Expression, refolding, purification and characterization of single chain antibody fragment produced in *Escherichia coli*

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Therapeutic applications of immunoglobulins and their derivatives are continuously increasing. Herein, feasibility to express single chain antibody fragment against oxazolone (scFvOx) as inclusion bodies and its *in vitro* refolding were investigated. The recombinant protein was expressed at a very high level in *Escherichia coli* cytoplasm as inclusion bodies. A stepwise recovery protocol showing yield estimates at various stages of processing was developed. The refolded protein was found to be purified to homogeneity and biologically active as determined by its antigen binding activity in enzyme linked immunosorbent assay (ELISA). Limited refolding yield and purification efficiency were identified as rate limiting steps during production of scFvOx. A simple protocol for production of scFvOx was developed.

**Keywords:** Single chain antibody fragment, scFvOx oxazolone, protein refolding, ELISA, inclusion bodies

**Introduction:**
There is an ever-growing list of products and solutions based on immunoglobulin fold proteins and their recombinant derivatives. As a result, there is an increased interest in studying production methodologies for these molecules. Therefore, it is useful to have more detailed possible information about their production and purification process and protocols under varying expression constructs and experimental conditions. Full length antibodies are produced in mammalian cell culture media. An idea of producing antibodies using yeasts faced challenges due to different glycosylation pattern from that in animal cells. In these circumstances, *Escherichia coli* remains a dominant host for production of non glycosylated antibody derivatives. A comprehensive account of various recombinant derivatives of immunoglobulin fold proteins with varying therapeutic payloads have been summarized by Holliger and Hudson.

The periplasmic and cytoplasmic expression of recombinant antibody fragments in *E. coli* has been discussed earlier. Functional expression in the cytoplasm of *E. coli* has been improved by using mutant genes coding for thioredoxin reductase and glutathione oxidoreductase. These mutant cells create an oxidising cytoplasm capable of forming disulfide bridges in proteins. As an alternative, the production yields of functional antibody fragments in the cytoplasm can also be significantly improved by co-expression with chaperones and foldases or by a fusion protein strategy. In some instances, where antibody fragments are sufficiently stable without the conserved disulfide bonds and thus, their folding is possible under the redox conditions of the cytoplasm, functional expression can be achieved without resorting to *in vitro* renaturation. Such disulfide-free antibody fragments are also important tools for the intrabody technology where antibody fragments fold in the reducing environment of the cytoplasm.

The cytoplasmic approach benefits from a high expression level of antibodies using a strong promoter. An advantage of cytoplasmic expression is that following lysis of bacterial cells, inclusion bodies (IB’s) can easily be separated from other cellular components because of their large size and high density. Moreover, this approach is useful for producing antibody-based fusion proteins such as immunotoxins that might be toxic for bacterial cells or antibody fragments that are unstable due to intracellular degradation when expressed in a soluble or secreted form. However, correct *in vitro* renaturation and purification of functional product are a complex and time-consuming process, requiring expertise and involving many steps. Problems and limitations commonly encountered with this approach, for antibodies in particular, are (i) difficulties in predicting the tendency of different sequences to form inclusion

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bodies (IBs) and their susceptibility to proteases, renaturation efficiency which is highly variable depending on the specific antibody fragment with yields varying from 10-40% for Fab and Fv fragments, and the need for separation of correctly and incorrectly folded fractions of the protein.

Renaturation of scFv’s can be achieved by gradual removal of denaturant by stepwise dialysis. However, these protocols have not been found to be applicable to other tested scFv constructs, where severe aggregation problems were observed. Successful matrix-assisted renaturation of scFv fragments was reported recently. However, for renaturation by dialysis as well as for matrix-assisted renaturation, process scale-up may represent problems due to sample volume limitations. For these reasons, dilution protocols for the in vitro renaturation of antibody fragments remain in widespread use.

Recently, the cytoplasmic expression of scFvs as a C-terminal fusion to maltose-binding protein provided the high-level production of soluble, functional fusion protein. In another report the efficient refolding and site-specific immobilization of scFvs fused with a poly(methyl methacrylate)-binding peptide were reported. Although, fusion tags do promote yield of scFvs, from process development point of view, it must be understood that it makes fusion protein technique additional steps for proteolytic digestion and removal of fusion tag. Earlier authors reported protocols for the renaturation of scFvOx in the presence of new class of chemicals such as ionic liquids and aromatic thiols as redox buffers have been reported. Recently, effect of pH on stability of scFvOx and its detailed biophysical characterization during refolding were reported.

Increasingly higher demands of recombinant therapeutic proteins have clearly indicated the need for systematic optimization of renaturation conditions. In the present work, findings on expression, isolation of inclusion bodies, renaturation and purification was reported. Thus, the purified protein was further characterized for activity by ELISA and analytical reversed phase high performance liquid chromatography and mass spectrometry. Hence, the study reports simple and scalable protocol for production of scFvOx.

Materials and Methods

Chemicals and Consumables

All reagents, chemicals and consumables were of analytical grade or of higher purity. Source of specialty chemicals and instruments are indicated alongside in the text where they appear. Milli-Q water was used for media and buffer preparations.

Expression Construct and Strain

The coding DNA sequence of recombinant anti-oxazolone single-chain antibody fragment (scFvOx), fused to sequences coding for an N-terminal hexahistidine tag and a C-terminal myc tag, was obtained from the expression construct scFvOx/pHEN-1, kindly provided by Dr. U. Fiedler. The hexahistidine tag was introduced for simplifying purification using immobilized metal affinity chromatography. For cytoplasmic expression in E. coli the coding sequence was inserted between the unique NdeI and BamHI restriction sites of the bacterial expression vector pET15b(+). ScFvOx protein was expressed in E. coli BL21(DE3) (Promega, USA) cells that had been co-transformed with the helper plasmid pUBS520. This vector carries the dnaY gene encoding the tRNA for the arginine codons AGA and AGG, which are rare in E. coli and thus often limit expression of genes containing these codons. Thus, the final expression strain was E. coli BL21 (DE3): pUBS520:pET15b(+):scFvOx.

Expression of scFvOx

Recombinant proteins expression was carried out in Luria Bertani (LB) media at 37°C. The final concentrations of the antibiotics were 100 μg mL⁻¹ for ampicillin and 70 μg mL⁻¹ for kanamycin. Depending on need of biomass expression was carried out in multiples of 1 L. For shake flask experiments, preculture and main cultures were grown in LB medium supplemented with the appropriate antibiotics. For the pre-culture, complex medium was inoculated with a single colony from an LB agar plate and incubated overnight on a rotary shaker at 30°C, 140 rpm. For the main culture, complex medium in a shake flasks was inoculated with 1% (v/v) of the overnight pre-culture and was incubated at 37°C to an OD₆₀₀ of about 0.5-0.7. After this, scFvOx synthesis was induced by 1 mM of isopropyl-β-D-thiogalacto-pyranoside (Merck, Germany). Post induction culture was incubated for 3-4 h. For harvesting E. coli cells were centrifuged at 13,000 rpm for 10 min and pellet was stored at -20°C till further use.

Inclusion Body Isolation

The biomass was homogenized (Ultra-Turmax, IKA Labortechnik, Germany) at 4°C in 5 mL of re-suspension buffer (0.1 M Tris/HCl, pH 7.0, containing 1 mM EDTA) per 1 g of E. coli wet cell weight in the presence of 0.3 mg mL⁻¹ lysozyme (Merck, Germany), and incubated for 30 min at 4°C. The cells were
mechanically broken open using a French press (Gaulin, Germany), and cellular DNA was digested for 30 min with 10 µg mL\(^{-1}\) Benzonase (Merck, Germany) in the presence of 3 mM MgCl\(_2\). The solution was mixed with 0.5 volumes of 60 mM EDTA, 6% (v/v) Triton X-100, 1.5 M NaCl, pH 7.0, and incubated for a further 30 min at 4°C. The IB’s were pelleted by centrifugation at 12,000 rpm for 10 min at 4°C, washed with 40 mL of re-suspension buffer per each g wet cell weight, and harvested by a final centrifugation step.

**Inclusion Body Solubilisation**

The inclusion bodies were solubilised in 1 mL of solubilisation buffer (0.1 M Tris/HCl, pH 8.0, containing 6 M guanidinium hydrochloride (GdnHCl) (Nigu Chemie, Germany), 0.1 M diethyliotiol (DTT), (Merck, Germany) and 1 mM EDTA) per 10 mg wet weight for 2 h at 25°C. The solubilisation mixture was acidified to pH 4.0 by the addition of HCl and cleared by centrifugation, dialysed twice against 100 volumes of 4 M GdnHCl, 10 mM HCl at room temperature and once against 200 volumes of 4 M GdnHCl at 4°C.

**Renaturation of scFvOx**

Preparative renaturation of scFvOx was performed by dilution of scFvOx IB solubilisate to a final concentration of 140 µg mL\(^{-1}\) in degassed renaturation buffer (100 mM Tris/HCl, pH 8.5, containing 2.5 mM GSH, 2.5 mM GSSG, 1 mM EDTA and 1 M L-Arg/HCl). Renaturation was allowed to proceed for 96 h at 15°C.

**Purification of scFvOx**

Following renaturation, the mixture was concentrated using a Filtron minisette cross-flow device (Filtron Technology Corp., MA, USA). The resulting solution was dialysed against 10 volumes of 25 mM sodium phosphate pH 6.0, containing 10 mM NaCl and 10% glycerol at 4°C (3 changes), and clarified by centrifugation at 10,000 rpm in a JA-10 rotor for 15 min. Protein purification process was carried out using Äktapnrifier basic system (GE Healthcare, Sweden). Subsequently, the clarified protein sample was loaded onto a 1 mL HiTrap SP sepharose HP column (GE Healthcare, Sweden) that had been pre-equilibrated with 20 column volumes (CV) of loading buffer (25 mM sodium phosphate, pH 6.0, containing 10 mM NaCl). The column was then washed with 10 CV of loading buffer, followed by elution with a linear gradient of 5-40% elution buffer (25 mM sodium phosphate, pH 6.0, containing 2 M NaCl) in 15 CV. Fractions containing pure scFvOx were identified by SDS-PAGE, silver staining and ELISA, then pooled and dialysed against 50 mM sodium phosphate, pH 7.0, containing 50 mM NaCl and stored at -80°C. SDS PAGE and silver staining techniques were performed as per standard protocols without any modifications.

**Protein Quantification**

The protein concentration in different samples was determined by Bradford’s using BioRad protein assay reagent (BioRad, USA), according to the manufacturer’s instructions, with bovine serum albumin as the standard. In order to determine protein concentrations in samples containing GdnHCl, the respective standard curve was measured in the same concentration of GdnHCl.

**ELISA Procedure**

Preparation of the bovine serum albumin (BSA) oxazolone conjugate for ELISA was prepared according to Alfthan et al. Concentrations of active scFvOx were determined by ELISA. For this purpose, 96-well ELISA microtiter plates were coated overnight with 120 µL per well of 25 µg mL\(^{-1}\) BSA-oxazolone conjugate in 0.1 M sodium carbonate, pH 9.6. The coated plates were washed three times with blocking reagent (1.5% BSA, 0.05% Tween 80 in PBS), followed by blocking for 90 min at room temperature. Samples and standards were diluted in blocking reagent to a final volume of 100 µL per well, loaded onto the plates, incubated for 90 min, and unbound material was removed by three washes with blocking reagent. For the detection of bound scFvOx, the plates were incubated for 1 h with 100 µL per well of a 1:5000 dilution of mouse anti-c-myc tag antibody in blocking reagent. After one washing with blocking reagent, the plates were incubated with horseradish peroxidase-coupled chicken anti-mouse-IgG (1:3000). The plates were washed with substrate buffer (3.25 mM sodium perborate, 40 mM sodium citrate, 60 mM sodium phosphate, pH 4.5) before applying 1 mg mL\(^{-1}\) 2, 2'-azino-di-(3-ethylbenzthi-azoline-6-sulfonic acid) (ABTS) (Roche, Germany) in substrate buffer. Colour development was followed at 405 nm in a Sunrise micro plate reader (Tecan, Switzerland). Bound scFvOx was determined from the increase in the absorption at 405 nm over 30 min. Purified protein obtained as above served as the reference, and used at concentrations of 250, 125, 65, 30, 15, 7 and 3 ng mL\(^{-1}\) to generate the standard curve.
Reversed Phase High Performance Liquid Chromatography

Reversed phase HPLC (RP-HPLC) was performed using a 4.6 mm x 250 mm diphenyl column. The protein was eluted at 60°C with a flow rate of 0.5 mL min⁻¹ and a linear gradient from 20 to 60% acetonitrile in 0.1% trifluoroacetic acid (TFA) over 40 min.

Mass Spectroscopy

After separation with RP-HPLC peaks were manually collected and submitted for mass spectrometric analysis. These analyses were performed by Dr. Angelika Schierhorn, Max Plank Research Unit for Enzymology of Protein Folding, Halle, Germany.

Results and Discussion

Production of scFvOx Inclusion Bodies

ScFvOx was produced in *E. coli*. Cells were harvested 4 h post induction and inclusion bodies (IB’s) prepared. Pre-induction, post-induction and IB samples were analysed by SDS-PAGE (Fig. 1). No scFvOx production was observed in the pre-induction sample (Fig. 1, lane 1). Post-induction, the crude cell extract showed strong expression (Fig. 1, lane 2), corresponding to ~30-50% of the total cellular protein. Apparently, the strong T7 promoter drove very high protein production. scFvOx was produced as insoluble IB’s and the preparation contained ~ 80% scFvOx polypeptide (Fig. 1, lane 3).

Preparative Renaturation of scFvOx from IB’s

Isolation of IB’s was done from 20 g of cell biomass (Fig. 2), resulting in 1.12 g of IB’s. After reduction, denaturation and dialysis, 267 mg of IB solubilisate in a total volume of 126 mL was obtained (Fig. 2). From this reduced protein, a 1.9 L renaturation experiment was performed. After cross-flow concentration, 400 mL of renatured sample was obtained, corresponding to 255 mg of protein (Fig. 2). This concentrated protein was dialyzed and clarified by centrifugation to remove aggregates, resulting in 45 mg of protein (Fig. 2). Purification by ion exchange chromatography (IEC) resulted in 13 mg of pure scFvOx, corresponding to 5% of the total IB protein obtained. The yield estimates were in good agreement with refolding estimates reported earlier for scFvOx²².

Purification of scFvOx

After production, isolation and renaturation of scFvOx, the protein was purified by IEC, using a Hi-Trap SP-sepharose column. The renatured protein applied for purification was relatively pure (Fig. 3 B, lane 4). Forty five mg of the protein was loaded on the column. No scFvOx was detected in the unbound and washing fractions (Fig. 3 B, lanes 5 and 16). Bound protein was eluted with a gradient of increasing NaCl concentration. Eluted scFvOx protein was found in the fractions 23-31 (Fig. 3 B) corresponding to NaCl concentrations between 0.23 and 0.6 M. The protein was collected and the purification process was repeated.

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**Fig. 1** — Production and isolation of scFvOx inclusion bodies: Proteins were analysed by 12% SDS-PAGE and staining with Coomassie Blue. Where, M, low molecular weight marker; lane 1, whole cell extract from un-induced cells; lane 2, whole cell extract from induced cells (4 h, post-induction); lane 3, IB preparation. For each lane the pellet of 200 µL culture volume was applied.

**Fig. 2** — Production of inclusion bodies, preparative renaturation and purification of scFvOx.
eluted in three overlapping peaks. All peak fractions contained an apparently homogeneous pure protein with a relative molecular weight of 29 kDa as observed by silver stained polyacrylamide gel. The activity (CD) assay by ELISA revealed scFvOx antigen binding activity in all peak fractions (Fig. 3, C), with specific scFvOx activities between 0.16 to 0.19 mg mL$^{-1}$.

Characterization by circular dichroism (CD) spectroscopy, analytical size-exclusion chromatography, RP-HPLC and mass spectroscopy failed to detect any differences between the protein in the different elution fractions (data not shown). This indicates that the observation of three overlapping elution peaks was not due to heterogeneity of the purified protein. Instead, it is conceivable that scFvOx adopted different conformations with different elution properties upon binding to the ion exchange matrix, in agreement with conformational flexibility of antibody fragments reported in the literature$^{27, 28}$. The overall recovery from this process was around 4.86%, which was considered a good recovery since the value gives the estimate of active and purified protein.

In order to achieve maximum renaturation yield, systematic optimization of a series of parameters such as the denatured protein concentration, pH, temperature, time, redox buffers and effect of different additives is required$^{18}$. Certain additives such as L-Arg (for suppression of aggregation), sucrose and glycerol (for stabilization of the native state) have been widely used to enhance renaturation yields of the proteins. Also reduced and oxidized glutathiones and small molecular weight thiols are commonly used as redox buffers for disulfide shuffling$^{18}$. In order to select the right low-molecular weight compounds as co-solvents for renaturation reactions, it is crucial to maintain the balance between several factors such as preservation of the stability of the native state of a protein and stabilization of denatured polypeptides and intermediates.

Recently, a new class of chemicals called ionic liquids (IL’s) was reported as refolding additives$^{29}$. In that, butyl ammonium nitrate and ammonium nitrate marginally increased renaturation yield of lysozyme$^{29}$. To extrapolate these findings, we showed that the IL’s from the series of N’-substituted N-methyl imidazolium chlorides and N-(2-hydroxyethyl) – pyridinium chloride promote the renaturation of scFvOx$^{20, 21}$. On the other hand, higher concentrations of these compounds exert a destabilizing effect on the native state of the protein, as loss in the renaturation yield of scFvOx was observed. This effect was more pronounced in case of compounds with longer alkyl side chains$^{20}$.

**Reversed-Phase HPLC Analysis**

The purity and homogeneity of isolated purified protein was further assessed by RP-HPLC. Different RP-HPLC column materials (C18, C8 and C4) and buffer systems (acetonitrile, methanol and acetone) were tested, but the observed resolution and
reproducibility were not satisfactory. A reversed phase di-phenyl solid phase column (VyDac, USA) was found to produce good resolution with acetonitrile/TFA buffer system, when the experiment was performed at 60°C (Fig. 4 A). Under these conditions, the separation of purified protein samples gave rise to single sharp peaks, indicating that the protein had been purified to homogeneity.

Mass Spectrometry

The mass of the recombinantly expressed and purified scFvOx was determined by Electron Spray Ionization-Time-of-Flight (ESI-TOF) mass spectrometric analysis of the collected peak fractions from the RP-HPLC runs. The mass of the protein was found to be 28,958 Da (Fig. 4 B). This was in good agreement with the theoretically expected mass of scFvOx (28,960 Da). Besides this no other contaminating protein peaks were detected confirming that the sample was purified to homogeneity.

These present study represent simple and one step purification of biologically active and purified protein which is desired for process development point of view. In case of single chain antibody fragments and antibody derivatives, ample information is available on constructs, designs, new expression tags and variety of experimental conditions; however, there is lack of knowledge about process, yield and challenges in the recovery. Therefore, in these circumstances the study represented in this reports provide simple protocol for production of single chain antibody fragment.

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References: