work. Antigen was administered intraperitoneally (100 μg per animal). The first immunization was performed in the complete, two next – in incomplete Freund ajuvant (Sigma, USA), the interval between immunizations was two weeks.

Construction of immune phage library. Total RNA, which was used as a matrix in kDNA synthesis, was isolated from the spleen tissue of immunized mice by TRI Reagent (Sigma, USA). The sequences encoding the variable domains of the light and heavy

immunoglobulin chains (VH and VL) were amplified with the set of specific primers designed according to [9]. The high-fidelity polymerase AccuTaq LA DNA Polymerase (Sigma, USA) was used for amplification. Nucleotide sequences VH and VL were assembled by SOE-PCR (splicing by overlap extension PCR). DNA sequences of scFv were inserted into the phagemide vector p*CANTAB*-5E by *Sfi*I and *Not*I restriction sites. The ligase mixture was used for transformation of *Escherichia coli* XL1-blue by the electroporation.

The positive clones were selected by the phage display method as described before [10]. Peptide which imitates Pro^{144}-Leu^{155} sequence of protein C conjugated with BSA was used for screening.

Enzyme-linked immunosorbent assay (ELISA). As antigens were used conjugate Pro^{144}-Leu^{155} peptide with BSA, native protein C (Sigma, USA) and BSA. Monoclonal antibodies against marker sequence fused with scFv (E-tag) (Amersham, USA) were used to determine scFv antibodies; and anti-mouse IgG HRP were used as the secondary antibodies. O-phenylenediamine (Sigma, USA) was used as chromogen substrate. The color reaction was quantified by measuring the absorbency at 490 nm.

Periplasmic extracts were prepared by the osmotic shock method. The extracts were 8-fold concentrated.

Immunoblotting was performed as described previously [11]. For detection of scFv were used monoclonal antibodies against E-tag (Amershem, USA). The method of enhanced chemoluminescence was used for visualization of results.

Subclonning of selected scFv into pET-22b vector. DNA sequences of scFv were ligated with p*ET-22b* vector by *Not*I/*Nco*I restriction sites using T4 DNA ligase (Fermentas, Lithuania). The *E. coli* Rosetta were transformed by this ligase mixture.

Separation and purification of scFv antibodies. ScFv were purified from insoluble fraction by metal affine chromatography method with farther refolding [12].

The affinity constant was determined as described previously [13] according to the formula $A_0/A_x = 1+K_a \cdot a_0$, where K_a is the affinity constant, A_0 – the value of absorption in the well without a competitive antigen, A_x – the value of absorption in the well with certain concentration of a competitive antigen, a_0 – concentration of competing antigen.

Results and Discussion

One of the most important characteristics of antibodies is their specificity, i. e., the capacity to bind with the target antigen without crossed reactions with other antigens. The carboxy-terminal part of protein C light chain (sequence Pro^{144}-Leu^{155}: PWKRMEKKRSHL) was chosen for generation of highly specific scFv to protein C. In accordance with the analysis in the BLAST search system this protein C sequence is unique. The only blood protein with the partial homology is erythropoetin (sequence Trp^{78}-Glu^{82}: WKRME), but its concentration in the blood plasma is significantly lower than the protein C concentration.

Synthesis of peptide Pro^{144}4-Leu^{155} and its conjugation with carrier proteins. The solid-phase synthesis of the peptide, which mimics the Pro^{144}-Leu^{155} sequence of protein C, was performed in the Laboratory of Peptide Immunogens of the State Scientific Centre Institute of Immunology FMBA. According to results of mass-spectroscopy and analytical HPLC the synthesized peptide had the expected molecular weight and purity above 92% (Fig. 1). The peptide was conjugated with carrier proteins (KLH and BSA). The peptide conjugated with KLH was used for mice immunization, and conjugate with BSA for measurement of antibody level.

Immune library of murine recombinant antibodies. According to ELISA the titer of antibodies against peptide Pro^{144}-Leu^{155} conjugated with BSA in the serum of immunized mouse was about 1:32000 (Fig. 2).

Total RNA from splenocytes of immunized mouse was used as the matrix for amplification of sequences encoding the variable domains of the heavy and light immunoglobulin chains. Electrophoresis on agarose gel showed 380 and 400 b.p. PCR products corresponding in size to VL and VH, respectively (Fig. 3, *a*).

The purified VH and VL were assembled in a single step of assembly PCR. The linker encoding $(Gly_4Ser)_3$ was generated by overlap of the two inner primers. In assembled scFv VL was located on the 5′-end and VH- on the 3′-end. After SOE-PCR were obtained PCR-products about 800 b.p. that corresponded to expected size of scFv (Fig. 3, *b*).

For library construction the p*CANTAB* 5E phagemid vector was used and scFv were inserted by *Sfi*I and *Not*I sites. These endonucleases are rare

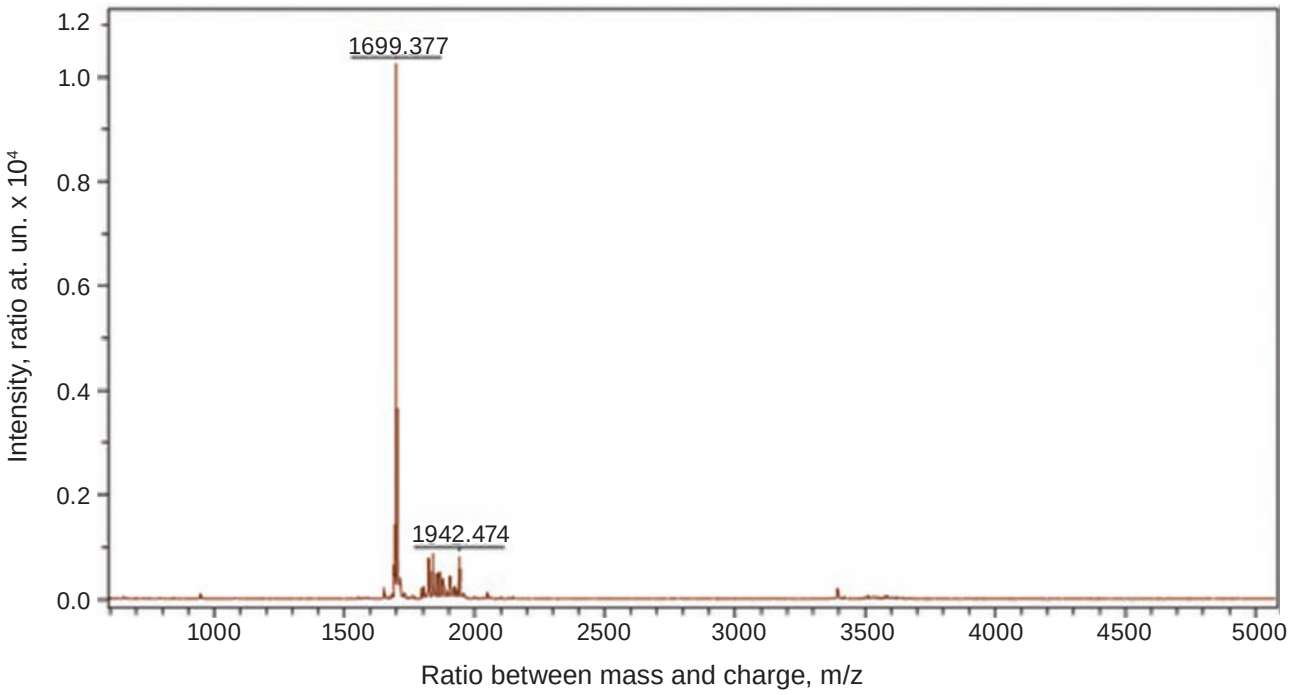

Fig. 1. Mass-spectroscopy of peptide Pro[144]-Leu[155]

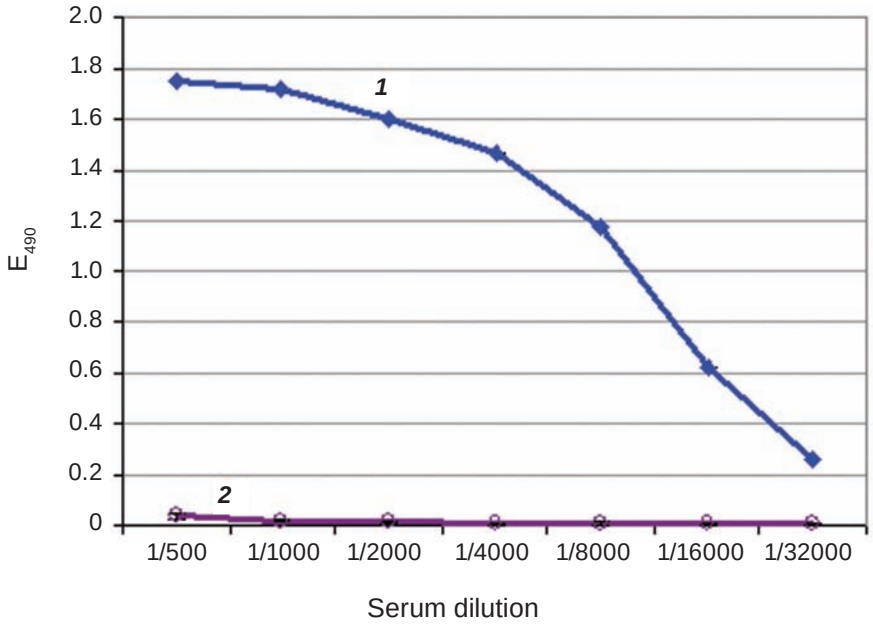

Fig. 2. IgG level to the target and control antigens in the serum of immunized mouse: 1 – peptide which mimics protein C Pro[144]-Leu[155] sequence conjugated with BSA; 2 – BSA. The results of a typical experiment are presented

for V-segments sequences, thus their use minimize the loss of diversity during restriction. As host cells *E. coli* XL1-blue *supE*+ strain were used. In these cells translation could continue through the amber stop codon to produce scFv fused to phage protein g3p displayed on the phage tip. Suppression of the amber stop codon is about 20%.

The size of constructed library was about 8.5×10^5 individual clones that is enough for selection of specific antibodies from immune libraries.

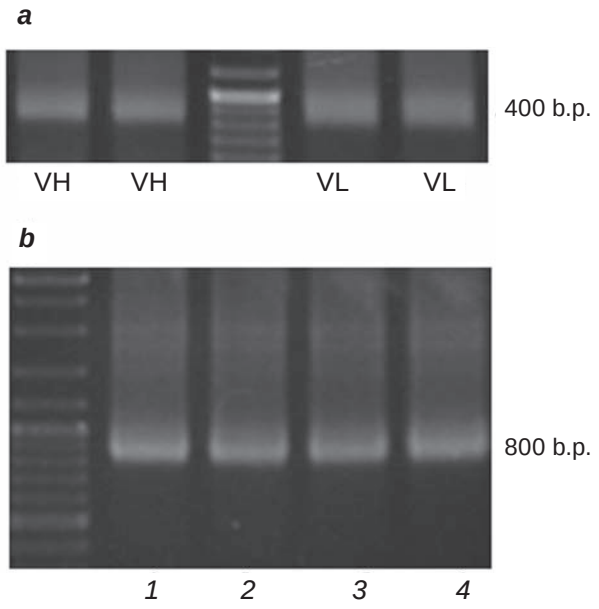

Fig. 3. PCR-amplification of VH- and VL-domains (a) and their assembling into scFv (b)

Characteristics of scFv antibodies specific for protein C. After selection of library against protein C 9E clone was isolated. By ELISA was shown that scFv 9E was specific both for peptide which mimics the protein C Pro^{144}-Leu^{155} sequence conjugated with BSA and for the native protein C (Fig, 4, *a*).

After immunoblotting of periplasmatic cell extract additional bands probably correspond to products of degradation of scFv and their protein g3p fusion have been found (Fig. 4, *b*). This means that the system based on the vector p*CANTAB* 5E and strain XL1-blue optimized for selection but not expression of recombinant antibodies was low-productive for scFv 9E. That is why another expression system based on the p*ET-22b* vector and strain *E. coli* Rosetta was used for further studying of selected recombinant antibody fragments. The p*ET-22b* vector contains a strong promoter of bacteriophage T7 as well as the Pe1B-signal for transporting of recombinant protein to periplasmatic space, where formation of disulfide bonds is possible [14]. Strain *E. coli* Rosetta designed to enhance the expression of eukaryotic proteins that contain AGG, AGA, AUA, CUA, CCC, GGA codons rarely used in *E. coli* [15].

DNA-sequence of scFv 9E was inserted into the p*ET-22b* vector by the *Nco*I/*Not*I sites (Fig. 5) and *E. coli* Rosetta cells were transformed by ligase mixture. After electrophoresis of obtained clones lysates bands with expected for scFv 9E molecular weight (about 30 kDa) were found (Fig. 6, *a*). Part of the target protein was about 27% of total bacterial proteins. So the scFv 9E production was considerably intensified. Expectedly, scFv 9E were synthesized mainly in a form of inclusion bodies and thus they were purified by further refolding *in vitro* (Fig. 5, *b*).

Specificity of scFv 9E to human protein C was confirmed by ELISA (Fig. 7). Dissociation constant of scFv 9E was about $2 \cdot 10^{-9}$ M. Thus, the obtained recombinant antibody fragments to C-terminus of protein C light chain were characterized by quite high affinity.

It is important that the antibodies, which could be used to study protein C properties, were capable to identify it not only in immunoenzyme analysis

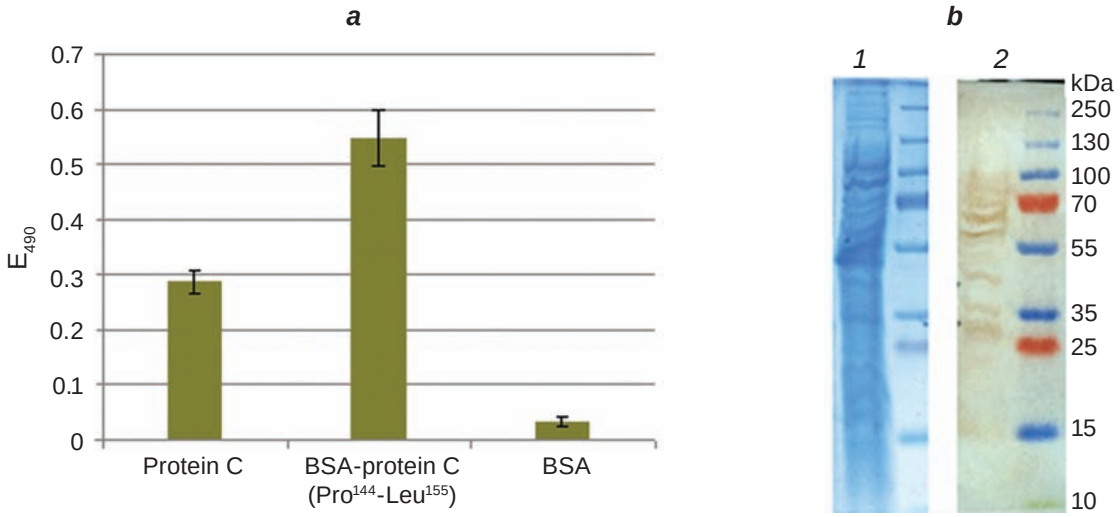

Fig. 4. ELISA of periplasmatic cell extract from 9E clone (a). Electrophoresis (b1) and immunoblotting (b2) of periplasmatic extract from 9E clone. ScFv 9E were detected with monoclonal antibodies to E-tag

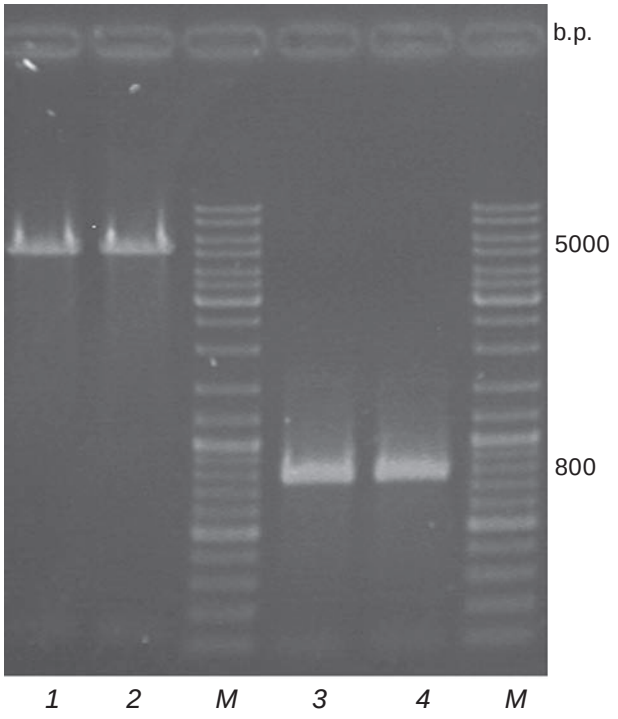

Fig. 5. Subclonning of scFv 9E into pET-22b vector: 1-2 – DNA-sequence of pET-22b vector, digested by the restriction endonucleases NcoI/NotI; 3-4 – DNA-sequence of scFv 9E, digested the restriction endonucleases NcoI/NotI, M – markers

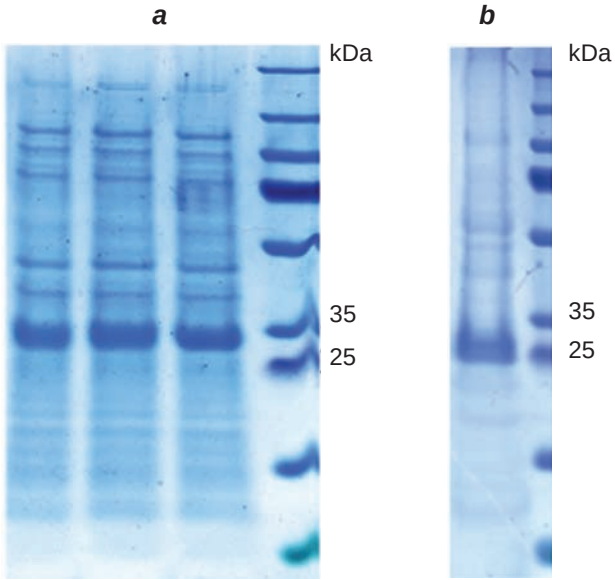

Fig. 6. Analysis of scFv 9E expression (a). scFv 9E after purification and refolding (b)

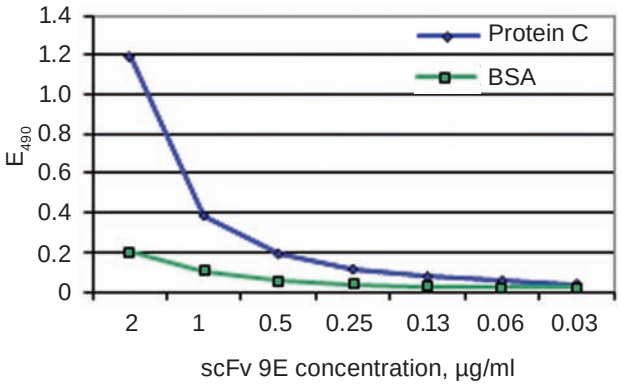

Fig. 7. The binding scFv 9E antibodies to target and control antigens. The results of a typical experiment are presented

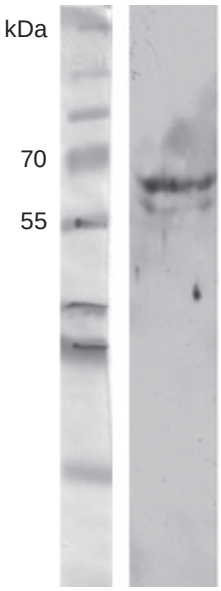

Fig. 8. Protein C detection by immunoblotting with obtained scFv 9E

but also in immunoblotting. Since a synthetic peptide was used for selection of scFv 9E, they had to be specific for linear epitope. And as was shown, scFv 9E recognized protein C in immunoblotting.

Therefore, the recombinant scFv antibodies to important component of anticoagulation blood system protein C were obtained. It was shown that the selected antibodies could be used in enzyme-linked immunosorbent assay and immunoblotting for protein C detection. The obtained scFv antibody could also be further used for studying protein C as well as for design of the diagnostic systems for its quantitative determination.

РЕКОМБІНАНТНІ ОДНОЛАНЦЮГОВІ ВАРІАБЕЛЬНІ ФРАГМЕНТИ АНТИТІЛ (scFv) ПРОТИ Pro^{144}-Leu^{155} ДІЛЯНКИ ПРОТЕЇНУ С ЛЮДИНИ

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Метою роботи було одержати рекомбінантні одноланцюгові варіабельні фрагменти антитіл (scFv) проти протеїну С людини, ключового компонента антикоагуляційної системи крові. Для цього було синтезовано пептид, що імітує послідовність Pro^{144}-Leu^{155} протеїну С, та сконструйовано бібліотеку scFv антитіл миші, імунну до цього пептиду. Методом фагового дисплею зі сконструйованої бібліотеки відібрано scFv 9E, специфічні до протеїну С. Константа дисоціації scFv 9E становила $2 \cdot 10^{-9}$ М. Показано, що scFv 9E можуть застосовуватись для визначення протеїну С в імуноензимному аналізі та імуноблотингу. Відібрані scFv у подальшому можуть бути використані в дослідженнях протеїну С, а також для розробки методу кількісного визначення протеїну С у плазмі крові людини.

Ключові слова: протеїн С, scFv антитіла, фаговий дисплей.

РЕКОМБИНАНТНЫЕ ОДНОЦЕПОЧЕЧНЫЕ ВАРИАБЕЛЬНЫЕ ФРАГМЕНТЫ АНТИТЕЛ (scFv) ПРОТИВ Pro^{144}-Leu^{155} УЧАСТКА ПРОТЕИНА С ЧЕЛОВЕКА

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Целью работы было получить рекомбинантные одноцепочечные фрагменты антител (scFv) против протеина С человека, ключевого компонента антикоагуляционной системы крови. С этой целью был синтезирован пептид, имитирующий последовательность Pro^{144}-Leu^{155} протеина С, и сконструирована библиотека scFv антител мыши, иммунная к этому пептиду. Методом фагового дисплея из сконструированной библиотеки были отобраны scFv 9E, специфичные к протеину С. Константа диссоциации scFv 9E составляла $2 \cdot 10^{-9}$ М. Показано, что scFv 9E могут применяться для определения протеина С методами иммуноэнзимного анализа и иммуноблоттинга. Отобранные scFv в дальнейшем могут быть использованы для исследования протеина С, а также для разработки метода его количественного определения в плазме крови человека.

Ключевые слова: протеин С, scFv антитела, фаговый дисплей.

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