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Enhanced expression and purification of camelid single domain VHH antibodies from classical inclusion bodies

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ABSTRACT

Single domain antibodies (sdAbs) are small antigen-binding domains derived from naturally occurring, heavy chain-only immunoglobulins isolated from camelid and sharks. They maintain the same binding capability of full-length IgGs but with improved thermal stability and permeability, which justifies their scientific, medical and industrial interest. Several described recombinant forms of sdAbs have been produced in different hosts and with different strategies. Here we present an optimized method for a time-saving, high yield production and extraction of a poly-histidine-tagged sdAb from *Escherichia coli* classical inclusion bodies. Protein expression and extraction were attempted using 4 different methods (e.g. autoinducing or IPTG-induced soluble expression, non-classical and classical inclusion bodies). The best method resulted to be expression in classical inclusion bodies and urea-mediated protein extraction which yielded 60–70 mg/l bacterial culture. The method we here describe can be of general interest for an enhanced and efficient heterologous expression of sdAbs for research and industrial purposes.

1. Introduction

Antibodies are molecules whereby our immune system intercepts antigens thus protecting us from non-self dangers. Because of their specificity and selectivity for the target, they have today a great relevance in diagnostic and therapeutic applications. Conventional antibodies of the IgG type are formed by two identical light chains (L) and heavy chains (H). Their N-terminal domains (VL and VH, respectively) are variable in sequence and, combined, form the antigen binding site, whose repertoire is estimated to reach about 10^7 in both humans and mice [1,2]. The remaining C-terminal domains are referred to as constant (C) domains and do not participate in antigen binding.

Camelid (dromedaries and llama) antibodies also belong to the IgG heavy chain isotype, with the IgG1 isotype being composed of two light chains and two heavy chains. A significant proportion (about 50%) of camel antibodies are however composed of heavy chains only, without light chains [3]. They are known as IgG2 and IgG3 heavy chain antibodies depending on the length of the hinge region connecting the VH domain to the first constant domain. Camel VH domains show five replacements in critical residues (11, 37, 44, 45 and 47) with respect to human ones. These substitutions have important consequences, as they prevent an association with a conventional VL domain and explain solubility both of heavy chain antibodies and of their recombinant single domains counterparts (VHHs [4,5]). The main feature of these antibodies is that antigen binding is reduced to a single domain. Indeed, so called single domain antibodies (sdAbs) are heavy-chain only antibody fragments that are capable of binding antigens with high specificity, a feature camelids share with sharks.

Several molecules derived from both species have been recently described, with applications in the biotechnological, medical and industrial fields [6-8]. In all of them, antibody production at high yield is essential for any applicative purpose. The simple nature and small size of sdAbs allows for efficient expression of functional molecules in different hosts like bacteria, yeast and mammalian cells. Production of sdAbs in yeast can be obtained but, in some cases, it results in reduced or absent functionality of the molecule due to the O-glycosylation operated by yeast that can limit or block its antigen binding capability. Production in mammalian cells can be obtained, but has the same limitations of the yeast system as for glycosylation and, moreover, is quite an expensive method for large-scale production of recombinant proteins. Production of sdAbs in filamentous fungi has been described only once and has resulted in the production of partially degraded sdAbs because of the presence of fungi-secreted proteases in the extracellular space

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[9]. Presently, heterologous production of sdAbs in bacteria is the more efficient and cost-effective method. sdAbs have a highly conserved disulfide bridge that is required for proper folding, therefore production of functional sdAbs requires an oxidative environment. *Escherichia coli* periplasmic environment is oxidative and, therefore, sdAb expression is more favorable in the periplasm rather than in the cytoplasm. Indeed, many described sdAbs express in *E. coli* periplasm with a yield of 1–10 mg *per* liter bacterial culture [10]. Recombinant, cytosolic and partially folded proteins expressed in *E. coli* can be accumulated as aggregates in vesicles, the so-called inclusion bodies. Recently, the first purification of sdAbs from inclusion bodies has been described by Bao et al. with a 10-times improvement in functional protein yield compared to the previously mentioned methods [11].

In this work, we have optimized and simplified production and extraction of a sdAb from classical inclusion bodies increasing yield by 7 times with respect to the canonical periplasmic production described so far for similar proteins.

2. Materials and methods

2.1. Materials

HisTrap FF, HiTrap Q XL and Superdex 75 GL 10/300 columns were purchased from GE Healthcare. Pierce bicinchoninic acid protein assay kit and the chemiluminescent substrate for detection of horseradish peroxidase (HRP) activity (ECL) were purchased from Thermo Fisher. Monoclonal HRP-conjugated anti-poly-Histidine antibody, glutathione (GSH), and oxidized glutathione (GSSG) were obtained from Sigma-Aldrich. All the other reagents were of analytical grade.

2.2. sdAb cloning

The sdAb, an engineered version of a previously described [12], was obtained by gene synthesis (GeneArt™). The resulting construct was subcloned into the pET45b (+) expression vector using *Nco* I and *Xba* I as cloning sites for the gene 5′- and 3′-end, respectively. A 6xHis purification tag was inserted at the gene 3′-end. After sequencing of the insert, the vector was transformed into an *E. coli BL21(DE3) ΔansA/ΔansB* derivative strain kindly provided by Douglas Scott Merrell (University of the Health and Sciences, Bethesda, MD, US) for protein expression. The strain was engineered to lack *ansA* and *ansB* genes coding for *E. coli* type I and type II L-Asparaginase, an amidohydrolase (EC 3.5.1.1) involved in bacteria ammonia metabolism.

2.3. Cytosolic protein expression by autoinducing

A starting culture was obtained by inoculating one single clone of *E. coli BL21(DE3) ΔansA/ΔansB* transformed with the expression construct into 50 ml Luria-Bertani (LB) medium containing 100 μg/ml ampicillin (Amp) and 100 μg/ml streptomycin (Str). According to Studier [13], 10 ml of overnight pre-inoculum were diluted into 500 ml ZYP-5052 reach medium and incubated at 37 °C for 3 h and then at 17 °C for 21 h with vigorous shaking (250 rpm). The next day, cells were collected by centrifugation (8000 rpm, 4 °C for 10 min). Typically, 5.0–6.0 g of wet cells were obtained from 1 l culture and the obtained pellet was dissolved in resuspension buffer using a 1:10 (g:ml) ratio. Accordingly, cells were, resuspended in buffer A (50 mM Na-phosphate, 300 mM NaCl, 10 mM imidazole, pH 8.0) and processed as described below.

2.4. Cytosolic protein expression by IPTG induction

A starting culture was obtained as described above. Eight ml of *E. coli BL21(DE3) ΔansA/ΔansB* pre-inoculum were diluted in 500 ml LB medium containing 100 μg/ml Amp and 100 μg/ml Str and the culture was incubated at 37 °C with shaking at 250 rpm. At an OD_{600} of 0.6, the culture was induced with 1 mM IPTG for 5 h. After induction, cells were collected by centrifugation at 8000 rpm, 4 °C for 10 min, roughly 2.0 g cells were resuspended in 25 ml buffer A (50 mM Na-phosphate, 300 mM NaCl, 10 mM imidazole, pH 8.0) and processed as described below.

2.5. sdAb purification from cytosolic fraction

The cell suspension obtained after auto- or IPTG-induction was kept on ice and sonicated using an Omni Sonic Ruptor 400 sonicator for 4 cycles, each consisting of 60% power for 1 min followed by a 1 min interval keeping the cell suspension on ice all the time. Cell debris were removed by centrifugations at 14000 rpm, 4 °C for 30 min, and the clear cell extract was 0.22 μm filtered. The cell extract was maintained on ice and loaded at 0.5 ml/min onto a 1-ml HisTrap column equilibrated in buffer A. The column was washed with buffer A for at least 10 column volumes. Bound proteins elution was obtained by an imidazole step gradient (25, 50, 150, 250 500 mM imidazole). According to the results of SDS-PAGE, fractions positive for the sdAb were pooled and their buffer was changed to 50 mM Na-phosphate, pH 7.4 before loading them onto a 1-ml HiTrap Q XL column. Buffer exchange was obtained using a 26/10 Desalting column (GE Healthcare) equilibrated in 50 mM Na-phosphate, pH 7.4. Elution was obtained with 50 mM Na-phosphate, pH 7.4, 1 M NaCl. Fractions positive for the sdAb both on SDS-PAGE and on western blot were analyzed by analytic gel-filtration using a Superdex 75 GL 10/300 column equilibrated in Na-phosphate 50 mM, NaCl 100 mM, pH 7.4. The overall purification yield of the homogenous sdAb solution was determined by assessing protein concentration by the bicinchoninic acid assay method.

2.6. sdAb expression in non-classical inclusion bodies (ncIBs)

E. coli BL21(DE3) ΔansA/ΔansB cells transformed with the expression vector containing the sdAb insert were grown at 37 °C shaking at 250 rpm overnight. Ten ml of starting culture were diluted in 500 ml LB media containing 100 μg/ml Amp and 100 μg/ml Str. The culture was incubated at 37 °C shaking at 250 rpm and protein expression was obtained by adding 0.4 mM IPTG when OD_{600} reached 0.6. In order to induce non-classical inclusion bodies (ncIBs) formation, the cells were grown at 18 °C with shaking at 250 rpm for 24 h [14]. Cells from 1 l culture were collected by centrifugation (8000 rpm, 4 °C for 10 min) and resuspended in 50 ml resuspension buffer A (RA, 50 mM Tris-HCl, 500 mM NaCl, pH 8.5). Usually, after centrifugation, 3.0–4.0 g of wet cells were obtained from 1 l culture. The cell suspension was subjected to 4 freeze-thaw cycles at −80 °C. After the last cycle, cells were disrupted by sonication (10 cycles of 1 min at 40% power with 1 min interval keeping the cell suspension on ice all the time). The cell lysate was centrifuged (13000 rpm, 4 °C for 20 min) and washed three times with ice-cold buffer RA. Washed inclusion bodies were resuspended in 30 ml ice-cold buffer RA added with 2 M L-arginine [15] and a redox couple (5 mM GSH, 0.5 mM GSSG), and incubated in agitation at 4 °C for 72 h. The solution was then diluted 100-times, centrifuged (18000 rpm, 4 °C for 10 min) and 0.45 μm filtered. The cleared inclusion bodies extract was loaded onto a 5 ml HisTrap column.