Development of the chromatographic medium for the affinity isolation of the recombinant hIFN-β1b based on immobilized single-chain antibodies

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Aim. The development of a laboratory method for the production of immunoaffinity chromatography medium for the purification of the recombinant human IFN-β1b. Methods. A gene of the chimeric protein ScFvβinf-CBD was constructed using the DNA sequences encoding ScFv specific to hIFN-β1b and the cellulose-binding domain (CBD) of Clostridium thermocellum. The developed chimeric protein was expressed in Escherichia coli cells. The target protein was obtained in soluble, functionally active form by its renaturation from the bacterial inclusion bodies in vitro. The ScFvβinf-CBD was immobilized on a chitin carrier. Results. The introduction of CBD by gene engineering techniques enabled the oriented non-covalent immobilization of ScFvβinf-CBD on chromatographic matrix. The developed immunoaffinity medium allowed isolating the rhIFN-β1b from the complex mixture, after its renaturation from the inclusion bodies, with more than 89 % purity. Conclusion. The designed immunoaffinity medium provides isolating the rhIFN-β1b from the complex protein mixtures.

Keywords: Interferon-β1b, single-chain antibodies, immunoaffinity purification, cellulose-binding domain.

Introduction

The purification of a recombinant protein by immunoaffinity chromatography is based on the interaction of an antibody, which is covalently immobilized on the matrix, with its antigen that separates the latter from the multicomponent mixture. The main advantage of this method is a potentially high selectivity allowing the isolation of trace quantities of a target product from the mixture. However, the generation of antibodies in bulk quantities, for the development of such media, is a difficult and costly process that requires the maintenance of either the significant livestock of immunized animals or the hybridoma cell line, which produces the antibodies specific to the target antigen, and their further expression and purification. Special efforts also should be taken to the elaboration of the isolated antibody immobilization on the media, because the existing methods of covalent immobilization (by crosslinking reagents such as glutaraldehyde and etc.) are unable to provide the strictly oriented linkage of the antibody to the matrix, and hence a significant quantity of antibodies loses their antigen binding properties owing to the blocking or modification of the antigen binding site of antibody, and etc. due to the immobilization procedure. All facts mentioned above influence both the technical characteristics of media, and their
cost. A technology of the recombinant single-chain antibodies (ScFv), developed in the last decade, enables to simplify significantly the process and reduce the cost of obtaining antibodies. In addition, it is possible to introduce the DNA sequence for a protein, which will provide oriented immobilization of ScFv on a matrix, into the encoding sequence of the antibody by the genetic engineering techniques [1, 2].

The recombinant human interferon-β1b (rhIFN-β1b) is the first medicine approved for the treatment of multiple sclerosis (MS). MS is an autoimmune disease, which is characterized by the neurons demyelination in the central nervous system that leads to the development of a wide spectrum of severe neurological disorders [3]. Today, according to the data presented by Multiple Sclerosis International Federation [http://www.msif.org], 2.3 million people worldwide have MS and the number of patients is continuously growing. The disease mainly affects young people: from 25 to 40 years old. The therapeutic effect of rhIFN-β1b on the disease is attributed to its anti-inflammatory properties, to the influence on the endothelial cells, and on the permeability of the blood brain barrier [4].

The present study is focused on the development of the laboratory method for the production of immunoaffinity chromatography medium for the purification of the rhIFN-β1b. The method is based on the oriented non-covalent immobilization of the chimeric protein, which consists of ScFv against rhIFN-β1b (ScFvbINF) and Clostridium thermocellum cellulose-binding domain (CBD), on a chitin carrier.

**Materials and Methods**

ScFv against the rhIFN-β1b, obtained earlier at our department from the plasmid vector pCANTAB-5E-ScFv-β1INF, was subcloned into the plasmid vector pET24-CBD-(Gly-spacer) via restriction sites SfiI and NotI. As a spacer for the spatial separation of two affinity centers of the chimeric protein (CBD and ScFv) a sequence of 13 amino acids was introduced [5, 6]. Professor Y. Shoham (Israel) kindly provided the plasmid vector pCBD with the cellulose binding domain (CBD) sequence from Clostridium thermocellum for our research. The resulting plasmid pET-24-ScFv-CBD was used for the transformation of E. coli cells, BL21(DE3) strain.

For the expression of the target protein, an auto-induction method described by Studier [7] was applied. The localization and the content of the target protein in the total lysate of E. coli cells were determined by electrophoretic separation of the soluble and insoluble fractions of the cell cytoplasmic proteins [8]. E.coli cells were disrupted with lysozyme as described below [9].

The isolated inclusion bodies were solubilized in 20 mM Tris-HCl (pH 8.0), 7 M guanidine-HCl, 15 mM 2-mercaptoethanol for 1 h at room temperature and filtered through a 0.45 μm PVDF membrane filter (Millipore). ScFvbINF-CBD was purified under denaturing conditions using the Ni²⁺-immobilized affinity chromatography on Ni-NTA agarose (Qiagen).

Refolding of the purified ScFvbINF-CBD was performed by stepwise dilution [6]. After renaturation ScFvbINF-CBD was immobilized on chitin beads (New England Biolabs, UK). The resulting immunoaffinity medium was washed with PBS pH 7.2, containing 0.1 % Tween-20 and 0.14 M NaCl. Afterwards, the rhIFN-1β renatured by stepwise dilution was applied to the column [5]. After washing of the immunoaffinity medium with buffer PBS pH 7.2, containing 0.1 % Tween-20, 0.14 M NaCl to remove unbound proteins, the rhIFN-β1b was eluted from the column by decreasing pH of elution buffer: (0.1 M glycine and 0.5 M NaCl, pH 3.0). The rhIFN-β1b concentration in the fraction eluted was quantified by measuring absorbance of the solutions at 280 nm. The rhIFN-β1b purity in the samples studied was determined by densitometry of polyacrylamide gel electrophoregrams. For this purpose the SDS-PAGE gels were stained with Coomassie Brilliant Blue according to the manufacturers instructions, documented by ChemiDoc™ XRS+ System («Bio-Rad», USA), and subsequently analyzed with «Image Lab SoftwareTM» («Bio-Rad»).

**Results and Discussion**

1. Construction of chimeric protein and expression in E. coli BL21 (DE3)

The CBD from the S1 subunit of the cellulolytic
Development of medium for isolation of rhIFN-β1b based on immobilized ScFv complex of Clostridium thermocellum has been selected as a partner protein for the fusion with the previously developed ScFv against the rhIFN-β1b [10]. It is worth mentioning, that the main features of the domain are its ability to create a stable complex with the hydrocarbon backbone of cellulose or chitin under both native and denaturing conditions, and a high efficiency of the restoration of the molecule functional structure after the renaturation from inclusion bodies [11, 12]. After the subcloning, the E. coli cells of the BL21(DE3) strain were transformed by the developed pET24-ScFvbinf-CBD. It is known that the components of the nutrient medium, the nature of inducer substance, and the cultivation conditions (temperature, aeration intensity, etc.), greatly influence the level of expression of the recombinant proteins in E. coli cells [13]. To enhance the yield of the chimeric protein ScFvbinf-CBD we have tested the influence of the complex nutrient medium for producing strain BL21 (DE3)/pET 24–ScFvbinf-CBD on the yield of the chimeric protein. Thus, the developed producing strain was cultivated on two different media, namely LB and 2xYT. The expression of ScFvbinf-CBD was induced by the addition of α-lactose. It was shown that the cultivation on the complex nutrient medium LB results in the synthesis of the target chimeric protein with the expected molecular weight of 45 kDa, while the yield of the chimeric protein after cultivation in 2xYT was scarce and hardly detectable. The accumulation of the target protein was ~0.1 g out of 1 L of the bacterial cells suspension in case of the LB medium. The study on the distribution of the ScFvbinf-CBD in E. coli cells demonstrated that it was accumulated in the inclusion bodies, which were subsequently isolated after the lysis of cells (Fig. 1.)

2. Purification and renaturation

The isolated inclusion bodies were solubilized in the buffer containing 7 M guanidine hydrochloride and 0.015 M 2-mercaptoethanol. After solubilization the ScFvbinf-CBD was purified by the metal ion affinity chromatography using the Ni-NTA agarose (Qiagen). The purity of the obtained target protein was 95 % as determined by the densitometry of polyacrylamide gel electrophoregrams. The ScFvbinf-CBD was renatured by step-wise dilution. The decrease of the concentration of guanidine hydrochloride was carried out by step-wise dilution of the protein solution with the renaturation buffer 20 mM Tris-HCl pH 8.0, 100 mM NaCl, 1 mM EDTA in order to provide the following concentration levels of the chaotropic agent: 6 M → 3 M → 2 M → 1 M → 0.5 M. At the concentrations of 2 M → 1 M → 0.5 M the 0.4 M L-arginine and the redox pair (GSH and GSSG) were added. The GSH/GSSG pair catalyzes the disulfide interchanges and thus ensures the correct folding of the chimeric protein and the proper spatial arrangement of both affinity sites: CBD and the active site of ScFv. The renaturation efficiency of the affinity sites was evaluated in accordance with their functions: the ability of CBD to interact with the cellulose carrier and the ScFv ability to bind rhIFN-β1b.

3. Immobilization of ScFvbinf-CBD on chitin beads

It has been demonstrated that the affinity interaction of CBD with the cellulose or chitin leads to the formation of the complex stabilized by the hydrophobic interaction of certain amino acid residues and hydrocarbon...
The chitin beads have been selected as the solid phase for immobilization of the chimeric protein. The protein solution obtained after the renaturation procedure, was added to the slurry of chitin beads, equilibrated with buffer 0.1 M Tris-HCl (pH 8.0) containing 0.1 M NaCl, and incubated with continuous agitation at 22°C for 2 hours. Thereafter the medium was packed in the column and washed by 5 volumes of PBS pH 7.2, in order to wash away the unbound proteins. The proteins attached to the chitin carrier were eluted under denaturing conditions and separated by SDS-PAGE. The analysis of electrophoregrams revealed the presence of chimeric protein in eluate. The binding capacity of the chitin beads for the target protein was ~0.6–1 mg of the ScFvβINF-CBD/mL of the medium as determined by the densitometry of polyacrylamide gel electrophoregrams.

4. Purification of rhIFN-β1b on developed medium

The solution of the rhIFN-β1b (obtained after its renaturation) containing a significant amount of E.coli proteins was applied of the column with developed affinity medium. The rhIFN-β1b was eluted by the pH change: from pH 7.2 to pH 3.0. The analysis of electrophoregrams obtained after the separation of eluted proteins by SDS-PAGE (Fig. 2) revealed that the purity of the rhIFN-β1b, eluted under conditions studied, was 89 %, and the additional minor band was also detected. Desorption of the ScFvβINF-CBD from the medium was not observed under the conditions studied. The data obtained by western-blot analysis (Fig. 2.b) revealed that the additional band with the molecular weight ~ 36 kDa represented the dimer of rhIFN-β1b, originated from its oligomerization during renaturation. It should be noted that the affinity media based on the use of antibodies do not allow separating the monomer from the oligomer forms of the same protein in case of the preservation of the epitope to which the antibody is specific. The final polishing of the eluate containing the rhIFN-β1b from its oligomer forms can be carried out by...
the fractionation of the protein mixture by a size-exclusion chromatography on Superdex 75 10/300 GL column.

Concluding Remarks

The present study is focused on the development of the laboratory method for obtaining the immunoaffinity medium for the chromatographic purification of the rhIFN-β1b. The proposed method is based on the oriented non-covalent immobilization of the chimeric protein, consisting of the ScFv specific to the rhIFN-β1b and the CBD from Clostridium thermocellum, on the chitin carrier. The developed immunoaffinity medium allows isolating the rhIFN-β1b from the complex protein mixture after its renaturation from the inclusion bodies, and purification to 89%. It is noteworthy that the developed media does not separate the oligomeric forms of rhIFN-β1b, appeared during the renaturation.

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Створення хроматографічного сорбенту на основі іммобілізованих одноланцюгових антитіл для афінітного виділення рекомбінантного IFN-β1b людини

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Мета. Розробити лабораторний метод створення імуноафінного хроматографічного сорбенту для виділення та очищення рекомбінантного IFN-β1b людини. Методи. Використовуючи послідовності ДНК, які кодують ScFv специфічні до hIFN-β1b, та цепеполоз́ “зважаний” домен (CBD) Clostridium thermocellum, створено ген химерного протеїну ScFvScFvCBD. Проведено експресію створеного химерного протеїну в клітинах Escherichia coli. Цільовий білок одержували у розчині, функціонально активні форми шляхом ренатурації з бактеріальних тілець включення в vitro. ScFvScFvCBD іммобілювали на хітіновому носії. Результати. Введення CBD до складу химерного білка забезпечує орієнтовану, не ковалентну іммобілізацію ScFvScFvCBD на хроматографічній матриці. За допомогою створеного імуноафінного
сорбенту было выделено rhIFN-β1b из складной суммации белков, одержанной после его ренатурации с телец включения, с чистотой более 89 %. Выводы. Створенный иммуноаффинный хроматографический сорбент позволяет выделять рекомбинантный IFN-β1b человека из сложных белковых смесяй.

**Ключевые слова:** интерферон-β1b, одноцепочечные антигены, иммуноаффинная очистка, целлюлозосвязывающий домен.

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