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Improvement in production and purification bioprocesses of bacterially expressed anti-alphaIIbbeta3 human single-chain FV antibodies

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Abstract

Production of anti-alphaIIbbeta3 (anti-αIIbβ3)-binding single-chain FV (scFv) fragments obtained from combinatorial libraries of IgG human antibodies is of broad interest for imaging and treatment of acute coronary syndromes. The objective of our work was to design an optimized production of one selected anti-αIIbβ3-binding scFv fragment for subsequent in vivo animal studies. Fed-batch fermentation was initiated with 2TY media supplemented with 0.1 M glucose. This growing batch culture was used as a starting point for further fed-batch induction, in which a media without glucose containing 1 mM IPTG and 0.4 M saccharose was continuously added. Subsequent purification was performed on the whole cell extract in native conditions over an immobilized copper-ion affinity column. The improved conditions allowed the recovery of 5 mg of highly purified scFv fragments as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The bioactivity of the scFv fragments was further monitored by ELISA, cytometric and immunohistochemical methods.

Keywords: IMAC purification; Single-chain Fv antibody fragment; AlphaIIbbeta3 integrin; Fermentation

1. Introduction

Antibody molecules and their fragments represent over 30% of the proteins currently undergoing clinical trials [1]. We recently isolated human IgG anti-alphaIIbbeta3 (anti-αIIbβ3)-binding fragments using combinatorial libraries of single-chain IgG created from the B cells of a polytrans-fused Glanzmann thrombasthenia patient and an autoimmune thrombocytopenic purpura patient (AITP), both with serum Abs directed against the αIIbβ3 integrin [2]. Platelet αIIbβ3 antagonists are potential therapeutic and diagnostic reagents for treating and imaging acute coronary syndromes [3]. Three of the anti-αIIbβ3 phage-Abs were selected for further cloning into the pHOG21 vector and expressed as single-chain Fragment variable (scFv) fragments. scFv[TEG4] was chosen for further investigation and large-scale production. The advantage of *Escherichia coli* as an expression system is its ability to produce proteins in relative large amounts [4]. Moreover, *E. coli* requires simple inexpensive media for rapid growth and *E. coli* transfected with plasmids of interest can easily be cultured in fermenters, thus allowing large-scale production of proteins [5].

Before deciding on a purification strategy, it is important to check for scFv cellular location. The first approach involves the secretion of the scFv fragment in standard lab-scale conditions into the oxidizing medium of the periplasmic space, i.e. the compartment between the inner and outer membrane [6]. Indeed, the intra-chain disulfide bonds of the antibody are not usually formed in the reducing environment of the cell cytoplasm, hence hampering the functional activity of the scFv fragment [7,8]. It is believed that the loss of disulfide is directly due to poor expression of folded fragments and

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further aggregation [9]. Unfortunately, the scFv could not be detected in the culture medium or in the periplasmic compartment. This was not surprising since the amount of proteins is currently limited by the export process [10]. We thus explored other possibilities of cellular expression allowing higher production levels, such as soluble or insoluble fractions in bacteria whole extract. We cultivated scFv[TEG4] in a bioreactor to allow growth at a higher cell density before induction. Moreover, fed-batch fermentation was undertaken to control the induction process. Extraction was enhanced in the cytoplasm of bacteria transformed with plasmid-containing scFv[TEG4] when a fed-batch bioprocess was used in 2TY medium supplemented with 0.1 M glucose and IPTG induction at 30 °C in 2TY medium supplemented with 0.4 M saccharose. As a result of this approach, we successfully purified the scFv[TEG4] fragments by immobilized metal ion affinity chromatography (IMAC). The fragments were then refolded in vitro by a simple dialysis procedure, as testified by immunological studies.

2. Materials and methods

2.1. Chemicals

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 fiber bioreactor (Tecnomouse, Integra Biosciences, Eaubonne, France).

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

All reagents were of analytical grade and purchased from Sigma-Aldrich (St. Quentin, France) unless otherwise specified. Ultra-pure Imidazole was from BDH Merck (VWR International, Strasburg, France).

XL1-Blue *E. coli* bacteria (Stratagene, La Jolla, CA, USA) were used as a host for cloning and protein expression.

The pHOG21 vector was kindly provided by M. Little (Affimed Therapeutics, Lademburg, Germany) [11].

2.2. Instruments

The fermenter used for cultivation was a New Brunswick Bioflow 3000 bioreactor (New Brunswick Scientific, Fisher Bioblock Scientific, Illkirch, France).

Biopilot chromatographic systems were used throughout this study (Amersham Biosciences). Data were evaluated using the Unicorn Data system.

Sonication was performed using a Vibracell 72427 sonicator (Fisher Bioblock Scientific, Illkirch, France).

Absorbance readings were performed with an Emax precision microplate reader (Molecular Devices, CA, USA).

Gel scanning was evaluated using the Kodak Biostation IS2000R (SienceTec, Les Ulis, France).

A vertical minigel electrophoresis apparatus used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and a semi-dry electrophoretic transfer system used for Western blotting were from BioRad (Marnes la Coquette, France).

For cytometric studies, mean fluorescence intensity (MFI) was measured on a FACScan fluorescence cytometer (BD BioSciences, Le Pont de Claix, France) using Cell Quest Pro software.

Immunohistochemical sections were examined using a light microscope (Nikon Microphot Fx).

2.3. Expression of recombinant scFv[TEG4] fragments

XL1-Blue *E. coli* bacteria transformed with the pHOG21 vector containing the TEG4 scFv fragment were grown for 15 h in LB-Lennox, 2TY or TB medium (Invitrogen, San Diego, CA) supplemented with 50 μg/ml ampicillin and 100 mM glucose (LB_{GA}, $2TY_{GA}$ or TB_{GA}) at 37 °C. Dilutions (1/60) of the cultures were grown as 50 ml culture in shake-flasks at 37 °C with shaking at 280 rpm. When the culture reached an absorbance of 0.7, bacteria were pelleted by centrifugation at $1500 \times g$ for 10 min and re-suspended in the same volume of fresh LB, 2TY or TB medium containing 50 μg/ml ampicillin and 0.4 M saccharose [12] (for culture in 2TY medium only). The increase in osmotic pressure induced by adding saccharose leads to the accumulation of osmoprotectants in the cell, which stabilize the native protein structure. scFv expression was induced by adding IPTG (isopropyl-β-D-thiogalactopyranoside) or lactose. The culture was allowed to grow for 7–15 h at 25 or 37 °C.

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

Bacteria pellet and culture supernatant were harvested by centrifugation at $5000 \times g$ for 15 min at 4 °C. To isolate soluble periplasmic proteins, the pelleted bacteria were re-suspended in 5% of the initial volume of ice-cold 50 mM Tris–HCl, 20% sucrose, 1 mM EDTA, pH 8.0. After 1 h incubation on ice with occasional stirring, the spheroplasts were centrifuged at $20{,}000 \times g$ for 30 min at 4 °C [12]. The scFv-TEG4 culture supernatant and the soluble periplasmic extract were clarified by additional centrifugation ($20{,}000 \times g$, 4 °C, 30 min) and passed through a 0.2 μm 1-l filter (Nalgene, Poly Labo, Strasbourg, France). The scFv-TEG4 soluble periplasmic extract was dialyzed against phosphate-buffered saline (PBS, Dulbecco buffer) pH 7.4 using Slyde-A-Lyser Dialysis products (Pierce, Besons, France).

2.5. Purification of scFv[TEG4] from whole cell extract under native conditions

In the pHOG21 vector, a c-myc tag recognized by the mAb 9E10 and six C-terminal histidine residues immedi-

ately follow the scFv fragment. The 6-His-tagged scFv was purified by Immobilized Metal Affinity Chromatography (IMAC) [13] on Ni-NTA spin columns (Qiagen, Courtaboeuf, France). To isolate soluble scFv fragments in native conditions, bacteria harvested by centrifugation at 5000 × *g* for 10 min after induction were re-suspended in lysis buffer (50 mM NaH_2PO_4, pH 8, 300 mM NaCl, 10 mM Imidazole) to which was added 1 mg/ml lysozyme. After 30 min on ice and sonication, the lysate was centrifuged at 10,000 × *g* for 30 min at 4 °C and the supernatant loaded on a Ni-NTA spin column. scFv fragments were isolated under the conditions recommended by the supplier with the lysis buffer containing 250 mM Imidazole. Eluted material was dialyzed against PBS and used for functional analysis by ELISA.

2.6. Purification of scFv[TEG4] from whole cell extract under denaturing conditions

An alternative technique for antibody fragment production in *E. coli* is preparation of inclusion bodies with subsequent in vitro folding.

The insoluble material obtained after sonication of bacteria re-suspended in lysis buffer and further centrifugation was treated under the conditions recommended by the supplier (Qiagen). Briefly, the pellets were solubilized in 50 mM Tris–HCl pH 8.0 containing 8 M urea. After 60 min gently stirring, the lysate was centrifuged at 10,000 × *g* for 30 min at 4 °C and the supernatant applied on a Ni-NTA spin column firstly equilibrated with the urea buffer. The column was then washed until the absorbance (280 nm) of the effluent was below 0.01. scFv fragments were isolated with the urea buffer containing 250 mM Imidazole. Urea was removed from the eluted material by dialysis against a renaturating buffer at pH 7 containing 0.1 M HCl, 5 mM EDTA and 400 mM L-arginine followed by dialysis against the PBS buffer.

2.7. Purification of scFv[TEG4] on the pelleted transfected bacteria grown in batch fermentation

E. coli XL1-Blue cells transfected with pHOG21[scFv-TEG4] were cultivated in the bioreactor at 37 °C in 1.5 l of LB medium containing glucose (1 g/l) and ampicillin (100 μg/ml). When the absorbance at 600 nm was 0.3, IPTG (isopropyl β-D-thiogalactopyranoside) was added to a final concentration of 0.3 mM and the temperature was decreased to 30 °C. After 15 h of protein induction, the cells were pelleted and re-suspended in 1/60 volume of the original culture in the lysis buffer containing 50 mM Tris–HCl (pH 8.0), NaCl 0.5 M and left for 30 min on ice with occasional shaking. Following the disruption of the cell through cycles of sonication and centrifugation, the supernatant representing the whole cell extract fraction was stored at −20 °C.

The thawed cleared lysate containing protease inhibitor was gently mixed for 15 h with 100 ml of Cu^{2+} charged gel equilibrated with 50 mM Tris–HCl (pH 8.0), NaCl 0.5 M buffer by shaking at 200 rpm on a rotary shaker at 4 °C. The protein/resin complex was then packed into a column and washed with the starting buffer containing 25 mM Imidazole. scFvs were then eluted with buffer containing 0.5 M Imidazole. Antibody-containing fractions were dialyzed for 15 h at 4 °C against 100 volumes PBS. scFv fractions were concentrated with Centricon plus-80 centrifugal filters (Millipore, St. Quentin Yvelines, France).

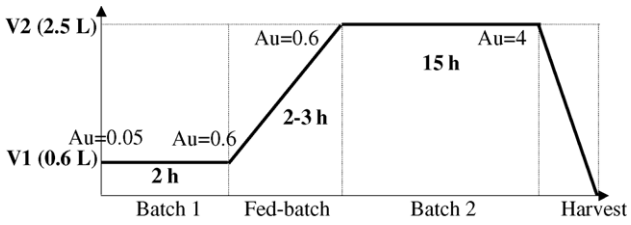

Fig. 1. Large-scale production of soluble scFv[TEG4] in fed-batch fermentation. Two fed-batch conditions were run sequentially in the Bioflow 3000 fermenter with TB or 2TY medium supplemented with 5 mM glucose or 100 mM glucose. Batch phase 1: bacteria are inoculated into 600 ml and grown in the same medium; fed-batch phase: induction is performed at 37 or 30 °C by adding 5.5 mM lactose or 1 mM IPTG in the medium. Batch phase 2: growth and production of scFv continued for 15 h at 25 or 30 °C.

2.8. Purification of scFv[TEG4] on the pelleted transfected bacteria grown in fed-batch fermentation

E. coli XL1-Blue cells transfected with pHOG21[scFv-TEG4] were respectively inoculated into 600 ml in the Bioflow 3000 fermenter for the batch phase of the culture and grown with TB (condition 1) or 2TY medium (condition 2) at 37 °C until the absorbance at 600 nm reached 0.6. During the fed-batch phase, a continuous flow rate of induction medium was injected to maintain the absorbance around 0.6 until the fermenter was full. At this point, growth continued under stirring at 200 rpm until the absorbance reached 4 (Fig. 1).

2.8.1. Fed-batch 1

Induction was performed at 37 °C by adding 5.5 mM lactose in the TB medium supplemented with ampicillin only. At the end of the fed-batch phase, growth continued for 15 h at 25 °C.

2.8.2. Fed-batch 2

Induction was performed at 30 °C by adding IPTG 1 mM in the 2TY medium supplemented with ampicillin and 0.4 M saccharose. Growth continued for 15 h at 30 °C.

2.8.3. Purification of scFv[TEG4] in fed-batch 1 and 2 conditions

After the harvest, cells were resuspended in 1/30 volume of the original culture in the lysis buffer and disrupted through cycles of sonication.

One hundred ml chelating Sepharose fast flow were loaded with 0.3 M copper sulfate metal ions in water (50 ml). The

column was washed with elution buffer (0.5 M Imidazole, 0.5 M NaCl, 50 mM Tris–HCl, pH 8.0) to remove the excess of copper sulfate metal ions and was then equilibrated with 0.5 M NaCl, 50 mM Tris–HCl, pH 8.0. The cleared lysate obtained after sonication was diluted 1:2 in equilibration buffer and loaded at a flow rate of 5 ml/min. The column was then washed with 20 column volumes of starting buffer containing 25 mM Imidazole until the absorbance (280 nm) of the effluent was minimal. Absorbed material was eluted with 0.5 M Imidazole, 0.5 M NaCl, 50 mM Tris–HCl, pH 8.0. Fractions corresponding to the A_{280} peak were pooled, dialyzed and concentrated against PBS using Spectrum MW8000-diam 10 mm and Macrosep^{TM} centrifugal concentrators (Filtron, VWR International).

2.9. Protein determination

The scFv concentration was calculated by determining the total protein concentration using the Bradford assay (Pierce, Perbio Science, Brebières, France) with bovine serum albumin as standard [14] and by measuring the percentage of scFv by gel scanning.

2.10. Analysis of scFv[TEG4] reactivity by ELISA

The wells of 96-well microtiter plates were coated with 5 μg/ml of purified αIIbβ3. Control wells were coated with 3.5 μg/ml BSA (Sigma-Aldrich). Washed platelets were coated 15 h at 4 °C at 10^7 platelets/well. Each well was blocked with 2% milk–PBS. scFv[TEG4] fragments isolated by IMAC were tested on purified αIIbβ3, human platelets and control wells using the anti-c-myc mAb 9E10. After incubation for 2 h, a 1/1000 dilution of horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Immunotech, Marseille, France) was added and incubated for 90 min. Color was developed with 2;2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) and the absorbance was read at 405 nm.

2.11. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot analyses.

The molecular masses and purity of scFv[TEG4] were monitored by SDS-PAGE under reducing conditions [15]. The gels were stained with Coomassie Blue staining solution. Alternatively, the protein bands were transferred to a nitrocellulose membrane (Millipore) using a semi-dry electrophoretic transfer system. The transblotted membrane was probed with the 9E10 anti-c-myc antibody, washed and incubated with sheep anti-mouse IgG labeled with peroxidase (Immunotech), as specified by the manufacturer. Bound antibody was detected with chemiluminescence ECL Western blotting detection reagents (Pierce).

2.12. Preparation of whole blood samples for flow cytometry

Platelets were isolated from ACD-A anti-coagulated blood group O. Platelet-rich plasma (PRP) was prepared by adding100 nM prostaglandin E_1, 0.05 U/ml Apyrase^{Grade7}, and ACD-A (1:9, v/v, PRP) before centrifugation at 120 × *g* for 10 min at room temperature [16]. Platelets were sedimented by centrifugation at 1100 × *g* for 15 min in the presence of prostaglandin E_1 and ACD-A, washed and adjusted at 10^8 ml^{-1} in 137 mM NaCl, 2.7 mM KCl, 12 mM $NaHCO_3$, 0.3 mM NaH_2PO_4, 1 mM $MgCl_2$, 5.5 mM glucose, 5 mM HEPES, 0.1% (w/v) bovine serum albumin (BSA), pH 7.4 (HEPES buffer-modified tyrode [HBMT]). One aliquot is activated with 0.5 U/ml human α-thrombin (Fibrindex, Ortho-Diagnostics, Raritan, NJ) and samples of both activated and non-activated platelets were fixed for 30 min with an equal volume of paraformaldehyde (PFA) 2%. Four hundred microliters of this suspension were incubated with 100 μl of diluted scFv[TEG4] (30 μg/ml) for 20 min at 37 °C. Platelet suspensions were washed with PBS. Detection of scFv binding was performed first with the 9E10 anti-c-myc antibody (30 min) and with a fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG antibody specific for Fc fragment diluted at a 1:80 (Immunotech). Reactions in the absence of scFv were used as negative controls. Cells were analyzed on the BD FACScan cytometer.

2.13. Immunohistochemical analysis of arterial thrombi formed in rabbits

Adult male Bourgogne brown rabbits were submitted for six months to an atherogenic diet consisting in 0.3% cholesterol [16]. Aortic thrombi were induced by percutaneous angioplasty. Thrombi obtained in this model are similar to those obtained in humans after atherosclerotic plaque disruption has occurred. The animals were sacrificed and aorta excised for immunohistochemical analysis.

Paraffin-embedded sections of arterial tissue with the thrombus were prepared by standard procedures. Tissue sections (10 μm thick) were further re-hydrated before endogenous peroxidase was quenched with 0.03% (v/v) hydrogen peroxide. The samples were blocked for 30 min with PBS–5% BSA. Tissue sections were next incubated for 2 h with 500 μl of scFv[TEG4] (30 μg/ml) diluted in PBS–1% BSA. Detection of scFv binding was performed with the 9E10 anti-c-myc antibody. A third incubation was carried out with a biotinylated antibody to mouse IgG (Immunotech) diluted 1/100 followed by peroxidase-labeled streptavidin biotin (Immunotech) diluted 1/100. Control tissue sections were incubated with the 9E10 anti-c-myc antibody, the biotinylated antibody to mouse IgG and with peroxidase-labeled streptavidin biotin. Staining was developed in DAB (3,3′-diaminobenzidine) dissolved in PBS, pH7.2 containing 0.03% (v/v) hydrogen peroxide. After washing with distilled water, the tissue sections were dehydrated in successive

baths of ethanol and xylene and sections were mounted in DePeX medium buffered with 10% PBS.

3. Results

3.1. Output of various small-scale expression and purification experiments for selected anti-αIIbβ3-binding scFv fragments to check for optimal scFv cellular location

Optimal growth and expression conditions for scFv protein should be established with small-scale cultures before large-scale antibody purification is attempted.

The expression and purification of scFv[TEG4], scFv[EBB3], scFv[EBB10] were first evaluated from culture supernatant and periplasmic extract. Indeed, for proteins with cysteine residues like scFv fragments, improper formation of disulfide bonds in the reducing environment of the cytoplasm may contribute to incorrect folding and formation of inclusion bodies [17]. Unfortunately, the scFvs could not be detected in the culture medium or in the oxidizing medium of the periplasmic compartment. Purifications from whole cell extract under native conditions and from whole cell extract under denaturing conditions were therefore tested. Soluble and insoluble extracts were applied on Ni-NTA spin columns (Qiagen) and isolated with buffers containing 250 mM Imidazole. Purification under denaturing condition implies renaturation after elution with a buffer at pH 7 containing 0.1 M HCl, 5 mM EDTA and 400 mM L-arginine.

Purification of the scFv fragments in insoluble fraction gave the best OD values, showing that a lot of the scFv proteins are expressed as inclusion bodies and that refolded antibodies maintained their bioactivity (Fig. 2). However, purification in the presence of urea requires laborious in vitro refolding (denaturation and renaturation). Hence, the final yield of fragments was only a small percentage of the protein that was initially present in the inclusion bodies, even though His_6-purification is facilitated with unfolded proteins [18]. Considering these time-consuming steps and the good bioactivity also encountered in the soluble fraction of the whole cell extract, we choose to develop large-scale productions of soluble recombinant scFvs in the cytoplasm.

3.2. Immobilized metal affinity chromatography purification of large-scale productions of soluble scFv[TEG4] in batch and fed-batch fermentations

3.2.1. One-step purification of soluble scFv[TEG4] grown in batch fermentation

A mixture consisting in the sonicated pellet resuspended in 1/60 volume of the original culture and 0.3 M Cu^{2+} charged gel was poured into a column. A starting buffer containing 25 mM Imidazole was applied in order to minimize binding of untagged, contaminating proteins. scFvs were eluted with buffer containing 0.5 M Imidazole. Batch purification, illustrated in Fig. 3A, showed a very poor elution profile with an elution peak under 0.2 and a very high value of absorbance for the 25 mM Imidazole washing step.

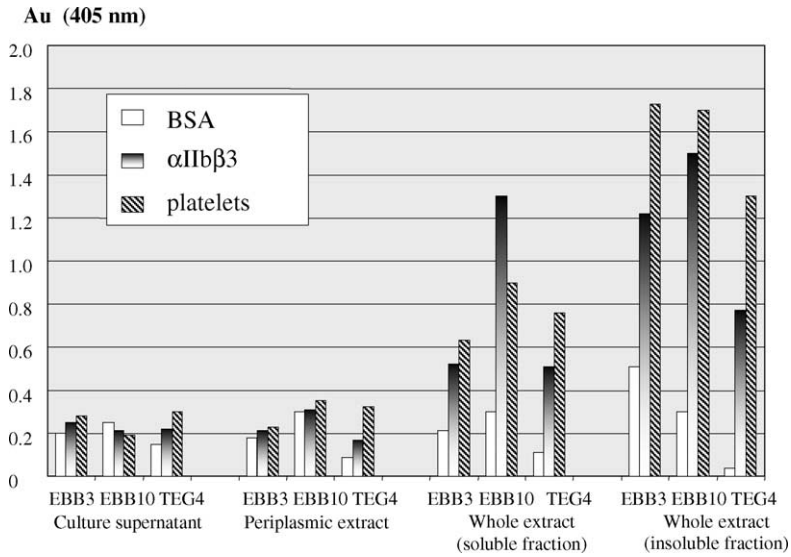

Fig. 2. Output of various small-scale expression and purification experiments for selected anti-αIIbβ3-binding scFv fragments. *Soluble periplasmic extract*: cell pellet was incubated in ice-cold 50 mM Tris–HCl, 20% sucrose, 1 mM EDTA, pH 8.0. *Soluble whole cell extract (native condition)*: bacteria were resuspended in lysis buffer (50 mM NaH_2PO_4, pH 8, 300 mM NaCl, 10 mM Imidazole, 1 mg/ml lysozyme) and sonicated. *Insoluble whole cell extract (denaturing condition)*: pellet after sonication was solubilized in 50 mM Tris–HCl, pH 8.0 + 8 M urea. Soluble and insoluble extracts were applied on Ni-NTA spin columns (Qiagen) and isolated with buffers containing 250 mM Imidazole. Purification under denaturing condition implies renaturation after elution with a buffer at pH 7 containing 0.1 M HCl, 5 mM EDTA and 400 mM L-arginine.

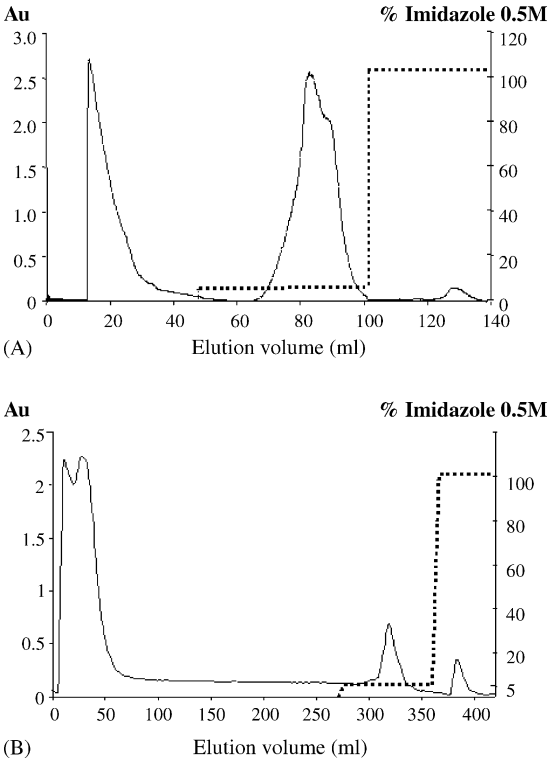

Fig. 3. Elution profile of the IMAC purification of large-scale production of soluble scFv[TEG4] in batch fermentation (A) and in fed-batch 2 fermentation (B). The cleared lysate obtained after sonication was diluted 1:2 in 50 mM Tris–HCl (pH 8.0), NaCl 0.5 M buffer and loaded on the Cu^{2+} charged Sepharose fast flow column. It was then washed with 20 column volumes of starting buffer containing 25 mM Imidazole. Absorbed material was eluted with 0.5 M Imidazole, 0.5 M NaCl, 50 mM Tris–HCl, pH 8.0.

3.2.2. *One-step purification of soluble scFv[TEG4] grown in fed-batch fermentation*

The growing culture of the first batch fermentation is used as a starting point for further fed-batch bioprocess, in which a media without glucose containing IPTG and saccharose [2,12] or lactose is continuously added until the fermenter is full. After the second batch phase, cells were re-suspended in 1/30 volume of the original culture in the lysis buffer and disrupted through cycles of sonication. Purification was done by IMAC using a chelating Sepharose fast flow column loaded with 0.3 M copper sulfate metal ions. After sample loading, the column was washed with 20 column volumes of starting buffer containing 25 mM Imidazole until the absorbance of the effluent was minimal. The scFv of fed-batch 2 fermentation were eluted under native conditions with 0.5 M Imidazole as a single peak around Au = 0.5 (Fig. 3B).

3.2.3. *Purity of batch and fed-batch purifications determined by SDS-PAGE and Western blotting*

This His_6-tagged scFv[TEG4] obtained in batch fermentation was recovered, dialyzed against PBS and its purity was monitored by 12% SDS-PAGE analysis under reducing conditions with Coomassie Brilliant Blue staining (Fig. 4A). Monomeric scFv yielded a specific band at M_r 32,000. Truncated monomeric and dimeric forms yielded bands below M_r 32,000 and above M_r 60,000 (Fig. 4A, lane c). These different expression forms of scFv[TEG4] were confirmed by Western blotting of proteins onto nitrocellulose membranes and labeling with mouse anti-c-myc antibody (Fig. 4A, lane d). Moreover, analysis by SDS-PAGE revealed many contaminating bands that remained after purification.

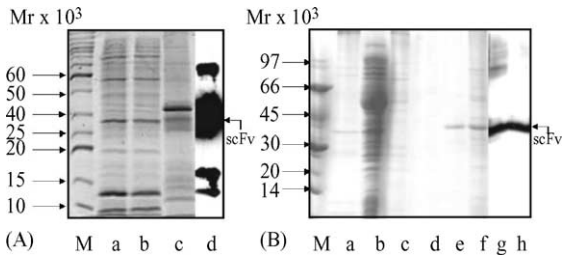

Fig. 4. SDS-PAGE and immunoblotting analysis of one step purification of soluble scFv[TEG4] grown in batch (A) and fed-batch fermentation (B). (1) SDS-PAGE stained with Coomassie Brilliant Blue. An arrow indicates the position of the scFv; (2) Immunoblotting of the purified scFv[TEG4] stained with mouse anti-c-myc antibody (IgG) and peroxidase-conjugated anti-mouse IgG. (A) Lanes: (M) Molecular mass marker; (a) flow-through; (b) 25 mM Imidazole washing fraction; (c) 500 mM Imidazole elution fraction; (d) elution fraction visualized by Western blotting. (B) Lanes: (M) Molecular mass marker; (a) fed-batch 1 elution; (b) soluble cell extract fraction (fed-batch 2); (c) flow-through (fed-batch 2); (d) 25 mM Imidazole washing fraction (fed-batch 2); (e) fed-batch 2 elution; (f) insoluble cell extract fraction (fed-batch 2); (g) fed-batch 1 elution visualized by Western blotting; (h) fed-batch 2 elution visualized by Western blotting.

The quality of the purified His_6-tagged scFv[TEG4] obtained in conditions 1 and 2 of fed-batch fermentation was also monitored by 12% SDS-PAGE analysis and subsequent immunoblotting with mouse anti-c-myc antibody. The purification in fed-batch 2 conditions (Fig. 4B, lane e) resulted in 5 mg of high purity monomeric scFv[TEG4] at a specific band at M_r 32,000. The protein content was estimated by the Bradford assay. On the other hand, in fed-batch 1 conditions, background contaminants remained after purification (Fig. 4B, lane a). Lane f in Fig. 4B illustrates the presence of scFv[TEG4] in inclusion bodies (insoluble cell extract). Immunoblotting confirmed the presence of purified scFv[TEG4] in fed-batch 2 conditions (Fig. 4B, lane h) and revealed dimeric forms of purified scFv[TEG4] in fed-batch 1 conditions (Fig. 4B, lane g).

3.3. *ELISA analysis of active scFv[TEG4] in fed-batch processes 1 and 2*

ELISA was performed to measure the binding of IMAC purified scFv[TEG4] expressed in fed-batch processes 1 and 2 on purified αIIbβ3 and coated human platelets (Fig. 5). It was found that scFv[TEG4] maintained a binding activity in the ELISA assay, essentially following fed-batch 2 fermentation.

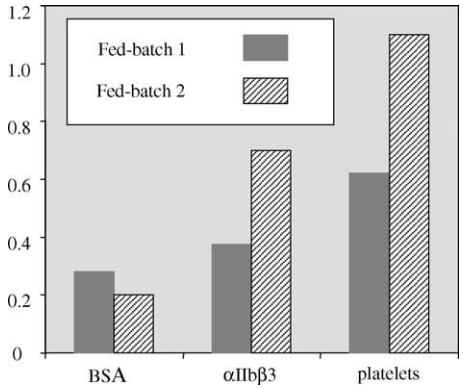

Fig. 5. Analysis of active scFv[TEG4] purified from fed-batch processes 1 and 2. Microplate wells, coated with purified αIIbβ3, human platelets and BSA as a control, were treated with purified scFv[TEG4] and then subjected to sequential incubation with mouse IgG anti-c-myc antibody and goat anti-mouse IgG conjugated with peroxidase. The binding activity was measured at 405 nm.

3.4. Assessment of scFv[TEG4] binding on platelets by flow cytometry

Human platelets isolated from ACD-A anti-coagulated blood group O donors as described [16] were adjusted at 10^8 ml^{-1} and were activated or not with 0.5 U/ml human α-thrombin. Samples of both activated and non-activated platelets were fixed v/v with 2% PFA. The functionality of scFv[TEG4] was characterized by its ability to bind human platelets. Diluted scFv[TEG4] was incubated with 400 μl of fixed activated and non-activated platelets. Detection of scFv binding was performed with the anti-c-myc antibody followed by a fluorescein isothiocyanate-labeled goat anti-mouse IgG antibody. Reactions in the absence of scFv were used as negative controls.

Thrombin activated and non-activated human platelets were labeled with scFv[TEG4]. Unlike in the non-activated population, the scFv fragment homogeneously stained platelets in the activated form (Fig. 6). scFv[TEG4] appeared

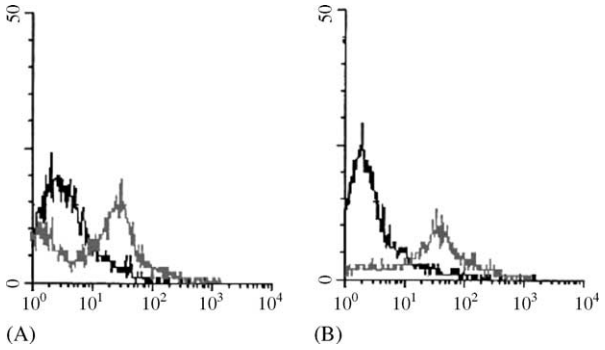

Fig. 6. Assessment of the binding of scFv[TEG4] on platelets by flow cytometry analysis. Gray line: Thrombin activated (B) and non-activated human platelets (A) are labeled with scFv[TEG4]. Black line: Reactions in the absence of scFv were used as negative controls.

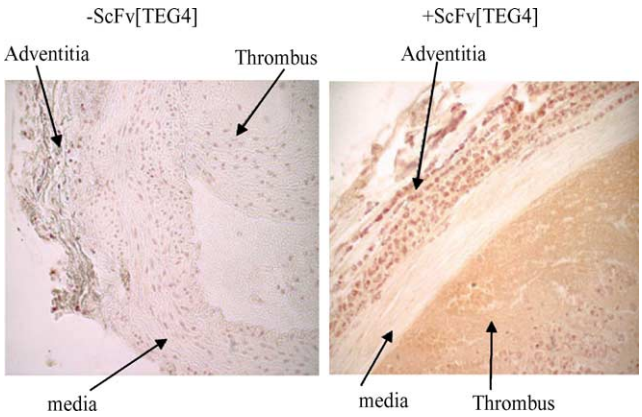

Fig. 7. Assessment of the binding of scFv[TEG4] on a thrombus induced in rabbits submitted to an atherogenic diet and percutaneous angioplasty. scFv binding was detected by immunohistochemical analysis using mouse anti-c-myc antibody and biotinylated antibody to mouse IgG followed by peroxidase-labeled streptavidin biotin.

to recognize an epitope shared by the whole activated platelet population.

3.5. Assessment of scFv[TEG4] binding on an induced rabbit thrombus by immunohistochemical analysis

Aortic thrombi were induced by percutaneous angioplasty in Bourgogne brown rabbits submitted for six months to an atherogenic diet. Paraffin-embedded sections of the thoracic aorta were stained with scFv[TEG4] followed by labeling with anti-c-myc antibody, biotinylated antibody to mouse IgG and peroxidase-labeled streptavidin biotin. Control tissue sections were incubated with anti-c-myc and secondary antibodies only.

scFv[TEG4] stained the large thrombus in the thoracic aortic fragment at a site of angioplasty-induced injury with no labeling of the media. Note the significant uptake of the anti-c-myc antibody in the cells of the adventitia and the thrombus (Fig. 7; scFv[TEG4]) that gave a contaminant labeling when using the scFv[TEG4] anti-αIIbβ3 fragments.

4. Discussion

Antagonists of integrin αIIbβ3 are potential therapeutic and diagnostic reagents for acute coronary syndromes [3]. In this study, our aim was to establish a high-cell-density fermentation strategy to recover the scFv anti-αIIbβ3 fragments in yields and purity sufficient for further animal in vivo studies. In comparison to the much larger Fab′, Fab′$_2$ and IgG forms of antibodies, scFvs have lower retention times in non-target tissues and exhibit more rapid blood clearance and better penetration into targeted tissues [11,12]. First of all, small-scale cultures of three anti-αIIbβ3 scFv fragments were performed to check for scFv cellular location. The scFv could not be detected in the culture medium or in the periplasmic compartment. Soluble periplasmic ex-

pression is very difficult to achieve, even if plasmid vectors have been designed for such a purpose via a sequence encoding for the *pelB* signal peptide. The *pelB* leader sequence probably remains unprocessed, as often reported [19]. Active fragments were in fact detected both in the soluble and insoluble fractions of whole cell extract. However, the refolding process does not always produce completely native protein and often yields low amounts of refolded proteins with poor specific activity [18]. Thus, even if in this particular case, inclusion bodies were formed and refolded antibodies showed preserved bioactivity, our aim was to adjust the expression conditions such that significant amounts of recombinant scFvs might be produced in a soluble, native form in the cytoplasm.

scFv[TEG4] was chosen for further investigation and large-scale production. Batch and fed-batch fermentations in the Bioflow 3000 bioreactor were compared. The multiplicity of bands observed in SDS-PAGE after purification in batch conditions may be regarded as truncated monomeric and dimeric forms of scFv fragments or incorrect folding species with differential migration properties [20]. The use of the bioreactor, which allows growth at a higher cell density, may have favored the aggregation process, hence precluding a proper folding process. Fed-batch bioprocesses were further tested with different media and induction conditions. By using fed-batch cultivation, we were able to control the induction process. Fed-batch 1 conditions did not allow good recovery of purification nor sufficient purity (Fig. 4B, lane 1). The best results were obtained in fed-batch 2 conditions where 5 mg of scFv[TEG4] were recovered at a purity higher than 95%. A reduction in growth temperature following induction seemed helpful here. Indeed it is known that lower temperatures reduce expression levels and provide a higher amount of soluble proteins [21]. Moreover, the higher concentration of glucose and the addition of saccharose [12] may have prevented too high an induction of the *lac* promoter and thus decreased the effects of protein aggregation. The recombinant fragments were produced in a soluble, native form in the cytoplasm, as demonstrated by ELISA study. Bioactivity was further confirmed by cytometric and immunocytochemical studies.

However, a particular point remains to be clarified. The oxidizing environment of the periplasm allowed the formation of disulfide bonds. This did not occur in the reducing environment of the cytoplasm. On the other hand, disulfide bond formation is one of the hallmarks of the antibody domain architecture [22]. Nevertheless, the biological activity was recovered in our fed-batch 2 fermenter conditions in the cytoplasm of the cell. One explanation for this could be that the scFv[TEG4] is able to recover its active form without the need for disulfide bond formation, as previously reported [23–27]. More probably, correct disulfide bond formation was obtained after dialysis [19]. Moreover, we cannot rule out that inactive scFv[TEG4] remained in the total purified scFv population. Improvement in the purification of fully biologically active fragments could thus be achieved by testing different *E. coli* strains for expression. Particularly, it would be interesting to use AD494 and Origami *E. coli* strains for expression studies. Indeed, mutations in the active thioredoxin reductase (trxB) for AD494 and trxB and glutathione reductase (gor) for Origami enable efficient disulfide bond formation and isomerization in the *E. coli* cytoplasm, thus providing the potential to produce properly folded, active proteins [4,8,28]. Further work in our laboratory is underway to test these *E. coli* strains for scFv[TEG4] expression and to explore its applicability to the other anti-αIIbβ3-binding scFv fragments.

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