Chapter 27
Improving Expression of scFv Fragments by Co-expression of Periplasmic Chaperones

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Abbreviations

IPTG	Isopropylthiogalactoside
PBS	Phosphate buffered saline
scFv	Single-chain Fv fragment
tet	Tetracycline
ELISA	Enzyme-linked Immunosorbent Assay
LB	Luria–Bertani media
SB	Super broth media

27.1 Introduction

For more than 20 years now, periplasmic expression in *Escherichia coli* has become the standard technology for preparing functional antibody fragments in a rapid and convenient way (Skerra and Plückthun 1988; Plückthun et al. 1996). The criteria of choosing either the Fab or single-chain Fv fragment (scFv) format, the properties of suitable expression vectors, as well as the influence of the *E. coli* strain used have been extensively summarized elsewhere (Plückthun et al. 1996). However, even when considering all these components and experimental conditions, the yield of recombinant antibody fragments is still highly variable, mainly being a direct consequence of the primary sequence and its sequence-dependent propensity to lead to aggregation-prone folding intermediates. In general, periplasmic folding is the yield-limiting step, being strongly influenced by the amino acid composition of the antibody to be expressed (Wörn and Plückthun 2001; Ewert et al. 2004). However, yield is not the only property influenced as the protein sequence also

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determines stability and resistance against aggregation upon storage of the purified protein. Since these properties cannot be changed by expression conditions, antibody sequence alteration must be seen in conjunction with choosing an appropriate expression system, and this includes chaperone co-expression.

Two principal methods have proven to be successful for improving antibody sequences: a "rational" approach and a "directed evolution" one. The rational approach is based on alignments of the particular antibody sequence to that of well-expressing fragments (Knappik and Plückthun 1995; Wörn et al. 2000; Ewert et al. 2003, 2004; Honegger et al. 2009), an analysis of exposed hydrophobic residues (Nieba et al. 1997), or the grafting of CDRs onto a stable and well-folding framework (Jung and Plückthun 1997; Willuda et al. 1999; Kügler et al. 2009). In a directed evolution approach, the protein is subjected to an evolutionary pressure, which rewards stability and expression (Jung et al. 1999; Jermutus et al. 2001; Schimmele and Plückthun 2005). When starting from a given antibody, such protein engineering constitutes — undeniably — a significant effort. The ability to rapidly characterize the given antibody fragment will be critical whenever a choice between various fragments with different binding properties has to be made. For this purpose, significant amounts of properly folded protein are necessary.

Therefore, we discuss here the co-expression of periplasmic factors improving the yield of soluble and correctly folded antibody. Because of their conserved intradomain disulfide bonds, antibody fragments need to be secreted to an oxidizing compartment for correct folding (Skerra and Plückthun 1988), this being the periplasmic space in bacteria. While some antibodies have been engineered to fold in the absence of disulfides (Proba et al. 1998) and others have been expressed (Proba et al. 1995; Levy et al. 2001) in *E. coli* mutant strains with altered cytoplasmic redox machinery where cytoplasmic disulfides can accumulate to some extent (Ortenberg and Beckwith 2003), this chapter deals with periplasmic expression.

The effect of overexpressing molecular chaperones and other folding modulators on the yield of foreign proteins has been reviewed (Wall and Plückthun 1995; Kolaj et al. 2009). Since the folding of the antibody takes place *after* its secretion, periplasmic factors are of greatest interest in this regard. Nonetheless, the overexpression of cytoplasmic factors has also been attempted in the hope of improving yield of soluble antibody (Söderlind et al. 1995; Hu et al. 2007).

In the bacterial periplasm, three types of folding modulators have been identified that may play a role with the folding of exogenous proteins in *E. coli*: (1) the disulfide-bond-forming (Dsb) machinery (Kadokura et al. 2003; Ortenberg and Beckwith 2003), with the periplasmic proteins DsbA and DsbC, and to some degree the specialized DsbE and DsbG (DsbB and DsbD being transmembrane proteins for regenerating the periplasmic factors); (2) the four periplasmic proteins with peptidyl prolyl *cis/trans* isomerase (PPI) activity (Galat 2003), PpiA (RotA), PpiD, FkpA, and SurA; and (3) the protein Skp with chaperone activity (see below), and finally the protease DegP (Skorko-Glonek et al. 2008) suspected to also have chaperone activity at low temperature.

There is clear evidence that the spectrum of activities of these proteins is overlapping. The dimeric peptidyl prolyl *cis/trans* isomerase FkpA has chaperone

activity, most clearly shown by its improvement of the periplasmic expression of scFv fragments that do not even have a *cis* proline (Bothmann and Plückthun 2000; Ramm and Plückthun 2000). The dimeric DsbC, while showing disulfide isomerase activity, is also thought to have chaperone activity (Chen et al. 1999; Zhao et al. 2003; Segatori et al. 2004) and has been observed to help against periplasmic lysis. However, clear evidence is lacking that an increased peptidyl prolyl *cis*/*trans* isomerase activity and an increased disulfide isomerization activity are actually *per se* beneficial for antibody scFv fragments, as opposed to the observed favorable effects being entirely due to the built-in chaperone activities of FkpA and DsbC, and possibly other factors (Bothmann and Plückthun 2000; Ramm and Plückthun 2000; Sandee et al. 2005). These enzymatic activities may become of great importance in other antibody constructs, however, e.g., those with additional disulfide bonds.

We have taken two approaches to tackle the problem of soluble expression of scFvs. First, we have previously identified factors that increase the functional expression of antibody constructs by a selection approach and have designed appropriate co-expression vectors. Second, we have created a modular system that allows a flexible co-expression of many factors with virtually any antibody expression vector.

For identification of the crucial factors for antibody expression, we used a phage display system, which displayed a constant, poorly folded antibody fragment and a library of co-expressed genes (Bothmann and Plückthun 1998; Bothmann and Plückthun 2000). With this enrichment strategy, we identified two periplasmic factors with beneficial, chaperone-like properties, both increasing the folding efficiency of scFvs and, consequently, their yield in the periplasm. The first factor identified, Skp (for 17 kDa protein), is a basic periplasmic protein that has been found to specifically interact with outer membrane proteins, assisting their transport through the periplasm (De Cock et al. 1999; Schäfer et al. 1999), and it may similarly interact with the folding antibody. It has recently been found to interact also with some periplasmic *E. coli* proteins (Jarchow et al. 2008). The second factor, FkpA, is a periplasmic peptidyl prolyl *cis*/*trans* isomerase, which also acts as a chaperone (Bothmann and Plückthun 2000; Ramm and Plückthun 2000), perhaps the more important property. The effects of Skp and FkpA (increasing the scFv yield by up to a factor 10) appear to be specific for every antibody variant, as neither additivity nor synergy was observed. However, we never noticed a negative influence of the co-expression of either Skp or FkpA onto the scFv level up to now — in some cases it had simply no effect, notably when the antibody did not show significant aggregation tendencies to begin with.

While Skp and FkpA have been experimentally identified by an enrichment strategy to be helpful for antibody fragments, a more generic co-expression strategy can be useful as well. Therefore, we designed a plasmid series, named pCH, for the overexpression of the thiol-disulfide oxidoreductases DsbA and DsbC, based on the pTUM4 vector (Schlapschy et al. 2006). The coding sequence for Skp, as well as FkpA and SurA (another peptidyl prolyl *cis*/*trans* isomerase with suspected chaperone activity, implicated in the delivery of proteins across the periplasm to

the outer membrane),was also included. The main reason for altering the existing pTUM4 plasmid was to create a new modular structure that should be compatible to virtually any antibody expression plasmid. This was achieved using a modular design previously utilized by Lutz and Bujard (1997), allowing a convenient exchange of both the origin of replication as well as the genes conferring antibiotic resistance by unique restriction sites. Thus, we created a set of plasmids, carrying different combinations of origin of replications (ColE1, p15A, and pSC101, each resulting in a different number of intracellular plasmid copies) in conjunction with the genes conferring resistance to ampicillin, kanamycin, chloramphenicol, or tetracycline, respectively. With this variety of origins and resistance genes, the pCH series is compatible with virtually all conventional antibody expression vectors. The choice of different origins allows one to control the level of chaperone co-expression based on different plasmid copy numbers. It also safeguards against plasmid incompatibility (which can lead to the loss of one of the plasmids), even though this may be less of a concern in high copy number plasmids (Velappan et al. 2007). Initial results indicate an overall yield increase of antibody fragments in a variety of formats upon co-transformation of suitable *E. coli* hosts with members of this vector series.

27.2 Materials

- Standard molecular biology equipment and reagents for
  - Isolating genomic DNA from *E. coli* (e.g., Qiagen DNeasy Blood & Tissue Kit),
  - Performing PCR reactions,
  - Cutting and gel-purifying DNA (e.g., Sigma-Aldrich GenElute Gel Extraction Kit),
  - Ligating and transforming DNA,
  - Conducting an enzyme-linked immunosorbent assay (ELISA), and
  - Performing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequent immunoblotting;
- An appropriate expression system to produce histidine-tagged antibody fragments in the periplasm, such as the pAK system (see Chap. 3 or Krebber et al. 1997);
- Cell disrupting instrument like a French Press (Aminco Rochester, NY, USA) with 4 ml cell and 40 ml cell or a TS 1.1 benchtop (Constant Systems Ltd. UK);
- An automated LC-System: e.g., BioCAD workstation (e.g., PerSeptive Biosystems, acquired by Applied Biosystems) with dual-channel variable-wavelength UV/visible detector, semipreparative flow cell (Perkin Elmer), fraction collector Advantec SF-2120 (Toyo Roshi International), or equivalent system;
- POROS20 MC/M 4.6 mm/100 mm (metal chelate) (Applied Biosystems);
- POROS20 HQ/M 4.6 mm/100 mm (anion exchange) (Applied Biosystems);
- POROS20 HS/M 4.6 mm/100 mm (cation exchange) (Applied Biosystems);

- Imidazole stock solution (1 M) adjusted to pH 7 with acetic acid.
  Note: Make sure to adjust the pH of the imidazole stock solution using *acetic acid*, and not with HCl, in order to keep the ionic strength low (otherwise the protein might run through the coupled downstream ion exchange column);
- NaCl stock solution (3 M);
- $NiCl_2$ (200 mM);
- Distilled water.

PBS (PBST)
Na_2HPO_4 (10 mM);
KH_2PO_4 (1.8 mM);
KCl (2.7 mM);
NaCl (137 mM, pH 7.4);
for PBST, also add Tween 20 to a final concentration of 0.05%

Extraction Buffer
Sucrose, 20% (w/v);
EDTA (1 mM);
Tris-HCl (100 mM, pH 8.0).

Solubilization Buffer
Urea (2 M);
EDTA (1 mM);
Glycylglycine (10 mM, pH 7.5).

MHA Buffer (5× stock solution is given)
Mes (33 mM);
Hepes (33 mM);
Na-acetate (33 mM; adjust to pH 7.5 with NaOH unless a different pH is indicated below).

27.3 Procedure

27.3.1 Construction of Vectors for the Co-expression of Periplasmic Chaperones

Co-expression the chaperones mentioned above can either be driven from expression cassettes within the same vector or from separate plasmids used in co-transformations. In the following, the design and cloning of such vectors is described.

27.3.1.1 Cloning of scFv Fragments from pAK/pJB into Vectors Overexpressing Periplasmic Chaperones (pHB110, pHB610, pJB33)

1. Excise the expression cassette coding for the scFv antibody fragment from the relevant pAK/pJB vector (described in detail in Chap. 3) by digestion with *Xba*I and *Hin*dIII. Use 2 µg purified plasmid DNA and incubate at 37°C for 2 h in a

total volume of 50 μl containing 5 μl 10 × NEBuffer 2 (NEB), 5 μl 10 × BSA, and 20 units of each *Xba*I (NEB) and *Hin*dIII (NEB).

Note: Procedure 27.3.1.1 describes the co-expression of periplasmic chaperones on the expression vector itself. These vectors have compatible restriction sites with the phage display vectors described in Chap. 3. The vectors differ (Fig. 27.1) in whether they also allow phage display and thus have a moderately strong translation initiation region (pHB110, pHB610), or only allow periplasmic expression and have a strong translation initiation region (pJB33), and in whether they co-express Skp or FkpA. All vectors have compatible restriction sites.

2. Digest appropriate amounts of vector (pHB110, pHB610, or pJB33) with *Xba*I and *Hin*dIII (removing the *tet*-cassette, see Fig. 27.1) for 2 h at 37°C under the same conditions as above. Also, dephosphorylate the cut vector by adding calf intestinal alkaline phosphatase (CIP, NEB; 0.5 unit/μg vector) to the digestion after 1 h.

   Note: Dephosphorylation should not be necessary because of the asymmetric overhangs. However, we always include this step to eliminate any risk of religation of single-cut vector.

3. Purify the digested scFv antibody genes and vector by preparative agarose gel electrophoresis in combination with the GenElute gel extraction kit (Sigma-Aldrich).

   Note: For pure preparations of a completely digested vector, it is very important not to overload the agarose gel. Furthermore, the gel electrophoresis has to be run long enough to separate small amounts of undigested or single-cut vector from the digested vector band.

4. Ligate 50 ng cut vector with the scFv expression cassette (molar ratio vector to insert 1:5) with 5 units T4 DNA ligase (NEB) in the presence of 1 × T4 DNA ligase buffer in 10 μl volume. Incubate for 2 h at room temperature or overnight at 16°C.

5. Transform 50 μl chemocompetent *E. coli* host cells suitable for periplasmic expression (e.g., JM83 (Yanisch-Perron et al. 1985), RV308 (Maurer et al. 1980), or SB536 (Bass et al. 1996)) with 5 μl of the ligation mix by heat-shock for 45 s at 42°C; add 500 μl of Luria–Bertani media (LB) media after 2 min incubation on ice and incubate for 60 min shaking at 37°C. Plate all transformed cells on LB, 1% glucose, chloramphenicol (30 μg/ml) agar plates and incubate overnight at 37°C.

   Note: JM83 is a generally robust strain that appears to lead to less lysis of the outer membrane upon periplasmic expression of some antibody fragments than some other strains. RV308 is a strain that produces very little (inhibitory) acetate during growth to high cell densities and thus supports fermentation very well. SB536 is deficient in two periplasmic proteases, HhoA (or DegQ) and HhoB (or DegS).

27.3.1.2 Cloning of *skp*/*fkpA* in Other Expression Vectors

Both the *skp* and *fkpA* genes can be conveniently obtained by digestion and purification from the vector pHB110 or pHB610, respectively (digested either

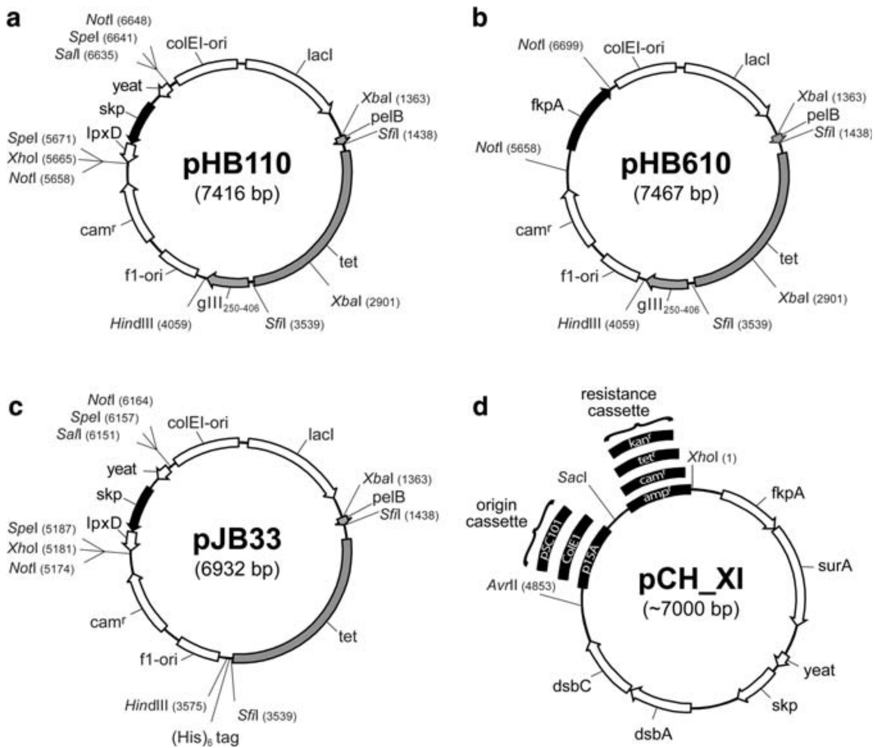

Fig. 27.1 *Vectors and cloning strategies*. The vectors pHB110, pHB610, and pJB33 all contain a chloramphenicol resistance gene (*cam*^{r}) as well as a tetracycline resistance "stuffer" cassette (*tet*, 2101 bp) (Krebber et al. 1997), which will be replaced by the antibody fragment (see Fig. 2.4). This stuffer is shown only schematically and contains the genes for *tetA* and *tetR* without making any fusion protein with upstream or downstream elements in the vector (for details, see Chap. 3). Vectors pHB110 and pHB610 allow either phage display (upon introducing an scFv cassette without stop codon, resulting in a fusion with the phage gene III) or periplasmic expression (if a stop codon is present at the end of the scFv gene), leading to moderate translation levels. In contrast, vector pJB33 leads to an enhanced periplasmic expression (due to the strong Shine–Dalgarno sequence SDT7g10 from T7 phage) and permits subsequent IMAC purification of the antibody fragment (see Chap. 3). Because of their compatible design, elements (e.g., the strong Shine– Dalgarno sequence) can be exchanged between vectors. (**a**) Vector pHB110 containing the *skp* cassette with flanking genes (in the form it was enriched during panning (Bothmann and Plückthun 1998)). This vector can also be used as a source of *skp* after digestion with *Not*I, *Spe*I, and *Sal*I/*Xho*I. (**b**) Vector pHB610 containing *fkpA*, excisable using *Not*I. (**c**) Vector pJB33 with stronger translation initiation region for high yield expression of scFv (see Chap. 3). (**d**) Schematic overview of the pCH series, encoding five different chaperones. As indicated, both the cassette for the origin of replication as well as that for the antibiotic resistance is exchangeable using *Avr*II/*Sac*I or *Sac*I/*Xho*I, respectively. *lpxD*: the first 65 aa of UDP-3-O-[hydroxymyristoyl]-glucosamine-*N*-acyltransferase, *yeat*: the last 49 aa of YeaT (outer membrane proteins involved in the insertion of other outer membrane proteins). The sequence of the vectors is available from the authors upon request

skp-for:	5'	NNN	NNN	XXX	XXX	GAT	CCA	AGC	AAT	ATC	CGT	ATG	TCT	GC	3'
skp-rev:	5'	NNN	NNN	XXX	XXX	TTA	TTT	AAC	CTG	TTT	CAG	TAC	GTC	GGC	3'
fkpA-for:	5'	NNN	NNN	XXX	XXX	GAT	TCA	CCT	CTT	TTG	TCG	AAT	GGT	C	3'
fkpA-rev:	5'	NNN	NNN	XXX	XXX	TTA	TTT	TTT	AGC	AGA	ATC	TGC	GGC	3	3'

Fig. 27.2 *Primers used for the amplification of skp and fkpA*. XXX XXX stands for the restriction site used for subcloning, while NNN NNN represents the additional bases flanking the restriction sites necessary for efficient cleavage (see e.g., the New England Biolabs catalog)

with *Not*I or alternatively with *Spe*I and *Xho*I/*Sal*I; see Fig. 27.1). By PCR amplification, new restriction sites can, of course, be added to insert them into any desired expression vector. These amplified fragments might thus also be useful for insertion into vectors used for expressing antibody fragments other than scFvs, and other periplasmic protein altogether (Fig. 27.1).

As neither for *skp* nor for *fkpA* the exact limits of their promoters have been experimentally verified, we recommend using the PCR primers specified in Fig. 27.2 for amplifying the genes (if they are to be expressed under their own promoters) from the vectors pHB110 or pHB610 or, alternatively, from genomic *E. coli* DNA:

1. Isolate genomic DNA from *E. coli* using the DNeasy Blood & Tissue Kit (Qiagen) as described by the manufacturer.
2. Perform PCR amplification of *skp* or *fkpA* with the above mentioned primers according to standard protocols.

   Note: We recommend using high-fidelity, proofreading polymerases (e.g., Phusion High-Fidelity DNA Polymerase from Finnzymes). At the beginning of the PCR reaction, the annealing temperatures for the above mentioned primers are set to the theoretical values of 56–59°C. However, we recommend increasing this temperature after the first five cycles (depending on the additional nucleotides added as overhang), as the amplified PCR product including this overhang will serve itself as template DNA for further amplification.
3. Digest the PCR product with the appropriate restriction enzymes, and ligate it into your favorite expression vector (also see steps 27.3.1.1.4–5).

27.3.1.3 Co-expression of Chaperones Encoded on a Second Plasmid

As an alternative to co-expressing the desired chaperones from the same plasmid, double transformation of *E. coli* hosts with two plasmids (one encoding the antibody fragment to be expressed and the second one harboring the genes for the chaperone(s)) is an option. However, the plasmids must possess different antibiotic resistance and preferentially different origin of replications, even though this is not strictly required when they are of high copy number (Velappan et al. 2007). The choice of different origins can be beneficial since chaperone expression can be tuned by differences in the plasmid copy number. Therefore, we used a modular design based on the pZ vector system developed by Lutz and Bujard (1997) for the pCH vector series leading to the constitutive overexpression of the chaperones

DsbA, DsbC, FkpA, SurA, and Skp. As mentioned above, this modular structure provides the chance to choose between the ColE1 origin of replication, (resulting in 50–70 intracellular plasmid copies), p15A origin (20–30 copies), and pSC101 origin (~10 copies), as well as cassettes encoding resistance to ampicillin, kanamycin, chloramphenicol, or tetracycline. These vectors should therefore be compatible with virtually any existing expression plasmid. For further details also refer to the legend of Fig. 27.1.

1. Transform suitable *E. coli* host cells with both plasmids, coding for the scFv and chaperones, respectively, as described in 27.3.1.1.5. For periplasmic expression, JM83 (Yanisch-Perron et al. 1985) is a robust host, but many other strains can be used (see 27.3.1.1.5).
2. Plate all transformed cells on LB, 1% glucose agar plates containing both appropriate antibiotics and incubate overnight at the desired temperature. It may be useful to test the effect of co-expression both at room temperature and at 37°C.

27.3.2 Small-Scale Expression of scFv Antibody Fragments

1. Inoculate 10 ml SB medium (per l, 35 g tryptone, 20 g yeast extract, 5 g NaCl, pH 7.5), containing the appropriate antibiotic(s) and 0.1% glucose, with a single colony of transformed *E. coli*, harboring the plasmid encoding the respective scFv fragment, and, if applicable, the plasmid co-expressing the chaperones. Grow the culture at 24°C and induce with 1 mM isopropylthiogalactoside (IPTG) (final concentration) at an OD_{600} of 0.5.

   Note: This procedure aims at analyzing the relative amounts of soluble protein for different constructs and/or chaperone co-expression.

   Note: The growth at room temperature is in general very beneficial for increasing the yield. At higher temperature, not only does a more significant portion of many antibody fragments end up in insoluble periplasmic fractions, but also incorrectly folded antibody fragments (or aggregates) interfere with membrane assembly, leading to an induced leakiness of the outer membrane and product loss.

   Note: Use only 0.1% glucose or less in the expression culture upon starting. This amount of glucose is enough to efficiently repress protein expression for 3–4 h until the culture has reached the OD required for induction. If higher concentrations of glucose are used, IPTG-induced protein expression might fail or be delayed.

   Note: When analyzing many constructs in parallel, it might be beneficial to grow overnight pre-cultures and inoculate the final expression culture at a starting OD_{600} of 0.1. This will lead to growth synchronization of the cultures and therefore synchronize the time points where the OD for IPTG induction has been reached.

2. Harvest the cells 4 h after induction by centrifugation (5,000 *g* for 10 min at 4°C).

   Note: This expression time is an average value, which depends on the aggregation properties of the construct and any proteolytic degradation, e.g., in linker regions of fusion proteins. Robust constructs can be expressed for longer times.

   Note: For troubleshooting, aliquots of the original culture and the supernatant after centrifugation should be kept and analyzed for scFv expression by SDS-PAGE and immunoblotting. These samples could pinpoint problems of the expression itself, compared to difficulties with the isolation and purification steps afterwards.
3. Resuspend the cells carefully in 0.5 ml pre-cooled extraction buffer on ice, and measure the OD_{600}. Do not lyse the bacteria. Add lysozyme (Sigma-Aldrich; 100 μg/ml) and incubate for 1 h on ice.

   Note: This procedure will destabilize both the *E. coli* peptidoglycan and the outer membrane, allowing soluble contents of the periplasm to leak out.
4. Centrifuge bacteria at 5,000 *g* for 10 min at 4°C and carefully transfer the supernatant (soluble periplasmic fraction) to a fresh Eppendorf tube.
5. Dissolve the pellet in 0.5 ml solubilization buffer (insoluble fraction).

   Note: This solubilization can be performed overnight, shaking at 4°C if necessary. This concentration of urea will in general be sufficient to dissolve periplasmic aggregates.
6. Normalize all fractions to the same OD_{600} of the original culture.

   Note: Make fractions comparable between cultures (correct for OD_{600}) and within a culture such that aliquots from the soluble and insoluble fractions can be compared easily.
7. For ELISA, coat suitable microtiter plates with the appropriate antigen overnight at 4°C according to standard protocols (see, e.g., Thorpe and Kerr 1994). Mix a defined amount of normalized soluble fraction with 2% skimmed milk in PBST and apply to the blocked ELISA plate. Subsequently, perform detection as, e.g., described in Thorpe and Kerr (1994).

   Note: If soluble antigen is available, include a competition ELISA control showing that free antigen is able to compete with bound antigen for binding to distinguish nonspecific "sticky" from specifically binding scFvs.
8. For western blot analysis, load defined amounts of soluble and insoluble protein fractions (also including samples taken in step 27.3.2.2 boiled in SDS-loading buffer to have a control of the total expression) on a 15% SDS-PAGE under reducing conditions. Perform standard immunoblotting according to the protocols described in Sambrook and Russell (2001).

   Note: To judge the effect of a construct or chaperone co-expression, it is important to evaluate both the total amount in the soluble fraction, as well as the ratio of soluble to insoluble protein.

   Note: The detected soluble protein may not necessarily be functional as it might consist of soluble aggregates (see next section).

   Note: Successful transport to the periplasm can be inferred from the correct processing of the signal sequence. This can be detected by the M1 antibody (Sigma-Aldrich) recognizing the processed FLAG tag at the very N-terminus

($^{+}H_3N$-DYKD...) (Knappik and Plückthun 1995), as the antibody does not recognize the tag when it is not at the N-terminus. This N-terminal short FLAG is present in the vector systems used here (this chapter, Chap. 3 and 7).

27.3.3 Large-Scale Expression

The single-chain Fv fragment carrying a C-terminal hexa-histidine tag (e.g., after expression from plasmid pJB33) can by purified by rapid two-column chromatography as described below (Sect. 27.3.3 and 27.3.4). This protocol is designed for 5–10 g wet weight of *E. coli* cells, corresponding to about 1 l of baffled shake-flask culture.

1. Inoculate a pre-culture of 10 ml SB medium, containing the appropriate antibiotic(s) and 1% glucose, with a single colony of *E. coli*, harboring the plasmid encoding the respective scFv fragment, and optionally a co-expression plasmid for chaperones. Incubate at 24°C overnight.
2. From this overnight culture, inoculate the main culture of 1 l SB medium containing 0.1% glucose at a starting OD_{600} of 0.1. Grow the culture at 24°C in a baffled shake flask for higher final cell densities and induce with 1 mM IPTG (final concentration) at an OD_{600} of 0.5.

   Note: Use only 0.1% glucose in the expression culture upon starting. This amount of glucose is enough to efficiently repress protein expression for 3–4 h until the culture has reached the OD required for induction. If higher concentrations of glucose are used, IPTG-induced protein expression might fail or be delayed.

   Note: The growth at room temperature is in general very beneficial for increasing the yield. At higher temperature, not only does a more significant portion of many antibody fragments end up in insoluble periplasmic fractions, but also incorrectly folded antibody fragments (or aggregates) interfere with membrane assembly, leading to an induced leakiness of the outer membrane and product loss.
3. Harvest the cells ca. 4 h after induction by centrifugation (5,000 *g* for 10 min at 4°C).

   Note: This expression time is an average value, which depends on the aggregation properties of the construct and any proteolytic degradation, e.g., in linker regions of fusion proteins. Robust constructs can be expressed for longer times. Ideally, this should be checked before on a small scale.
4. Resuspend the cell pellet in 40 ml 1× MHA buffer containing 0.5 M NaCl and add Benzonase (Merck) to a final concentration of 10 U/ml for removal of nucleic acids.

   Note: To reduce protein degradation, protease inhibitors can be added to the solubilized cells. Proteolysis is usually only an issue for some fusion proteins, especially with positively charged residues in or near the linker region. It should

be kept in mind that most scFv fragments are not readily degraded by proteases. The commercial protease inhibitor cocktails are mostly targeting eukaryotic proteases and are thus not very effective against *E. coli* proteases. Also, proteolysis, if it occurs by periplasmic enzymes, frequently begins during the induction phase, and can therefore only partially be combated with inhibitors.

5. Disrupt the cells using a French Press (20,000 psi, 4°C in a cold room) or the TS 1.1 benchtop. For the French Press, perform at least three passages for optimal lysis of the cells.

   Note: The large-scale protocol consists of a lysis of the whole cells, not a periplasmic extraction. The latter can be done as an alternative, but is usually more difficult to do reproducibly on large scales.
6. Centrifuge the crude extract in order to separate insoluble cell debris from soluble protein (20,000 *g*, 30 min at 4°C). Carefully separate supernatant from pellet and transfer it to a new tube.
7. Filter the supernatant through a 0.22-μm filter (use filters with low protein binding properties, e.g., Durapore filters from Millipore). Save an aliquot for subsequent analysis by SDS-PAGE.

27.3.4 Purification of scFv Fragments

The purification scheme described below includes immobilized metal affinity chromatography (IMAC) as the main step in combination with a directly coupled ion-exchange (IEX) chromatography for separation of the scFvs from bacterial proteins. It is, in general, difficult to get a very highly pure product after a single step of IMAC. Also, such preparations frequently contain a significant amount of RNA or DNA. This motivated the use of the coupled system (Fig. 27.3)

For IEX chromatography, calculate the isoelectric point (pI) of the scFv on the basis of its amino acid composition (e.g., using the website www.expasy.org/cgi-bin/protparam), as this value is important for deciding which ion exchange matrix and buffer system to use: for scFvs with pI values below 7.0 we recommend using an anion exchanger, while for values higher than 7.0, cation exchange chromatography should be performed. Purification on the BioCAD 700E (PerSeptive Biosystems, acquired by Applied Biosystems) over both columns can be done within only 30 min either by manual operation or by running a program automatically.

Note: If possible, perform all chromatography steps at 4°C. Use only buffers of highest purity, properly degassed and filtered (0.22 μm) prior to use. The system should be completely purged with 1× MHA buffer before the start of purification to avoid any air bubbles in the tubings, which might subsequently get trapped on the columns.

1. Prepare the Ni-IDA POROS MC column (having a column volume (CV) of 1.7 ml) by preloading it with 10 CV 200 mM $NiCl_2$ and subsequent washing with 10 CV sterile distilled water to remove the excess Ni^{2+} ions. Equilibrate the column with 10 CV 1× MHA buffer, 150 mM NaCl, pH 7.0.

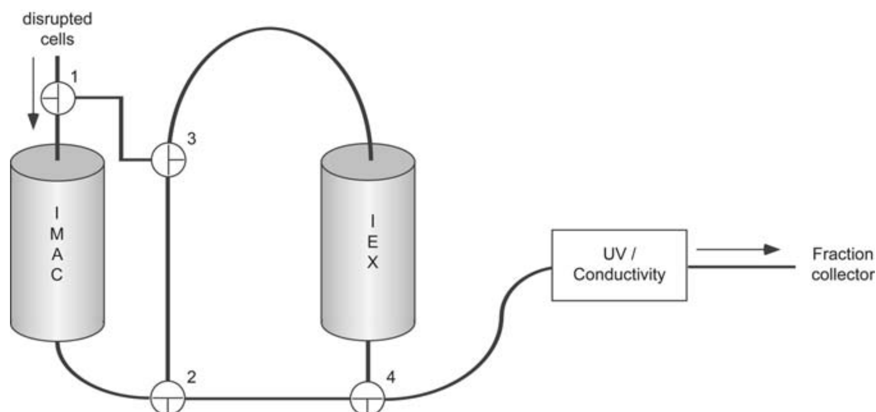

Fig. 27.3 *Tubing diagram for rapid two-column purification of antibody fragments*. The disrupted and filtrated cells are loaded onto the immobilized metal ion affinity chromatography (IMAC) column first. Upon antibody elution by increased imidazole concentration, the eluant flow is redirected onto the ion-exchange (IEX) column by turning valves 2 and 4. The adsorbed protein is finally eluted by applying a salt gradient and reversing/switching valves 1 and 3. Please note that it is essential that the imidazole used for elution does not have a high ionic strength, requiring that its pH is adjusted with acetic acid (see note in Sect. 27.2 "Materials")

Note: Similar chromatographic materials can be used with other chromatography systems.

2. Load the filtrated antibody sample onto the POROS MC column. During sample loading, the flow rate – otherwise being 3 ml/min – should be reduced to 1.5 ml per minute.
3. Wash the column with 15 CV 1 × MHA buffer containing 150 mM NaCl. The UV absorption signal at 280 nm should have reached its baseline by then.
4. Wash the column with 10 CV 1 × MHA buffer containing 30 mM NaCl, pH 7.0.
5. Wash the column with 10 CV 1 × MHA buffer containing 1 M NaCl, pH 7.0.

   Note: Washing with low and high salt concentrations assists in removing unspecifically bound material. If the protein of interest is present only in a small amount, several contaminating bacterial proteins can bind to the IMAC column under purification conditions and would finally coelute with the scFv if these stringent washing steps were omitted.
6. Wash column with 10 CV of 30 mM imidazole, 150 mM NaCl, pH 7.0.
7. Elute specifically bound scFv by either applying an imidazole gradient from 30 to 250 mM imidazole (pH 7.0) (no salt) (10 CV) or a step elution with 250 mM imidazole (pH 7.0) (no salt) (10 CV).
8. Directly load the elution on the downstream IEX column by using the BioCAD workstation or equivalent (for tubing diagram see Fig. 27.3). This column can either be a cation or an anion exchanger (see note at the beginning of this subsection).

Note: The pH for the following washing step and the final elution depends on the pI of the antibody fragment and on the type of the column used (i.e., if the antibody has a pI of 8.5, the pH should be adjusted to 7.0 and the sample should be applied to a cation exchange column; however, if the scFv fragment's pI is lower than 7.0, work with an anion exchanger at pH 8.0).

9. Wash the column with 1× MHA buffer, containing 30 mM NaCl, at the appropriate pH until the UV 280 nm baseline is reached.
10. Elute the scFv from the ion exchange column with a salt gradient from 30 to 750 mM NaCl with the appropriate pH (15 CV). Monitor the elution by its UV absorbance at 280 nm and collect 0.5 ml fractions. Analyze each of them by SDS-PAGE and pool those containing pure scFvs.

    Note: The imidazole stock solution used to elute the protein from IMAC must be pH-adjusted by using acetic acid and not with HCl, in order to keep the ionic strength low (otherwise, the protein might run through the coupled downstream ion exchange column).
11. Finally, determine the concentration of this protein solution using standard procedures, and store the purified scFv at 4°C after addition of 0.05% sodium azide. For long-term storage at −80°C, it might be beneficial to stabilize the purified scFv by adding human serum albumin to a final concentration of 10 mg/ml.

27.4 Comments

This part of the protocol contains general comments about the recommended standard method. The most critical steps were already highlighted directly following the instructions in the different subsections.

(a) The methods outlined in this chapter will almost certainly be used on antibody fragments that are intrinsically aggregation-prone, this being their main motivation. It must be kept in mind that a tendency for aggregation is an intrinsic property of the protein, and cannot be overcome upon successful expression by whatever method. Such antibody fragments tend to form *soluble* aggregates, and thus a mere inspection of soluble protein on western blots after expression may be very misleading. Molecular chaperones can prevent the formation of large, insoluble aggregates, but sometimes not of smaller, soluble aggregates. Therefore, a serious characterization of an scFv fragment must include gel chromatography, ideally coupled with multi-angle light scattering. This will give a very clear description of the amount of soluble aggregates in a preparation, or their development over time.

(b) Co-expressing Skp together with an antibody fragment might sometimes result in a prolonged lag phase and slower doubling time of the bacterial cells. However, upon reaching an OD_{600} of 0.8, these cells recover, possess a higher doubling rate, and finally lead to higher yield of recombinant protein.

(c) In contrast to the production of poorly folding antibodies in the absence of chaperone, scFv expression in their presence also offers the advantage of the ability to increase the time of expression. As chaperone expression results in less cell lysis, the final cell density can be increased, which also results in increased total scFv yield.
(d) We previously demonstrated that the co-expression of the periplasmic PPIase SurA produced no increase in the functional scFv fragment level in the periplasm, at least for the scFv fragments tested (Bothmann and Plückthun 2000; Ramm and Plückthun 2000). However, we decided to retain its gene in the pCH vector series, as we did not observe any disadvantage of SurA expression. In addition, we wanted these vectors to be as generally applicable as possible, also being able to assist the folding of proteins other than scFv.
(e) As most of the *E. coli* host proteins co-purified in IMAC have a pI of less than 6.5, they will bind to anion-exchange columns. Therefore, these columns can also be used in an inverse setup for scFv constructs with high pI, trapping the *E. coli* proteins while leaving the scFv in the flow-though.
(f) As imidazole slowly catalyzes the hydrolysis of acid labile bonds and can interfere with many subsequent assays, its presence is not ideal for long-term storage. Therefore, the two-step method presented in this protocol helps as built-in buffer exchange. Alternatively, the IMAC eluate can be dialyzed against a physiological buffer such as PBS immediately after purification.

Acknowledgments This protocol has evolved over the years, and heavily relies on the research and the original versions developed by Hendrick Bothmann.

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