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Refolding of scFv mini-antibodies using size-exclusion chromatography *via* arginine solution layer

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ABSTRACT

The method for refolding of mini-antibodies using size-exclusion chromatography *via* arginine solution layer was developed. This method allows to refold scFv, to separate both aggregated protein and low molecular weight compounds and to isolate functionally active protein preparation in monomeric form. The comparison of various scFv preparations isolated either from inclusion bodies or from soluble fraction revealed that refolded mini-antibodies demonstrate higher antigen-binding activity. Mini-antibodies refolded in the presence of arginine also demonstrate higher electrophoretic mobility during native PAGE in comparison with soluble cytoplasmic antibodies. Both soluble as well as refolded antibodies had similar CD spectra. Refolded mini-antibodies are storage-stable.

1. Introduction

The use of various bacterial systems to express recombinant protein genes is one of the most impressive developments of modern biotechnology [1]. *Escherichia coli* cells are the most commonly used bacterial system for the large-scale production of proteins useful in scientific investigations as well as in industry and in medicine [2,3]. One of the main problems of recombinant protein production is that alien proteins expressed in *E. coli* are often found within so-called inclusion bodies in hardly soluble form. There are a wide variety of methods to refold proteins from inclusion bodies and the methods utilizing the refolding inside chromatographic carriers are of particular interest among them [4–8].

The removal of denaturant and protein structure transformation into the native state takes place during chromatography process. In this case, protein allocation inside hydrophilic carrier limits the interaction between inverted hydrophobic regions of folding protein molecules and thereby limits the ability of their non-specific association [9,10]. However, even under these conditions correct complete protein folding does not take place always, and sometimes an aggregation is observed. To avoid an aggregation, low concentrations of denaturants such as 0.6 M guanidine hydrochloride [11,12] or 1–2.5 M urea [13,14] are maintained in renaturation medium. At the same time it is widely known that arginine, when added at intermediate and/or final steps of protein renaturation, facilitates its folding and stabilization of its native conformations. In some cases the addition of arginine solution during renaturation process increases the yield of functionally active protein [15–19], hypothetically due to the inhibition of its aggregation [20]. However, there are many examples, when it is necessary to obtain the recombinant protein free from any extraneous impurities.

Hereinafter, the novel method for refolding of scFv, which are synthesized in *E. coli* cells as inclusion bodies, is worked out. The method is based on size-exclusion chromatography, which is the procedure for gradual exchange of denaturant solution with arginine solution and then with physiological saline.

scFv are the group of biologically active protein molecules, generated using gene engineering techniques based on natural immunoglobulins [21]. Among the large diversity of mini-antibody forms [22], the single-chain fragments of scFv, generated by the expression of the corresponding genes, encoding variable domains of heavy (V_H) and light (V_L) immunoglobulin chains, linked together *via* oligonucleotide bridge, encoding flexible hydrophilic peptide $(Gly_4Ser)_3$, are widely used in modern biotechnology. The use of antigen-binding part of immunoglobulin molecule instead of whole molecule allows reducing molecular size, which leads to the facilitation of mini-antibody permeation into tissues and at the same time to the drop in their immunogenicity [23]. The unique features

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of mini-antibodies make them an important tool both in therapy and in diagnostics of antigens of different nature [24].

The pesticin – monomeric cytoplasmic protein from *Yersinia pestis* – was used as an antigen against which the mini-antibodies were produced. This bacteriocin is revealed in the vast majority of natural isolates of *Y. pestis* [25,26], that makes it an attractive tool for the indication and identification of plague agent. Previously, the pesticin production by *Y. pestis* cells was determined using microbiology techniques during several days. Therefore, the production of recombinant single-chain antibodies against pesticin for short-time immunochemical diagnostics is of actual interest at this time.

2. Materials and methods

2.1. Materials

Urea, tris(hydroxymethyl)aminomethane (Tris), hen egg white lysozyme, monobasic sodium phosphate (NaH_2PO_4), dibasic sodium phosphate (Na_2HPO_4), ethylenediaminetetraacetic acid (EDTA), imidazole, Tween-20, Coomassie G-250, were purchased from (AppliChem, Germany). Sephacryl S200 Super Fine size-exclusion resin was obtained from Amersham Biosciences (UK). NiNTA–Agarose were purchased from Quagen (USA). DNAse I, T4DNA ligase and kanamicine from Fermentas (Lithuania). NcoI and NotI from New England Biolabs (USA). All other chemicals were of analytical reagent grade (Sigma, USA). Recombinant Pesticin was gifted by Dr. A. Anisimov, SCRAM, Obolensk, Russian Federation.

2.2. Cloning of scFv genes

Genes, encoding scFv against pesticin, were isolated by affinity selection on antigen technique using previously created antibody phage-display library [27,28].

Anti-pesticin scFv genes were subcloned into the pET 28 (myc) expression vector, designed on the basis of pET 28(+). ScFv coding sequence was cloned under the control of T7 promoter at the NcoI and NotI restriction sites. Besides that, scFv sequence comprised myc tag at the C-terminus.

2.3. Expression and isolation of scFv from inclusion bodies

E. coli BL21DE3Rosetta cells, carrying single-chain antibody genes as a part of pET 28 (myc) expression vector, were cultured at 37 °C in 2YT medium supplemented with 0.1% glucose and 50 μg/ml kanamycin. When the optical density of the culture became 0.6–0.8, the induction of recombinant protein expression by adding of 1 mM IPTG was carried out during 5 h at 37 °C, 200 rpm. The cells were then collected by centrifugation during 15 min at 4000 × *g* and stored at −20 °C. Cell pellets were thawed at room temperature and soaked in lysis buffer (LB) containing phosphate-buffered saline (PBS 2.68 mM KCl, 1.47 mM KH_2PO_4, 137 mM NaCl, 4.29 mM Na_2HPO_4, pH 7.4) and 0.5 mg/ml lysozyme, 1 mM EDTA, 1 mM PMSF, 1 μg/ml leupeptin. After cell lysis, 20 μg/ml DNAseI and 10 mM $MgCl_2$ were added. Cell lysate was incubated 15 min, and then centrifuged at 12,000 × *g* during 30 min. The pellet, containing inclusion bodies, was dissolved during 2 h in denaturizing buffer (DB): 8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris–HCl, pH 8. The sample was centrifuged at 12,000 × *g* 30 min, supernatant was collected and applied onto NiNTA–Agarose column, USA) (V = 1 ml), equilibrated with DB. The column was washed with five volumes of buffer WB: 8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris–HCl, 20 mM imidazole. Elution was carried out by 10 ml of elution buffer (EB): 8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris–HCl, 200 mM Imidazole and pH 7.5.

2.4. Expression and isolation of soluble scFv

To synthesize and to isolate soluble mini-antibodies protein expression was carried out during 5 h at 29 °C, 200 rpm. After cell disruption in LB buffer (for buffer composition see above) and centrifugation soluble protein was purified on NiNTA–Agarose column, equilibrated with PBS, containing 300 mM NaCl, pH 8. The column was washed with five volumes of PBS, containing 300 mM NaCl and 10 mM imidazole. Elution was carried out by 10 ml of PBS, containing 200 mM imidazole.

2.5. Refolding of denaturated scFv

2.5.1. Refolding of scFv by serial dilution

Inclusion bodies, dissolved in 8 M urea according to previously described technique, were applied onto the NiNTA–Agarose column (V = 1 ml), equilibrated with DB. Washing out non-specifically absorbed protein was combined with gradual renaturation of scFv. For this purpose the carrier with bound protein was gradually washed with WB buffer, containing stepwise decreasing concentrations of denaturant: 8 M → 4 M → 2 M → 1 M → 0.5 M urea. ScFv were eluted with PBS, containing 0.5 M urea and 0.2 imidazole. To change the buffer protein preparation was dialyzed against 100 volumes of PBS at 4 °C during 24 h.

2.5.2. Refolding of mini-antibodies using size-exclusion chromatography

An aliquot of 0.4 M arginine solution was applied onto Sephacryl S200 Super Fine column (5% of column volume). Mini-antibodies, previously isolated by metal–chelate chromatography in a buffer, containing 8 M urea, were concentrated by ultrafiltration using YM10 membranes (Millipore-Amicone, USA) and antibody solution was centrifuged at 13,000 × *g* during 5 min. Mini-antibody concentration in solution ranged from 0.5 to 7.5 mg/ml. Supernatant comprising antibodies was applied onto the column with Sephacryl S200 (sample volume ranged from 1.5% to 2.5% of column volume), and chromatographic fractionation together with refolding were carried out simultaneously. Flow rate was 6 ml/h/cm^2. Size-exclusion chromatography combined with refolding was carried out using various columns: the length was either 60 cm or 1000 cm and the diameter was 0.9 cm (Watman, UK), 1.6 cm, 2.6 cm or 5 cm (Pharmacia Biotech, Sweden). The elution profile was registered by UV-absorption at 280 nm with using UV1 monitor (Pharmacia Biotech, Sweden). During the course of analytical experiments, using columns with the diameter of 0.9 cm, fraction size was 2 ml.

2.6. Arginine detection in refolded protein fractions

Arginine content in chromatographic fraction was assayed using thin-layer chromatography on TLC aluminum sheets Kiesel gel 60 F_{254} or Silica gel 60 F_{254} (Merck, Germany), EtOAc:n-BuOH:AcOH:H_2O (2:1:1:1). To visualize the chromatogram 5% ninhydrin in acetone/acetic acid (30:1) was used.

2.7. Amino acid analysis

Protein hydrolysis was carried out using the mixture of hydrochloric acid and propionic acid [29]. Amino acid analysis was performed using LC 3000 analyser (Biotronik, Germany).

2.8. Electrophoresis in polyacrylamide gel

Gel-electrophoresis in the presence of SDS (SDS-PAGE) was carried out in 10% polyacrylamide gel [30], protein bands were stained by Coomassie G-250

Native electrophoresis was carried out in same Laemmli system in 7% polyacrylamide gel. Neither polyacrylamide gel, nor electrode buffer contained SDS or reducing agents.

2.9. Spectroscopic techniques

Protein concentration was determined by far UV absorbance using Carry 100 Bio spectrophotometer (Varian, USA) in combination with a Pierce Protein Assay kit (Pierce, USA), according to the manufacture instruction, as well as by amino acid analysis. Circular dichroism spectra were recorded on a J500 spectropolarimeter (Jasco, Japan) using 0.1 mm quartz cells at protein concentrations from 0.5 to 1 mg/ml.

2.10. Studies of scFv-binding activity and stability

Activities for soluble and refolded antibodies were assayed by ELISA [7] using Medium Sorb immunoplates (Costar, USA). ScFv were detected using anti-myc tag monoclonal antibodies (Invitrogen, USA) and anti-mouse Horseradish peroxidase conjugate (Dako, Denmark). *O*-phenylenediamine as a substrate was added and reaction was stopped by the addition of H_2SO_4. The absorbance at 492 nm was measured by ELISA reader Titertek Multiscan (Flow, UK).

2.11. Aggregation potency of refolded protein

scFv samples in PBS were stored during 1 month at 4 °C, and every 10 days the aliquots were taken to estimate the ratio of aggregated protein. For this purpose, antibodies were centrifuged at 12,000 × *g* during 10 min and protein concentration in supernatant was measured by absorbance at 280 nm (Ultraspec II LKB, Sweden).

3. Results and discussion

Recombinant scFv against pesticin were produced in *E. coli* cells, because they are most suitable system for heterologous protein expression. The advantages of this system are: high level of expression of desired product; process scaling-up feasibility; low cost and high culture growth rate [1–3]. However, there is a clearly defined tendency: the higher is an alien gene expression level in *E. coli* cells, or the higher is protein hydrophobicity, the higher is aggregation potency of synthesized proteins and "inclusion bodies" formation possibility. It is known that inclusion bodies comprise recombinant proteins folded in conformations that differ from native one [12,31,32]. Reactivation of recombinant proteins from "inclusion bodies" is one of the key problems of cell biotechnology [33,34].

Indeed, as it can be seen from Fig. 1, essentially entire portion of synthesized recombinant protein was found in inclusion bodies. Such a kind of expression was typical for most part of examined scFv. Meanwhile, high protein content inside the cell, as well as the existence of 6 His tag in molecular structure made it possible to isolate efficiently the protein from inclusion bodies after dissolving them in a buffer, containing 8 M urea (Fig. 1).

The renaturation of mini-antibodies purified from solutions containing 8 M urea was carried out by two different methods. At initial steps of current investigations the method for scFv refolding at NiNTA–Agarose [4,35] was used. This method (see Section 2) allows performing protein isolation simultaneously with protein refolding. However, thus obtained scFv contained significant amount of oligomeric forms resulting in their accelerated aggregation during storage at 4 °C and therefore in loss of their functional activity. Therefore, an additional step of purification and isolation of monomeric forms using size-exclusion chromatography was necessary.

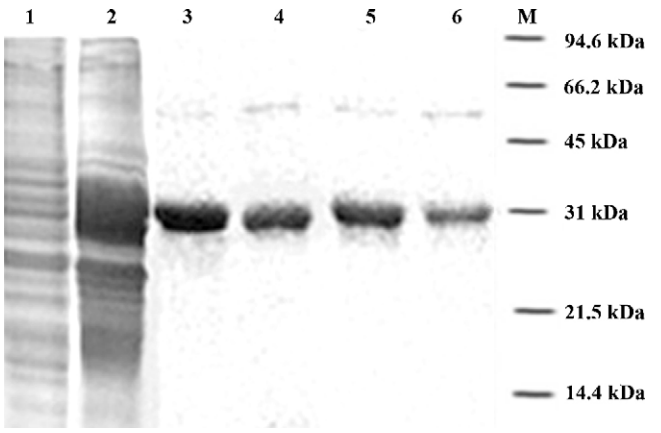

Fig. 1. SDS-PAGE of cell lysate soluble fraction (1), insoluble fraction containing inclusion bodies (2), scFv purified by Ni–NTA affinity chromatography (3–6), and protein molecular weight markers (7). Insoluble fraction of cell lysate was dissolved in buffer: 8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris–HCl, pH 8. Ni–NTA affinity chromatography with reducing concentration of urea (from 8 to 0.5 M) was used to purify and refold scFv from inclusion body.

Another possible variant of refolding is size-exclusion chromatography of the protein dissolved in a denaturant such as 8 M urea or 6 M guanidine hydrochloride. In this case the buffer used to equilibrate the carrier (meaning that it is the buffer for refolded protein to transfer in) contained some amount of denaturants such as 6 M guanidine hydrochloride or 0.2–1.0 M urea [6,10,13]. The use of low molecular additives is of great importance to avoid protein aggregation, to increase the yield of refolded proteins and to produce them in stable soluble form. These additives are of different chemical nature. They could be chemical denaturants at low concentration, such as 0.6–2 M urea or guanidine hydrochloride, detergents or polyethyleneglycol, amino acids and their derivatives [36,37,38], di- and polyamines [39,40], glycerol or sugars [41] and salts [8].

Among large diversity of such additives [42] one of the most efficient is arginine [16,17], and several its derivatives such as arginine amide [43]. It was described that arginine prevents protein aggregation during thermodenaturation [44] and increases the yield of stable proteins during refolding. The role of arginine in protein folding is not clear to date. It was assumed that arginine could alter local environment of aromatic amino acids interacting *via* its guanidine moiety with tryptophan residue and, presumably, with tyrosine residue [45,16]. It was shown in the review paper [36] that most amino acid side chains interact with arginine or guanidine hydrochloride and both of these interactions are similar. The exception is Val. Furthermore, arginine and guanidine hydrochloride preferably interact with aromatic amino acids such as Tyr, Trp and, in a lesser degree, with Phe. Arginine interaction with hydrophobic amino acids, such as Leu, Ile, Val, is rather less preferable, or is not observed at all. However, the interaction of arginine comparing with guanidine hydrochloride is limited by several arginine molecules per one protein molecule, whereas for guanidine hydrochloride greater ratio is typical (100–200 molecules per one protein molecule at 6 M guanidine hydrochloride), and thus arginine inhibits protein aggregation not destabilizing it upon that [36]. It was supposed that arginine plays a role of neutral crowding agent for protein molecule, exerting so-called steric "gap effect". In the presence of arginine in solution, Gibbs free energy of associated protein complexes increases, resulting in dissociation of such complexes [20]. Therefore, it is assumed that arginine inhibits protein–protein association reaction.

Arginine addition in the final steps of protein refolding increases the yield of properly folded protein, and in this case protein incu-

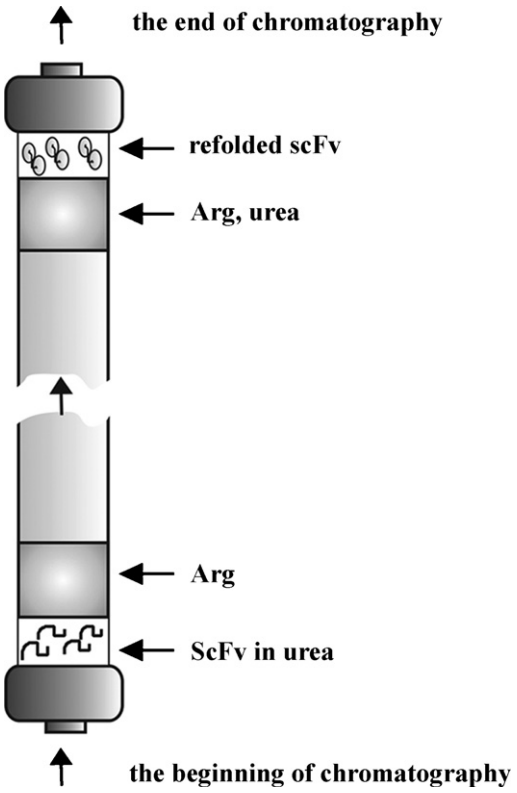

Fig. 2. Schematic representation of scFv refolding using size-exclusion chromatography *via* arginine solution layer. Column 0.9-cm × 97-cm Sephacryl S200 was loaded with PBS and then superloaded with 0.4 M arginine. ScFv were denaturated with 8 M urea and loaded onto column. Protein was eluted with refolding buffer PBS.

bation in arginine solution could be of short-term [31]. To simplify monomeric scFv production and to increase their yield by herein described method for refolding of mini-antibodies using size-exclusion chromatography *via* arginine solution layer several steps were combined in one stage: the step of urea removal from protein solution to initiate protein folding; the step of monomeric protein form separation from oligomeric one; and the step of protein transfer to necessary physicochemical conditions. As a carrier for size-exclusion chromatography the Sephacryl was chosen, which was successfully used for similar protein refolding procedure [10]. In this work Sephacryl S200 Super Fine was used, which is a composite medium prepared by covalently cross-linking of allyl dextran with N,N′-methylene bisacrylamide to form a hydrophilic matrix of high mechanical strength, capable of efficient separation of globular proteins within molecular weight range from 5 to 250 kDa. The molecular weight of mini-antibodies being studied is about 30 kDa that allows using such a carrier to separate monomeric and aggregated forms of folding protein and low molecular solution constituents such as urea and arginine. Schematically this process could be represented as follows (Fig. 2): at the first step of chromatography the removal of denaturant and its exchange for arginine occur simultaneously, and during all this process folding protein molecule remains in hydrophilic surrounding of arginine and carrier. During the next step, the transfer of folded molecule from arginine surrounding to buffer constituents' one takes place. At the same time in the course of protein renaturation inside a carrier, the formation of protein monomers and oligomers is taking place, and they could be separated during chromatography either totally or partially.

To optimize scFv refolding using size-exclusion chromatography *via* arginine solution layer the effects of various factors were examined. The basic requirement for refolding in the presence of arginine is the choice of its proper concentration. In the course of protein refolding by dialysis, arginine solutions with the concentrations ranged from 0.1 to 2 M are used [17,32]. Even at relatively low concentration (2.5 mM), arginine prevents protein aggregation [44]. During this work the effect of arginine concentrations of 0.1, 0.2, 0.4 and 1 M upon refolding was examined and it was found that the use of arginine solution with the concentration about 0.4 M is most effective.

Another parameter important for proper refolding is volume ratio of arginine solution to protein one when applying onto the chromatography column. It was found that optimal volume for arginine solution is up to 5% of column volume. The volume of protein sample applied ranged from 1.5% to 2.5% of column volume. Selected volume ratio of protein solution to arginine one allowed to perform the refolding and to isolate mini-antibodies from arginine and other low molecular impurities.

Most often used methods for protein refolding from inclusion bodies are dialysis, dilution or their combination [4,46]. In the course of protein refolding using these methods only dilute protein solutions (10–100 μg/ml) are used [47]. This accompanies with manipulations with large solution volumes that need to be further concentrated to obtain final product. One of the advantages of chromatographic refolding is the possibility to use concentrated protein solutions. In the method presented herein, antibody concentrations in solutions used ranged from 0.1 to 7.5 mg/ml. Optimal concentrations were found to be 3–7 mg/ml, when the yield of monomeric protein was maximal.

Another important factor, affecting refolding using size-exclusion chromatography, is column size. It is known that the longer is column length, the higher is the yield of active protein as a result of chromatography isolation [12]. Two columns (60 and 100 cm in length) were tested in current work. The longer column (100 cm) demonstrated better separation of oligomeric antibody forms from monomeric ones, increased yield of latter ones and better cut-off low molecular fractions, including arginine.

The efficiency of protein renaturation using size-exclusion chromatography *via* an arginine solution layer was controlled directly using the protein elution profiles recorded by absorbance at 280 nm. A number of scFv were tried to refold in the course of this work. Depending on antibody nature (most likely on hydrophobicity of the molecule), variable efficiency of protein renaturation was observed. Fig. 3 represents chromatography elution profiles at optimal conditions for various types of scFv. Fig. 3A represents chromatography elution profile of refolded mini-antibodies characterized by negligible degree of aggregation and efficient separation of monomeric form from the aggregated one. Generally, the yield of monomeric form comparing with initial antibody amount was about 50–60%. Fig. 3B represents chromatography elution profile of the antibodies characterized by higher aggregation potency. It was a problem to isolate monomeric antibody form in this case, because it was difficult to achieve efficient separation of aggregated form from monomeric one. The yield of monomeric form ranged from 10% to 30%. Fig. 3C represents elution profile of mini-antibodies for which monomeric form is the dominant one and thus the yield of such an antibody was maximal (70%). One can see that in all cases an essential part of protein molecules refolds in a globular monomeric form (the elution volume corresponds to 18 fraction (36 ml), but the content of this form depends on the protein primary structure. Higher molecular weight associates (elution fractions from 13 up to 15 (26–30 ml)) as well as low molecular weight compounds are well separated from refolded monomeric forms.

It was important to produce antibody preparations that comprise neither aggregated protein forms, nor low molecular constituents of refolding medium. The efficiency of mini-antibody separation from arginine was controlled using ninhydrin test demonstrated that major part of arginine was eluted after

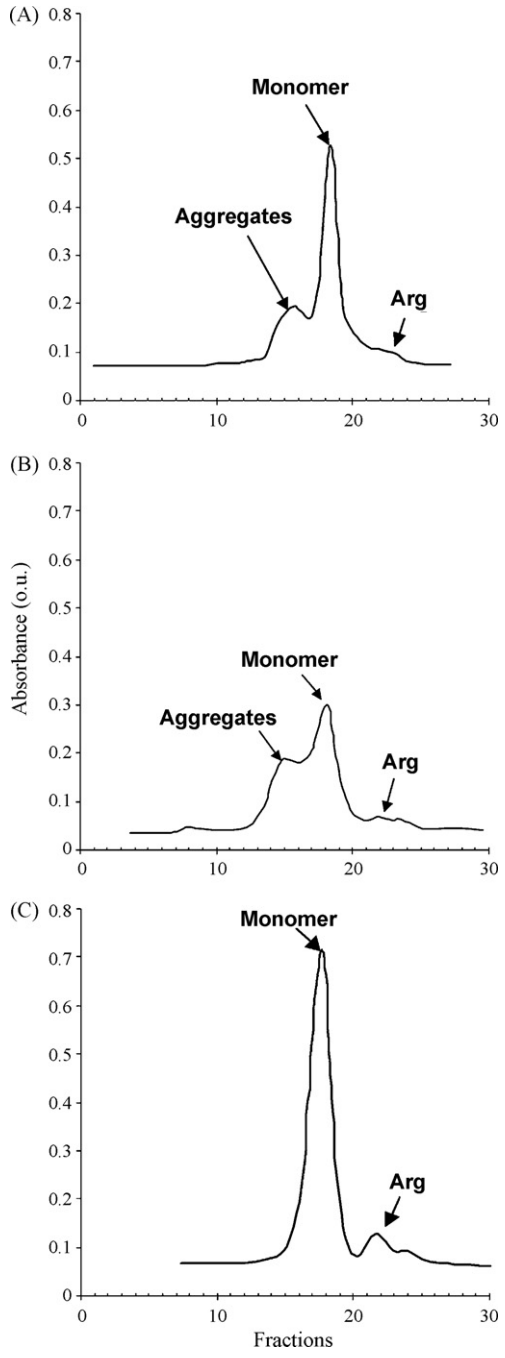

Fig. 3. Elution profiles size-exclusion chromatography of scFv purified from inclusion bodies. Aggregated scFv fractions, monomeric scFv and fraction of Arg indicated by arrows. A, B and C are three types of scFv with different refolding efficiency.

monomeric antibody fraction recovery from the column, and arginine content in protein sample corresponded to background level (Fig. 3A–C). For supplemental check for impurities of free arginine in protein fraction the amino acid analysis of the antibody preparation obtained was performed. According to amino acid analysis data, arginine content in amino acid hydrolyzate of scFv preparation was similar to the same one, calculated basing on scFv primary structure thus confirming successful antibody separation from arginine during chromatography process and the absence of additional impurities in refolding product obtained.

To evaluate the efficiency of scFv renaturation from inclusion bodies, some physical–chemical properties of these proteins were compared with those of scFv purified from soluble fraction of cell

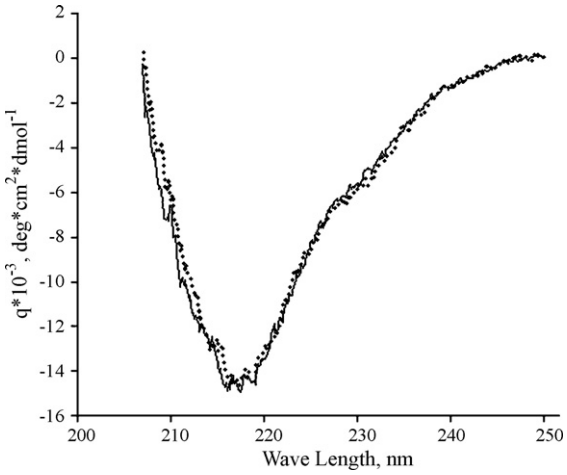

Fig. 4. Far-UV CD spectra of scFv purified from cell lysate soluble fraction (–) and refolded from inclusion bodies by size-exclusion chromatography through a layer of arginine (···).

lysate. To increase scFv content in soluble form during the expression, cell growth after IPTG induction was carried out at 29 °C. In this case, total amount of synthesized proteins was essentially lower, but scFv content in the soluble form was higher than in inclusion bodies. This allowed the purification of appropriate quantity of soluble proteins using Ni–NTA affinity chromatography. Fig. 4 represents far UV circular dichroism spectra of scFv (group 1, see Fig. 3) isolated from inclusion bodies and from soluble fraction of cell lysate. One can see that two spectra are very similar suggesting that secondary structures of both proteins are similar too.

To detect eventual formation of soluble protein aggregates in the samples just after refolding polyacrylamide gel-electrophoresis in native conditions was performed. In native non-denaturing conditions, electrophoretic mobilities of antibodies studied were different (see Fig. 5). The mini-antibodies purified and refolded from inclusion bodies had higher mobility (Fig. 5, lanes 2 and 5) than the other ones purified from soluble fraction (Fig. 5, lanes 1 and 4). This unexpected result suggests that soluble scFv mini-antibodies might be non-monomeric, or less compact than other ones refolded from inclusion bodies, or for some reasons they bear an altered surface-bound charge. Aggregated antibody forms were less mobile (Fig. 5, lanes 3 and 6).

The principal attribute of antibodies is their antigen-binding capacity. In the course of activity rating of mini-antibody prepa-

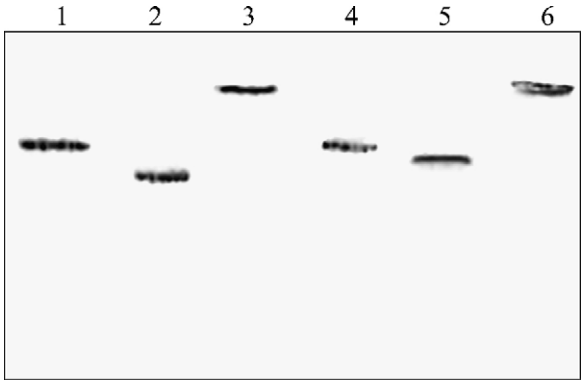

Fig. 5. Native PAGE of scFv. Comparative PAGE mobility of scFv from soluble fraction and refolded scFv. Lanes 1 and 4 represent scFv purified from cell soluble fraction. Lanes 2 and 5 represent monomeric forms of scFv purified from inclusion bodies and refolded by size-exclusion chromatography (see correspondingly Fig. 3C and A). Lanes 3 and 6 represent scFv aggregates (see correspondingly Fig. 3A and B).

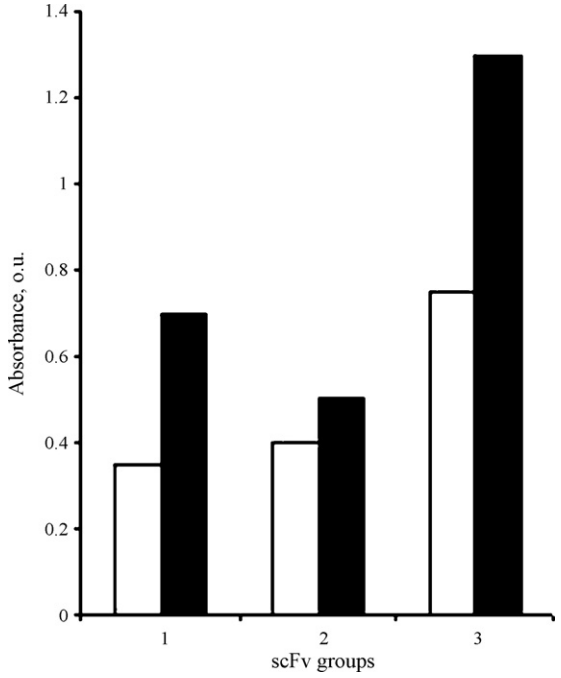

Fig. 6. ELISA activities of different scFv. (□)—scFv purified from soluble fractions and (■)—scFv monomeric forms purified and refolded from inclusion bodies. 1—scFv type A, 2—scFv type B group, 3—scFv type C of refolded scFv.

rations obtained from inclusion bodies, namely oligomeric and monomeric protein forms, soluble scFv from *E. coli* cytoplasmic fraction unaltered upon denaturants were used as a control. It was found that oligomeric fraction of several antibodies demonstrated similar or even higher activity, than corresponding monomeric form, but after short-term storage, the aggregates were formed resulting in loss of antigen-binding activity (data not shown). The enhancement effect after refolding was observed for several other antibodies: their monomeric forms were more active, than preparations of the same antibodies, isolated from soluble cytoplasmic fraction (see Fig. 6). It could be supposed that different activities of antibodies obtained depend on their amino acid sequence and aggregation potency.

There is a probability that in the course of renaturation when protein molecules passed through arginine layer their exposed hydrophobic regions capable for their aggregation tendency were hided inside the molecule. Such a transformation did not result in altering of molecular secondary structure, as it could be seen from CD spectra, but affected the compactness of the molecule and led to the increase of its electrophoretic mobility.

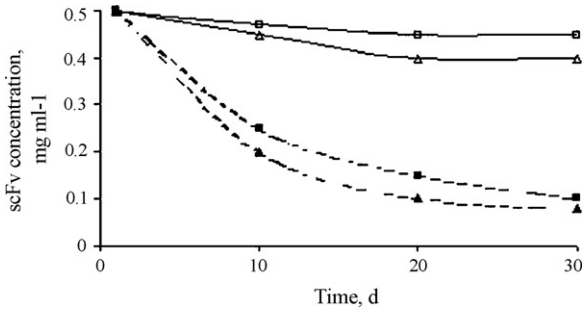

Fig. 7. Aggregation of scFv purified from inclusion bodies (□, △) and from cell soluble fractions (■, ▲) on the storage time at 4 °C. The samples of scFv (0.5 mg/ml) were stored at 4 °C. Every 10 days the protein solution aliquots were centrifuged at 12,000 × *g* and the supernatant absorbance at 280 nm was measured.

Very important biotechnological and pharmacological feature of recombinant therapeutic preparation is the stability of its functional and physical–chemical characteristics in storage [48]. To evaluate this feature antibody storage stability was examined. ScFv isolated from soluble cytoplasmic fraction exhibited high tendency to aggregate formation and to loss of functional activity in storage. At the same time, monomeric protein preparations obtained by renaturation using size-exclusion chromatography *via* an arginine solution layer retained their functional activity for a long period of time and did not form aggregates (see Fig. 7).

4. Conclusions

The large-scale production of recombinant proteins by means of cell biotechnology for industry and medicine is very often complicated by the aggregation of the majority of newly synthesized polypeptides and formation of hardly soluble inclusion bodies. Development of techniques for recombinant protein reactivation from inclusion bodies is still of great importance. The most popular methods for protein renaturation from inclusion bodies dissolved at denaturing conditions are: a dilution of protein solution containing high concentration of denaturing agent with native buffer to initiate the protein refolding; step-by-step dialysis; or their combination. Less popular approaches exploit the removal of denaturants directly on the Ni–NTA affinity column, or gel-filtration of recombinant proteins dissolved under denaturing conditions on a column equilibrated with a native buffer, or using urea gradient. In the present work, all these approaches were applied to produce active scFv mini-antibodies in a preparative quantity. Unfortunately, all known approaches to reactivate recombinant mini-antibodies from inclusion bodies have failed. Only a combination of size-exclusion chromatography involving initiation of protein refolding in the presence of 0.4 M arginine at the initial step of chromatography was quite effective. The described method allowed refolding scFv in one step, employing concentrated protein solutions (up to 8 mg/ml). Proteins, isolated from inclusion bodies and renatured by size-exclusion chromatography *via* an arginine solution layer, were monomeric and their secondary structure was similar to that of the same proteins purified from soluble fraction of cell lysate. Besides, scFv were stable upon storage at 4 °C without the loss of antigen-binding activity. The approach described in the present work could be useful for large-scale reactivation of other recombinant proteins from inclusion bodies.

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