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Efficient refolding of a hydrophobic protein with multiple S–S bonds by on-resin immobilized metal affinity chromatography

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ABSTRACT

The efficient refolding of recombinant proteins produced in the form of inclusion bodies (IBs) in *Escherichia coli* still is a complicated experimental problem especially for large hydrophobic highly disulfide-bonded proteins. The aim of this work was to develop highly efficient and simple refolding procedure for such a protein. The recombinant C-terminal fragment of human alpha-fetoprotein (rAFP-Cterm), which has molecular weight of 26 kDa and possesses 6 S–S bonds, was expressed in the form of IBs in *E. coli*. The C-terminal 7× His tag was introduced to facilitate protein purification and refolding. The refolding procedure of the immobilized protein by immobilized metal chelating chromatography (IMAC) was developed. Such hydrophobic highly disulfide-bonded proteins tend to irreversibly bind to traditionally used agarose-based matrices upon attempted refolding of the immobilized protein. Indeed, the yield of rAFP-Cterm upon its refolding by IMAC on agarose-based matrix was negligible with bulk of the protein irreversibly stacked to the resin. The key has occurred to be using IMAC based on silica matrix. This increased on-resin refolding yield of the target protein from almost 0 to 60% with purity 98%. Compared to dilution refolding of the same protein, the productivity of the developed procedure was two orders higher. There was no need for further purification or concentration of the renatured protein. The usage of silica-based matrix for the refolding of immobilized proteins by IMAC can improve and facilitate the experimental work for difficult-to-refold proteins.

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1. Introduction

Many, if not most, expressed proteins are found in an insoluble form called inclusion body (IB). The target protein is often relatively pure in washed IBs. So the challenge is not just to purify a protein, but rather to solubilize IBs and refold the protein into its native biologically active structure.

IBs can vary from being mostly native protein, relatively easily solubilized under mild conditions to being misfolded, insoluble material that requires high concentrations of denaturants to be solubilized [1,2]. The latter is most common, so the main challenge is to find conditions under which the denatured protein can be refolded efficiently.

Upon folding *in vitro*, misfolding as well as aggregation competes with the correct folding pathway [3,4]. It is commonly accepted that renatured soluble protein molecules do not aggregate in the medium; in contrast, unfolded or partially structured intermediates are prone to aggregation. To achieve high yield of refolding, the main aim is to reduce interactions among unfolded and partially folded protein molecules during renaturation. As a consequence, renaturation must often be performed at extremely high dilutions, at protein concentrations not higher than 10–50 μg/ml and long incubation time [5,6].

Conventional protein-folding protocols in most cases do not yield efficient refolding of hydrophobic, multidomain, multisubunit and/or highly disulfide-bonded proteins [7].

The refolding on a solid phase is a relatively new approach. The basic principle is that denatured protein is immobilized on a resin and then allowed to refold by removing denaturant still keeping the protein bound to the solid phase. During the procedure the protein molecules are sequestered and less likely to aggregate. This idea was first proposed by Tom Creighton in the late 1980s [8]. To this time, for demonstration of the principle, usually relatively simple model proteins have been chosen where unproductive side reactions do not occur to any significant extent. Successful solid phase refolding on ion-exchange chromatography resin has been achieved for such highly disulfide-bonded proteins as a bovine serum albumin and recombinant human full-length AFP (hAFP) [9,10].

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An intriguing approach is accommodation of size-exclusion chromatography for protein refolding as successfully demonstrated by Chen and Leong for recombinant hAFP [11].

One of the approaches to the refolding on a solid phase is the immobilization of the denatured protein on a metal affinity resin. The main advantage of this approach is that protein molecule is immobilized by His-tag and the molecule body can easily fluctuate in the medium during renaturation. Thus, hydrophobic interactions among unfolded or partially structured protein molecules are reduced. It should be noted that using immobilized metal affinity chromatography (IMAC) possesses certain limitations as a target protein should carry His-tag, in addition, protein intended use can be affected by metal ion carryover. This may require further tag removal and cleanup of the final product.

The majority of proteins that were renatured by IMAC are hydrophilic and have one or two or no disulfide bonds at all as followed from the analysis of the protein refolding data base at http://refold.med.monash.edu.au. One of the main problems in similar renaturation of hydrophobic proteins is that unfolded or partially folded protein molecules may irreversibly bind to the resin decreasing the yield of the refolding procedure. Almost all IMAC refolding protocols utilize carbohydrate matrix-based resins. The use of an inert material such as silica-based matrix may reduce the aggregation and increase the efficacy of the refolding procedure. Here we describe a method of the *in vitro* renaturation for the hydrophobic highly disulfide bonded protein on a silica-based matrix.

2. Materials and methods

2.1. Plasmid constructs expressing rAFP C-terminal fragment

The plasmid expressing rAFP-Cterm was constructed as described earlier [12].

2.2. Expression of rAFP-Cterm

The rAFP-Cterm gene construct was expressed in *E. coli* BL21 (DE3) strain (Novagen) following induction with 0.4 mM IPTG (Sigma–Aldrich) at 37 °C. Cells were grown in Luria Broth (LB) to a density of 0.4 OD_{600} prior to adding IPTG (isopropyl β-D-1-thiogalactopyranoside) and then grown further for 3 h.

After induction cells were harvested by centrifugation and stored at −20 °C.

2.3. Purification of inclusion bodies

The IBs were purified as described earlier [12].

2.4. Refolding of rAFP-Cterm by IMAC on-resin method

IMAC on-resin refolding was conducted with 1 ml of HisLink resin (Promega). Purified inclusion bodies were resuspended in solubilization buffer (0.05 M Na_3BO_3, pH 8.6, supplemented with 8 M urea and 12 mM β-mercaptoethanol) and incubated for 5 h at RT. The suspension was centrifuged at 13,000 rpm (Beckman, JA-14) for 15 min. The supernatant was added to HisLink resin equilibrated with solubilization buffer. The rAFP-Cterm was allowed to bind to the resin for at least 0.5 h, then the unbound material was washed three times during 10 min each by 10 volumes of PBS (1.7 mM KH_2PO_4, 5.2 mM Na_2HPO_4, 150 mM NaCl), pH 8.0, containing 8 M urea, 10 mM imidazole and 12 mM β-mercaptoethanol. The 10× excess of the refolding buffer (PBS, pH 8.0, 5 mM reduced glutathione and 1 mM oxidized glutathione) was added to the resin, the protein was allowed to refold during 4 h at constant stirring at +4 °C. The protein was eluted two times during 10 min by 1 volume of PBS, pH 8.0, 800 mM imidazole at +4 °C. If necessary, imidazole was removed by dialysis against 1000× excess of PBS, pH 8.0 overnight at +4 °C. The refolded protein was stored at −20 °C.

2.5. Purification of rAFP-Cterm on Ni-NTA

Purified inclusion bodies were solubilized as described above (see Section 2.4). The supernatant was added to Ni-NTA resin (Qiagen) equilibrated in solubilization buffer. The rAFP-Cterm was allowed to bind to the resin for at least 1 h, then the unbound material was washed by 30 volumes of PBS, pH 8.0, with 8 M urea and 10 mM imidazole. The protein was eluted in PBS, pH 8.0, with 8 M urea and 300 mM imidazole.

2.6. Refolding of rAFP-Cterm by dilution

The protein purified on Ni-NTA (1–2 mg/ml) was dropwise diluted at fast mixing in 100× excess of renaturation buffer (PBS, pH 8.0, 5 mM reduced glutathione and 1 mM oxidized glutathione) to final concentration 0.01–0.02 mg/ml and incubated for 48 h at 4 °C with constant stirring. Renatured protein was concentrated on Ni-NTA and then eluted in PBS, pH 8.0, containing 300 mM imidazole. The protein was dialyzed against PBS pH 8.0 at 4 °C for 24 h.

2.7. Analytical methods

2.7.1. Free SH-groups identification

Free SH-groups in renatured protein were identified using Ellman's method [13].

2.7.2. Analytical chromatography

The reverse-phase chromatography was performed using Symmetry300 C4 3.9 mm × 150 mm column on a Breeze HPLC (Waters). Trifluoroacetic acid (0.1% v/v) was added to all HPLC buffers. The column was equilibrated in water, followed by 0–100% (v/v) water–acetonitrile gradient for 60 min at 0.5 ml/min flow rate. Absorbance was measured at 214 and 280 nm.

The size-exclusion chromatography was performed using BioSuite 450 HR SEC 7.8 mm × 300 mm column on a Breeze HPLC (Waters), in PBS buffer, pH 7.4, with solvent flow rate 1 ml/min. Absorbance was measured at 280 nm. Molecular weight standards were ferritin (440 kDa), bovine serum albumin (67 kDa), human CD81 (30 kDa), lysozyme (14 kDa) and NaN_3 solution.

2.7.3. Circular dichroism (CD)

Far UV CD spectrum of renatured rAFP-Cterm was measured on Jasco-810 spectral polarimeter (Jasco Corp.). Quartz cuvettes with 1 mm path length were used. Protein was used at a concentration of 0.03 mg/ml in PBS, pH 7.4.

Protein amounts and purity were determined by SDS-PAGE followed by densitometry using OnedScan software (Stratagen).

2.8. Functional assay

2.8.1. Preparation of FITC-labeled rAFP-Cterm protein derivative

FITC-labeled protein was prepared according to Pierce manual. The FITC/protein molar ratio was 2.1:1.

2.8.2. Cell culture

Human ovarian carcinoma SKOV3 (cell line expressing receptor for hAFP (RAFP)) cells were maintained in DMEM medium (ICN) containing 10% fetal bovine serum (FBS, Gibco) and 50 μg/ml of gentamicin (ICN) in a CO_2 incubator at 37 °C in a humidified atmosphere containing 5% CO_2.

Table 1
Mass balance for rAFP-Cterm IMAC on-resin refolding.

Protein load (mg)[a]	Protein in flow through (mg)	Protein lost in wash buffer (mg)	Refolded protein in elution fraction (mg)	Protein left on resin after elution (mg)	Refolding yield (%)	Refolded protein purity (%)
1	<0.01	<0.01	0.60	0.40	60	98

[a] Protein amount and purity were determined by densitometry of SDS-PAGE using OnedScan software (Stratagen).

2.8.3. Binding assay

Twenty-four hours prior to the experiment, the cells were seeded in six-well plates. Before the experiment, the cells were incubated for 2 h in FBS-free DMEM medium. The specificity of rAFP-Cterm-FITC binding to the RAFP was determined by the incubation of cells with rAFP-Cterm-FITC (30–4000 nM) in the presence of a 30-fold excess of native human AFP (hAFP) in PBS for 1 h at 37 °C. After incubation, the cells were washed twice with cold PBS and fixed with 2% paraformaldehyde. Fluorescence was measured using an EPICS XL flow cytometer (Beckman–Coulter) equipped with an argon laser ($k_{excit.}$ 488 nm, bandpass 515–520 nm). Samples (10^5 cells/sample) were analyzed using XL SYSTEM II software.

3. Results and discussion

3.1. Refolding by metal-chelating chromatography

In the previous study [12], the purification of rAFP-Cterm was performed on Ni-NTA resin. So initially the IMAC solid-phase refolding was performed using the same resin. Unfortunately, most of the protein was not eluted from the resin after the renaturation procedure by high imidazole concentration under a variety of buffer conditions (high salt concentration, reducing agents, chaotropic agents, etc.). It can be concluded that during renaturation protein molecules irreversibly bind to the resin's carbohydrate matrix, agarose. Agarose is a polymer made of disaccharides, so it is not absolutely inert material. It is possible that unfolded protein during renaturation binds to the agarose matrix by hydrophobic interactions, via chemically reactive groups (SH–, NH_2–, etc.) or both and cannot be subsequently eluted from the resin. The other commonly used resin matrix is silica gel, chemically more inert material. The HisLink™ silica resin has been chosen for rAFP-Cterm refolding. The renaturation buffer and conditions were the same as successfully used by us before in refolding by dilution [12] (see Section 2.6).

A mass balance of the refolding procedure with rAFP-Cterm refolding concentration 1 mg/ml and incubation in refolding buffer during 4 h was calculated to determine protein recovery and purity (Table 1 and Fig. 1). The refolding yield of rAFP-Cterm reached 60% with purity 98% of the final product. This is a dramatic advance over refolding of rAFP-Cterm on the previously used agarose-based resin, especially considering that it is a hydrophobic highly disulfide bonded protein.

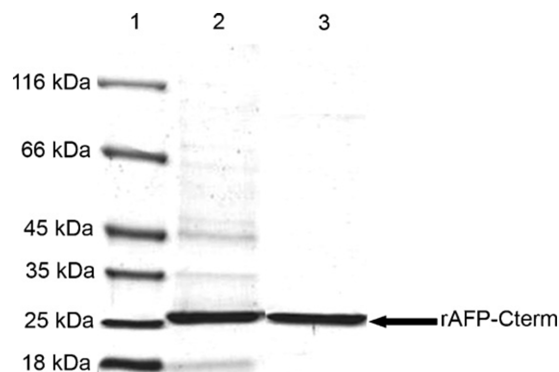

Fig. 1. SDS-PAGE analysis of the rAFP-Cterm before (lane 2) and after (lane 3) renaturation by IMAC. Lane 1 – molecular weight markers (Fermentas). Arrow points at the rAFP-Cterm band.

The effect of incubation time in refolding buffer on refolding yields was studied. Upon varying incubation time from 1 to 24 h, the best refolding yield was achieved after 4 h shown in Table 1. The protein loss was due to its irreversible binding on the resin during refolding. Stacked protein could have been eluted only by incubation in hot SDS with reducing agents, i.e., β-mercaptoethanol.

It is believed that, with the increase of protein loads in on-column solid-phase refolding procedure, the aggregation also increases, so the refolding yield consequently decreases [14]. This fact can be explained by non-uniform distribution of protein molecules across the column during loading. A high local protein concentration at any section of the column may promote aggregation and hence decrease refolding yield [9]. Consequently, the effect of rAFP-Cterm load on refolding yields in solid-phase refolding was studied. Different amounts of the purified protein (0.5–10.0 mg) were loaded on the same amount of the resin (1 ml) and refolded under identical conditions (refer to Section 2.4). Refolding yields were independent on the amount of rAFP-Cterm loaded and reached 60–65% in all cases. Possibly, protein distribution in off-column IMAC refolding procedure in batch format, as in our experiments, is more uniform than in on-column format so the protein loads less influence the refolding yields.

3.2. Analytical chromatography

Based on the results of size-exclusion chromatography calculated molecular weight of the protein was about 26 kDa. This agrees with rAFP-Cterm theoretical molecular weight. Aggregates of the protein were not detected by size-exclusion chromatography (Fig. 2). The homogeneity of the protein was confirmed by reverse-phase chromatography (Fig. 3).

3.3. Free SH-groups identification

Using Ellman's method, it was demonstrated that renatured rAFP-Cterm did not contain detectable amounts of free SH-groups.

3.4. Circular dichroism

The secondary structure of refolded rAFP-Cterm was determined by measuring its CD spectrum in the far-UV region (190–250 nm). Fig. 4 shows that secondary structure of the refolded protein is predominantly alpha-helix as seen from its strong characteristic absorption at 199, 208 and 222 nm. These data agree with the prevalence of alpha-helical structure in native hAFP [15]. The far-UV spectrum of rAFP-Cterm is very similar to that of native AFP and rAFP-Cterm previously refolded by dilution [12,16,17]. This confirmed that the secondary structure of refolded rAFP-Cterm is very much like that of native hAFP.

3.5. Cell binding and uptake assays

To analyze functional properties of rAFP-Cterm, its binding with human ovary adenocarcinoma SKOV3 cells were studied. Binding of rAFP-Cterm-FITC with SKOV3 cells in the presence of 30-fold

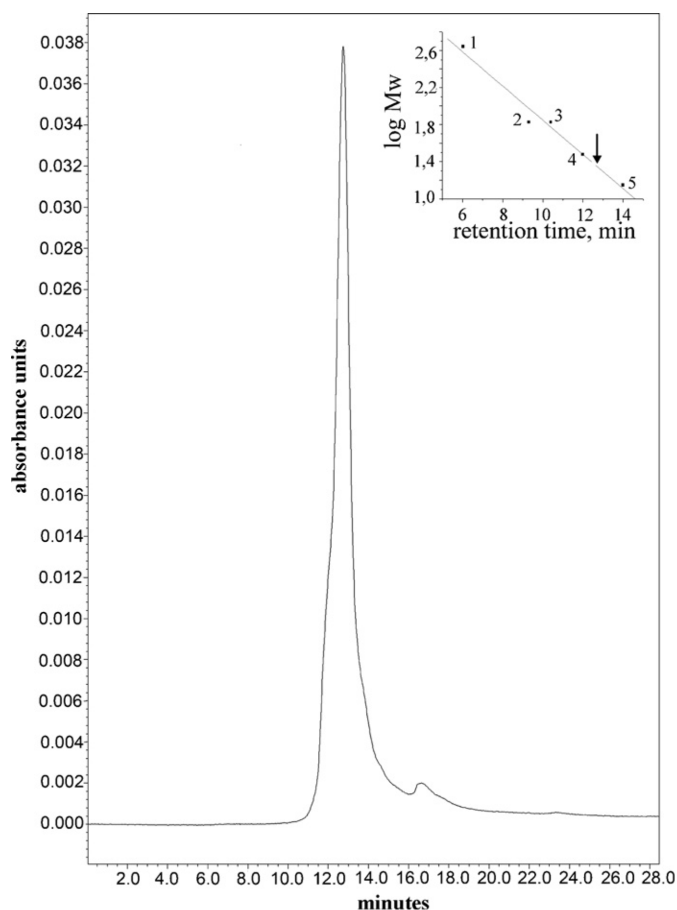

Fig. 2. Size-exclusion chromatography of refolded rAFP-Cterm in native conditions (PBS). Wavelength detection at 280 nm. Insert shows retention time dependence on molecular weight of reference proteins (1 – ferritin (440 kDa), 2 and 3 – bovine serum albumin dimer (134 kDa) and monomer (67 kDa), 4 – human CD81 (30kDa) and 5 – lysozyme (14 kDa)). Arrow points at the retention time of refolded rAFP-Cterm.

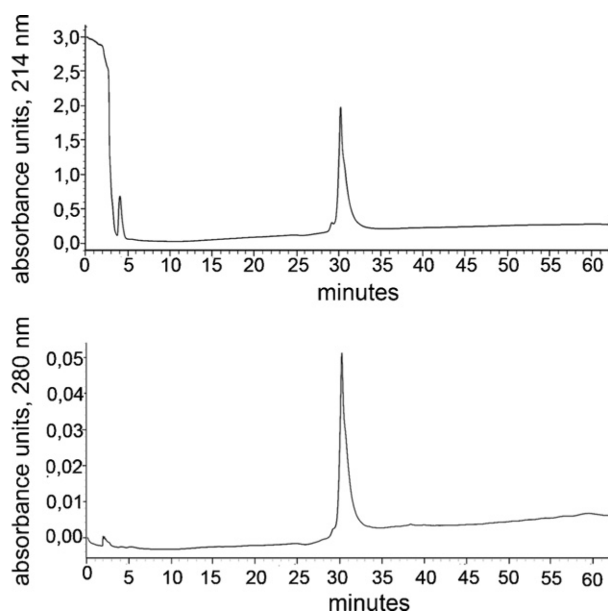

Fig. 3. Reverse-phase chromatography of rAFP-Cterm. Wavelength detection at 214 and 280 nm. Trifluoroacetic acid (0.1% v/v) was added to all HPLC buffers. The column was equilibrated in water, followed by 0–100% (v/v) water–acetonitrile gradient for 60 min at 0.5 ml/min flow rate.

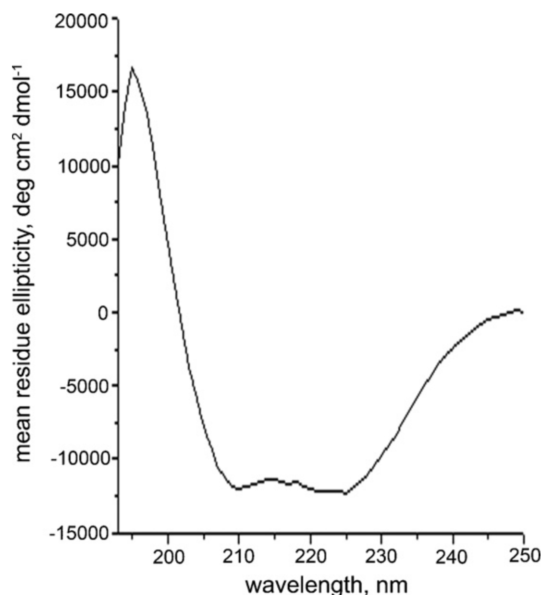

Fig. 4. Far-UV circular dichroism spectrum of refolded rAFP-Cterm. See Section 2.7.3 for details.

excess of the native hAFP was strongly inhibited by hAFP. These data indicate specific interaction of rAFP-Cterm-FITC with RAFP (data not shown).

3.6. Comparison of dilution-based and IMAC on-resin refolding

The refolding of rAFP-Cterm by dilution was conducted as a control study. Briefly, the purified protein at 1–2 mg/ml was dropwise diluted at fast mixing in 100× excess of renaturation buffer (see Section 2.6 for further details) to final concentration 10–20 μg/ml and incubated for 48 h at 4 °C with constant stirring. Renatured protein was concentrated on Ni-NTA and then eluted in the buffer containing 300 mM imidazole. It has been shown earlier that 10–20 μg/ml protein concentration range optimal for rAFP-Cterm refolding yield [12]. It is also consistent with the analysis of concentration (20–120 μg/ml concentration range) dependence of refolded hAFP yield upon its dilution refolding [16]. The highest refolding yield of about 70% was obtained at protein concentration 20–25 μg/ml [16]. The refolding of rAFP-Cterm by dilution gave a refolding yield of 50% with purity of about 95%. The yields of dilution-based and IMAC on-resin refolding procedures were similar, 50% and 60%, respectively. At the same time, the maximum protein concentration during refolding in dilution procedure was only 0.01–0.02 mg/ml, while in the IMAC refolding it could reach 0.5–1.0 mg/ml. The time required for the refolding was 48 h and 4 h for the dilution and IMAC refolding, respectively.

Electrophoretic analysis followed by densitometry showed the increase in refolded rAFP-Cterm purity from 90% in dilution refolding to 98% in IMAC refolding.

The yields and final protein purity in both techniques are close and the main difference between them consists in required refolding incubation time and buffer volumes, so the comparison of refolding productivity is more appropriate here. Refolding productivity was determined according to [9,11] with certain modifications as described in Eq. (1):

$$P = \frac{YM}{Vt\ 100\%} \quad (1)$$

where P is refolding productivity ($\mathrm{mg\,ml^{-1}\,h^{-1}}$), Y is refolding yield (%), M is the mass of total denatured rAFP-Cterm in the refolding

Table 2
Experimental parameters of IMAC on-resin and dilution-based refolding.

	IMAC	Dilution
Mass of rAFP-Cterm load (mg)	1	1
Refolding buffer volume (ml)	10	100
Refolding incubation time (h)	4	48
Refolding concentration of rAFP-Cterm (mg/ml)	0.1	0.01
Refolding yield (%)[a]	60	50
Refolded rAFP-Cterm purity (%)[a]	98	90
Refolding productivity (mg ml^{-1} h^{-1})	1.5×10^{-2}	1×10^{-4}

[a] Determined by densitometry of SDS-PAGE using OnedScan software (Stratagen).

system (mg), V is the volume of the refolding buffer (ml) and t is refolding incubation time (h). If the overall productivity is required, it should include all the procedures to obtain the final product. For measuring total productivity Y is final yield (%), M is the mass of total rAFP-Cterm taken for purification and renaturation (mg), V is the volume of all used buffers (ml), t is total time spent to obtain the final product.

Although the yields of both refolding techniques are close, the superiority of IMAC on-resin refolding in terms of productivity and procedures simplicity is obvious (Table 2). The refolding productivity was 1.5×10^{-2} mg ml^{-1} h^{-1} for the IMAC on-resin refolding and 1×10^{-4} mg ml^{-1} h^{-1} for the refolding by dilution.

Refolding productivity in IMAC refolding increased by two orders compared to dilution refolding. The increased productivity of IMAC refolding is attributed mainly to higher refolding protein concentration in the procedure, lower buffer consumption and shorter incubation time compared to refolding by dilution.

It is important to mention that in IMAC refolding the eluted protein was the final product that did not require any additional purification or concentration. At the current research stage, there was no need for His-tag removal from the target protein. The protein after refolding by dilution required the additional concentration step on Ni-NTA, which further increased time and buffer consumption. This step also additionally purified protein (the final purity was 95%), although the final protein purity in IMAC refolding procedure was still a little higher compared to dilution. The total buffer requirement for IMAC refolding is substantially lower than for refolding by dilution. The overall productivity for our target protein starting from harvested cells was 1.2×10^{-3} mg ml^{-1} h^{-1} for IMAC on-resin refolding and 2.9×10^{-5} for refolding by dilution.

Earlier Chen and Leong described the on- and off-column refolding of recombinant hAFP by anion-exchange chromatography on cross-linked agarose matrices [9]. Productivity of these refolding procedures after the adjustment of the original experimental parameters from [9] to the productivity equilibrium described above was within range from 2.5×10^{-3} to 1.4×10^{-2} mg ml^{-1} h^{-1}. These results are comparable with refolding productivity obtained by us in this study (1.5×10^{-2} mg ml^{-1} h^{-1}). Refolding yields for rhAFP in [9] varied from 15 to 42% compared with 60% for IMAC refolding in this study.

4. Conclusion

This work demonstrates the effectiveness of the IMAC on-resin refolding technology for refolding of a hydrophobic highly disulfide bonded protein, at refolding concentration 1 mg/ml. The influence of the resin matrix on rAFP-Cterm refolding yield is significant. The usage of silica gel instead of agarose matrix allowed dramatic increasing of the refolding yield (from almost 0 to 60%). The refolding yield did not depend on the protein load onto the resin. The productivity of IMAC refolding compared to refolding by dilution was two orders higher, due to high protein concentration and low buffer consumption in the procedure. The refolded eluted protein did not require any additional purification or concentration steps. The protein, rAFP-Cterm, was fully functional with regard to the binding to the target cells possessing specific AFP receptors. At the current research stage, there was no need for His-tag removal from the target protein.

The highly productive technology for the hydrophobic, highly disulfide bonded proteins is demonstrated in this study. The main achievement is the usage of silica gel matrix instead of agarose-based one. This promoted higher protein recovery in the refolding procedure and, as a consequence, higher refolding productivity. The usage of the silica gel matrices for the protein refolding could improve and facilitate the experimental work for many proteins with similar properties.

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