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Antibody Engineering

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Protocol

Improving Expression of scFv Fragments by Coexpression of Periplasmic Chaperones

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reprinted from:

H. Bothmann and A. Plückthun, Improving expression of scFv fragments by coexpression of periplasmic chaperones. In: Antibody Engineering, R. Kontermann and S. Dübel, Eds. (Springer-Verlag, Berlin Heidelberg, 2001) pp. 307-317.

Introduction

The periplasmic expression in *E. coli* has become the standard technology for preparing functional antibody fragments in a rapid way (Skerra and Plückthun 1988, Plückthun et al. 1996). The consequences of choosing Fab or scFv fragments, the properties of suitable expression vectors or the influence of the E. coli strain have been extensively summarized elsewhere (Plückthun et al. 1996). Even when paying attention to all these components and experimental conditions, it has become clear that the yield of recombinant antibody fragments is variable and these variations are a direct consequence of their primary sequences (Wörn and Plückthun 2001). The periplasmic folding is the yield limiting step and is most strongly influenced by the sequence. Consequently, antibody expression can be greatly improved by altering the sequence. This is possible without losing affinity, and two principal approaches have been found to be successful, a "rational" approach and a "directed evolution" approach. The rational approach gives the choice between aligning the antibody sequence to well-expressing ones (Knappik and Plückthun 1995, Wörn and Plückthun 2001) together with exchanging exposed hydrophobic residues (Nieba et al. 1997) and the possibility of grafting the CDRs directly to a very stable and well expressing framework (Jung and Plückthun 1997, Wörn and Plückthun 2001, Willuda et al. 1999). Alternatively, the protein can be subjected to a directed evolution approach (Proba et al. 1998, Jung and Plückthun 1999). While a picture of important residues and interactions for folding is slowly emerging (Wörn and Plückthun 2001), these approaches are still

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time consuming and worthwhile only if the antibody will be used frequently and in important applications or if the initial yield is extremely poor.

A faster method, which can of course be used in conjunction with sequence engineering, is to coexpress periplasmic factors which may improve the yield of folded antibody. Up to now, two periplasmic factors have been discovered by using phage display for selection for the improved expression of a poorly folded antibody fragment on filamentous phage (Bothmann and Plückthun 1998, 2000). The first factor identified, Skp, is a basic periplasmic protein of unknown function, which has been implicated in outer membrane protein biogenesis (de Cock et al. 1999, Schäfer et al. 1999). The second factor, FkpA, is a periplasmic peptidylprolyl cis/trans isomerase, which also acts as a chaperone even on antibodies not containing cis-prolines (Bothmann and Plückthun 2000, Ramm and Plückthun 2000). Both factors were found to increase the yield of periplasmic antibody fragments. Which one is to be preferred, however, appears to be molecule-specific among antibodies. Neither additivity nor synergy was observed between the factors, the more effective one of the two appears to set the expression yield. We have never found a negative influence of the coexpression of either Skp or FkpA up to now. However, in some "well-behaving" antibodies, the effect of coexpression is very small, while in other cases it can increase the yield up to 10-fold. Therefore, it seems to be a good strategy to have them both routinely present on antibody expression plasmids or work with a second compatible plasmid. While the latter strategy might seem more versatile, working with single vectors is generally more convenient, as the loss of a second replicon is a potential source of irreproducibility.

Undoubtedly, many variations of the method of coexpressing Skp and/ or FkpA are possible (Bothmann and Plückthun 1998, 2000, Hayhurst and Harris, 1999), and not all have been experimentally tested up till now. The procedure we discuss uses the natural promoters of Skp and FkpA, but other strategies may be possible as well (Bothmann and Plückthun 2000). Here, we describe the vectors pHB100s, pHB110 and pHB610, which are compatible with the pAK series for phage display (Chapter 2) and miniantibody production (Chapter 43), and as an alternative, the PCR procedure for cloning the genes into another vector. We give the procedure for a small scale experiment to test the effect of the chaperone coexpression and that of a large scale expression for purification. In contrast to the production of poorly folding antibodies in the absence of the chaperone, the expression in the presence of the chaperone can be carried out for longer times, as it leads to less cell lysis, which in turn increases the reachable cell density, again increasing the yield.

Materials

- French Press (Aminco) (Rochester, NY, USA) with 4 ml cell and 40 ml cell
- Optima TLX Ultracentrifuge (Beckmann Instruments) with TLA-100.3 rotor
- DNeasy Tissue Kit (50) (QIAGEN or equivalent)
- Appropriate expression system to produce his6-tagged antibody fragments, such as the pAK system (Chapter 2, Krebber et al. 1997)
- Automated LC-System: BioCAD workstation with dual channel variable wavelength UV/visible detector, semi-preparative 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)
- POROS20 HQ/M 4.6 mm/100 mm (anion exchange)
- POROS20 HS/M 4.6 mm/100 mm (cation exchange)
- 200 mM imidazole adjusted to pH 7 with acetic acid
- 3 M NaCl stock solution
- distilled water

Solubilization buffer

- 2 M urea
- 1 mM EDTA
- 10 mM glycylglycine (pH 7.5)

PBS (PBST)

- 8 mM Na₂HPO₄
- 1.8 mM KH₂PO₄
- 3 mM KCl
- 137 mM NaCl (pH 7.4)
- For PBST add Tween 20 to 0.05% final concentration

100 mM MHA-buffer (stock solution)

- 33 mM Mes
- 33 mM Hepes
- 33 mM Na-acetate (adjust to pH 7.5 with NaOH)

Hepes extraction buffer

- 20 mM Hepes
- 0.5 M NaCl (adjust to pH 7.0 with NaOH)

Procedure

Cloning of scFv fragments from pAK/pJB vectors in pHB100s/pHB610 vectors

1. Excise the the antibody fragment expression cassette with XbaI/ HindIII (Fig. 4, Chapter 2).

Note: The vector system and primer are described in detail in Chapter 2

- 2. Prepare the vector fragment by removing the *tet*-cassette from pHB100s, pHB610 or pJB33 with *XbaI/Hind*III (Fig. 1).
- **3.** Ligate the antibody fragment expression cassette into the appropriate vector (pHB100s, pHB610 or equivalent).

Fig. 1. Vectors and cloning strategies. All vectors contain a chloramphenicol resistance cassette (camR) and a tetracycline resistance "stuffer" cassette (tet, composed of tetA and tetR; 2101 bp) (Krebber et al. 1997), which will be replaced by the antibody fragment. This stuffer, shown here only schematically, contains the genes for *tetA* and *tetR*, and it does not make a fusion protein with upstream or downstream elements in the vector. For details, see Chapter 2. The vectors pHB110, pHB100s and pHB610 allow phage display, if an scFv cassette without stop codon is introduced, as detailed in Chapter 2, or periplasmic expression, if a stop codon is present at the end of the scFc gene, and the g3p gene can be replaced by a tag sequence. Because of its strong ribosome binding site vector pJB33 allows strong periplasmic expression and the tag allows IMAC purification of the antibody fragment (see Chapter 2). (a) Vector containing the *skp* cassette with flanking genes, as it was enriched during panning (Bothmann and Plückthun 1998), allowing isolation and subsequent recloning of skp after digestion with NotI, SpeI and SalI/XhoI. (b) Vector containing skp without the truncated lpxD. (c) Vector containing fkpA. (d) High yield expression vector (see Chapter 2). *lpxD*: the first 65 aa of UDP-3-O-[hydroxymyristoyl]-glucosamine-N-acyltransferase, *yeat*: the last 49 aa of Yeat (unknown function).







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Cloning skp/fkpA in other expression vectors

- *skp* and *fkpA* can be cut out from vectors pHB100s and pB610 by *Not*I (Fig. 1).
 - skp can also cut out from vector pHB110 with SpeI and XhoI/SalI (Fig. 1)
 - *skp* and *fkpA* can be amplified from genomic template DNA or from the above vectors pHB100s, pHB610 or equivalent and inserted in any expression vector.

For neither *skp* nor *fkpA* has the exact position of the promotor been experimentally determined and we had to rely on predictions. Therefore, we recommend the following PCR-primers for amplifying the genes from the vectors pHB110 or pHB610 or from genomic *E. coli* DNA.

fkpAseq: 5' NNNNNXXXXXXTTATTTTTTAGCAGAATCTGCGGC 3' fkpArev: 5' NNNNNXXXXXGATTCACCTCTTTTGTCGAATGG 3' skpseq: 5' NNNNXXXXXGATCCAAGCAATATCCGTATG 3' skprev: 5' NNNNXXXXXTTATTTAACCTGTTTCAGTACGTC 3'

- XXXXXX stands for the restriction site used for subcloning.
- NNNNN stands for additional bases flanking the restriction sites, which are necessary for efficient cleavage (see, e. g. the New England Biolabs catalog for more information).
- 4. Isolate genomic DNA from *E. coli* as described in the manual of the DNeasy Tissue kit or equivalent.
- 5. Perform PCR with primers for *skp* or *fkpA* as according to standard protocols.
- 6. Digest the PCR-product with the appropriate restriction enzymes and clone it into the expression vector. Another strategy is to subclone the PCR-product first in a vector such as pCRscript (Stratagene) and use this construct as starting point for further subcloning.

Small scale expression

7. Inoculate 50 ml LB medium, containing the appropriate antibiotic, with a single colony of *E. coli* JM83 or SB536 (see Chapter 43, harboring a plasmid encoding the respective scFv fragment.

- 8. Grow the culture at 24°C or 37°C to an OD_{550} of 0.5 and induce with 1 mM IPTG (final concentration).
- **9.** Harvest the cells after 1 h induction at 37°C or 3 h induction at 24°C.
- 10. Resuspend the cells carefully in 2 ml PBS.
- 11. Measure OD_{550} .
- 12. Lyse the cells by two passages through the French Press (20,000 psi).
- 13. Centrifuge 1 ml of the whole cell extract in an Eppendorf tube for 30 min at 50,000 rpm in a TLA-100.3 rotor at 4° C.
- 14. Transfer the supernatant carefully to a fresh Eppendorf tube (soluble fraction).
- 15. Dissolve the pellet in 1 ml solubilization buffer (shaking overnight at 4°C, if necessary) (insoluble fraction).
- 16. Normalize all fractions to the same OD_{550} of the original culture.
- 17. For crude extract ELISA, use normalized soluble fraction, for Western blots normalized soluble and insoluble fractions.
- 18. Coat ELISA plates with antigen ON at 4°C using standard protocols (see, e. g. Thorpe and Kerr 1994).
- 19. Mix a defined amount of normalized soluble fraction with 2% skimmed milk in PBST in the presence and absence of soluble antigen and apply to the blocked ELISA plate.
- 20. Perform detection as e. g. described in Thorpe and Kerr 1994.
- 21. For Western blots load defined amounts of soluble and insoluble fractions on a reducing SDS-PAGE.

22. Perform standard Western blot, according to the protocols described in Sambrook et al. 1989.

Large scale expression

The single-chain Fv fragment with 6-histidine tag can by purified by rapid two-column chromatography. This protocol is given for 5-10 g wet weight of *E. coli* cells, corresponding to about 1 l of baffled shake-flask culture.

23. Resuspend the cell pellet in 30 ml Hepes extraction buffer.

- 24. Add DNase to a final concentration of 10 μ g/ml.
- 25. Disrupt the cells in a French Press (20,000 psi, 4°C in a cold room). At least two passages are required for optimal lysis of the cells.
- Centrifugation of the suspension (Sorvall SS-34, 48 200 g, 4°C, >30 min).
- Filter the supernatant (0.22 μm, use filter with low protein binding properties).
- 28. Load the filtrate on an Ni-IDA POROS column (1.66 ml) (preloaded with 3 ml 0.1 M NiCl₂), pre-equilibrated with 20 mM MHA-buffer, 0.15 mM NaCl, pH 7.0. The flow rate should be 5 column volumes (CV) per minute (Fig. 2).

Note: The whole procedure with both columns takes only 30 minutes.

- Wash column with 20 mM MHA-buffer, containing 0.15 mM NaCl until the baseline is reached.
- 30. Wash column with 10 CV 20 mM MHA-buffer, containing 30 mM NaCl, pH 7.0.
- Wash column with 10 CV 20 mM MHA-buffer, containing 1 M NaCl, pH 7.0.
- 32. Wash column with 30 mM imidazole, 0.15 mM NaCl, pH 7.0 for 10 CV.



Fig. 2. Tubing diagram for rapid two-column purification of antibody fragments. At the beginning of the chromatography, all flow is through the IMAC column (valve positions as shown). Upon antibody elution, the flow is redirected to the ion-exchange column (IEX), by turning valves 2 and 4. The adsorbed protein is then eluted with a new gradient, by turning valves 1 and 3.

- 33. Elute either using an imidazole gradient from 30 to 150 mM imidazole (pH 7.0) (no salt) (10 CV) or a step elution with 150 mM imidazole (pH 7.0) (no salt) (6 CV) (Fig. 2).
- 34. Load the IDA-elution directly on a second column, without collecting the samples, by using the BIO-CAD workstation or equivalent. This column can be either a cation-exchange or an anion-exchange.

Note: For the tubing diagram, see Fig. 2. The pH for the second washing step and the elution depends on the pI of the antibody fragment and on the type of the second column (i. e. if the antibody has a pI of 8.5, the pH should be adjusted to 7.0 and the sample should applied to a cation exchange column.

35. Wash the column with 20 mM MHA-buffer, containing 30 mM NaCl,

pH 7.0 until the baseline is reached.

36. Elute the cation-exchange column with a salt gradient from 30 to 750 mM NaCl (15 CV). Collect the samples in 1 ml fractions and analyze each of them by SDS-PAGE.

Comments

- Coexpressing Skp together with an antibody fragment sometimes results in a prolonged lag phase and slower doubling phase of the cells. Nevertheless, when the OD_{550} reaches 0.8, these cells recover, showing a higher doubling rate and, finally, higher yield of recombinant protein.
- Most of the *E. coli* host proteins co-purified in IMAC have a pI less then 6.5, therefore they will bind to an anion-exchange column. A salt gradient for separation usually works very well, in conjunction with a running buffer of pH 7.0.

Imidazole as a storage buffer and as a sample component in SDS-PAGE is not desirable, because it will slowly catalyze the hydrolysis of acid labile bonds. Therefore, the 2-step method might even be useful for those antibody-fragments which are already pure enough after the IMAC step. Alternatively, the IMAC eluate can be dialyzed against a physiological buffer such as PBS immediately after purification.

Acknowledgements. We wish to thank Stefan Ewert for helpful contributions to the methods presented here.

References

Bothmann H, Plückthun A (1998) Selection for a periplasmic factor improving phage display and functional periplasmic expression. Nature Biotechnol 16:376-380
Bothmann H, Plückthun A (2000) The periplasmic *E. coli* peptidylprolyl cis, trans-isomerase FkpA (I): increased functional expression of antibody fragments with and without *cis*-prolines. J Biol Chem 275:17100-17105

de Cock H, Schäfer U, Poteger M, Demel R, Müller M, Tommassen J (1999) Affinity of the periplasmic chaperone Skp of Escherichia coli for phospholipids, lipopolysac-charides and non-native outer membrane proteins. Eur J Biochem 259:96-103
Hayhurst A, Harris WJ (1999) Escherichia coli Skp chaperone coexpression improves solubility and phage display of single-chain antibody fragments
Jung S, Plückthun A (1997) Improving *in vivo* folding and stability of a single-chain Fv antibody fragment by loop grafting. Prot Eng 10:959-966
Jung S, Honegger A, Plückthun A (1999) Selection for improved protein stability by

Jung S, Honegger A, Plückthun A (1999) Selection for improved protein stability by phage display. J Mol Biol 294:163-180

- Knappik A, Plückthun A (1995) Engineered turns of a recombinant antibody improve its *in vivo* folding. Prot Eng 8:81-89
- Krebber A, Bornhauser S, Burmester J, Honegger A, Willuda J, Bosshard HR, Plückthun A (1997) Reliable cloning of functional antibody domains from hybridomas and spleen cell repertoires employing a reengineered phage display system. J Immunol Meth 201:35-55
- Missiakas D, Betton JM, Raina S (1996) New components of protein folding in extracytoplasmic compartments of *Escherichia coli* SurA, FkpA and Skp/OmpH. Mol Microbiol 21:871-884
- Nieba L, Honegger A, Krebber C, Plückthun A (1997) Disrupting the hydrophobic patches at the antibody variable/constant domain interface: improved *in vivo* fold-ing and physical characterization of an engineered scFv fragment. Prot Eng 10:435-444
- Plückthun A, Krebber A, Krebber C, Horn U, Knüpfer U, Wenderoth R, Nieba L, Proba K, Riesenberg D (1996) Producing antibodies in *Escherichia coli*: From PCR to fermentation. In: McCafferty J, Hoogenboom H, Chiswell D (eds) Antibody Engineering. A practical approach, Oxford University Press, Oxford, pp 203-252

Proba K, Wörn A, Honegger A, Plückthun A (1998) Antibody scFv fragments without disulfide bonds made by molecular evolution. J Mol Biol 275:245-253

- Ramm K, Plückthun A (2000) The periplasmic *E. coli* peptidyl-prolyl isomerase FkpA (II): isomerase-independent chaperone activity *in vitro*. J Biol Chem 275:17106-17113
- Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, New York
- Schäfer U, Beck K, Müller M (1999) Skp, a molecular chaperone of Gram-negative bacteria, is required for the formation of soluble periplasmic intermediates or outer membrane proteins. J Biol Chem 274:24567-24574
- Skerra A, Plückthun A (1988) Assembly of a functional immunoglobulin F_v fragment in *Escherichia coli*. Science 263:14315-14322
- Thorpe SJ, Kerr MA (1994) Common immunological techniques: ELISA, blotting, immunohistochemistry and immunocytochemistry. In: Kerr MA, Thorpe R (eds) Immunochemistry. Labfax, BIOS Scientific Publishers Limited, Oxford, pp 175-209

Willuda J, Honegger A, Waibel R, Schubiger PA, Stahel R, Zangenmeister-Wittke U, Plückthun A (1999) High thermal stability is essential for tumor targeting of antibody fragments: Engineering of a humanized anti-epithelial glycoprotein-2 (epithelial cell adhesion molecule) single-chain Fv fragment. Cancer Research 59:5758-5767
Wörn A, Plückthun A (2001) Stability engineering of antibody single-chain Fv fragments. J Mol Biol, 305: 989–1010