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Journal of Chromatography B, 786 (2003) 161–176
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Optimizing expression and purification from cell culture medium of trispecific recombinant antibody derivatives

An Willems, Jannick Leoen, Steve Schoonooghe, Johan Grooten, Nico Mertens*

Department of Molecular Biomedical Research, Flanders Interuniversity Institute for Biotechnology (VIB), University of Ghent, K.L. Ledeganckstraat 35, 9000 Ghent, Belgium

Received 2 July 2002; received in revised form 23 September 2002; accepted 10 October 2002

Abstract

Antibody fragments offer the possibility to build multifunctional manifolds tailored to meet a large variety of needs. We optimized the production of a manifold consisting of one (bibody) or two (tribody) single-chain variable fragments coupled to the C-terminus of Fab chains. Different strong mammalian promoters were compared and the influence of expression media on production and recovery was investigated. Since the physical and chemical nature of these molecules largely depends on the nature of the antibody building blocks incorporated, a generally applicable process for the purification of recombinant antibody derivatives from serum containing mammalian cell culture medium was designed. To this end we compared protein L, hydroxyapatite, immobilized metal affinity chromatography, cation-exchange chromatography and hydrophobic charge induction chromatography.

Keywords: Expression; Purification; Bispecific antibodies; Fab fragment; Single-chain variable fragments

1. Introduction

Monoclonal and recombinant antibodies have proven to be of interest to develop novel and improved biologicals for use in medical treatment. Monoclonal antibodies are relatively large molecules that have difficulty in penetrating the extracellular matrix. They are protected from degradation in the liver through an interaction involving their Fc-tail and the FcRn receptor present in liver cells. IgG molecules can have serum circulation times of several days to weeks. This persistence in non-specific tissue impairs specific targeting, for example at tumor cells. A more specific target/blood ratio is desired for recombinant antibodies engineered to perform an active function, or to carry a toxin in order to avoid toxicity in peripheral tissues. Antibody fragments, such as single-chain variable fragments (scFv) [1,2], diabodies [3] and Fab fragments are smaller molecules without an Fc-tail, showing a more rapid clearance from the blood. Because of their smaller size, they have a better tissue penetration potential. However, since the glomerular pores in the kidney have an average size of 6–10 nm in size, a protein with an M_r<60 kDa results in extremely rapid kidney clearance [4]. With a glomerular filtration rate of 7 l/h, and given the average size of 5×8 nm for a Fab molecule, a rapid

*Corresponding author. Tel.: +32-9-264-5134; fax: +32-9-264-5348.
E-mail address: nico.mertens@dmb.rug.ac.be (N. Mertens).

1570-0232/02/$ – see front matter
doi:10.1016/S1570-0232(02)00813-9

removal of this molecule from the blood is expected. The observed serum half-life of these fragments of down to 30 min was noticed to be too short to permit sufficient accumulation at the tumor site, thus limiting the therapeutic potential of these small molecules. In order to avoid this rapid kidney clearance (but also the extremely long serum persistence imposed by the Fc-tail) intermediate-sized molecules might be the better candidates to allow for better target cell binding and specificity [4–7]. We previously presented a novel model to engineer bi- or trifunctional antibody fragments (bi- or tribodies) of intermediate size (75–100 kDa), based on fusion of single–chain variable fragments (scFv) to the C-terminus of one or each of the Fd and L chains of a Fab fragment [8]. By making use of the disulfide-stabilized Fab fragment as a heterodimerization scaffold, the need for an extra antigenic heterodimerization domain is circumvented, and three or more functions can be exactly determined in the molecule.

A variety of expression systems have been used to produce antibody fragments. Expression in *Escherichia coli* provides important advantages, mainly due to the fast growth and easy handling of the organism. After translocation of the polypeptide chains into the bacterial periplasm, protein folding and formation of the disulfide bonds can take place and functional protein can be obtained. This technique is widely used for the production of scFv [9,10] and Fab fragments [11,12]. It has become clear that the yield of recombinant antibody fragments is variable and that these variations are a direct consequence of their primary sequences [13]. Periplasmic folding is a yield-limiting step and is strongly influenced by the sequence of the complementarity-determining region loops, which are unique for each individual antibody. Bacterial expression of more complex fusions of antibody fragments, such as bispecific antibodies, often leads to the formation of insoluble inclusion bodies [14] or rapid protein degradation (our own unpublished results). Although the in vitro refolding from intracellular inclusion bodies is possible, this often leads to low quality of the refolded protein, appearing in several isoforms. Thus, the use of a eukaryotic expression system (such as the yeast *Pichia pastoris*, insect cells or mammalian cells) can be more advantageous, and since less process steps are needed, in the end it might be a cheaper production method.

Eukaryotic secretion systems provide an extensive quality control of protein folding in the endoplasmatic reticulum. One of the key components of this system is BiP, a member of the HSP70 chaperone protein family. BiP binds to the heavy chain of antibodies and is replaced by the interaction of the light chain, ensuring an efficient control of the required heterodimerization of the L and Fd parts of the Fab molecule [15].

For the purification of antibodies and their fragments, a wide range of strategies has been developed, employing different adsorption–desorption principles. At present, the most wildly used technique to capture monoclonal antibodies and antibody derivatives comprising the Fc-tail, is affinity chromatography on immobilized protein A sorbents. Protein A binds primarily to the Fc-portion of most IgG antibodies, but can also bind to V_HIII domains present in some Fab and scFv fragments [16]. For the purification of scFv and Fab fragments, a protein L matrix was developed, with specificity for human λ light chains of subclasses I, III and IV and mouse κ light chains of subclass I and V [17,18]. Another classical method for antibody purification is hydroxyapatite affinity chromatography (HAC), which has a high selectivity for different IgG subtypes [19]. Recently, a new antibody separation technique called hydrophobic charge induction chromatography (HCIC) has been introduced [20–22]. HCIC has been proposed as a cost-effective, process-compatible alternative to protein A affinity chromatography for purification of monoclonal antibodies. When His tag fusion proteins can be used in recombinant antibodies, immobilized metal affinity chromatography (IMAC) is a well-established technique [23]. The affinity chromatography techniques mentioned above have been used for the purification of antibody derivatives in single-step protocols [24–28], in combination with each other [21,29–31] and combined with classical chromatography techniques such as ion-exchange chromatography [32] and hydrophobic interaction chromatography (HIC) [33].

In order to develop an efficient production system for manifolds using recombinant antigen-binding fragments in eukaryotic cell culture, expression of the Fab–scFv bibodies and Fab–(scFv)2 tribodies

was optimized. Also, a generally applicable purification process that can be used for a broad class of antibody derivatives, such as the bi- and tribodies, has been designed.

2. Experimental

2.1. Plasmids and gene assembly

Restriction enzymes and DNA modifying enzymes were used as recommended by the manufacturers. DNA amplification was performed with Vent-DNA polymerase (New England Biolabs, Beverely, MA, USA). E6, B1 and 2c11 denote the gene fragments of an anti-human alkaline phosphatase (anti-hPLAP), an anti-B-cell lymphoma idiotype (anti-BCL1) and an anti-murine T-cell receptor associated CD3ϵ chain (anti-CD3) monoclonal antibody, respectively. Expression plasmids were constructed in pEF1/V5-His A, pCDNA3.1zeo- (both from Invitrogen, San Diego, CA, USA) and pCAGGS [34]. The cloning of the light chain of the parental murine anti-hPLAP monoclonal antibody (IgG2b/κ) in the vector pSV51E6L as well as the cloning of the heavy chain E6Fd fragment and of the E6Fd fragment extended with the anti-BCL1-scFv, in the pCAGGS, have been described previously [8,35]. A hybrid anti-BCL1 Fab was constructed by grafting the VL and VH domains of the anti-BCL1 moAb onto the CL and CH1 domains of the E6 Fab. Different peptide linkers were incorporated using modifying polymerase chain reaction primers. All PCR-derived fragments were sequence verified after cloning.

2.2. Bacterial expression of scFv

The *E. coli* strain MC1061(pICA2) was used for isopropyl β-D-thiogalactopyranoside (IPTG) induction of the pLSO32scB1E expression plasmid containing the tightly repressed λP_L promoter and an ompA signal sequence [36], and grown in a Luria–Bertani (LB) broth, supplemented with 100 μg/ml ampicillin and kanamycin. Induction was carried out in the exponential phase with 1 m*M* IPTG, for 16 h at 20 °C.

2.3. Production of antibody fragments

For transient expression, HEK293T cells were transfected according to the $Ca_3(PO_4)_2$ precipitation method [37]. HEK293T is a human embryonic kidney cell line transfected with SV40 large T-antigen (SV40T tsA1609) [38]. Cells were seeded at 4×10^6 cells/175 cm^2 20 h before transfection. DNA (14 μg) of each expression plasmid was added to the cells for 24 h, after which the cells were covered with supplemented Dulbecco's modified Eagle's medium containing 10% fetal calf serum (FCS) or 5 mg/l bovin insulin, 5 mg/l transferrin and 5 μg/l selenium (ITS). Medium was harvested every 48 h after transfection. For stable expression lines, SP2/0-Ag14 cells (non-Ig secreting myeloma cells) were electroporated, cultured in selective medium, subcloned and screened for production.

2.4. Analysis of production efficiency

Medium fractions (50× concentrated) of transfected cells, corresponding to 1 ml supernatant, were diluted with non-reducing sodium dodecyl sulfate (SDS) sample buffer, boiled for 5 min, fractionated by 10% SDS–polyacrylamide gel electrophoresis (PAGE), and blotted onto a nitrocellulose membrane. Subsequent immunodetection of the proteins on blot was as described previously [35]; goat anti-mouse Ig κ serum (Sera-Lab, Crawley Down, Sussex, UK) and anti-goat IgG serum conjugated to alkaline phosphatase were used.

2.5. Protein purification

2.5.1. Chemicals and equipment

The following chemicals were used: sodium acetate trihydrate, potassium chloride and sodium dihydrogen phosphate monohydrate (Merck, Darmstadt, Germany); ammonium sulfate, imidazole, nickel sulfate hexahydrate and sodium caprylate (Sigma Chemical Co., St. Louis, MO, USA); sodium chloride (Acros Organics, Geel, Belgium); disodium hydrogenphosphate monohydrate (Merck Eurolab, Leuven, Belgium); Tris (USB, Cleveland, OH, USA); ethylene glycol (Aldrich Chemical Co., Milwaukee, WI, USA).

All liquid chromatography (LC) runs were performed at room temperature on a BioLogic LP chromatography system (Bio-Rad Laboratories, Richmond, CA, USA) or a ProSys™ Workstation (Biosepra, Paris, FR). Column hardware was from Amersham Biosciences, Uppsala, Sweden.

2.5.2. Chromatographic methods

For protein L affinity chromatography (protL AC), cell culture supernatant was loaded directly, without pH adjustment, dialysing or addition of any component. ProtL AC was performed on a 5-ml CBinD™ L resin (i.e. rCBD-Protein L-cellulose; CBD Technologies, Rehovoth, Israel) packed in a C 10/10 column, run at a flow-rate of 1 ml/min. For IMAC, the sample was supplemented with 20 m*M* Imidazole pH 7.5 (final pH adjusted to 7.5). IMAC was performed on a 30-ml C16/20 Chelating Sepharose Fast Flow (Amersham Biosciences, Uppsala, Sweden), charged with Ni^{2+} metal ions and run at 2 ml/min. For cation-exchange chromatography (CEC), the pH of the sample was adjusted to pH 5.5 before loading on a 400-ml XK50/20 SP Sepharose Fast Flow (Amersham Biosciences) column, run at 8 ml/min. For HIC, the sample was supplemented with 1 *M* ammonium sulphate before loading on a 30-ml XK16/20 Phenyl Sepharose High Performance (Amersham Biosciences), run at 3 ml/min. For HAC, cell culture supernatant was loaded directly, without pH adjustment, dialysis or addition of any component. HAC was performed with 5 ml CHT® Ceramic hydroxyapatite (Bio-Rad Laboratories), packed in a C10/10 column, run at 1 ml/min. For HCIC, cell culture supernatant was loaded directly, without pH adjustment, dialysing or addition of any component. HCIC was performed with respectively 5 ml MEP HyperCel (BioSepra, Paris, FR), packed in a C10/10 column, run at 1 ml/min. For size exclusion chromatography (SEC), the sample was concentrated to 3 ml using Centriprep centrifugal filter devices (Amicon Bioseperations, MA, USA), with a cut-off of 10 kDa. SEC was performed on a 500-ml C26/100 Superdex 200 prep grade column, run at 2 ml/min with phosphate buffered saline (PBS). The column was calibrated before each run with Gel Filtration Standard (Bio-Rad Laboratories).

2.5.3. Endotoxin removal

All buffers used were made in endotoxin-free glassware; residual endotoxin in stock solutions was removed by filtration over two consecutive carbon filters (Scleicher & Schuell, Dassel, Germany), followed by a glass fiber prefilter and a 0.22-μm filter (Millipore, Bedford, MA, USA). Columns and devices were treated with 1 *M* NaOH overnight and abundantly flushed with endotoxin-free buffer. Detoxi-Gel™ AffinityPak™ 1 ml columns (Pierce Chemical Company, Rockford, IL, USA) were run at 0.2 ml/min with PBS. Sartobind Membrane Adsorbers Q5 (Sartorius, Goettingen, Germany) were used with PBS at 1 ml/min. Alternatively, 50 mg PROSEP-Remtox beads (Bioprocessing, Scarborough, ME, USA) were added to the protein solution and incubated for 3 h at room temperature. The beads were removed by passing the solution over an endotoxin-free empty column. A 9-ml prepacked Acticlean Etox column (Sterogene, Carlsbad, CA, USA) was initially run with PBS at 0.2 ml/min. Sodium acetate (10 m*M*) was added to the protein solution (PBS) and the pH was lowered to 5.0 by adding acetic acid. This solution was passed over the Acticlean Etox column at 0.2 ml/min.

2.6. Analysis of chromatographic fractions

The protein fractions collected were analyzed by 10% SDS–PAGE, after precipitation with trichloroacetic acid (TCA) of a fixed amount of each fraction. Test runs were performed with 500 μg of protein; an equivalent fraction was taken, precipitated with TCA and analysed with 10% SDS–PAGE as a reference. Proteins were visualized using Coomassie Brilliant Blue (CBB) or were blotted onto a nitrocellulose membrane. Subsequent immunodetection of the proteins on the blot was as described previously [35]; goat anti-mouse Ig κ serum and anti-goat IgG serum conjugated to alkaline phosphatase were used. For determination of protein recovery after early purification steps, blots containing immunoreactive signals of serial dilutions were scanned and analysed with TotalLab software (Nonlinear Dynamics, Newcastle, UK). Protein concentrations were measured with the Micro BCA™ Protein Assay Reagent Kit (Pierce

Chemical Company) and using the Bradford method (Bio-Rad Laboratories) with IgG as standard protein.

3. Results

3.1. Optimizing production levels of bibodies and tribodies in HEK(293T) cells

When expressing the minimum binding region of antibodies in either the scFv or Fab format, the use of *E. coli* is usually sufficient for most research purposes. However, bacterial expression of more complex fusions of antibody fragments, such as bispecific antibodies, often leads to the formation of insoluble inclusion bodies [14]. Moreover, in *E. coli* heterodimerisation is not very specific [39,40]. In contrast to *E. coli,* mammalian cells have extensive control mechanisms that prevent incorrectly folded proteins to proceed along the secretory pathway [41]. Therefore, production in mammalian cells of CL–CH1 containing heterodimers should lead to more efficient and more specific heterodimerization, since this can be enhanced by the chaperoning of the heavy chain-binding protein (BiP), which binds to newly formed CH1 domain in the endoplasmic reticulum [42]. BiP is displaced after expression of the light chain (L), by proper CL–CH1 interaction. Secretion in mammalian cells can be even more efficient if in addition to the CL–CH1 interaction, also the VL–VH interaction can contribute to displace BiP [8]. By using Fab chains for heterodimerization of scFv molecules, an efficient model for controlled heterodimerization is established. Since the Fab moiety already contributes a binding specificity on its own, it suffices to fuse a scFv molecule to one of the Fab-constituting chains to obtain monovalent bispecific antibodies. Even more, trispecific (or trifunctional) antibodies can be formed efficiently by extending both Fab chains with a scFv molecule [8] (Fig. 1A).

By varying the length and composition of the peptide linker connecting the Fab chains with the various scFv we wanted to test the influence of linker length and composition on expression and folding of the bi- and tribody molecules. Either a long or short peptide linker connecting the heavy chain with a scFv was combined with five different peptide linkers connecting the light chain with a scFv, the latter varying in length and amino acid sequence (Fig. 1B). The expression of the tribody with a long heavy chain peptide linker was consistently higher when compared to a short heavy chain linker, independently of the length or amino acid sequence of the light chain linker (Fig. 1C). The molecules produced were fully functional for all three binding specificities, even when assayed for 3× bispecificity (along the three axes of the Tribody) using shorter linker sequences. This is consistent with the folding model of the tribody, where it can be seen that the connection of the N-terminus of the scFv with the peptide linker, is not compromising the binding site (Fig. 1B).

3.2. Comparing strong eukaryotic promoters for secretion of bi- and tribodies in cell culture medium

For standard expression of the bi- and tribodies, a strong promoter should be used. Several suitable strong promoters have been suggested before. Here we compare the Elongation Factor 1α (EF1α) promoter [43], with the cytomegalovirus (CMV) promoter and the Actin promoter [34] (Fig. 2A). The genes encoding light chain and heavy chain were introduced into the multiple cloning sites (MCS) of the respective vectors: pEF1/V5-H6 (Invitrogen), pCDNA3-zeo (Invitrogen) and pES31 (derivative of pCAGGS, unpublished results). Transient expression levels of the bibodies in HEK293T cells, under control of the different promoters, were compared. Transfection efficiencies were monitored by transfection of the lacZ gene and X-gal staining and showed 70–80% of the cells to be consistently transfected. Western blotting of the cell culture supernatant revealed a strong expression of the bibodies under the control of the actin promoter, while a lower expression level was seen with the EF1α promoter or CMV promoter (Fig. 2B).

The influence of serum supplements in the medium during the harvest phase was evaluated by comparing transient expression levels of a bibody in supplemented medium containing either 10% FCS or 5 mg/l bovin insulin, 5 mg/l transferrin and 5 μg/l

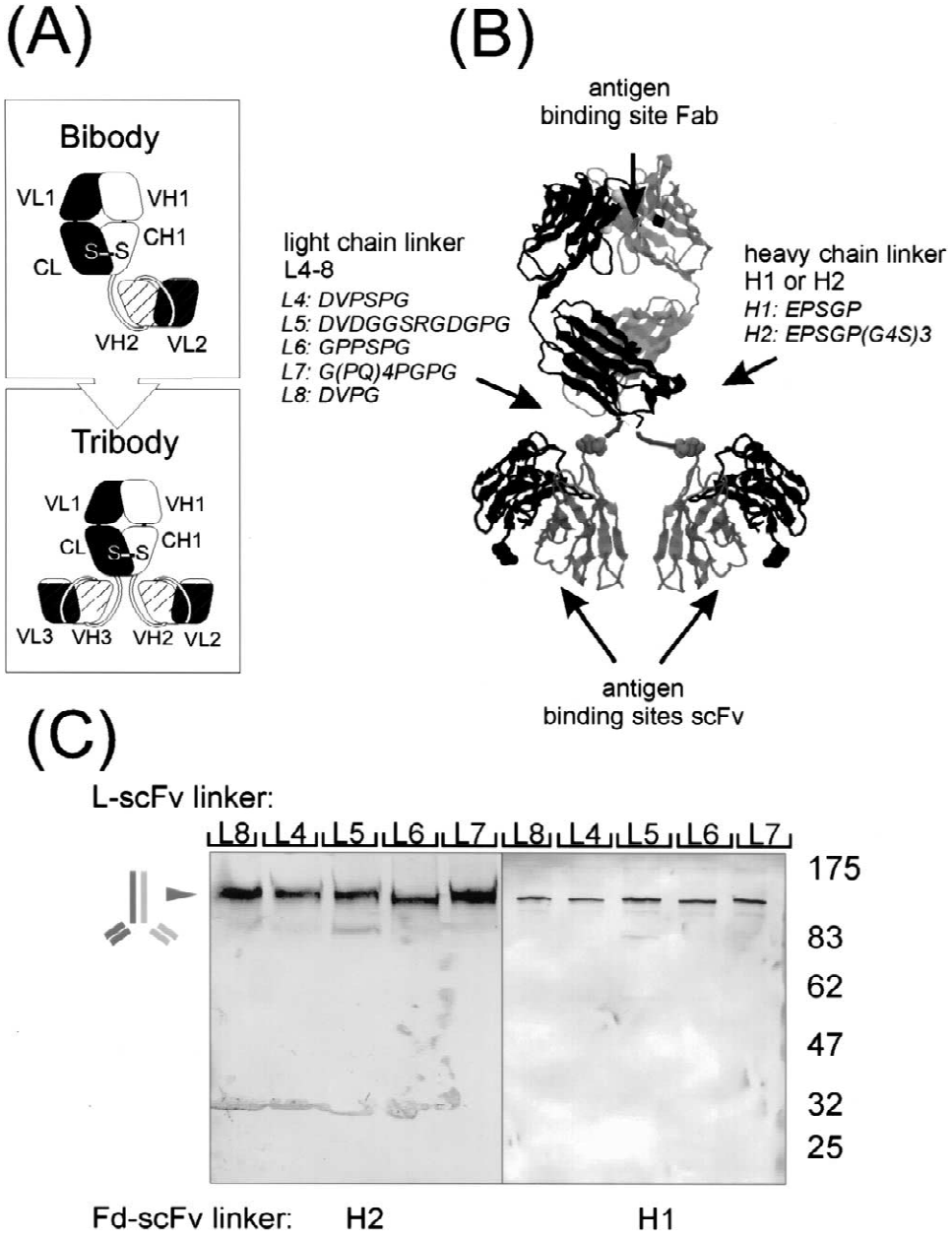


Fig. 1. The antibody model: influence of connecting peptide linkers on production of a Fab–(scFv)2 tribody. (A) Schematic view of the bi- and tribody model. (B) Structural model of a tribody, arrows indicate the antigen binding sites and the peptide linkers connecting the C-terminus of the Fab chains with the N-terminus of the scFv. (C) SDS–PAGE analysis (immunoblotting) of different combinations expressed: tribody with a long (H2) and a short (H1) heavy chain connecting linker in combination with different light chain connecting linkers (L4–8).

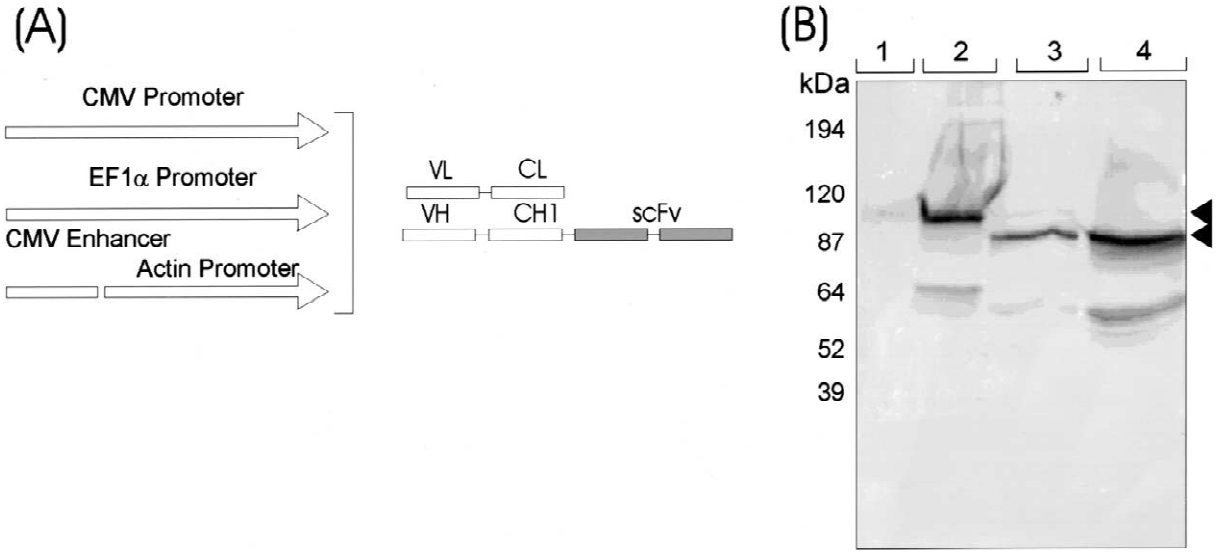


Fig. 2. Expression study of bi- and tribodies: influence of promoter on final production yield. Expression of two different antibodies in HEK(293)T cells, under the control of three different promoters. (A) Cloning of the genes encoding a bibody under the control of the CMV promoter, the EF1α promoter or the Actin promoter. (B) SDS–PAGE analysis of promoter study (immunoblotting detected with anti-mouse-κ light chain). Lanes: M, Molecular mass marker; 1, bibody A with EF1α-promoter; 2, bibody A with actin promoter; 3, bibody B with CMV-promoter; 4, bibody B with actin promoter.

selenium (ITS). The usage of ITS medium has the advantage that the subsequent purification of bi- and tribodies is simplified, since no serum albumin is present. However, expression levels in medium containing FCS remains high for a longer time, which results in a higher final yield. The same results were obtained when producing tribodies.

Stable cell lines of transfected SP2/0 myeloma cells could be obtained by electroporation. Expression levels of primary isolates varied between 5 and 100 mg/l, and could be improved by increasing the antibiotic concentration used for selection.

3.3. Use of protein L affinity chromatography as capture/purification step for bibodies and tribodies

Protein L is a surface protein from *Peptostreptococcus magnus* which binds to scFv and Fab fragments containing either human λ light chains of subclasses I, III and IV or mouse κ light chains of subclasses I and V [17,18]. rProtein L is a recombinant form of the native protein and contains only the four immunoglobulin binding domains. Protein L AC was evaluated for purification of different bi- and tribodies by spiking 500 μg of purified bibody in cell culture medium, containing 10% FCS. After sample loading, the column was washed with equilibration buffer (PBS). The bibody fraction was then eluted with 50 m*M* glycine pH 2.2. Based on SDS–PAGE analysis, the bibody retention and elution was successful, with a >90% recovery. Almost all contaminating medium proteins were lost, which resulted in a >90% purity (Fig. 3B). However, when using bibodies and tribodies containing a Fab-fragment of a chimeric type of mouse origin (different variable domains grafted on the CH1 and CL constant domains), no binding on the protein L matrix could be observed. The failure to apply protein L purification to all Fab chains, made it necessary to evaluate other purification methods for antibody manifolds only consisting of Fab chains and scFv's. Due to the heterogeneous nature of antibodies and their fragments, the methods were screened for their ability to be generally applicable, as opposed to really optimizing a single purification step by buffer and matrix scouting.

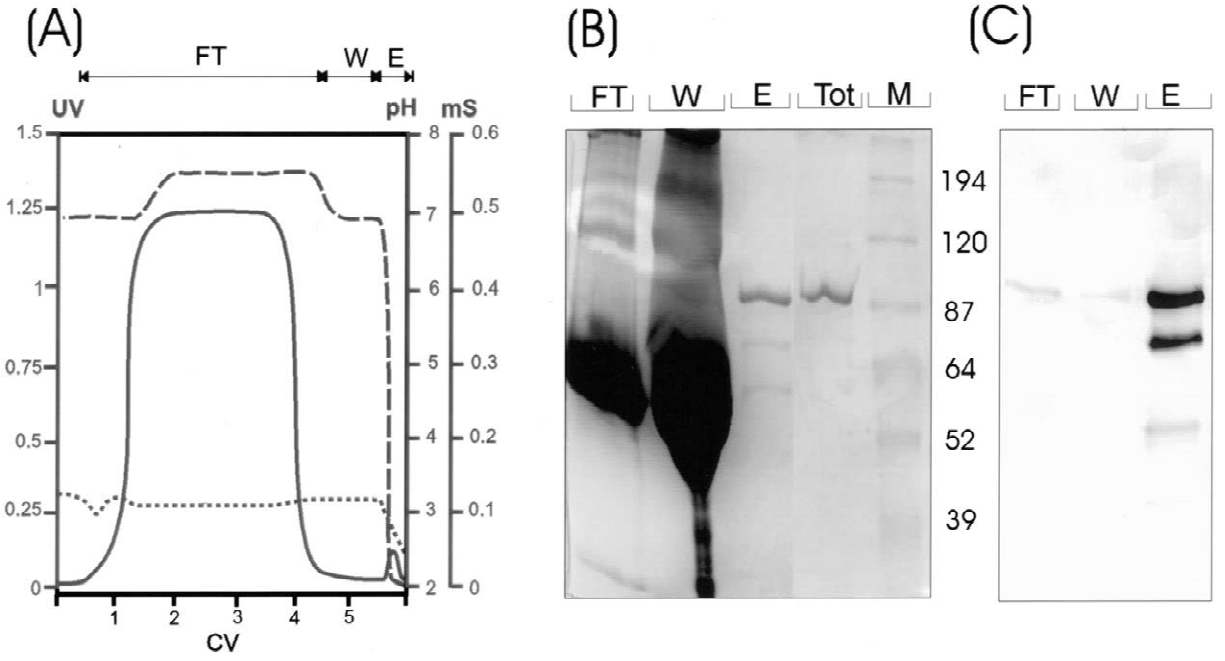


Fig. 3. Protein L affinity chromatography is effective and efficient for certain classes of antibody fragments. Prot L affinity purification of a bibody from medium with 10%FCS. (A) Chromatogram. (B) SDS–PAGE analysis (CBB staining). (C) SDS–PAGE analysis (immunoblotting). Lanes: FT, flow through; W, wash; E, eluate; Tot, total amount of bibody brought onto the column, M, molecular mass marker.

3.4. Immobilised metal affinity chromatography (IMAC) as a capture/purification step for recombinant His-tagged bibodies and tribodies

IMAC of His tag fusion proteins was first introduced in 1988 [23] as a method for the purification of recombinant proteins and was soon demonstrated to be of use for the purification of scFv fragments isolated from *E. coli* periplasm [44]. However, the use of IMAC for purification of antibody fragments composed of two different polypeptide chains, like Fv and Fab fragments, turned out to be more difficult. Fv fragments can only be purified when both of the variable domains are equipped with a His tag [45]. In Fab fragments the chain association is more stable due to the presence of the constant domains and of an interchain disulphide bond, which makes a single tag sufficient [46]. To avoid co-purification of free light chains (which have a natural tendency to form Bence–Jones dimers), the His tag was engineered at the C-terminus of the heavy chain Fd-fragment or the Fd–scFv fusion protein. In contrast to L-chain dimers, Fd-chain dimers are never formed in eukaryotic cells, so that only the functional Fd:L heterodimers (or the Fd–scFv:L–scFv) are isolated.

To prepare the test sample, 500 μg of already purified bibody was spiked in cell culture medium containing 10% FCS. After sample loading, the IMAC column was washed with equilibration buffer (20 m*M* phosphate pH 7.5, 0.5 *M* NaCl, 50 m*M* Imidazole pH 7.5). The bibody fraction was eluted with an increasing imidazole concentration for competitive replacement. Based on SDS–PAGE analysis, the bibody retention and elution were successful, but the obtained purity was poor, due to contamination of the abundant serum albumin protein (Fig. 4). Despite of its specificity, IMAC thus cannot be used as a single capture/purification step for isolating His tagged bi- and tribodies from FCS containing medium, and an additional capture step is needed to remove the bulk of the serum albumins. The desired capture step needs to be specific for a large variety of antibody fragments, while specifically separating them from the major contaminant (bovine serum albumin).

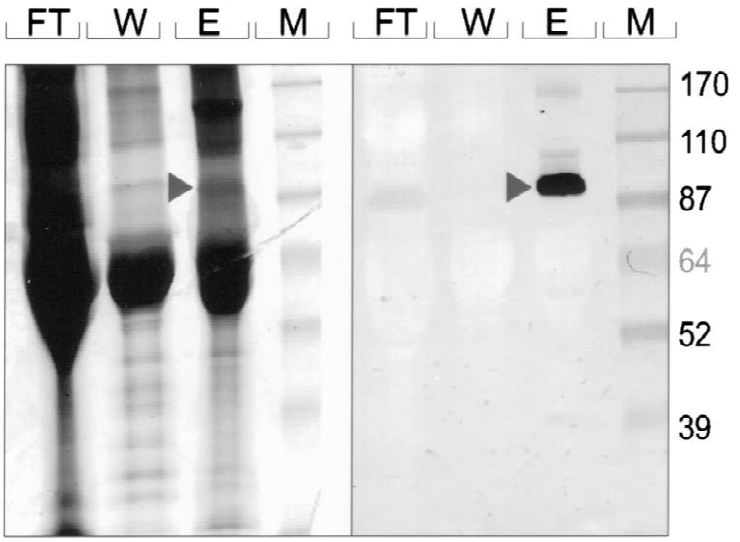


Fig. 4. Immobilised metal affinity chromatography: eluate is still contaminated with serum proteins. IMAC was used for purification of a bibody from medium with 10% FCS. SDS–PAGE analysis: Left: CBB staining, Right: immunoblotting. Lanes: M, molecular mass marker; FT, flow through; W, wash; E, eluate.

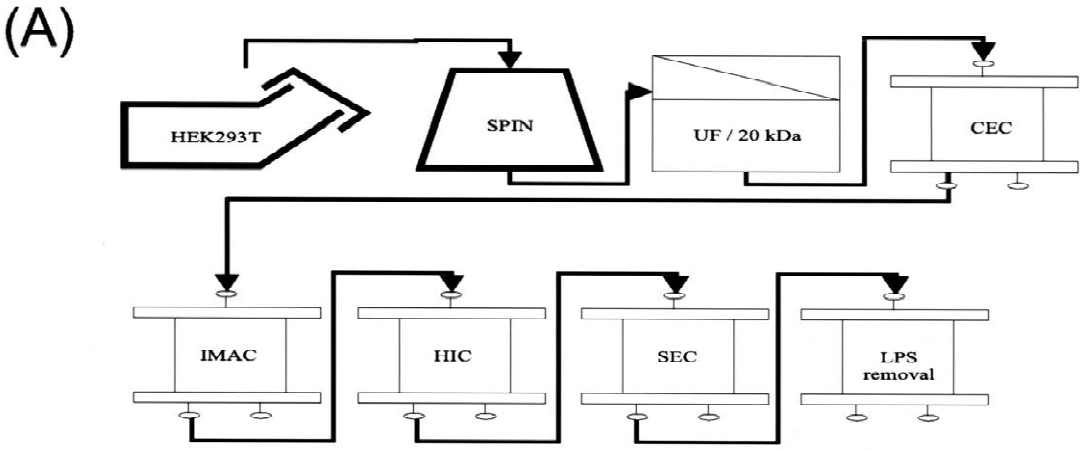
3.5. Cation-exchange chromatography as a capture step

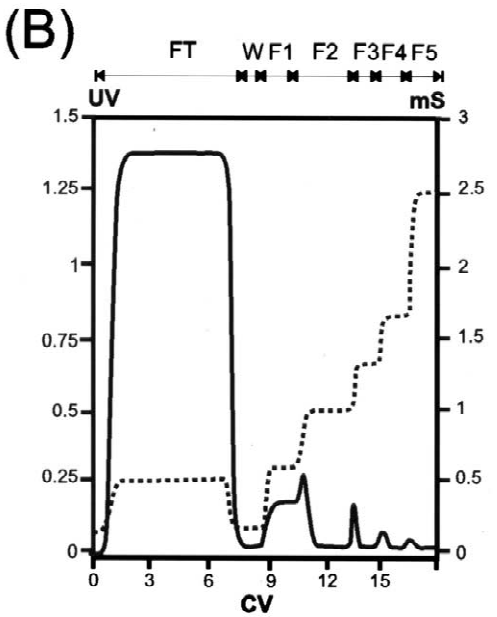
Since the p*I*-value of the tested bi- and tribodies is rather high, cation-exchange chromatography was evaluated as a capture step, especially to remove the contaminating serum albumins. Cell culture supernatant (containing 10% FCS) of HEK293T cells containing a tribody was loaded onto an SP sepharose column. After washing with sodium acetate pH 5.5, a step gradient elution with 150, 300, 450, 600, 750 and 1000 m*M* sodium chloride was performed. The prototype tribody used in this study was eluted when applying 450–600 m*M* sodium chloride, while the bulk of the contaminating albumins was lost in the flow-through and low-salt elution (Fig. 5C). An additional IMAC purification led to an almost pure product, with only one prominent contaminating band (data not shown), which could be removed by a HIC step on phenyl sepharose HP, developed with a 1–0 *M* gradient of ammonium sulfate (Fig. 5). However, some of our other tested recombinant antibody fragments used eluted at a lower salt concentration, together with the contaminating albumins (data not shown). For these antibody fragments a different capture step is required.

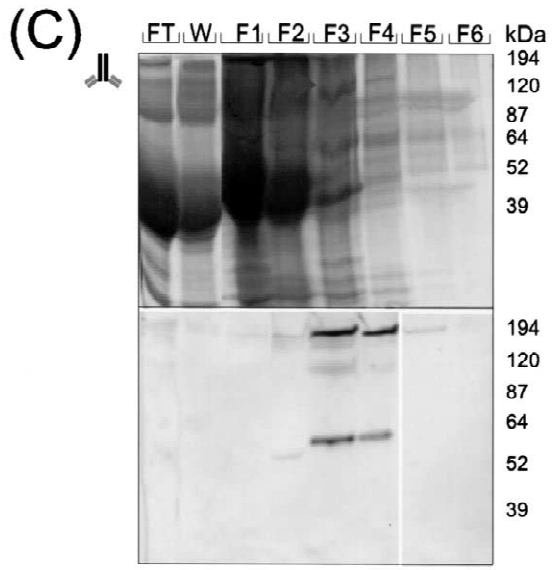
3.6. Hydroxyapatite affinity chromatography as a capture step

Hydroxyapatite $(Ca_5(PO_4)_3OH)_2$ (HA) is a form of calcium phosphate used in the chromatographic separation of biomolecules. It has unique separation properties and has been used for the purification of different subclasses of monoclonal and polyclonal antibodies, antibodies that differ in light chain composition, antibody fragments, isozymes, supercoiled DNA from linear duplexes and single-stranded from double-stranded DNA. CHT ceramic hydroxyapatite is a spherical, macroporous form. CHT Type II, is especially suitable for the purification of many Ig species and classes since it has a low affinity for serum albumin.

As a test sample, we used 500 μg of bibody, respectively in equilibration buffer (10 m*M* phosphate pH 6.8) and in cell culture medium (containing 10% FCS). The column was washed with equilibration buffer and the bibody-containing fraction was eluted with 400 m*M* phosphate pH 6.8. Based on SDS–PAGE analysis, the bibody retention and elution were successful, but only a poor product recovery was obtained, so the method was not efficient. This can partly be explained by the fact that a







(D)

Purification step	Total volume (ml)	Total protein (mg)	Tribody recovery (%)	Tribody recovery (mg)	Purification fold (step)	Purification fold (tot)	Purification purity (%)
Cell culture SN	2500	6878	100	7,5	1	1	0,11
SP FF	500	139	55	4,1	27	27	2,95
IMAC	100	6,5	43	3,2	17	450	49,23
HIC	100	3	40	3,0	2	920	>98,00

Fig. 5. Multi-step purification protocol for purification of a tribody from cell culture supernatant containing 10% FCS. (A) Flow chart of the purification protocol. (B) Chromatogram of the capture step with cation-exchange chromatography. (C) SDS–PAGE analysis of the capture step: CBB staining (1) and immunoblotting (2). Lanes: M, molecular mass marker; FT, flow through; W, wash; F1, 150 m*M* NaCl; F2, 300 m*M* NaCl; F3, 450 m*M* NaCl; F4, 600 m*M* NaCl; F5, 750 m*M* NaCl; F6, 1 *M* NaCl. (D) Purification table of the multi-step procedure.

small amount of bibody was found in the column flow-through. Subsequent elution steps with respectively a lower pH (400 m*M* phosphate pH 5.7) or with additional salt (400 m*M* phosphate with 1 *M* NaCl) did not lead to additional product recovery (Fig. 6A). Moreover, when starting from cell culture medium containing 10% FCS, only a fraction of the contaminating proteins was lost in the flow-through, resulting in poor product purity (Fig. 6B). However, as described for monoclonal antibodies [21], phosphate buffers may not be sufficient to separate albumins from the bibody. Cationic proteins, such as most of the antibody fragments, can be desorbed by a potassium chloride gradient, while all acidic contaminants remain adsorbed on the resin. Eluting the bibody with equilibration buffer supplemented with 500 m*M* potassium chloride led to an improved purity, but recovery remained low.

3.7. Hydrophobic charge induction chromatography as a capture step

The HCIC sorbent, MEP HyperCel, carries a ligand derived from 4-mercaptoethylpyridine (4-MEP). The pK_a of 4-MEP is 4.8. Thus, under near-neutral or alkaline conditions, the ligand is uncharged and behaves much like a hydrophobic group that binds antibody by hydrophobic interaction. However, the thiophilic nature of the ligand provides the antibody-selective characteristic for which this matrix was designed [47]. Due to this combination of hydrophobic and thiophilic interaction, immunoglobulins can be bound in normal physiological conditions, without the addition of ammonium sulfate. Desorption is achieved by reducing the pH of the mobile phase to pH 4.0. This induces a net positive charge on both ligand and antibody, resulting in electrostatic charge repulsion. The MEP-matrix was shown to be selective for monoclonal IgG [20,22] but also binds Fab fragments, although it was demonstrated that an Fc-tail binds MEP more tightly than a Fab fragment [21].

As a test sample, 500 μg of already purified bibody in equilibration buffer (50 m*M* Tris–HCl buffer, pH 8.0) or in cell culture medium containing 10% FCS was loaded on the MEP Hypercel matrix. After sample loading, the column was washed: once with equilibration buffer, once with water, and once with equilibration buffer containing 25 m*M* sodium caprylate. Before elution, a supplementary wash with equilibration buffer was required to prevent subsequent pH dependent precipitation of the sodium

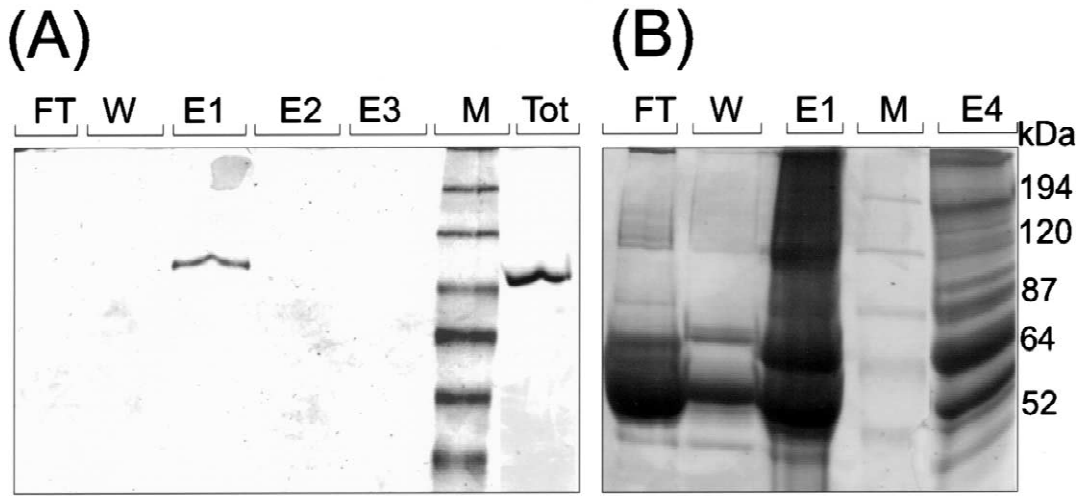


Fig. 6. Hydroxyapatite affinity chromatography as a capture step for purification of a bibody. (A) Purification of a known amount of bibody, spiked in equilibrating buffer. Recovery was estimated by direct comparison of initial amount on a CBB stained gel. (B) Purification of a bibody from cell culture supernatant, containing 10% FCS. Purity was estimated on a CBB stained gel. Lanes: FT, Flow through; W, Wash; E1, eluate with 400 m*M* phosphate pH 6.8; E2, eluate with 400 m*M* phosphate pH 5.7; E3, eluate with 400 m*M* phosphate pH 6.8 +1 *M* NaCl; E4, eluate with 10 m*M* phosphate pH 6.8 +1 *M* KCl.

caprylate (data not shown). The bibody fraction was then eluted by 50 m*M* sodium acetate buffer, pH 4.0. Based on SDS–PAGE analysis the bibody retention and elution were successful, with a product recovery of >90%. When starting from cell culture medium, the bulk fraction of contaminating albumins was lost in the column flow-through and the caprylate wash (Fig. 7B). Also an expected enrichment for bovine Igs from the FCS was noticed.

To test whether also the scFv fragments contribute to the affinity adsorption, the medium and periplasmic fraction of 100 ml *E. coli* cell culture producing

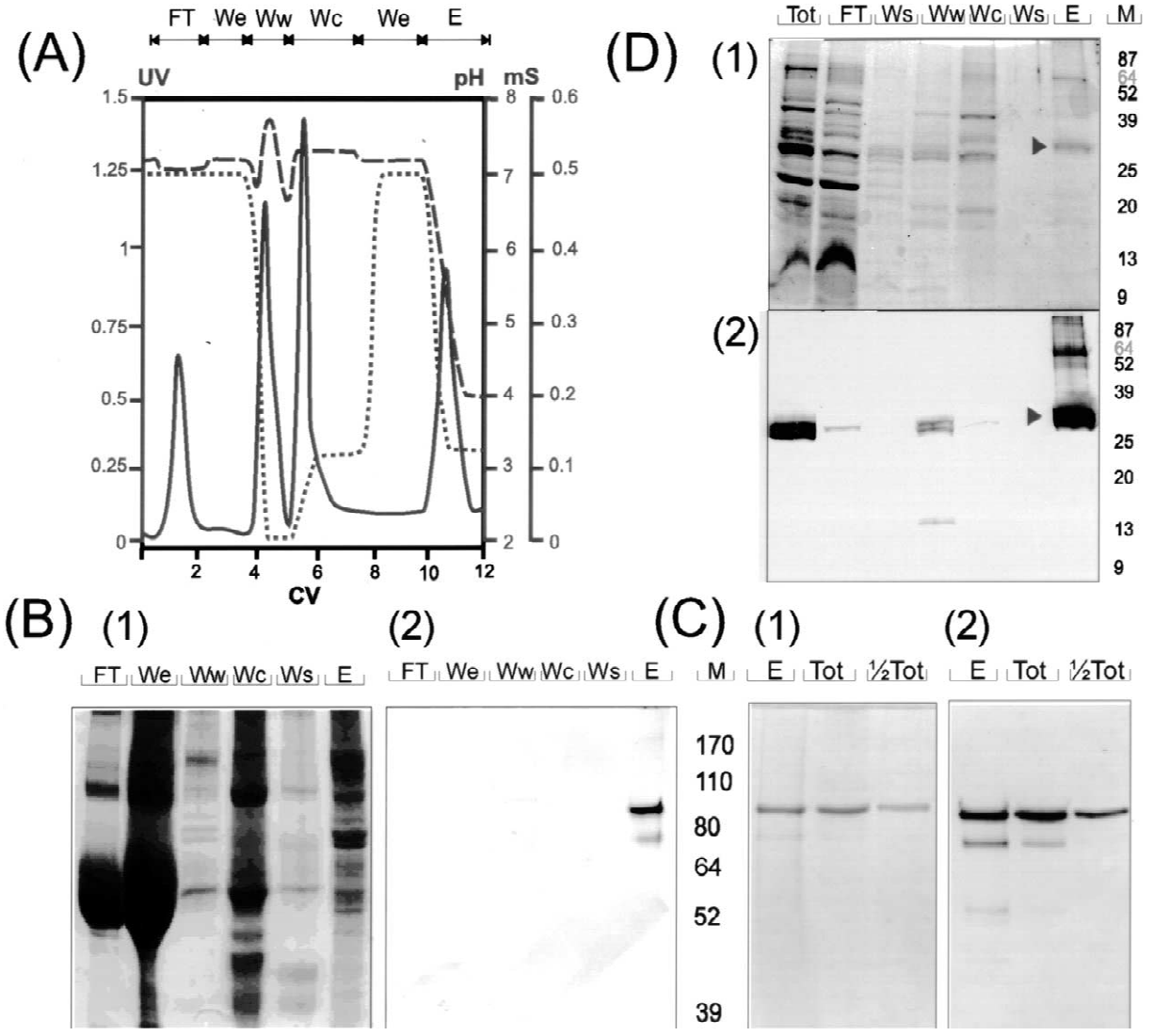


Fig. 7. Hydrophobic charge induction chromatography (on MEP HyperCel sorbent) as a capture step for purification of bibodies and scFv fragments. (A) Purification of a bibody from medium with 10% FCS: Chromatogram. (B) Purification of a bibody from medium with 10% FCS: SDS–PAGE analysis: CBB staining (1) and immunoblotting (2). (C) Recovery of known amount of pure bibody, spiked in equilibrating buffer, before and after purification: CBB staining (1) and immunoblotting (2). (D) Purification of a bacterially expressed scFv. SDS–PAGE analysis: CBB staining (1) and immunoblotting (2). Lanes: FT, flow through; We, wash with equilibrating buffer; Ww, Wash with water; Wc, Wash with sodium caprylate; E, eluate; Tot, total amount of bibody brought onto the column; M, molecular mass marker.

mouse scFv, were loaded onto the column. Based on SDS–PAGE analysis, the scFv retention and elution were successful, with good recovery estimated. Moreover, we found that the bulk of contaminating bacterial proteins was lost in the column flow through and washes, especially in the caprylate wash, leading to an estimated product purity of >70%, with only one prominent contaminating band (Fig. 7C).

HCIC thus appears to be a suitable general capture step for the purification of both Fab and scFv fragments, giving high product recovery while the bulk of either serum albumins (from mammalian cell culture medium) or bacterial proteins (from *E. coli* expression) was removed in the column flow through, but also by washing with 25 m*M* sodium caprylate.

3.8. Size exclusion chromatography to remove aggregates and dimers

Since bispecific antibody derivatives aim at targeting an immunological function towards a target cell, aggregation of the antibody manifolds can and will lead to aspecific activation in the periphery. So avoidance of aggregated products is essential both to study the real effects of only targeted protein in experimental animals, and to avoid peripheral toxicity. To separate monomers from dimers and aggregates, SEC was applied. Although a small fraction of the bi- and tribodies is present as dimers, the majority of the purified bi- and tribody protein is typically found to be monomeric (Fig. 8). No antibody-derived material was found in the void volume, suggesting that no protein aggregates were present.

3.9. Endotoxin removal: a necessary step for in vivo applications

Endotoxins (also known as pyrogens) are lipopolysaccharide–protein (LPS) complexes found in the cell wall of gram-negative bacteria. The LPS component contains the Lipid A moiety which is common to all endotoxins, and which is responsible for some of the pyrogenic activity. Endotoxins from different strains vary markedly in their pyrogenicity. The removal of endotoxins from solutions intended for therapeutic or other in vivo biological applications is crucial, because concentrations of ng/μl have been shown to cause shock reactions in humans and animals.

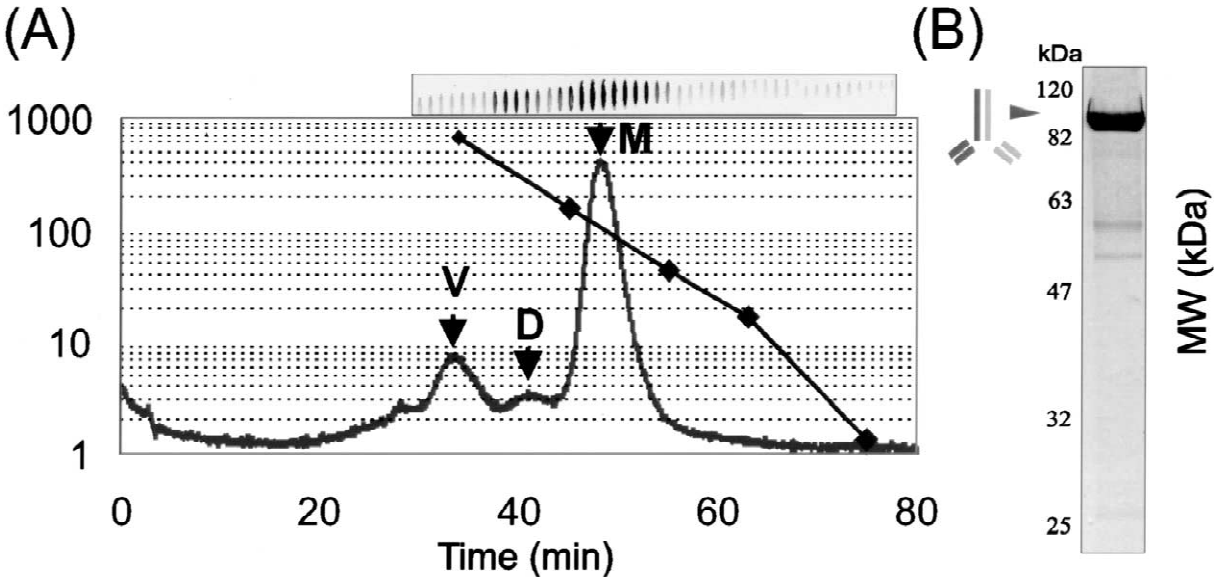


Fig. 8. Polishing with size exclusion chromatography. (A) Chromatogram with integrated molecular mass calibration curve. M, monomers; D, dimers; V, void. (B) CBB staining of the peak at approximately 50 min (monomers).

The most secure way to avoid any microbial contamination and the subsequent release of endotoxins is absolute sterility during the production process and all processes downstream. Yet complete prevention of endotoxin introduction is difficult in a laboratory environment. Typical sources of contamination are raw materials such as salt, water, serum as well as buffers, sorbents and devices used for purification. Hence, removal of endotoxins is a necessary safety step as a final polishing step in a purification process. It must, however, be ensured that any method employed for decontamination allows for high recovery of the target product.

Different methods for endotoxin removal were compared, including Detoxi-Gel (polymyxin B immobilised on agarose), Sartobind membrane adsorber Q5 (a strong basic anion-exchange filter), Acticlean-Etox and PROSEP-Remtox (both solid-phase reagents with a high affinity for endotoxins) and CEC. Two main problems were observed. In a first series of experiments, we found that little or no LPS was removed. This was the case with Detoxi-Gel, Sartobind membrane Q, Acticlean Etox and CEC. Alternatively, we lost the bulk of our protein with the LPS (PROSEP-Remtox). These results suggested that the endotoxin was interacting with the antibody fragments, making it necessary to dissociate it from the protein to allow efficient removal. This was achieved by adding 10 m*M* sodium acetate to the protein solution in PBS and lowering the pH to 5.0 with acetic acid. Passing this solution over an Acticlean Etox matrix resulted in selective removal of the contaminating endotoxins.

4. Conclusion

In order to set up a general production and purification protocol for antibody manifolds containing Fab and scFv fragments produced in mammalian cell culture media, we searched to optimize both the expression and purification of the bibody and tribody antibody manifold.

Different strong eukaryotic promoters (CMV, EF1α and actin) were compared side-by-side for the efficiency of inducing the antibody derivative. Transient expression of bibodies and tribodies in HEK293T cells is best performed under the control of the actin promoter, and in medium containing 10% FCS. For larger-scale experiments however, stable producing SP2/0 myeloma cells were established, using the same strong promoter and growth conditions.

Different strategies for purification of antibody fragments from serum-containing mammalian cell culture medium were subsequently compared. Protein L AC was found to give >90% recovery and >90% pure bibody in a single step. However, because the affinity of protein L is limited to certain classes of light chains, we developed a purification protocol that is more generally applicable, based on the introduction of a hexa-His tag to the C-terminus of the Fd–scFv fusion and a purification step with IMAC. To optimize a necessary capture step, we performed a comparative study of CEC, HAC and HCIC. Using CEC as a capture step, the bulk of the contaminating albumins eluted in the early fractions (150–450 m*M* NaCl), which implies that this is suitable only for antibody fragments eluting at a higher salt concentration, which is not always the case. The evaluation of HAC as a capture step suffered from two drawbacks. First, we found the product recovery to be low; secondly, we found poor product purity. This could be improved by eluting with potassium chloride, which causes cationic proteins (such as most of the antibody fragments) to be desorbed, while all acidic contaminants remain adsorbed on the resin. This approach, however, requires a high capacity column since contaminating proteins are separated from the antibody fragments after both are adsorbed onto the column, rather than by separating the bulk contaminant in the column flow-through. We found HCIC, using the MEP HyperCel matrix to be the method of choice for antibody fragment capture, giving >90% recovery and eliminating the bulk of the contaminating proteins (mainly bovine serum albumin) from cell culture supernatant. HCIC was also suitable to obtain highly purified, bacterially expressed scFv, thus efficiently eliminating the bulk of the contaminating bacterial proteins; this demonstrates that both the Fab and the scFv moieties contribute to the affinity of the charge-inducible, thiophilic matrix. It has been described before that Fab fragments can be eluted from a MEP Hypercel matrix at a higher pH than Fc-fragments and whole antibodies [21]. This feature

could allow for an even more specific elution of Fab-fusion proteins at pH 5.5, leaving the contaminating bovine Igs adsorbed on the resin.

To obtain aggregate- and endotoxin-free antibody derivatives suitable for in vivo experiments, further polishing was necessary. Possible aggregates and dimers were removed with SEC. Endotoxins were removed by dissociating the LPS from the purified proteins at pH 5.0 and passing the solution over a solid-phase reagent with a high affinity for endotoxin (Acticlean Etox).

Acknowledgements

An Willems is a research assistant with the Fonds voor Wetenschappelijk Onderzoek Vlaanderen.

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