

Molecular and Cell Biotechnologies

Immobilized single chain antibodies for affinity purification of recombinant human ifn- 2b

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A laboratory method for obtaining immunoaffinity media for chromatographic purification of proteins based on orienting and non-covalent immobilization of recombinant antibody fragments on cellulose matrix is described. The single-chain antibody (ScFv) against human IFN- 2b was genetically fused to cellulose-binding domain (CBD) from Clostridium thermocellum cellulosome and expressed in Escherichia coli. After the isolation of the target protein in functionally active form from bacteria cells the directed immobilization on microgranular cellulose has been carried out. The cellulose with immobilized ScFv-CBD-fusion was used as affinity media to perform the purification of recombinant human IFN- 2b.

Keywords: *single chain antibodies, immunoaffinity chromatography, protein immobilization, fusion protein.*

Recombinant antibody fragments (rAbs) construction and their expression in heterologous systems represents the modern approach of obtaining highly selective immunological reagents for fundamental researches, diagnostics and therapy [1, 2]. Single chain antibodies (ScFv) are the recombinant molecules, designed by the genetic fusion of variable domains from heavy and light antibody chains via the artificial linker, which are expressed by the producers

as one polypeptide chain [3]. Nowadays, ScFv antibodies are widely used in many applied and fundamental researches, which are conditioned by the capability of selecting the molecules with the desired binding characteristics and the possibility of their large-scale production using high-efficient expression systems [4].

One of the attractive directions of rAbs application is the development of inexpensive matrixes for immunoaffinity chromatography (IAC). Recently the principal applicability of antibody fragments for purification of target proteins by immunoaffinity chromatography

(IAC) has been shown [5, 6], and different strategies for their immobilization on chromatographic matrixes, including chemical coupling or indirect binding via some engineered protein partners, have also been reported [7, 8]. The most common disadvantage of chemical immobilization methods is the random orientation of antigen-binding site of antibody molecules on the matrix surface, which leads to its low availability for the interaction with antigen.

Current work presents the laboratory method of immunoaffinity media obtaining for chromatographic purification of human recombinant IFN- 2b, which is based on direct and oriented immobilization of ScFv antibodies on cellulose matrix via cellulose binding domain (CBD) *Clostridium thermocellum*. ScFv's against human IFN- 2b were selected out from an immune mouse cDNA library according to protocols as described in the Amersham Biosciences (Sweden) Recombinant Phage Antibody System manual [9]. The gene encoding anti-IFN- 2b ScFv was amplified from plasmid pCANTAB 5E and subcloned into plasmid pET-24a (+) (Novagen) as N-terminus fusion with cellulose binding domain - CBD (GenBank Accession

№X68233) (Fig.1). For the achievement of the high-level production of ScFv-CBD the corresponding plasmid was transformed into *E. coli* strain BL21 (DE3) carrying the T7 RNA polymerase gene and the fusion protein were expressed as inclusion bodies. After the isolation of inclusion bodies the ScFv-CBD was solubilized in 6 M GuHCl and purified under denaturing conditions by Ni²⁺-immobilized affinity chromatography (IMAC) [10]. The purified ScFv-CBD was refolded by gradual removing of solubilizing reagent using Quix Stand ultrafiltration system (Amersham Biosciences) and the method described in the work [11].

Refolded ScFv-CBD was directly immobilized on microgranular cellulose CC31 (Whatman, Great Britain) [12]. Prepared affinity cellulose (~40 ml) was washed several times with the phosphate-saline buffer PBS pH (8.0) and packed into the chromatographic column XK 16 (Amersham Biosciences). The purification procedure was carried out using the "Acta Prime" chromatography equipment (Amersham Biosciences) as follows. Clarified cell lysates of *E.coli* cells, containing recombinant human IFN-62b (VDNK PharBiotech, ВНДК «ФармБіотек», Ukraine), were loaded on the column. After washing the column for several times with PBS the bound IFN- 2b was eluted by the buffer, containing 0,1 M glycine-HCl, 0,5 M NaCl (pH 3,0). IFN- 2b concentration was determined by measuring adsorption at A₂₈₀, and the purity of eluted IFN- 2b was analyzed by electrophoresis in 15% SDS-polyacrilamide gel (SDS-PAGE).

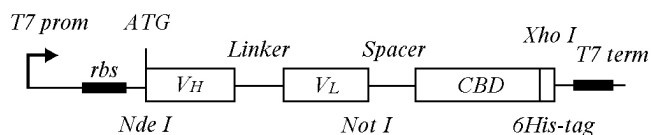


Fig.1. The scheme of "ScFv-CBD" expression cassette in plasmid vector pET-24 a(+)

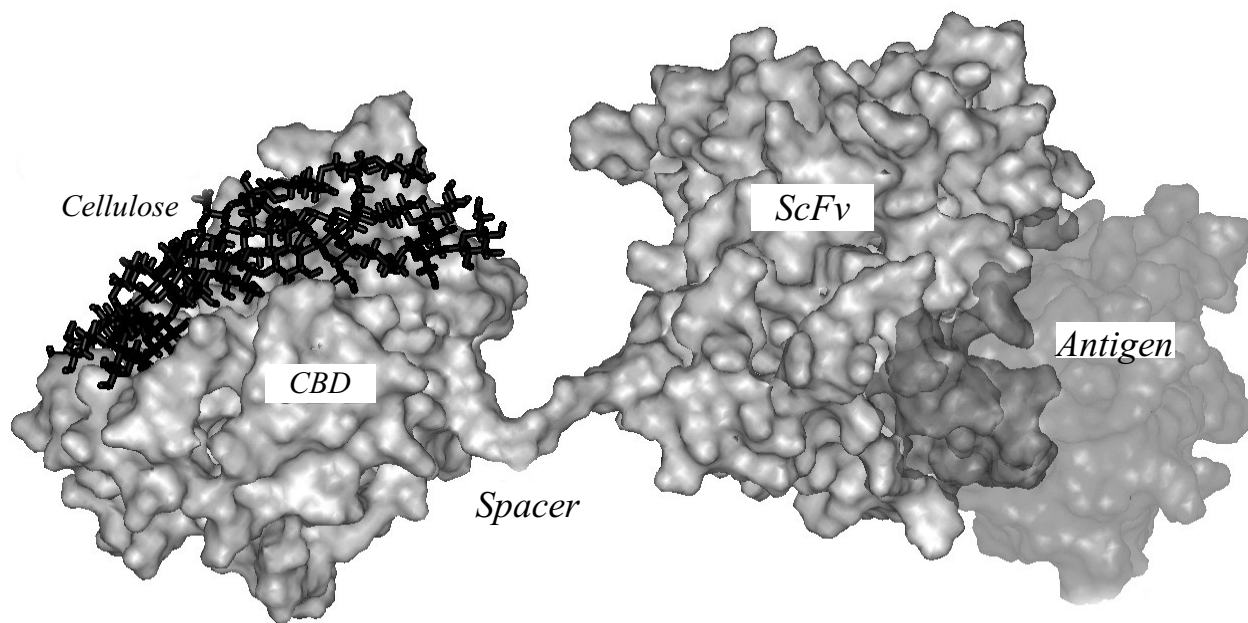


Fig.2. The schematic image of fusion protein ScFv-CBD, containing affinity moieties for antigen binding and immobilization on cellulose

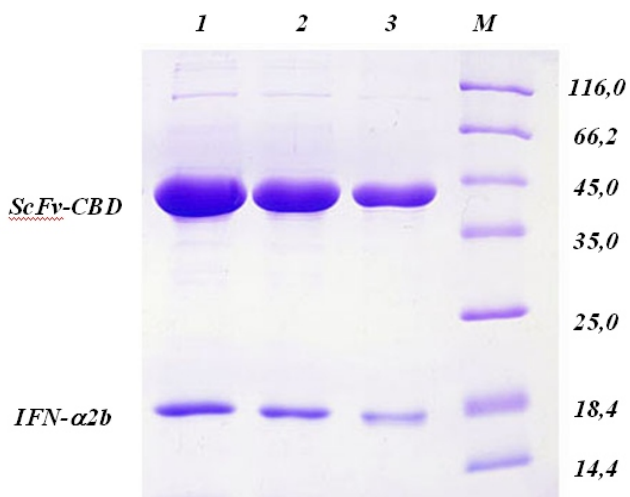


Fig.3. SDS-PAGE analysis demonstrating the protein complexes which are formed on cellulose with immobilized ScFv-CBD (~45 kDa) after binding of IFN- 2b (~18 kDa) to the column. Immune complexes were eluted from cellulose by the buffer containing 8 M of urea, 0,5% of SDS and 0,1 M of 2-mercaptoethanol. The amount of proteins, loaded to lines 1-3, corresponds to 10; 5 and 2,5 μ g of affinity cellulose; M - molecular weight marker; the gel was stained by Coomassie brilliant blue R-250.

The designed fusion protein (~46 kDa) contains two affinity moieties, represented by N-terminus of ScFv antibody and C-terminus of CBD, which are joining with 13 amino acid spacer- (-Gly₃-Ser-Glu-Gly₃-Ser-Glu-Gly₃-). Introduced affinity tag sequence “(His)₆-tag” from the expression vector facilitates one-step purification of ScFv-CBD from inclusion bodies by IMAC. The conducted design of fusion protein molecule provides stable complex formation between cellulose-binding domain and carbohydrate scaffold of cellulose, and exposes the antigen-binding site of the ScFv to the optimal position for the interaction with the antigen (Fig.2).

Biologically active ScFv-CBD was recovered from the *E.coli* inclusion bodies as described above. The refolding efficacy was calculated by the percentage of recovered biological activity separately for each moiety of fusion protein (ScFv and CBD) using the approach earlier developed by us (not published data). It has the following principle: the biological activity for ScFv is the capability of binding antigen, while for CBD it is the selective interaction with cellulose. Thus, the activity of CBD was calculated according to the percentage ScFv-CBD protein, which was selectively bound onto cellulose after the refolding. The analysis of ScFv activity was carried out by estimation of the protein

molar ratio in immune complexes ScFv-CBD / IFN- 2b, which were eluted from affinity cellulose under harsh denaturing conditions and loaded for separation to 15% SDS-PAGE (Fig.3). Using the described methods, it was determined that the refolding efficacy for ScFv and CBD moieties of fusion protein amounts to 98 and 45% respectively.

The usage of effective refolding method allowed recovery of soluble and active material from inclusion bodies which proved to be enough to obtain preparative affinity column (40 ml of affinity cellulose with capacity for the ScFv-CBD of ~2 mg/ml) and to fulfill the purification of recombinant IFN- 2b (molecular weight ~18 kDa).

Clarified lysate of *E.coli* cells, containing expressed recombinant human IFN- 2b, was loaded on the affinity column. The elution of bound IFN- 2b was carried out at mild conditions (pH 3,0) which provided its selective dissociation without significant “leakage” of immobilized ScFv-CBD (Fig.4, a). According to the SDS-PAGE data the purity of IFN- 2b in eluted fractions amounts to more than 95% and only one additional minor band is visible (~45 kDa), which is the product of ScFv-CBD dissociation from cellulose at pH 3,0 (Fig.4, b). Nevertheless, the amount of eluted fusion protein was less than 0,1% per one purification cycle, which practically did not influence the column performance. The column dynamic capacity for IFN- 2b was calculated according to the measurement data of purified IFN- 2b concentration, it amounted to about 0,1 mg of IFN- 2b per 1 ml of affinity cellulose. The important result is the fact that we demonstrated the possibility of repeated purification of IFN- 2b for the same affinity column after its subsequent reloading.

In current work we present the laboratory method for obtaining affinity media using single chain antibodies which immobilized via cellulose binding domain. The principal applicability of immunoaffinity column for one-step chromatographic purification of recombinant human IFN- 2b from clarified lysates of producer cells (purity ~95%) was demonstrated, and high stability of immobilized ScFv antibodies at several subsequent purification cycles was also shown.

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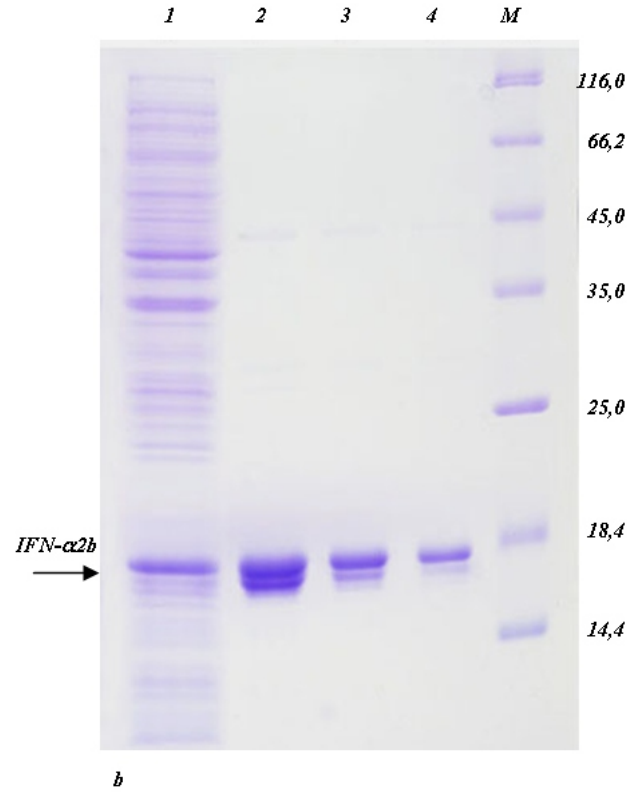
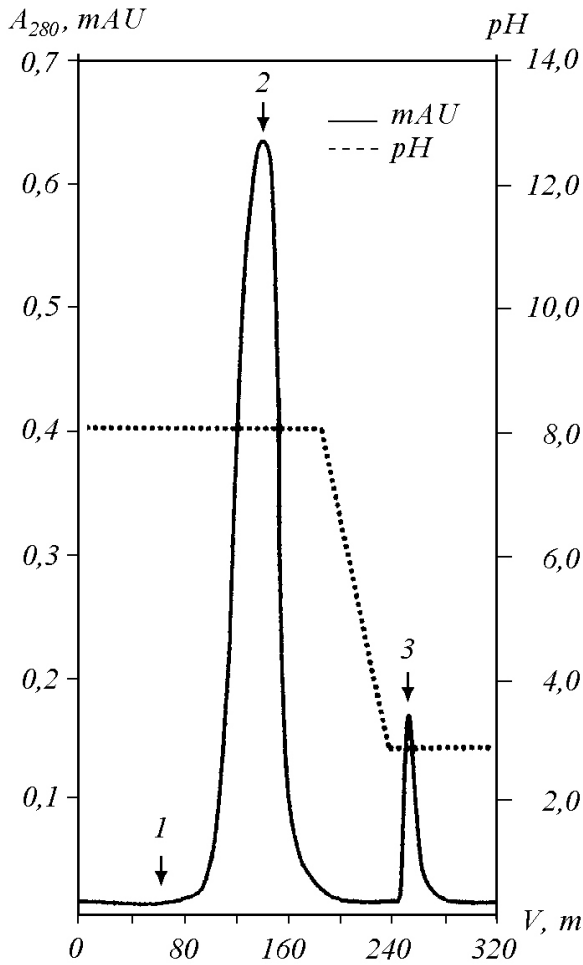


Fig.4. Purification of recombinant human IFN- 2b with immobilized on cellulose single chain antibodies: a – elution profile (1 – loading of clarified lysate from *E.coli* cells with IFN- 2b; 2 – proteins that did not bind to the column and were removed by washing with PBS; 3 – elution of bound IFN- 2b at pH 3,0); b – SDS-PAGE analysis (1 – proteins in clarified lysate from *E.coli* cells with IFN- 2b; 2-4 – proteins in eluted fractions; M - molecular weight marker; the gel was stained by Coomassie brilliant blue R-250.

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Иммобилизованные одноцепочечные антитела для аффинной очистки рекомбинантного IFN- 2b человека

Резюме

Представлен лабораторный метод получения иммуоаффинного сорбента для хроматографической очистки белков, основанный на ориентированной и нековалентной иммобилизации рекомбинантных фрагментов антител на целлюлозном носителе. На основе ДНК-последовательностей однонитчатых антител (ScFv) к IFN- 2b человека и связывающего целлюлозу домена (CBD) из целлюлозолитического комплекса *Clostridium thermocellum* конструирован слитый белок (ScFv-CBD). После экспрессии

ScFv-CBD в клетках *Escherichia coli* и выделения целевого белка в функционально активной форме проведена его прямая иммобилизация на микрогранулированной целлюлозе. Целлюлоза с иммобилизованным ScFv-CBD использована как аффинный сорбент для очистки рекомбинантного IFN- 2b человека.

Ключевые слова: одноцепочечные антитела, иммуоаффинная хроматография, иммобилизация белка, слитый белок.

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