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Production of single-chain Fv monomers and multimers

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1. Introduction

The Fv fragment (25 kDa) is a two-chain heterodimer of the antibody variable domains (V_L and V_H) and is regarded as the minimal structural component of an antibody required for antigen-binding activity. Fvs have been found to dissociate into V_L and V_H domains at low protein concentrations and under physiological conditions (1). This limited stability may be overcome by incorporation of a designed linker peptide to bridge isolated V_L and V_H domains into a single polypeptide (2). Basic research on recombinant single-chain Fv (scFv) proteins produced from *Escherichia coli* has recently provided some answers to at least two basic questions about this new antibody technology. First, may problems arise from linker obstruction of either antigen-binding sites or domain folding? Second, will scFv proteins display suitable *in vivo* stability, targeting, clearance, and tissue penetration?

Several antigen-binding studies reported for scFv proteins recognizing hapten antigens, polypeptide antigens, carbohydrate antigens, and tumour-associated antigens indicate that a similar monovalent binding activity and either equivalent or somewhat reduced K_a values, may be retained in diverse scFv proteins when compared to the monoclonal antibodies (mAbs) from which they were derived (3, 4). As further evidence for the identity of the binding site in mAbs and scFv proteins, an scFv version of a catalytic mAb was reported to produce nearly equivalent kinetic parameters (5). Finally, structural studies using NMR spectroscopy (6) or X-ray crystallography (7, 8) also support the fidelity of the scFv binding site.

scFv proteins lack the constant domains and bivalency of mAbs. mAbs (150 kDa) are six-fold larger than scFv proteins and display high avidity for repetitive epitopes. When compared to mAbs *in vivo*, anti-tumour scFv proteins clear more quickly from the blood and penetrate tumours with a rapid and even distribution (9, 10). A major application of scFv proteins will be in the production of recombinant single-chain immunoeffector proteins which link an scFv derived cell binding specificity to an effector protein domain,

such as *Pseudomonas* exotoxin A. Single-chain immunotoxins have been shown to regress established tumours in rats and mice (11, 12). Furthermore, since scFv technology allows single transcript expression of antibody specificities *in vivo*, single-chain Fv constructions may be valuable in gene therapy applications of intracellular antibodies (13) or targeted viral vectors (14).

Commercialization of scFv technology will require large-scale protein production and purification. It would be valuable to develop a generic production process protocol, lacking affinity chromatography or proteolytic clipping steps, which might be applied to diverse scFv proteins including scFv expressed as insoluble proteins. In this chapter we describe our protocols for the design, construction, and purification of single-chain Fv proteins. We also describe the phenomenon of aggregation of scFv proteins to form multivalent Fv. Although the presence of scFv dimers and higher aggregates can be troublesome in the purification of scFv monomers, these stable Fv multimers have recently been identified as rearranged multivalent antibody fragments which may significantly extend scFv technology into bispecificity and cross-linking capabilities (15–17).

2. Linker designs

In natural antibodies, the V_L and V_H domains associate through non-covalent interactions. The designed linker polypeptide in scFv proteins creates a single subunit protein with two variable domains. In the description of these recombinant antibody sequences, we will follow the numbering system of Kabat *et al.* (18).

Since selected scFv linkers were initially designed to covalently connect the two variable chains without participation in the final Fv conformation, it could be predicted that active single-chain Fv proteins can be constructed in either orientation. Either V_L is the N-terminal domain followed by the linker and V_H (V_L–linker–V_H construction) or V_H is the N-terminal domain followed by the linker and V_L(V_H–linker–V_L construction). A retention of monovalent binding specificities and affinities in both types of purified recombinant scFv proteins has been reported (2–4). The choice of variable domain orientation may be relevant to optimal binding activity and this may be characteristic of individual Fv binding sites and selected linkers. For example, Desplancq *et al.* (17) report binding activity for B72.3 scFv to be dependent on variable chain order and upon linker length. Batra *et al.* (19) found that variable domain orientation did not affect the cytotoxic performance of an anti-Tac scFv-based immunotoxin.

The polypeptide linker sequences in scFv proteins were designed to span the ~3.5–4.0 nm between the C terminus of V_L and the N terminus of V_H, or between the C terminus of V_H and the N terminus of V_L. To provide flexibility, the linkers may include a motif of alternating G and S residues. This rationale is followed in the $(G_4S)_n$ linkers described by Huston *et al.* (4) and

Table 1. Linker design in V_L–linker–V_H constructions

Linker name	Linker length[a]	Linker sequence[a]	Major oligomer forms of scFv	Reference
212	14	GSTSGSGKSSEGKG	Monomers, multimers	16, 21, 22
216	18	GSTSGSGKSSEGSGSTKG	Monomers	16, 21
217	12	GSTSGKPSEGKG	Multimers	16
218	18	GSTSGSGKPGSGEGSTKG	Monomers	21

[a] Linker peptide connects V_L residue 107 to V_H residue 1 (18)

serine-rich linkers described by Dorai *et al.* (20). However, our current linker designs include three charged residues (K, K, E) to enhance the solubility of the linker and its associated scFv. One of the K residues is placed close to the N terminus of V_H, to replace the positive charge lost when forming the peptide bond between the linker and the V_H. An additional consideration in linker design relates to proteolytic stability. Our studies with several scFv linkers that included the above selected characteristics have shown that protease susceptible sites may be identified and corrected. For example, the proteolytic clip in the 212 linker which occurred between K8 and S9 of the linker (see *Table 1*), may be protected by placing a proline residue at the position succeeding the K8 residue (21).

Single-chain Fv proteins are known to aggregate and form multimeric species. Recent reports (8, 15–17) have provided a better understanding of these aggregates. Multimeric scFv proteins result from intermolecular V_L/V_H pairings between two or more scFv polypeptides that produce multivalent Fv molecules from monovalent scFvs (see *Figure 1*). The degree of multimer Fv formation is linker dependent. In general, longer linkers have a decreased aptitude to form multimeric Fv. An scFv protein with a short linker (0–10 residues), which hinders intramolecular V_L/V_H domain pairing, forms predominately multimeric Fv (15). *Table 1* shows the linkers which we have recently utilized in *in vitro* and *in vivo* studies (5, 9, 10, 16, 21, 22). Since multivalent Fv formation is dependent upon individual variable domain sequences and buffer composition, as well as linker length, these are also important considerations in the interpretation of antigen-binding assays (16–17).

3. scFv gene construction from hybridoma cells

Large synthetic repertoires of variable genes which have been produced by bacteriophage surface display are increasingly the starting source for the isolation of an antibody specificity (Chapters 1 and 2, ref. 23). However, many researchers will wish to develop an scFv protein from a specific mAb produced by a hybridoma cell line. Sequence-specific DNA synthesis from mRNA isolated from mAb-producing hybridoma cells may be performed

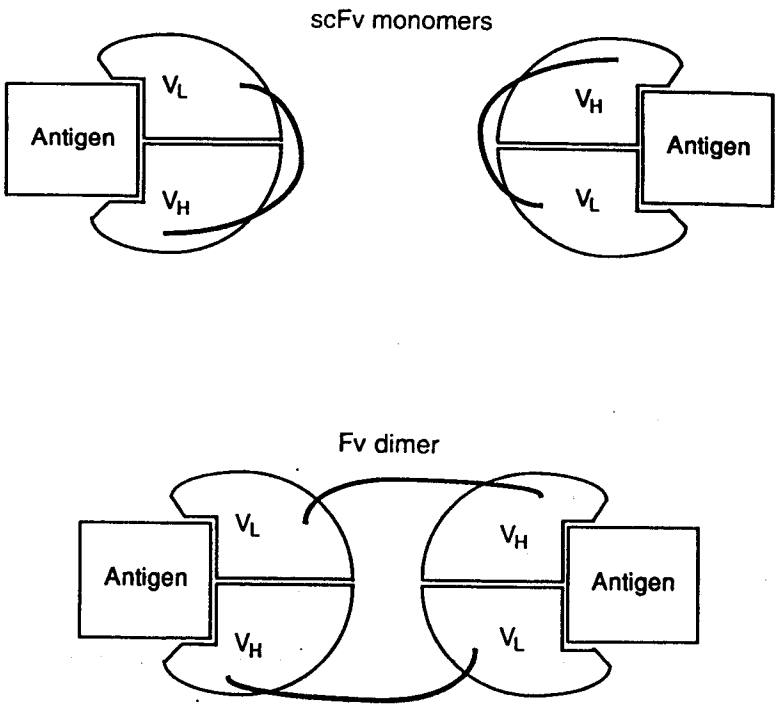

Figure 1. Schematic depiction of V_L/V_H rearrangement model. Top: Two single-chain Fv proteins bound to antigens. Linker peptides shown in bold print. Bottom: An scFv dimer with interchain V_L–V_H interfaces formed by V_L and V_H domains from two scFv monomer polypeptides.

using genetic cloning strategies which exploit the conserved sequences present in the signal and constant (C_H1 or C_L) domains which flank the variable domains, or alternatively, use the FR1 and FR4 framework regions which are on the boundary of either variable domain (see Chapters 1 and 6). Collections of oligonucleotide primers which are designed to prime synthesis into mouse or human antibody variable regions have been reported (e.g. ref. 23).

Whether PCR, oligonucleotide-directed mutagenesis, or wholly synthetic approaches are employed in scFv gene synthesis, it is convenient to design desired sequence features into the synthetic primers. The linker sequence itself and compatible restriction sites for insertion into the selected expression plasmid may be included in the oligonucleotides used. In the examples given below, the scFv gene has an *Aat* II site at the 5′ end; *Hin*dIII and *Pvu*II sites flank the linker; and a *Bam*HI site follows the translational stop codons. *Figure 2* shows the completed gene construction of an scFv protein derived from mAb CC49 which is currently in clinical trials for radioimmunodetection of colorectal cancer (9, 10). A PCR gene assembly method using 'splicing by overlap extension' (24–26) is one convenient approach to scFv gene synthesis. This general method can be used with previously isolated V_L and V_H cDNA clones or from total hybridoma first-strand cDNA. *Table 2* displays examples of oligonucleotide primers used in this protocol. Primers 1 and 2 are complementary to the FR1 and FR4 boundaries, respectively, of a specific

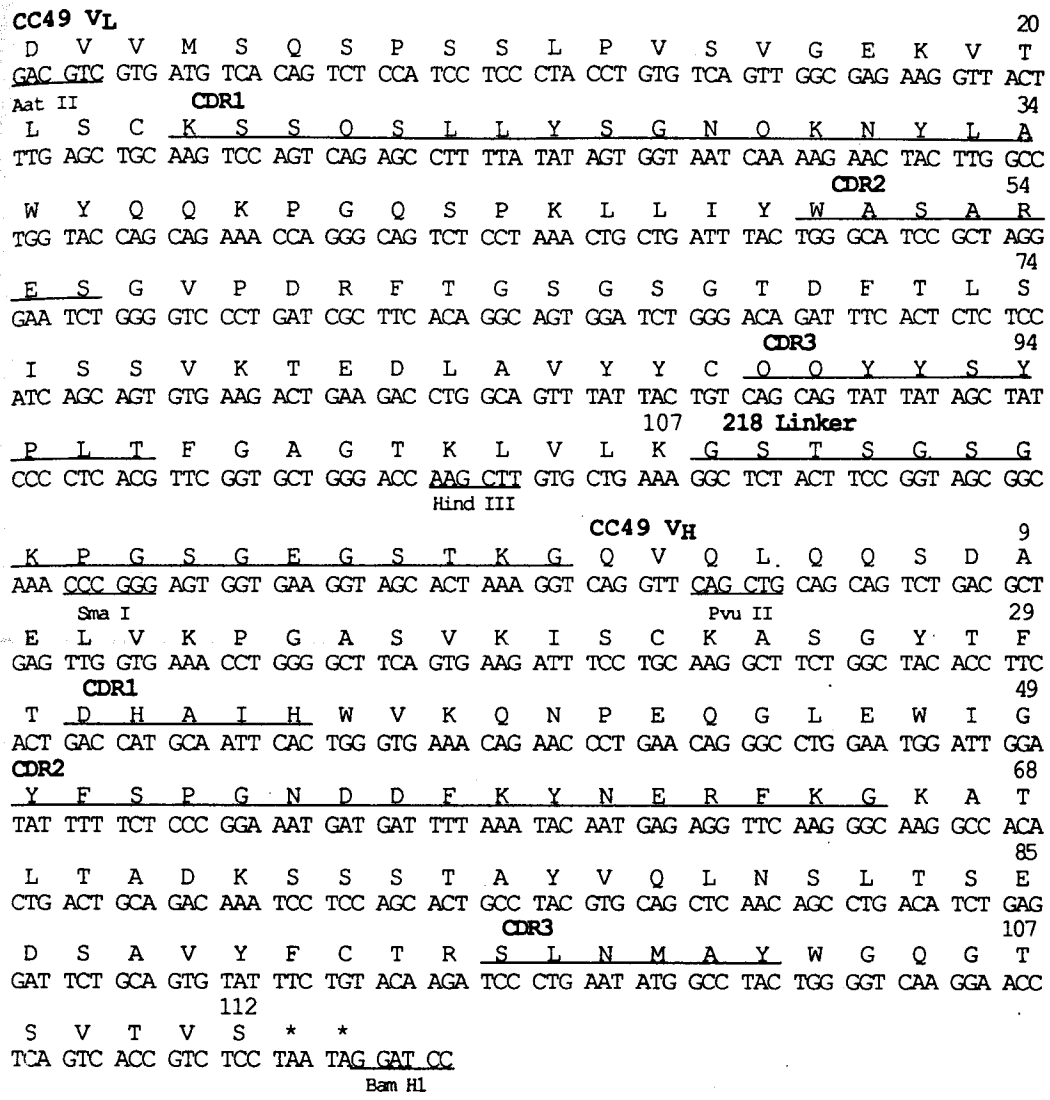

Figure 2. DNA sequence of CC49/218 scFv gene in V_L–218 linker–V_H orientation. The translated variable region sequences are numbered according to Kabat *et al.* (18).

mouse *kappa* V_L gene. Primers 3 and 4 are complementary to the FR1 and FR4 boundaries, respectively, of a specific mouse V_H gene. Primers 2 and 3 also have complementary 5′ extensions which encode the scFv linker segment. The PCR amplification may be performed in a single tube first with all four primers, followed by a second PCR assembly using only the two outside primers 1 and 4 (see *Protocol 1*). Alternatively, the use of separate PCR tubes for the V_L and V_H gene amplifications may simplify the optimization of reaction conditions. The optional, engineered *Hin*dIII and *Pvu*II sites, which facilitate subsequent linker interchange, do not alter the protein sequence. The N terminal *Aat* II site can be fused to the *omp*A signal peptide for expression in *E. coli* (see Section 5). Selection of alternate or degenerate PCR primers for various mammalian variable region subgroups is

Table 2. Examples of PCR primers used in CC49/218 scFv assembly[a]

V_L forward *Primer 1*	5'AACACCGACGTCGTGATGTCACAGTCTCCATCCTC 3', *Aat* II
Linker/V_L back *Primer 2*	5'CACCACTCCCGGGTTTGCCGCTACCGGAAGTAGAGCCTTTC-AGCACAAGCTTGGTCCCAGCACCGAACGTG 3', *Sma* I, *Hind*III
Linker/V_H forward *Primer 3*	5'AGCGGCAAACCCGGGAGTGGTGAAGGTAGCACTAAAGGTC-AGGTTCAGCTGCAGCAGTCTGACGCTGAG 3', *Sma* I, *Pvu*II
V_H back *Primer 4*	5' GCGGATCCTATTAGGAGACGGTGACTGAGGTTCC 3', *Bam*HI

[a] Restriction sites underlined and listed after sequence.

facilitated by the availability of antibody variable gene sequence databases (see, e.g., Chapter 6 and ref. 18). Direct RNA sequencing of V_L and V_H regions from hybridoma mRNA (27) can rapidly provide nucleotide sequence information useful for PCR primer design in subsequent scFv gene assembly.

As an alternative to PCR, further engineering of the cloned scFv gene can be readily accomplished by multiple primed oligonucleotide-directed mutagenesis using the Sculptor™ system (Amersham Corporation) as described (5, 26). We have found that two independently primed mutations can be simultaneously introduced at > 95% frequencies using this convenient commercial system.

Protocol 1. Polymerase chain reaction assembly of scFv gene

Equipment and reagents

- Agarose gel electrophoresis equipment
- PCR thermocycler instrument (Perkin Elmer Cetus, *GeneAmp* 9600)
- 0.2 ml reaction tubes (e.g. Perkin Elmer MicroAmp Tubes, cat. no. N801–0540)
- MuLV reverse transcriptase (BRL or Perkin Elmer)
- *AmpliTaq* DNA polymerase (Perkin Elmer Cetus)
- Restriction enzymes (Boehringer Mannheim)
- Random hexanucleotides $pd(N)_6$ (Boehringer Mannheim)
- Oligonucleotide primers 1, 2, 3, and 4 (see *Table 2*)
- NuSieve GTG agarose (FMC Corporation)
- TAE buffer: 40 mM Tris–acetate, 1 mM EDTA, pH 7.5
- Ethidium bromide
- RNasin (Promega)
- RT buffer: 10 mM Tris–HCl (pH 8.3), 1 mM DTT, 50 mM KCl, 5 mM $MgCl_2$, 1 mM dATP, 1 mM dGTP, 1 mM dCTP, 1 mM TTP
- PCR buffer I: 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$
- PCR buffer II: 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dATP, 0.2 mM dGTP, 0.2 mM dCTP, 0.2 mM TTP
- Ethidium bromide
- Phenol
- Chloroform
- Ethanol

Method

1. Combine, in a 0.2 ml tube, 1 μg of purified hybridoma mRNA, 50 pmol of $pd(N)_6$ primers, 50 U of *MuLV* reverse transcriptase, 20 U of RNasin in RT buffer to a total volume of 20 μl. Incubate for 10 min at 22°C.

2. Incubate for 1 h at 42°C, heat to 99°C for 5 min, and cool to 4°C.
3. Add 80 µl PCR buffer I containing 30 pmol of each of the four PCR primers (*Table 2*).
4. Heat the reaction mixture for 2 min at 95°C.
5. Add 2.5 U *AmpliTaq* DNA polymerase to the reaction mixture at 95°C.
6. Perform PCR synthesis in the thermal cycler. Each cycle consists of 30 sec denaturation at 95°C, 45 sec annealing at 62°C, and 30 sec extension at 72°C. Repeat each cycle 30 times and follow by a 5 min extension at 72°C. The optimal annealing temperature may be between 52°C and 72°C and can be determined empirically.
7. Remove a 1 µl aliquot from step 6 and add to 99 µl of PCR buffer II containing 30 pmol each of primer 1 and primer 4 (*Table 2*).
8. Perform the second PCR synthesis following steps 4, 5, and 6 in order.
9. Extract the PCR products with phenol and chloroform and precipitate the DNA with ethanol. Digest the resuspended DNA with *Aat*II plus *Bam*HI and purify the ~740 bp fragment by 4% NuSieve agarose electrophoresis in TAE buffer containing ethidium bromide (0.5 µg/ml).
10. Ligate, 'in gel' (3, 5), the fragment to the selected vector (see *Figure 3*).
11. Transform *E. coli* bacteria with the ligated DNA.

4. Expression of a single-chain Fv in *E. coli*

Since the first report of a low-yield secretion of functional scFv from *E. coli* (1), there have been continued efforts to achieve high yield (g/L) heterologous expression of scFv proteins which are soluble, active, and stable. Yeast (25), plants (28), baculovirus (29), bacillus (30), and mammalian cells (20) are all possible hosts. However, most work to date on scFv protein expression has employed *E. coli* systems. High-level cytoplasmic or periplasmic expression of scFv or scFv fusion proteins in *E. coli* may produce insoluble aggregates which can be renatured and purified. We will describe our *E. coli* production system which has provided us with 100 mg quantities of active clinical grade scFv protein following a protocol of dissolution, renaturation, and purification from an insoluble initial protein extract. Alternatively, research grade scFv proteins may be produced and rapidly purified using an engineered affinity tag (31) or fusion partner (32). For example, we have purified crude denatured or renatured scFv containing an engineered 5 histidine C terminal tail to about 90% purity on the nickel affinity resin Ni–NTA

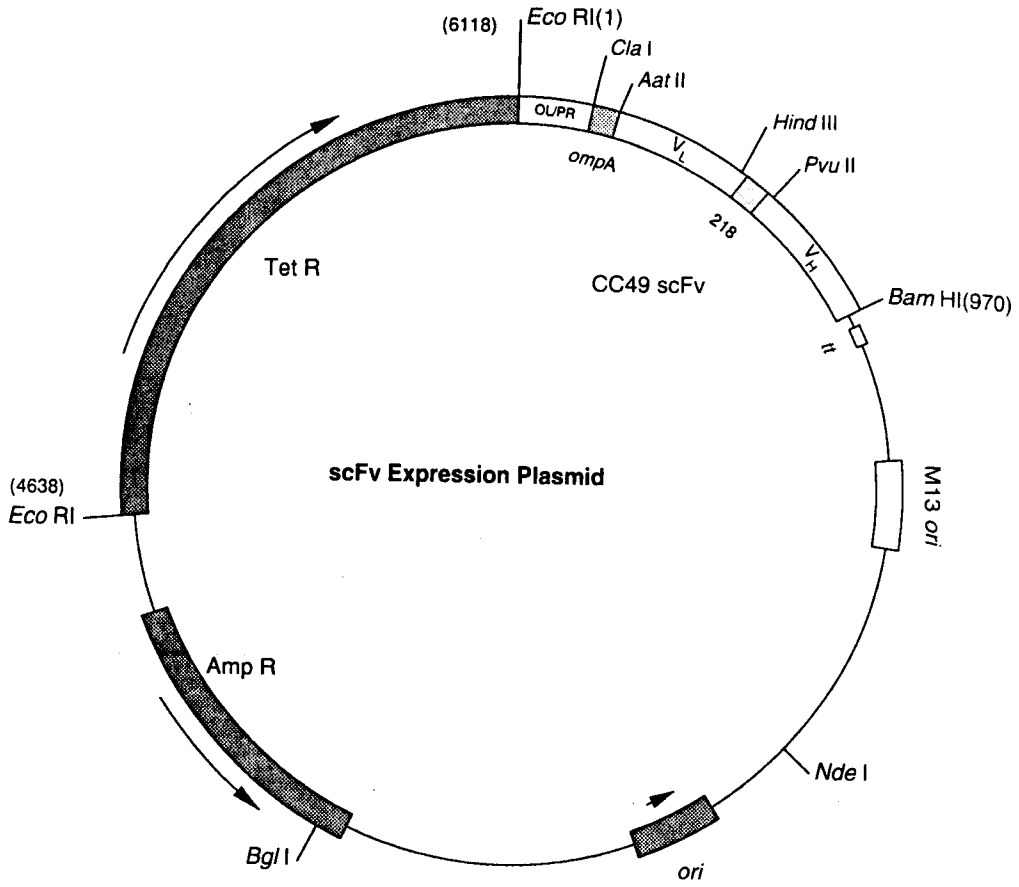

Figure 3. Enzon expression vector used for scFv production in *E. coli*. Contains the hybrid lambda phage promoter O_L/P_R, the *ompA* signal sequence, and a V_L–linker–V_H scFv gene.

(QIAGEN). However, we have thus far avoided affinity chromatography and fusion protein cleavage steps in the production of clinical grade samples which require stringent process validation.

The Enzon expression vector used for scFv expression in *E. coli* contains the hybrid lambda phage promoter O_L/P_R and the *ompA* signal sequence (see *Figure 3*). To produce the final expression strains, the completed scFv expression vectors are transformed into *E. coli* host strain GX6712 which contains the gene for the cI*ts*857 temperature-sensitive repressor. This provides a transcriptional regulation system in which induction of scFv synthesis occurs by raising the culture temperature from 32 °C to 42 °C. Fifteen independent murine-derived and one rat-derived scFv proteins have been expressed at 5–20% of total cell protein using this expression system. N terminal amino acid sequence analysis has confirmed the predicted signal sequence removal. High-level expression of secreted proteins in *E. coli* may result in the formation of protein aggregates in the periplasmic space. Since our expressed scFv proteins accumulate as insoluble aggregates, denaturation and refolding are required for purification (see Section 5).

Because expression of scFv proteins fused to the *omp*A signal sequence has resulted in mature scFv production at high levels for several distinct scFv proteins, we have included the signal sequence as part of our standard expression system.

5. Fermentation, renaturation, and purification of an scFv protein

In most reports, scFv and scFv fusion proteins have been produced in *E. coli* as insoluble aggregates that are subjected to denaturation and refolding prior to purification. Our heat-induction process (Section 4) results in partially lysed *E. coli* and the sedimented cellular debris also contains all of the expressed, insoluble scFv protein. When using alternate cytoplasmic expression systems, the insoluble scFv may accumulate in intracellular inclusion bodies. The purification process described here may be adapted to insoluble scFv derived from various crude extracts. In general, the insoluble pellet has been resuspended in a denaturant such as guanidine or urea and diluted 10- to 10000-fold with a renaturation buffer. We describe a generic production and purification protocol, which involves refolding and ion-exchange HPLC chromatographic steps, that we have used successfully on over ten different scFv proteins.

Protocol 2 presents a typical fermentation process for an *E. coli* GX6712 strain containing an scFv expression vector (*Figure 3*).

Protocol 2. Fermentation of scFv protein from *E. coli*

Equipment and reagents

- Chemap laboratory fermenter, 10 litre working volume (Chemapec Inc.)
- Inoculum (*E. coli* expression strain)
- M-63 (1 litre): 0.57 g H_3BO_3, 0.39 g $CuSO_4 \cdot 5H_2O$, 5 g $FeCl_3 \cdot 6H_2O$, 4 g $MnCl_2 \cdot 4H_2O$, 0.5 g $NaMoO_4 \cdot 2H_2O$, 5 g NaCl, 1 g $ZnSO_4 \cdot 7H_2O$, 2.9 ml H_2SO_4
- 5 M NaOH
- 2 M H_3PO_4
- Production medium (1 litre): dissolve in about 900 ml water, 3 g $(NH_4)_2SO_4$, 2.5 g K_2HPO_4. 30 g casein CE90MS (Deltown Specialties), 0.25 g $MgSO_4 \cdot 7H_2O$, 0.1 mg $CaCl_2 \cdot 2H_2O$, 10 ml M-63 salt concentrate, 0.2 ml MAZU 204 Antifoam (Mazer Chemicals). Adjust to pH 7.4 and bring to volume. Autoclave. Then add 30 g glucose, 0.1 mg biotin, 1 mg nicotinamide, 100 mg ampicillin (omit when inoculum is grown in LB medium plus tetracycline).

Method

1. Prior to fermentation, inoculate 0.5 litre of modified LB medium containing either 50 mg ampicillin or 25 mg tetracycline with one frozen 1 ml vial of *E. coli* expression strain. Shake flask at 32°C for 10 h.
2. Inoculate 9.5 litres of production medium in the Chemap fermenter with 0.5 L of inoculum.

Protocol 2. *Continued*

3. Adjust running parameters:
   (a) pH 7.2 ± 0.1 with titrants (5 M NaOH; 2 M H_3PO_4)
   (b) 1 volume air per volume medium per min aeration
   (c) 800 r.p.m. agitation
   (d) 32°C temperature.
4. At an absorbance at 600 nm of 18–20, raise fermentation temperature to 42°C. This temperature shift is achieved in 2 min in the Chemap unit.
5. At 1 h post shift-up, cool fermentation to 10°C, then harvest cell paste at 7000 *g* for 10 min. The wet cell paste can be stored at −20°C. Approximately 200–300 g of wet cell paste is normally recovered from a 10 litre fermentation.

Protocol 3 describes the resolubilization and renaturation of the scFv protein from the frozen cell pellet.

Protocol 3. Solubilization of scFv protein from *E. coli* cell paste

Equipment and reagents

- Cell homogenizer
- Chilling coil (Lauda/Brinkman)
- RC-5B centrifuge (Sorvall)
- Tissue homogenizer (Heat Systems Ultrasonics)
- UV spectrophotometer
- Cell lysis buffer: 50 mM Tris–HCl, 1.0 mM EDTA, 100 mM KCl, 0.1 mM PMSF (phenylmethylsulfonyl fluoride), pH 8.0
- Denaturing buffer: 6 M guanidine hydrochloride, 50 mM Tris–HCl, 10 mM $CaCl_2$, 50 mM KCl, pH 8.0
- Refolding buffer: 50 mM Tris–HCl, 10 mM $CaCl_2$, 50 mM KCl, 0.1 mM PMSF, pH 8.0
- 0.45 μm microporous membranes (Millipore, cat. no. HVLP0005)

A. *Cell lysis*

1. Thaw the cell paste from a 10 litre fermentation (200–300 *g*) overnight at 4°C.
2. Gently resuspend the wet cell paste in 2.5 litres of the cell lysis buffer at 4°C.
3. Pass the cell suspension through a Manton-Gaulin cell homogenizer three times. Because the cell homogenizer raises the temperature of the cell lysate to 25 ± 5°C, the cell lysate is cooled to 5 ± 2°C with a Lauda/Brinkman chilling coil after each pass.

B. *Washing the cell pellet*

1. Centrifuge the cell lysate at 24 300 *g* for 30 min at 6°C. Discard the supernatant, for the pellet contains the insoluble scFv.

2. Wash the pellet by gently scraping it from the centrifuge bottles and resuspending it in 1.2 litres of cell lysis buffer.
3. Repeat steps 1 and 2 as many as five times. At any time during this washing procedure the material can be stored as a frozen pellet at –20°C.

C. *Solubilization and renaturation of the scFv protein*

1. Solubilize the washed cell pellet in freshly prepared denaturing buffer at 4°C, using 6 ml of denaturing buffer per gram of cell pellet. If necessary, a few quick pulses from a tissue homogenizer can be used to complete the solubilization.
2. Centrifuge the resulting suspension at 24 300 *g* for 45 min at 6°C and discard the pellet.
3. Determine the optical density at 280 nm of the supernatant. If the OD_{280} is above 30, additional denaturing buffer should be added to obtain an OD_{280} of approximately 25.
4. Slowly dilute the supernatant into cold (4–10°C) refolding buffer until a 1:10 dilution is reached. We have found that the best results are obtained when the supernatant is slowly added to the refolding buffer over a 2 h period, with gentle mixing.
5. Allow the solution to stand undisturbed for at least a 20 h period at 4°C.
6. Filter the solution through 0.45 µm microporous membranes at 4°C.
7. Concentrate the filtrate to about 500 ml at 4°C.

The fraction of multimer can be increased by treating the solubilized scFv solution from *Protocol 3* with 20% ethanol. The ethanol treatment results in some precipitation which must be filtered prior to purifying the scFv multimers. The exact ethanol concentration required can vary depending on the scFv.

Protocol 4. 20% ethanol treatment of the solubilized scFv, used to increase the fraction of multimers

Equipment and reagents

- Solubilized scFv solution from *Protocol 3*
- Ethanol
- 0.45 µm microporous membrane filter (Millipore, cat. no. HVLP0005)

Method

1. Under slow mixing add sufficient quantities of pure ethanol to bring the solubilized scFv solution to 20% ethanol. Sixteen litres of solubilized scFv would require 4 litres of ethanol.

Protocol 4. *Continued*

2. Allow the solution to stand undisturbed for at least 2 h. Longer periods of time (12–18 h) allow the precipitate to settle to the bottom of the tank, which makes the filtration easier.
3. Filter the solution through a 0.45 μm microporous membrane. Drawing the solution from the top of the tank after the precipitate has settled avoids premature clogging of the filter.
4. Discard the precipitate.

Since most scFv proteins have an isoelectric point between 8.0 and 9.4, cation-exchange chromatography is an appropriate purification approach. *Protocol 5* describes the purification methods by cation-exchange HPLC and the further characterization of the scFv by size-exclusion HPLC. The purification protocol yields scFv proteins that are greater than 95% pure as examined by SDS-PAGE. We have observed that the Amp^r gene product β-lactamase co-migrates with some scFv proteins on SDS-PAGE. This can have relevance in attempts to quantitate expression yields by gel analysis.

Protocol 5. HPLC purification of scFv protein

Equipment and reagents

- HPLC buffer A: 60 mM Mops, 0.5 mM Ca acetate, pH 6.4
- HPLC buffer B: 60 mM Mops, 10 mM Ca acetate, pH 7.5
- HPLC buffer C: 60 mM Mops, 100 mM $CaCl_2$, pH 7.5
- HPLC buffer D: 50 mM Mops, 100 mM NaCl, pH 7.5
- Conductivity meter
- UV spectrophotometer
- PolyCAT A column (Poly LC Inc., Columbia, MD)
- Pre-cast 4–20% acrylamide SDS-PAGE slab gels (Novex)
- TSK G3000SW column (Toso Haas)
- Waters HPLC system (Millipore)
- MacIntosh SE (Apple Computer)
- Dynamax software package (Rainin Instrument Co.)

A. *Cation-exchange HPLC purification*

1. Dialyse the renatured scFv solution against HPLC buffer A, until the conductivity is lowered to that of buffer A.
2. Equilibrate the 21.5 mm × 150 mm polyaspartic acid PolyCAT A column with HPLC buffer A for 20 min.
3. Load the dialysed sample on the PolyCAT A column. If more than 60 mg is loaded on this column the resolution begins to deteriorate, thus the sample must usually be divided into several PolyCAT A runs.
4. Determine optical density at 280 nm and calculate the protein concentration. Most scFv proteins have an extinction coefficient of about 2.0 mg ml^{-1} cm^{-1} at 280 nm and this can be used to determine the protein concentration.

5. Elute the sample from the PolyCAT A column with a 50 min linear gradient between HPLC buffers A and B. Most of the monomer scFv proteins that we have purified elute between 20 and 30 min using this gradient, while multimers elute later. We normally collect 3 min fractions.
6. Apply a final 6 min linear gradient at 15 ml/min (90 ml) to the PolyCAT A column to remove the remaining protein with HPLC buffer C. 50 ml fractions are analysed.
7. Analyse the collected fractions on 4–20% Tris–glycine SDS-PAGE gels.

B. *Size-exclusion (SE) HPLC characterization*

1. Equilibrate the 7.8 × 300 mm TSK G3000SW column with HPLC buffer D for 20 min.
2. Load 10–50 µl samples on the SE-HPLC column.
3. Elute the column with HPLC buffer D at a flow rate of 0.5 ml/min. Most of the scFv proteins that we have examined elute at 17–21 min.
4. Collect data using Dynamax software package.

Figure 4A shows an example of a PolyCAT A HPLC where the separation of monomeric and dimeric scFv molecules is apparent. *Figure 4B* displays a representative SE-HPLC chromatograph of a highly aggregated scFv where monomer, dimer, and trimer species are identified. Note that in both examples, the scFv proteins contain the 212 linker (see *Table 1*). scFv aggregates have recently been shown to contain intermolecular V_L/V_H pairs from rearranged scFv proteins (15, 16). Multivalent Fv stability is dependent on individual V_L and V_H sequences as well as linker length. Disassociating agents such as 0.5 M guanidine hydrochloride with 20% ethanol can catalyse an interconversion between monomeric and dimeric scFv species (16). Significantly, the multivalent Fv represents a novel antibody fragment which provides new avidity and bifunctionality possibilities to scFv technology.

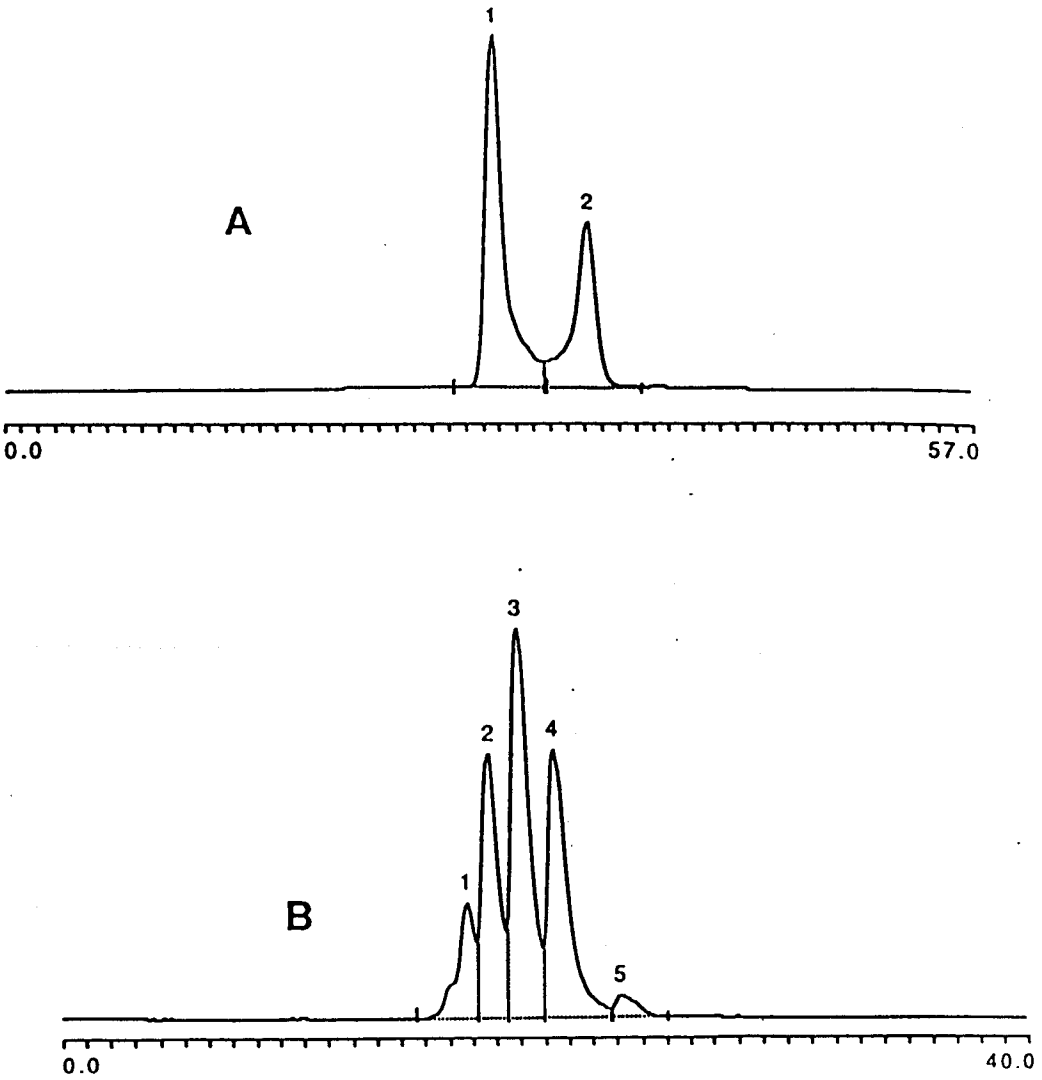

Figure 4. (A) PolyCAT A cation-exchange HPLC chromatographic separation of a 5 mg/ml sample of 4–4–20/212 scFv showing monomer (peak 1) and dimer (peak 2). Retention times of peaks 1 and 2 are 28.9 and 34.4 min, respectively. (B) HPLC size-exclusion chromatograph analysed on a TSK G3000SW column showing a highly aggregated CC49/212 scFv. The monomer (peak 4), dimer (peak 3), and trimer (peak 2) fractions elute at 20.4, 18.9, and 17.7 min, respectively.

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Antibody engineering

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