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Three-step purification of bacterially expressed human single-chain Fv antibodies for clinical applications

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Abstract

We have obtained a cell line which secretes a human monoclonal IgM (B7) reacting with the myosin heavy chain of human heart. We have constructed single-chain fragments (scFv) of B7. The scFv may be useful for the imaging of myocardial necrosis after myocarditis, cardiac drug toxicosis or graft rejection. The aim of our work was to purify the scFv for immunoscintigraphy. We describe several purification steps including immobilized metal affinity chromatography (IMAC), anti-c-myc monoclonal antibody affinity chromatography, size-exclusion chromatography with Superdex^{R} 75 HR 10/30 and ion-exchange chromatography (mini Q TM 30Q).

Keywords: Purification; Single-chain Fv antibodies

1. Introduction

Human monoclonal antibodies (mAbs) are a new class of diagnostic and therapeutic agents which have made a substantial impact in the biomedical field. Up to now, the majority of clinical studies have been performed with murine mAbs. For example, OKT3, a murine IgG which recognises an epitope on the ϵ-subunit of the human CD3 complex, has been shown to be effective in the treatment of renal, heart, lung and liver allograph rejection [1–4]. A murine anti-myosin mAb is a powerful probe for the imaging of myocardial necrosis [5]. Chimaeric 7E3 Fab fragments recognising the integrin αIIbβ3, composed of the murine antibody V_H and V_L domains and C_{H1} and C_L constant regions of human origin, have been used as an antithrombotic agent [6]. Nevertheless, for therapeutic and diagnostic uses, mAbs of entirely human origin offer considerable advantages over those from other species and are less likely to provoke the immune responses which can severely impair the efficiency of murine mAbs [7]. For example, a human anti-mouse antibody (HAMA) response may accelerate clearance of a circulating mAb, block binding of a mAb to its antigen and/or trigger allergic reactions. Because of the HAMA phenomenon, interest has increased in human mAbs.

We have previously described how the Epstein–Barr virus transformation of B lymphocytes from a Glanzmann's thrombasthenia patient (an inherited bleeding syndrome linked to decreased amounts or the absence of the integrin αIIbβ3 on platelets) led to the immortalisation of a B cell secreting a monospecific human anti-myosin IgM (referred to as B7) [8]. The monoclonal antibody B7 was extensively characterised by Western blotting, enzyme linked

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immunosorbent assay (ELISA) tests and immunohistochemistry [8]. In the latter procedure, B7 clearly recognised myosin filaments in longitudinal sections of myocytes; there was no labelling of intracellular spaces [8]. Because B7 is entirely human in origin and because it reacts with cardiac myosin with high avidity, it may be useful in the imaging of myocardial necrosis after graft rejection [5], cardiomyopathy [9], suspected myocarditis [10], or cardiac drug toxicosis [11]. However, owing to the fact that the relative molecular mass of an IgM is 900 000, poor diffusion and a slow penetration inside necrotic myocytes could reduce its sensitivity in scintigraphic detection. The development of genetically engineered scFv (M_r 32 000) molecules, which consist of only the antibody variable regions held together by a flexible linker to increase stability, has the potential to alleviate some of these problems. Indeed, these molecules have the advantage of rapid clearance from circulation, good penetration within solid tissue, and theoretically, low immunogenicity [12]. It is also possible to modify the scFv fragments to endow them with novel characteristics, such as the ability to chelate radiometals for imaging or therapeutic purposes [11]. Such molecules have been used in clinic with success for imaging tumours [13,14].

We have constructed the recombinant scFv fragments of the B7 human IgM into the expression vector pHOG21 [15]. The scFv are expressed in soluble form in *Escherichia coli* and are found both in the periplasmic space and the medium (manuscript in preparation). The aim of our work was to purify the B7 scFv fragments for immunoscintigraphy so that they could be used as an imaging agent to identify necrotic myocardium in a number of cardiac disorders. Different separation technologies have been described that permitted the purification of recombinant scFv to homogeneity [16–21]. One of them, largely used, immobilized metal affinity chromatography (IMAC), is based on the introduction of a sequence encoding multi-histidine domains at the N or C terminus of the protein of interest [22,23]. Although previous studies have described the purification of scFv fragments in a single-step by IMAC [15,24,25], we have found that IMAC purification can only be considered as a primary step in the purification. For this first step, the use of an anti-c-myc mAb 9E10 affinity column was also evaluated. To obtain a pure preparation of the scFv, additional steps such as size-exclusion fast protein liquid chromatography (FPLC) chromatography or anion-exchange chromatography followed by an anti-c-myc mAb 9E10 affinity chromatography were found to be necessary. In this paper, we describe how the bacterially expressed human scFv can be purified by a three-step purification procedure for clinical applications which usually require 99% purity.

2. Materials and methods

2.1. Expression of recombinant single-chain antibody fragments

The genes encoding for the B7 antibody V_H and V_L domains were cloned into the pHOG21 expression vector [15] (manuscript in preparation). In this vector, the scFv fragment is immediately followed by a c-myc tag recognised by the mAb 9E10 and by six C-terminal histidine residues. XL1-Blue *E. coli* bacteria (Stratagene, La Jolla, CA, USA) transformed with the pHOG21 vector containing the B7 scFv fragment were grown overnight in LB medium (Invitrogen, San Diego, CA, USA) with 50 μg/ml ampicillin and 100 m*M* glucose (LB_{GA}) at 37°C. The overnight culture was diluted (1:20) and culture continued in 5-l flasks at 37°C with shaking at 280 rpm. When the culture reached $OD_{600}=0.7$, bacteria were pelleted by centrifugation at 1500 *g* for 10 min at 20°C and resuspended in the same volume of fresh LB medium containing 50 μg/ml ampicillin and 0.4 *M* sucrose. The expression of the scFv was induced by the addition of 1 m*M* IPTG (isopropyl-β-D-thiogalactopyranoside). The culture was allowed to grow for 18–20 h at 37°C.

2.2. Preparation of periplasmic extract and culture supernatant containing the soluble scFv

Bacteria pellet and culture supernatant were harvested by centrifugation at 5000 *g* for 15 min at 4°C. To isolate soluble periplasmic proteins, the pelleted bacteria were resuspended in 5% of the initial volume of ice-cold 50 m*M* Tris–HCl, 20% sucrose, 1 m*M* EDTA, pH 8.0. After a 1 h incubation on ice with occasional stirring, the spheroplasts were cen-

trifuged at 30 000 *g* for 30 min at 4°C, yielding a soluble periplasmic extract in the supernatant and insoluble periplasmic material in the pellet. The B7 culture supernatant and the soluble periplasmic extract were combined, clarified by additional centrifugation (30 000 *g*, 4°C, 30 min) and passed through a 0.2-μm 1-l filter (Nalgene, Poly Labo, Strasbourg, France). The clarified supernatant was concentrated and dialyzed against 50 m*M* Tris–HCl, 1 *M* NaCl, pH 7.0 (starting buffer) using a Prep/Scale ultrafiltration system (Millipore, St. Quentin Yvelines, France) incorporating a cartridge with a molecular mass cut-off of M_r 5.

2.3. Purification of B7 scFv fragments

2.3.1. Chemicals

Chelating-Sepharose fast flow, Superdex^R 75 HR 10/30, mini Q^R PE 4.6/50 were purchased from Amersham Pharmacia Biotech (Saclay, France).

Ultralink Immobilized Hydrazide was purchased from Pierce (Interchim, Montluçon, France).

The mouse mAb, Myc-1 (9E10), was produced in our laboratory from the CRL-1729 clone purchased from ATCC (Rockville, Canada) and cultivated in a hollow fibre bioreactor (Tecnomouse, Integra Biosciences, Eaubonne, France).

A mouse anti-histidine mAb was purchased from Dianova (Hamburg,Germany).

Ovalbumin (M_r 45 000), β-lactoglobulin (M_r 35 000), chymotrypsinogen A (M_r 25 000) were purchased from Sigma (l'Isle d'Abeau Chesnes, France).

All salts were HPLC grade, and the buffers were filtered through a 0.22-μm membrane filter.

2.3.2. Instruments

FPLC systems were used throughout this study (Amersham Pharmacia Biotech). Data were evaluated using the FPLC Director Data System.

A Uvikon 930 spectrophotometer (Kontron, Montigny Lebretonneux, France) was used to monitor recovery by measuring absorbance at 280 nm.

A K16/20 column and the GradiFrac system from Amersham Pharmacia Biotech were used for the affinity chromatography.

2.3.3. Immobilized metal affinity chromatography

A 5-ml volume of chelating Sepharose fast flow was charged with 0.3 *M* copper sulphate metal ions (Sigma) in water (20 ml). The column was equilibrated with 10 m*M* imidazole, 1 *M* NaCl, 50 m*M* Tris–HCl, pH 7.0. The sample, diluted 1:2 in equilibration buffer, was loaded at a flow-rate of 0.6 ml/min. The column was then washed with 20 column volumes of starting buffer containing 40 m*M* imidazole until the absorbance (280 nm) of the effluent was minimal. Absorbed material was eluted with 100 m*M* imidazole, 1 *M* NaCl, 50 m*M* Tris–HCl, pH 7.0. Fractions corresponding to the A_{280} peak were pooled, concentrated and dialyzed against phosphate-buffered saline (PBS, Dulbecco buffer) (Eurobio, Les Ulis, France), pH 7.4 using Macrosep centrifugal concentrators (Filtron, Poly Labo).

2.3.4. Affinity chromatography on an anti-c-myc mAb affinity column

The mouse anti-c-myc mAb, 9E10, was coupled onto a hydrazide-derivatized azlactone-activated support according to the manufacturer's instructions.

The affinity column (2 ml) was equilibrated in PBS–0.5 *M* NaCl, pH 7.0. A 1-l volume of B7 culture supernatant mixed with the soluble periplasmic extract which had been concentrated 10 times and dialyzed against PBS–0.5 *M* NaCl (100 ml), was loaded at 0.6 ml/min at 4°C. Contaminants were eliminated by washing with 20 column volumes of PBS–0.5 *M* NaCl. Bound proteins were eluted with 0.1 *M* glycine–HCl, pH 2.8 at 1 ml/min. The eluted fractions were immediately neutralised with 2.5 *M* Tris-base, concentrated and dialyzed against PBS, pH 7.4.

2.3.5. Size-exclusion FPLC chromatography

Preparations of scFv, partially purified by IMAC, were further purified by size-exclusion on Superdex 75 HR10/30. The column was equilibrated with PBS, pH 7.4. Sample volume and flow-rate were 500 μl (100 μg protein/ml) and 0.2 ml/min, respectively. The flow through was calibrated for proteins of known molecular masses [ovalbumin (M_r 45 000), β-lactoglobulin (M_r 35,000) and chymotrypsinogen A (M_r 25 000)].

2.3.6. Anion-exchange chromatography

Anion-exchange chromatography was performed using a mini Q^R PE 4.6/50 column. The column was equilibrated with 4 ml of starting buffer (50 m*M* Tris–HCl, pH 8). A 1-ml volume of an IMAC flow through sample (100 μg protein/ml) was dialyzed against the starting buffer and loaded on the column at 0.2 ml/min. The proteins were eluted with a step gradient of up to 1 *M* NaCl.

2.4. Analytical

2.4.1. Protein determination

The concentration of scFv was calculated by determining the total protein concentration using the Micro BCA Protein Assay Reagent and the NanoOrange protein quantification kit (Pierce) and by measuring the percentage of scFv by gel scanning (Image Station 440 CF, Kodak, Rochester, NY, USA).

2.4.2. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblot analysis

Analysis of the fractions containing scFv was performed by electrophoresis on 12% SDS–polyacrylamide gels under reducing conditions [26]. The gels were silver stained (Silver Stain Kit) (Bio-Rad, Ivry Sur Seine, France) or the proteins transferred by electrophoresis to a nitrocellulose membrane for immunoblotting using the mAb, 9E10. Bound antibody was detected using peroxydase-labelled sheep anti-mouse IgG antibody (Jackson Immunoresearch, Marseille, France) and chemiluminescence ECL Western blotting detection reagents (Pierce).

3. Results

3.1. Primary step for B7 scFv purification:

3.1.1. IMAC

A 100-ml volume of the concentrated *E. coli* supernatant combined with the periplasmic extract (2 mg/ml of total protein), diluted (1:2) in equilibration buffer and containing the B7 scFv, around M_r= 35 000 as shown on immunoblot analysis (Fig. 2a, lane 3), was passed through a Cu^{2+}-charged chelating Sepharose column. After washing the column with buffer containing 40 m*M* imidazole, the scFv were eluted with 100 m*M* imidazole as a single peak in nine column volumes (Fig. 1). The eluted fraction was concentrated 10 times and dialyzed against PBS for size-exclusion FPLC, or 50 m*M* Tris–HCl, pH 8.0, for anion-exchange chromatography. The concentration of proteins in this IMAC fraction was calculated with the NanoOrange protein quantification kit and found to be 100 μg/ml. SDS–PAGE analysis of material purified by IMAC showed several major proteins of M_r 30 000, 35 000, 48 000 and 70 000 (Fig. 2a, lane 2) with only the 35 000 band reacting with the anti-c-myc antibody on immunoblot analysis (Fig. 2a, lane 4). The predicted relative molecular mass of the expected protein is 31 000. Thus, the B7 scFv fragments run 11% slower on SDS gels. The gel scanning and immunoblotting analysis showed that only a small proportion of this material corresponded to the recombinant scFv, the rest being contaminating material. The percentage of the scFv in the IMAC preparation estimated by gel scanning was around 17%. So, we obtained a preparation (4.5 ml) containing 76.7 μg of scFv.

The contaminant protein of M_r 30 000, co-purified by IMAC, representing 36% of the total protein, was very close to the predicted molecular mass of the B7 scFv fragment. One hypothesis could be that this protein was in fact a B7 scFv fragment in which the c-myc portion was obscured by folding. This was not the case for this protein did not react in Western blotting with the mAb against the C-terminal His tag (data not shown).

3.1.2. Affinity chromatography on an anti-c-myc mAB 9E10 affinity column

The crude concentrated preparation (100 ml containing 2 mg/ml of protein) was chromatographed on an anti-c-myc mAb 9E10 affinity column. The purity of scFv obtained by IMAC and by anti-c-myc affinity chromatography was compared. Fig. 2b shows that different contaminant proteins from those observed after IMAC were present in the anti-c-myc affinity chromatography purified preparation. A major protein of higher molecular mass (M_r 62 000) was seen. It is to be noted that fewer contaminants were present here than in the IMAC eluate. However, the scFv fragments purified by IMAC, gave a

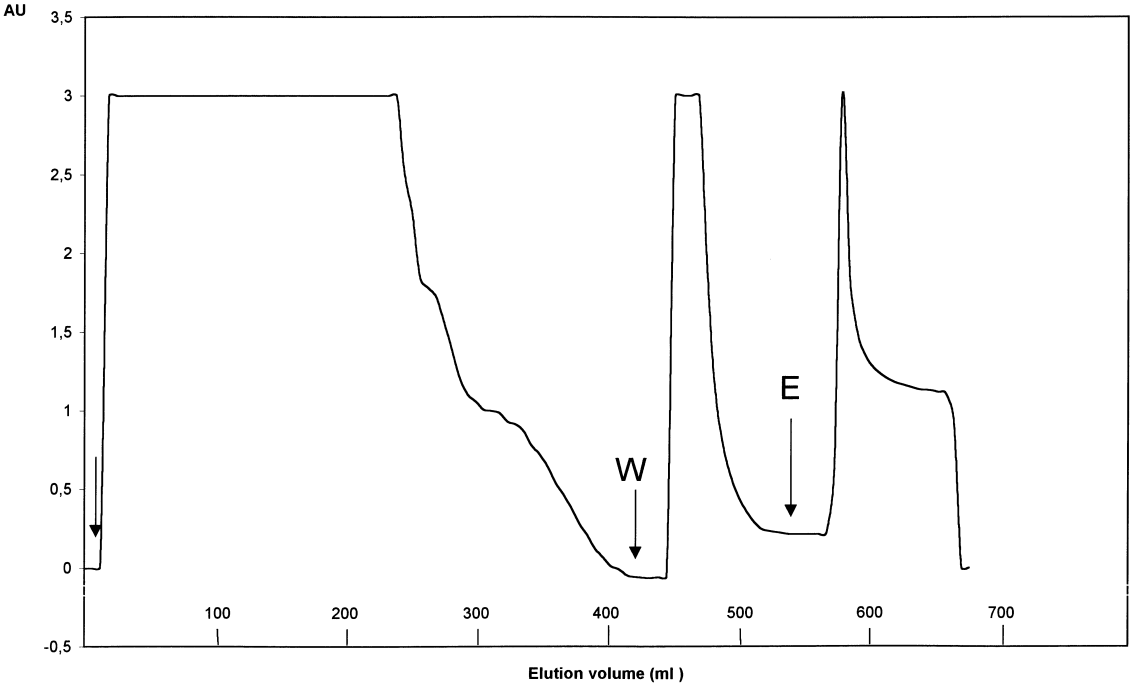

Fig. 1. Immobilized metal affinity chromatography (IMAC). The column was prepared by preloading with Cu^{2+} ions and equilibrated with a starting buffer (10 m*M* imidazole, 50 m*M* Tris–HCl, 1 *M* NaCl, pH 7). After passing the sample, the column was first washed with the starting buffer until the absorbance (280 nm) of the effluent was minimal and then with starting buffer containing 40 m*M* imidazole (W). Absorbed material was eluted with starting buffer containing 100 m*M* imidazole, pH 7 (E).

stronger intensity in chemiluminescence (Fig. 2a, lane 4 compared to Fig. 2b, lane 4). The percentage of the scFv in the fraction obtained by affinity chromatography on an anti-c-myc mAb affinity column, was 12% as determined by gel scanning. Finally, we obtained 500 μl of a concentrated and dialyzed preparation containing 60.5 μg/ml of protein and 3.63 μg of scFv, which is only 5% of scFv yield from IMAC. In view of these results, we selected the IMAC method for the first purification step.

3.2. Second step of B7 scFv purification:

3.2.1. Anion-exchange chromatography of IMAC-purified scFv

A 1-ml volume of the IMAC-scFv preparation (100 μg/ml) was passed through the mini Q^R PE 4.6/50 column. The elution profile (Fig. 3a) showed three peaks. Analysis by Western blotting (Fig. 4a) revealed the presence of the scFv in the fraction corresponding to the third peak (lanes 3 and 4). The percentages of the scFv were 16% for peak 3 and 23% for peak 4. Finally, we obtained a preparation of about 2 ml by mixing both fractions. The protein concentration was 33.6 μg/ml. Within this preparation, the quantity of the scFv was estimated at 13.1 μg. Nevertheless, SDS–PAGE analysis showed that the major contaminant protein (M_r 30 000) had simultaneously eluted with the scFv.

3.2.2. Gel permeation chromatography of IMAC-purified scFv on a Superdex 75 HR30/10 column

A 500-μl volume of the IMAC-purified scFv preparation (100 μg/ml) was fractionated by FPLC gel permeation on Superdex 75 HR30/10 at pH 7.0. Fig. 3b shows the elution profile and reveals the presence of two peaks. Peak 1 on SDS–PAGE gave a minor band at M_r 73 000 (Fig. 4b, lane 2). This protein did not react with the anti-c-myc mAb, 9E10, on Western blot analysis (data not shown). Peak 2 on SDS–PAGE gave two bands of M_r 35 000 and

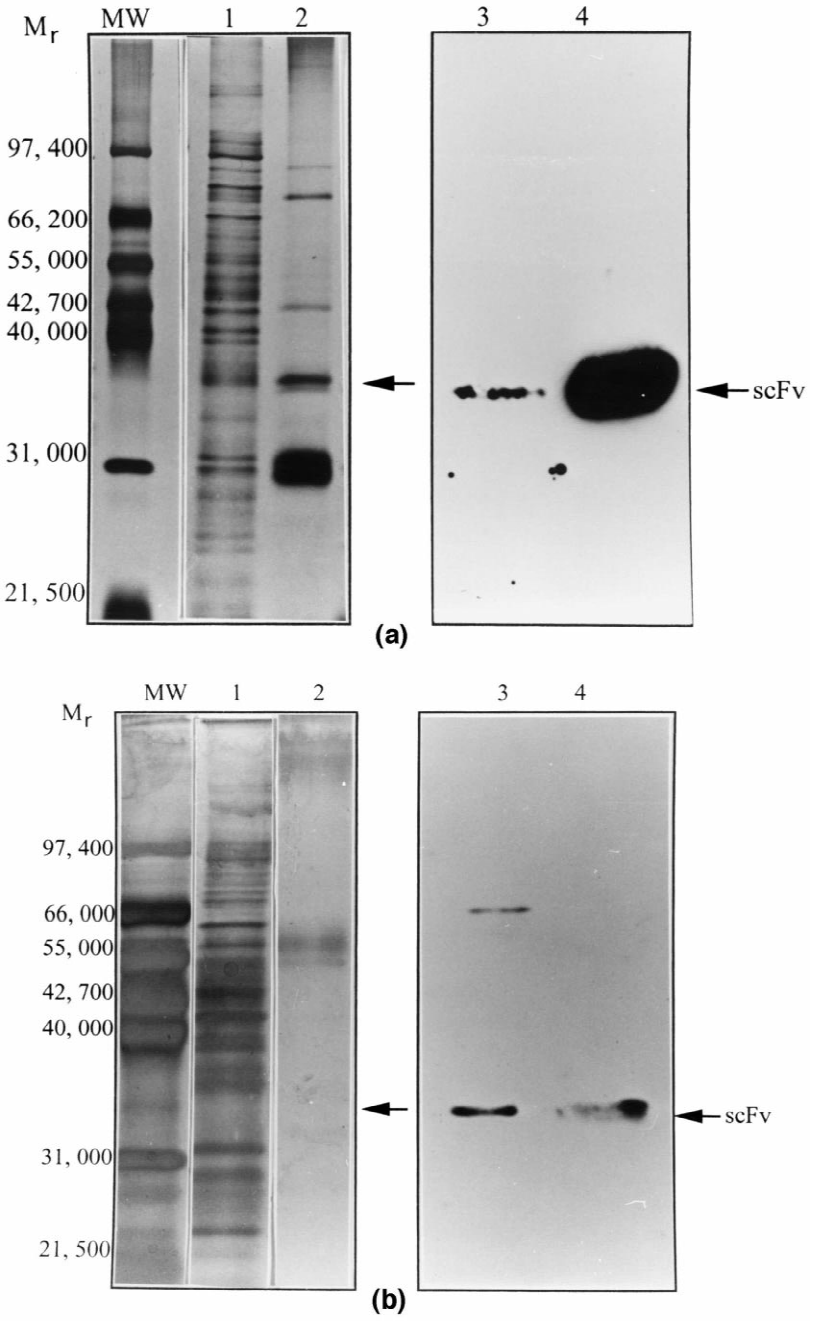

Fig. 2. (a) 12% SDS–PAGE and immunoblot analyses of scFv enriched by IMAC under reducing conditions. MW (molecular mass markers); lanes 1, 3: 20 μl concentrated culture medium and periplasmic extract (40 μg protein); lanes 2, 4: 80 μl of the scFv enriched fraction obtained by IMAC (1.36 μg scFv). MW, lanes 1, 2: silver staining; lanes 3, 4: Western blot analysis with anti-c-myc antibody. (b) 12% SDS–PAGE and immunoblot analyses of scFv enriched by affinity chromatography on an anti-c-myc column under reducing conditions. MW (molecular mass markers); lanes 1, 3: 20 μl concentrated culture medium and periplasmic extract (40 μg protein); lanes 2, 4: 20 μl of the scFv partially purified on the 9E10 affinity column (0.145 μg scFv). MW, lanes 1, 2: silver staining; lanes 3, 4: Western blot analysis with anti-c-myc antibody.

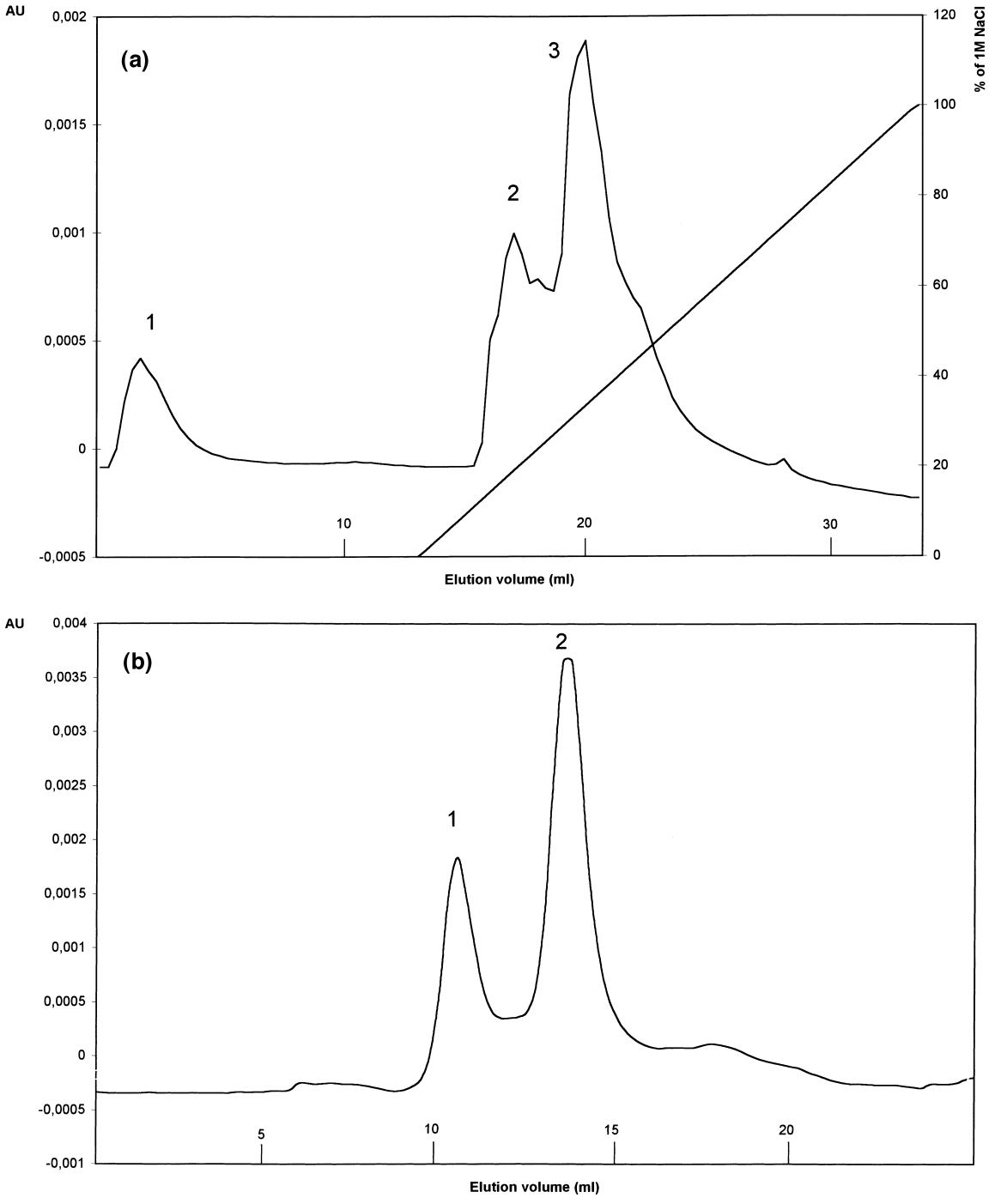

Fig. 3. (a) Anion-exchange chromatography on a mini Q^R PE 4.6/50 of B7 scFv isolated by IMAC. One ml (100 μg protein) of an IMAC purified sample was loaded on the column equilibrated with 50 m*M* Tris–HCl, pH 8.0 at 0.2 ml/min. The scFv was eluted with a step gradient of up to 1 *M* NaCl. The elution profile shows three peaks (1, 2, 3). (b) Size-exclusion FPLC on a calibrated Superdex 75 HR30/10 column of B7 scFv isolated by IMAC. The column was equilibrated with PBS, pH 7.4. Sample volume and flow-rate were 500 μl (100 μg/ml) and 0.2 ml/min, respectively. Molecular masses were calibrated with ovalbumin (M_r 45 000), β-lactoglobulin (M_r 35 000) and chymotrypsinogen A (M_r 25 000). The elution profile shows two peaks: (1) M_r 80 000, (2) M_r 35 000.

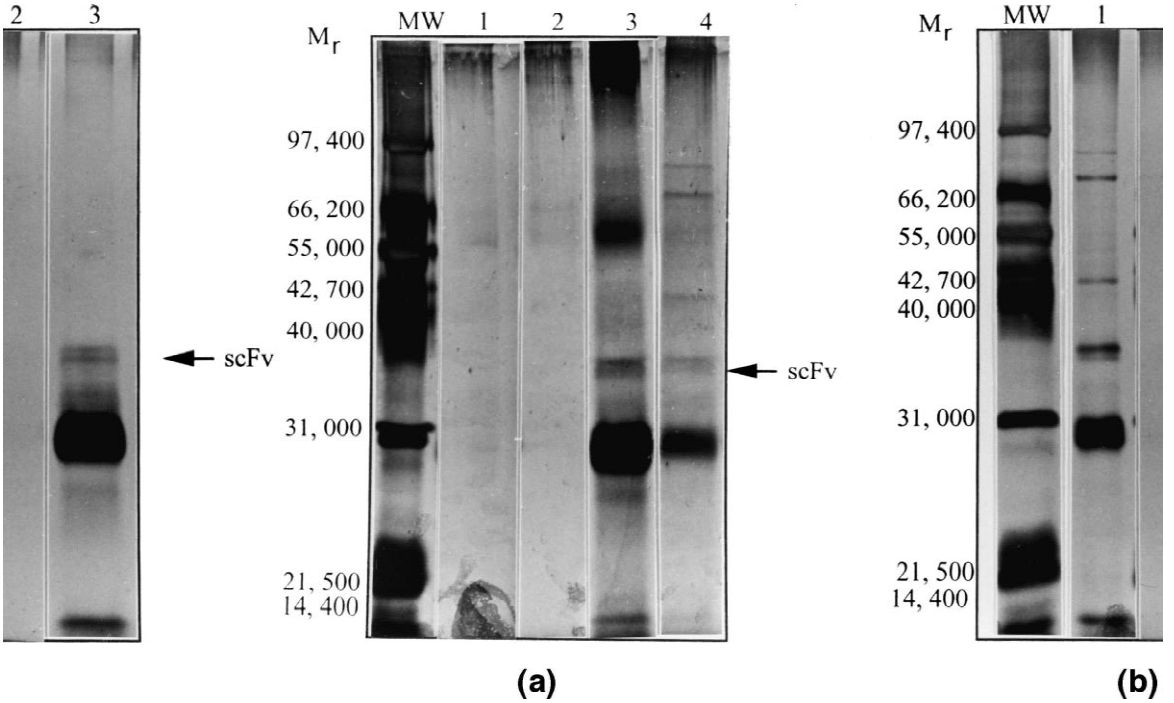

Fig. 4. (a) 12% SDS–PAGE of scFv from the IMAC eluate further purified by anion-exchange chromatography using a mini Q^R PE 4.6/50. MW (molecular mass markers), lanes 1–4: 80 μl of the eluted fractions, concentrated three times and dialyzed against PBS, pH 7, corresponding, respectively, to peak 1, peak 2, first part of peak 3, second part of peak 3. The gel was silver stained. An arrow head indicates the position of the scFv present in the fraction corresponding to the third peak. The quantity of the scFv was estimated to be 0.44 μg. A major contaminant protein (M_r 30 000) is present in the same scFv fraction. (b) 12% SDS–PAGE of scFv from the IMAC eluate further purified by size exclusion on Superdex 75 HR10/30. MW (molecular mass markers); lane 1: 40 μl of scFv enriched by IMAC (100 μg/ml); lanes 2, 3: 80 μl of the eluted fractions, concentrated three times and dialysed against PBS, pH 7, corresponding, respectively, to peaks 1 and 2. The gel was silver stained. The scFv is present in the second peak. An arrow head indicates the position of the scFv. Their quantity was estimated to be 0.6 μg. A major contaminant protein (M_r 30 000) is present in the same scFv fraction.

30 000 (Fig. 4b, lane 3). The band at M_r 35 000 reacting with the mAb 9E10 corresponded to the scFv fragments (data not shown). The percentage of the scFv was 23%. As the final preparation contained 32 μg of protein in 1 ml, the quantity of the scFv was estimated at 7.3 μg.

3.3. Third step for B7 scFv purification:

3.3.1. Affinity chromatography on an anti-c-myc mAb affinity column

A 3-ml volume corresponding to the second peak of the FPLC gel permeation performed on the Superdex 75 HR30/10 column, containing 21 μg of scFv, were chromatographed on an anti-c-myc mAb affinity column. The contaminating protein (M_r 30 000) was eliminated from the bound fraction as shown by SDS–PAGE analysis in Fig. 5 (lane 1) where only the scFv fraction was present. These purified scFv fragments, representing 6 μg of scFv in 1 ml, were also detected by Western blotting (Fig. 5, lane 2).

4. Discussion

For the in vivo detection of myocardial cell death, Khaw et al. [27] introduced the application of radiolabeled anti-myosin antibodies which bind to insoluble myosin after the loss of myocyte mem-

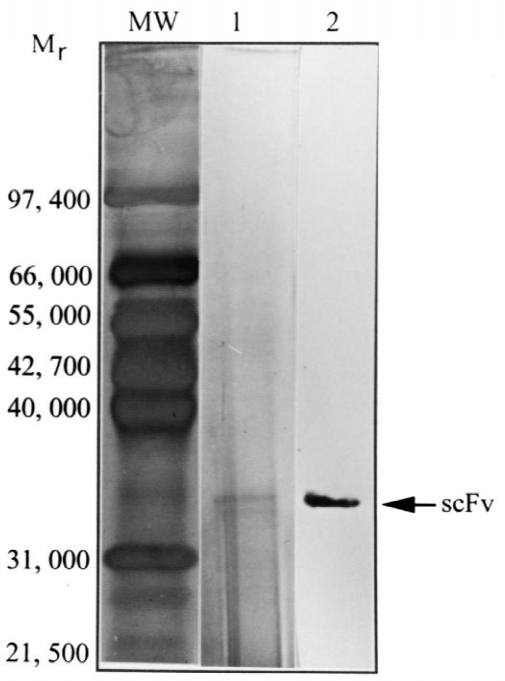

Fig. 5. 12% SDS–PAGE of the third step of purification by chromatography on an anti-c-myc mAb affinity column after IMAC and gel permeation. MW (molecular mass molecular mass markers); lane 1: 80 μl (0.480 μg scFv) of the eluted fraction after dialysis against PBS, pH 7. The concentration of the scFv was estimated at 6 μg/ml. Lane 1: silver staining; lane 2: Western blot analysis with 9E10.

brane integrity, therefore allowing the scintigraphic detection of myocardial necrosis. Anti-myosin imaging may also be helpful as a non-invasive method in identifying cardiac rejection and its progression in heart transplants [5], suspected myocarditis [10], muscular tumors [28] and cardiac side-effects of anthracycline treatment [11,29]. However, murine antibodies, as foreign, may elicit immune reactions that reduce or eliminate their therapeutic efficacy and/or evoke allergic or hypersensitivity reactions in patients upon readministration with the same antibody [30]. So, a human antibody to myosin might offer advantages in this type of imaging.

The Epstein–Barr virus transformation followed by somatic cell hybridisation of B lymphocytes from a Glanzmann's thrombasthenia patient permitted us to obtain a cell line which was found to secrete a human IgM (B7) that reacted with the myosin heavy chain in human heart [8]. Our aim was to evaluate whether this highly specific human IgM antibody was useful for the efficient imaging of myocardial necrosis. However, human monoclonal IgM antibodies may be poor imaging agents due to their large size [31]. The IgM molecule has a molecular mass of about M_r 900 000. It is generally assumed that due to steric hindrance, IgM has a slow tissue penetration rate [32]. The recent development of genetically engineered scFv, which consist of only the antibody variable regions held together by a flexible linker to increase stability [33], offer the potential to alleviate this problem. ScFv can be expressed in *E. coli* within the periplasmic space or are secreted into the medium [15]. Moreover, bacterial expression of scFv also offer other advantages, including cost and speed of production [34].

We have constructed a gene encoding a B7 antibody scFv fragment, with a C-terminal six-histidine domain. ScFv were expressed in soluble form in *E. coli* (manuscript in preparation). Here, the paper describes in detail the procedure used to process and purify this human scFv containing a c-myc tag and a 6XHis tail. Three purification steps were necessary to obtain our scFv free from contaminant proteins that were co-purified by IMAC. Two methods were tested as a second step of purification: gel permeation of IMAC-purified scFv on a Superdex 75 HR30/10 column and anion-exchange chromatography of IMAC-purified scFv. After the anion-exchange chromatography, many contaminant proteins were still present. While SDS–PAGE of the gel permeation eluate showed improvement, a major contaminant protein (M_r 30 000) is co-enriched with the scFv fragments. As this protein is present after either the anion-exchange chromatography or the gel permeation, we decided not to use these two chromatographies sequentially. So, we decided to use gel permeation as a second step as fewer contaminant proteins were present. As the result obtained following anti-c-myc affinity chromatography clearly showed that the protein of M_r 30 000 could be eliminated by this method, this step was chosen as a suitable third step purification for our scFv fragments, even if the yield of this process has previously proven to be low. We have now to determine in further experiments if inverting the second and third steps could represent a more suitable sequence for

the purification of the scFv. Indeed, a gel permeation as a third step could avoid a dialysis process after the affinity purification.

In view of our results, it appears that the B7 scFv fragments are poorly secreted either in the periplasmic space or culture medium (manuscript in preparation). This makes their purification difficult. Several studies have indicated that the yield of secreted recombinant antibody fragments depends on the primary sequence of the antibody in *E. coli*. For example, in identical *E. coli* host–vector systems, 1–5 mg/l of anti-glycophorin A 1C3 scFv were found in the culture medium [35] compared to 0.1–0.5 mg/l of anti-DNP U7.6 scFv and no anti-transferrin receptor OKT9 scFv [36]. Our results show that even scFv fragments poorly secreted by *E. coli* can be purified by stepwise purification protocol.

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