УДК 579.69 + 577.112

IMMOBILIZATION OF MOUSE SINGLE-CHAIN ANTIBODIES FOR AFFINITY CHROMATOGRAPHY USING THE CELLULOSE-BINDING PROTEIN

P. V. GILCHUK[1], G. L. VOLKOV[2]

[1]Taras Shevchenko Kyiv National University, Ukraine;
[2]Palladin Institute of Biochemistry, National Academy of Sciences of Ukraine, Kyiv;
e-mail: Gilchuk@ukr.net

A laboratory method for obtaining immunoaffinity medium for chromatographic purification of recombinant human interferon α2b (IFN-α2b) is described. The method is based on oriented and non-covalent immobilization of recombinant antibody fragments on cellulose. The single-chain fragment variable (ScFv) against human IFN-α2b 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 its bioaffinity immobilization on several forms of cellulose powders has been carried out. The crystalline microgranular cellulose with immobilized ScFv-CBD-fusion protein was used as affinity medium to perform the purification of recombinant human IFN-α2b directly from clarified extract of E. coli cells.

Key words: single-chain antibodies, immunoaffinity chromatography, protein immobilization, fusion protein.

Recombinant antibody fragments construction and their expression in heterologous systems is the modern approach to obtaining highly selective immunological reagents for fundamental researches, diagnostics and therapy [1, 2]. Single-chain fragment variable (ScFv) is the recombinant molecule, 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 due to the capability of selecting the molecules with the desired binding characteristics and the possibility of their large-scale production [4].

One of the attractive fields of ScFv application is the development of inexpensive matrices for immunoaffinity chromatography (IAC). The general applicability of single-chain antibodies for purification of target proteins has recently been shown [5, 6], and different strategies for their immobilization on chromatographic matrices, including chemical coupling or indirect binding via some engineered partners, have also been reported [7, 8]. The most common disadvantage of chemical immobilization methods is the random orientation and low yield of functional immobilized molecules. Here we present a versatile and inexpensive laboratory method for obtaining of immunoaffinity column from single-chain antibodies overexpressed in *Escherichia coli.*

Materials and Methods

ScFv against human IFN-α2b were selected out of the immune mouse cDNA library by the phage display method as was described before [9]. The gene encoding anti-IFN-α2b 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) [10, 11]. For high-level production the plasmid encoding ScFv-CBD was transformed in the *E. coli* K12 strain BL21 (DE3) and the fusion protein was overexpressed as inclusion bodies. After the isolation of the inclusion bodies the ScFv-CBD was solubilized and refolded by controlled dilution according to previously described method [11]. The refolding efficacy was calculated by the percentage of recovered biological activity separately for each moiety of fusion protein using the approach developed before [11].

The following refolding for determining the binding characteristics of ScFv-CBD to different forms of cellulose the aliquots of ScFv-CBD (100 μg) were adsorbed with 20 μl of chromatographic media, such as CF1 (long fibrous cellulose), CF11 (short fibrous cellulose) and CC31 (crystalline microgranular cellulose) which were available from «Whatman» (Great Britain). After 1 h the cellulose was recovered by centrifugation, washed twice with distilled water and loaded in 15% SDS-polyacrylamide gel (SDS-PAGE) for analysis.

For purification experiments refolded ScFv-CBD (3–6 mg) was immobilized on CC31 beads to reach the capacity of 1.5 mg of ScFv-CBD per 1 ml of settled cellulose. The prepared affinity cellulose was washed several times with TBS buffer (20 mM Tris/HCl, pH 7.5; 1 mM EDTA) containing 500 mM NaCl and packed onto empty NAP 10 column («Amersham Biosciences»). The purification procedure was carried out under gravity flow as follows. Clarified extract of *E. coli* cells, containing recombinant human IFN-α2b («ФармБіотек» «Farm-Biotek», Ukraine), was loaded on the column. After washing the column several times with TBS/500 mM NaCl the bound IFN-α2b was eluted at pH 3.0 (100 mM glycine/HCl, pH 3.0; 500 mM NaCl). The purity of eluted IFN-α2b was analyzed by electrophoresis in 15% SDS-PAGE.

Results and Discussions

Recently we produced several genetic fusions of single-chain antibodies to cellulose binding domain from *Clostridium thermocellum* [11]. All fusion proteins contain two affinity moieties, represented by N-terminus of ScFv and C-terminus of CBD, which are joined via flexible amino acid spacer (Fig. 1). The conducted design of fusion protein molecule provides stable complex formation between cellulose-binding moiety and carbohydrate scaffold of cellulose, and exposes the antigen-binding site of the ScFv to the optimal position for the interaction with antigen. The usage of effective refolding method allowed the recovery of soluble and active ScFv-CBD from *E. coli* inclusion bodies which proved to be enough to obtain the affinity column and to purify the recombinant human IFN-α2b.

We have determined the capacity of different cellulose media (crystalline and fibrous forms) for ScFv-CBD fusion protein (~ 46 kDa). Batch binding experiments suggested that the capacity of these media for ScFv-CBD is quite different

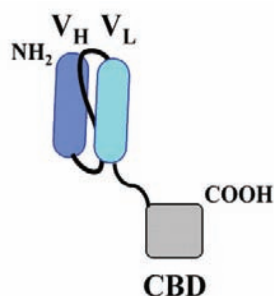

Fig. 1. Schematic representation of fusion protein, containing affinity moieties for antigen binding (ScFv) and immobilization on cellulose (CBD).

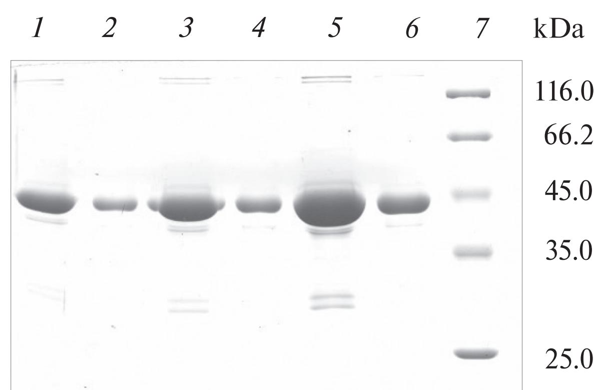

Fig. 2. SDS-PAGE analysis demonstrates the capacity of different cellulose media for ScFv-CBD fusion protein: Line 1,2 – long fibrous cellulose CF1; Line 3,4 – short fibrous cellulose CF11; Line 5,6 – crystalline microgranular cellulose CC31; Line 7 – molecular weight marker. 5 and 1 μl each of affinity cellulose was loaded in corresponding line. The gel was run under reducing conditions and stained with Coomassie brilliant blue R-250.

(Fig. 2), and in the range of 0.4 to 1.7 mg of fusion protein per 1 ml of settled CF1- and CC31-cellulose correspondingly. For this reason for preparation of the affinity column and performing the pu-

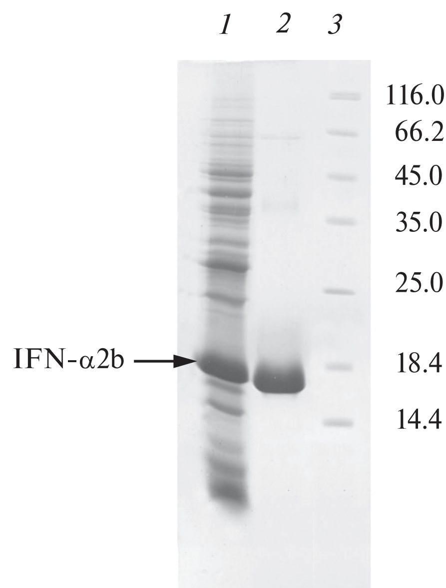

Fig. 3. SDS-PAGE analysis of purification of recombinant human IFN-α2b with immobilized on cellulose ScFv-CBD: Line 1 – clarified extract from E. coli cells containing IFN-α2b; Line 2 – IFN-α2b recovered from column by elution at pH 3.0; Line 3 – molecular weight marker. The gel was run under reducing conditions and stained with Coomassie brilliant blue R-250.

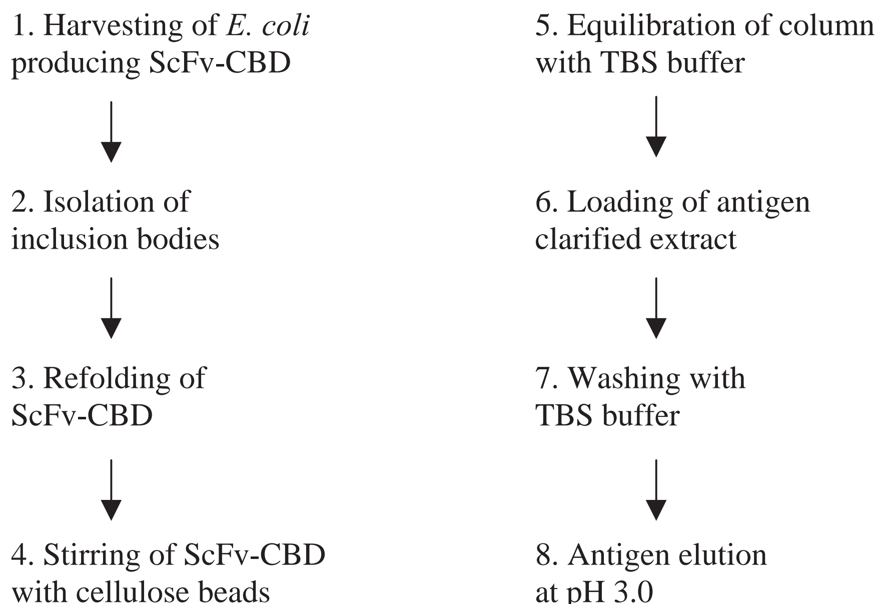

Fig. 4. Affinity chromatography principle using ScFv-CBD fusion protein on a cellulose column.

rification procedures the refolded ScFv-CBD was immobilized only on CC31 cellulose.

Clarified extract of *E. coli* cells, which contained 15–20% expressed recombinant human IFN-α2b (~18 kDa), was loaded on the affinity column. The elution of bound IFN-α2b was carried out under conditions (pH 3.0), which provided selective dissociation of IFN-α2b without significant leakage of immobilized ScFv-CBD [11]. According to the SDS-PAGE data the purity of IFN-α2b in eluted fractions amounts more than 95% (Fig. 3), that demonstrates the applicability of our method for one-step purification of IFN-α2b directly from clarified extracts of bacterial producers. The column capacity for IFN-α2b was calculated according to the measurement data of purified IFN-α2b concentration, it amounted to about 0,1 mg of IFN-α2b per 1ml of settled affinity medium. Previously we have demonstrated that reequilibrated column could be reloaded with extracts containing IFN-α2b and reused for several repeated purifications [12]. The steps illustrated in Fig. 4 were performed in various scales on gravity flow columns (1–4 ml of affinity media) and automated chromatography system (40 ml of affinity media) [12].

In this work we describe a versatile method for the directed and oriented immobilization of single-chain antibodies expressed in *E. coli* and for preparation of affinity column for one-step purification of recombinant IFN-α2b with high degree of purity. Conventional IAC methods requires for immobilization expensive chemically activated matrices. The CBD-cellulose affinity system is attractive because it does not require any derivatized matrix, and cellulose is available in a variety of inexpensive forms. The described approach can be useful for the preparation of immunosorbents from ScFv for rapid purification of target proteins on the milligram scales.

The authors express sincere gratitude to Professor Y. Shoham (Haifa, Israel) for the plasmid with CBD sent us for investigations as a gift.

ІММОБІЛІЗАЦІЯ ОДНОЛАНЦЮГОВИХ АНТИТІЛ МИШІ ДЛЯ АФІННОЇ ХРОМАТОГРАФІЇ ЗА ВИКОРИСТАННЯ ЦЕЛЮЛОЗО-ЗВ'ЯЗУВАЛЬНОГО БІЛКА

П. В. Гільчук[1], Г. Л. Волков[2]

[1]Київський національний університет імені Тараса Шевченка, Україна;
[2]Інститут біохімії ім. О.В. Палладіна НАН України, Київ;
e-mail: Gilchuk@ukr.net

Запропоновано лабораторний метод одержання афінного імуносорбенту для хроматографічного очищення рекомбінантного інтерферону IFN-α2b людини (IFN-α2b), який полягає в орієнтованій і нековалентній іммобілізації рекомбінантних фрагментів антитіл на целюлозному носії. На основі ДНК-послідовностей одноланцюгових антитіл (ScFv) проти IFN-α2b людини і целюлозозв'язувального домену (CBD) із целюлозолітичного комплексу *Clostridium thermocellum* сконструйовано гіб-

ридний білок ScFv-CBD. Після експресії ScFv-CBD в *Escherichia coli* і виділення цільового білка у функціонально активній формі здійснено його біоафінну іммобілізацію на різних типах целюлозних носіїв. Кристалічну мікрогранулярну целюлозу з іммобілізованим ScFv-CBD було використано як афінний імуносорбент для очищення рекомбінантного IFN-α2b людини безпосередньо з освітленого лізату клітин *E. coli*.

К л ю ч о в і с л о в а: одноланцюгові антитіла, імуноафінна хроматографія, іммобілізація білка, гібридний білок.

ИММОБИЛИЗАЦИЯ ОДНОЦЕПОЧЕЧНЫХ АНТИТЕЛ МЫШИ ДЛЯ АФФИННОЙ ХРОМАТОГРАФИИ ПРИ ИСПОЛЬЗОВАНИИ ЦЕЛЛЮЛОЗО-СВЯЗЫВАЮЩЕГО БЕЛКА

П. В. Гільчук[1], *Г. Л. Волков*[2]

[1]Киевский национальный университет имени Тараса Шевченко, Украина;
[2]Институт биохимии им. А. В. Палладина НАН Украины, Киев;
e-mail: Gilchuk@ukr.net

Описан предложенный авторами лабораторный метод получения аффинного иммуносорбента для хроматографической очистки рекомбинантного интерферона α2b человека (IFN-α2b), который основан на ориентированной и нековалентной иммобилизации рекомбинантных фрагментов антител на целлюлозном носителе. На основе ДНК последовательностей одноцепочечных антител (ScFv) против IFN-α2b человека и целлюлозосвязывающего домена (CBD) из целлюлозолитического комплекса *Clostridium thermocellum* сконструирован гибридный белок ScFv-CBD. После экспрессии ScFv-CBD в *Escherichia coli* и выделения целевого белка в функционально активной форме была проведена его биоаффинная иммобилизация на разных типах целлюлозных носителей. Кристаллическая микрогранулированная целлюлоза с иммобилизированным ScFv-CBD использована как аффинный иммуносорбент для очистки рекомбинантного IFN-α2b человека непосредственно из осветленного лизата клеток *E. coli*.

К л ю ч е в ы е с л о в а: одноцепочечные антитела, иммуноаффинная хроматография, иммобилизация белка, гибридный белок.

1. *Moroney S., Pluckhun A.* // Modern Biopharmaceuticals. — Weinheim: Wiley-VCH, 2005. — **4**. — P. 1147—1186.
2. Holliger P., Hudson P. J. // Nature biotechnol. — 2005. — **23**. — P. 1126—1136.
3. Huston J.S., Levinson D., Mudgett-Hunter M. et al. // Proc. Nat. Acad. Sci. USA. — 1988. — **85**. — P. 5879—5883.
4. *Pluckthun A., Krebber A., Krebber C. et al.* // Antibody Engineering / Eds J. McCafferty, H.R. Hoogenboom. — Oxford: IRL Press, 1996. — P. 203—252.
5. *Berry M. J., Davies J., Smith C. G. et al.* // J. Chromatogr. — 1991. — **587**. — P. 161—169.
6. *Schild A., Kleist D., Welschof M. et al.* // Cancer Res. — 2000. — **60**. — P. 4175 — 4178.
7. *Berdichevsky Y., Lamed R., Frenkel D. et al.* // Protein Express. Purif. — 1999. — **17**. — P. 249—259.
8. *Blank K., Lidner P., Diafenbach B. et al.* // Protein Express. Purif. — 2002. — **24**. — P. 313—322.
9. *Окунев О. В., Гильчук П. В., Иродов Д. М. и др.* // Укр. біохім. журн. — 2005. — **77**, № 5. — P. 106—115.
10. *Morag E., Lapidot A., Govorko D. et al.* // Appl. Envir. Microbiol. — 1995. — **61**, N 5. — P. 1980—1986.
11. *Гільчук П. В., Окунєв О. В., Павлова М. В. та ін.* // Укр. біохім. журн. — 2006. — **78**, № 2. — С. 18—27.
12. *Гільчук П. В., Окунєв О. В., Іродов Д. М. та ін.* // Біополімери і клітина. — 2006. — **22**, № 2. — С. 157—161.

Received 21.08.2006