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Production of a soluble and active MBP-scFv fusion: favorable effect of the leaky *tolR* strain

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Abstract The 6D6 anti-cortisol scFv was prepared as fusion protein with maltose-binding protein (MBP) to increase the amount of soluble product. This fusion was almost completely insoluble when produced in a wild-type strain of *Escherichia coli*. However, when MBP-scFv fusion was produced in a *tolR* leaky strain, it was secreted into the culture medium as an active, soluble protein. Production of recombinant proteins in the *tolR* strain greatly enhances the recovery of active protein and may be a useful system to produce MBP fusion proteins that would normally aggregate when produced in wild-type bacterial strains.

Key words: Secretion; Aggregation; Inclusion body; Antibody; Anti-cortisol; Steroid

1. Introduction

The production of immunoglobulin protein subunits in *Escherichia coli* is often problematical. The proteins may be toxic for the host cell, degraded in vivo or misfolded and directed to inclusion bodies. In the latter case, the proteins can be recovered by using strong chaotropic reagents followed by refolding in vitro. However, this step is not easily performed with large proteins [1,2]. In vivo, protein aggregation during induced production can be reduced by several different approaches. For example, higher yields of soluble protein may be achieved by using relatively low culture temperature and by reducing the concentration of the induction agent [3,4]. In addition, the use of different bacterial strains can result in changes in soluble protein production [5]. Another approach is to fuse the gene of interest to genes of periplasmic proteins such as the maltose-binding protein (MBP) [6,7] or alkaline phosphatase [8].

Isolation of single-chain immunoglobulin variable fragment (scFv) from bacteria is difficult because it forms aggregates in the periplasm [9]. The aggregates are likely formed by intermolecular hydrophobic contacts between folding intermediates [10,11]. If these contacts could be reduced by, for example, releasing the recombinant protein into the culture medium, it might be possible to produce greater quantity of functional protein. To test this hypothesis, we used a strain mutated in the gene encoding the TolR membrane protein. This protein is a component of the 'Tol complex' which required for the transport of group A colicins and filamentous bacteriophage DNA into the bacteria (see [12] for review). All *tol* strains are hypersensitive to SDS, indicating that their membrane structure is altered. Mutations in *tol* genes induce the release of periplasmic proteins into the extracellular medium [12,13].

We have prepared recombinant MBP-scFv fusion by using a leaky *tolR* bacterial strain. This approach resulted in the production of soluble, active antibody fragment; a product that was not obtainable by using wild-type bacteria strains.

2. Materials and methods

2.1. Bacterial strains, plasmids and media

TPS300, a *tolR* strain of *E. coli* K12, was obtained by insertion of a transposon encoding resistance to chloramphenicol into the ORF3 of the tol cluster of *E. coli* GM1 (*ara* △*(lac-pro) thi/F′ lac-pro*) [14].

The gene coding for the anti-cortisol scFv 6D6 was amplified from the pscFv plasmid [15], using the polymerase chain reaction (PCR) with the following oligonucleotides: 5′-GTTACTCGCTGAATTCCCGGCCATGGCGGC-3′ and 5′-AATCAATCAATCTAGATCAGATCTGGCAAAG-3′. After digestion at the *Eco*RI and *Xba*I restriction sites (underlined), the DNA fragment was inserted in frame at the 3′-end of the *malE* gene of the pMalp (New England Biolabs) to give the pMalscFv.

Wild-type bacteria were grown at 30°C [4] in 2YT-rich medium with ampicillin and *tolR* bacteria were grown in 2YT-rich medium with ampicillin and chloramphenicol. When cultures were at an optical density of 0.5 (A_{600nm}), recombinant protein production was induced by addition of 10 mM maltose or 100 μM IPTG to bacterial cultures harbouring the chromosome-coded MBP or bacterial cultures harbouring the pscFv or pMBP-scFv plasmids, respectively.

2.2. Preparation and characterization of cellular extracts

After 2 h of induction, the bacterial culture was centrifuged and the cell pellet resuspended directly in SDS loading buffer as described by Laemmli [16]. Supernatant proteins were concentrated and washed using Centricon 10 (Amicon). Cells were also resuspended in lysis buffer (lysosyme 1 mg/ml in 100 mM Tris-HCl, pH 7.5, 1 mM EDTA). After 30 min at 37°C, the resuspended cells were lysed by 10 freeze-thaw steps and centrifuged 30 min at 60 000×*g*. The supernatant and the pellet are referred to as the cellular soluble fraction and the cellular insoluble fraction, respectively.

Samples corresponding to 2×10^8 cells were analyzed by 12% SDS-PAGE and Western blotting. MBP and MBP-scFv were detected by anti-MBP serum (New England Biolabs). scFv was detected by a rabbit anti-Fab serum (Immunotech S.A.)

2.3. Purification of the MBP-scFv fusion

2.3.1. MBP-scFv purification by cortisol affinity chromatography.

Fifty millilitres of culture medium was centrifuged for 30 min at 60 000×*g* and the supernatant was concentrated to 1 ml, using an ultra filtration unit. At this step, samples were diluted 10 times in phosphate buffered saline (PBS), concentrated on Centriprep 10 (Amicon) to 1 ml and loaded onto an affinity chromatography column. The affinity column was prepared by adding cortisol conjugated to BSA (Sigma) to activated Affi-gel 10 resin (BioRad) described in the manufacturer's instructions. Binding efficiency of the column was checked

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Abbreviations: ABTS, 2,2′-azino-bis[3-ethylbenzthiazoline sulfonate]; ELISA, enzyme-linked immunosorbant assay; Fv, immunoglobulin-variable fragment; scFv, single-chain Fv; IPTG, isopropyl-β-D-thiogalactoside; MBP, maltose-binding protein; MBP*, MBP part of the degraded MBP-scFv fusion

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with Fab fragments prepared from whole monoclonal 6D6 antibody. Fab fragments were eluted with 100 mM triethanolamine.

2.3.2. MBP-scFv purification by amylose affinity chromatography. Cell-free extracts containing MBP-scFv were prepared as described above. The extracts were loaded onto amylose, washed extensively, and eluted with 10 mM maltose.

2.4. Immunological tests

Soluble fraction and culture medium activity were tested enzyme linked immunosorbant assay (ELISA). Biotinylated BSA-cortisol was bound on avidin-coated microwells (Immunotech S.A.). The wells were saturated with 2% milk in PBS. Fifty microlitres of each fraction (corresponding to 10^9 cells) was added to wells containing 50 μl of 4% milk PBS (each mix was also tested after a 1:10 or 1:100 dilution in 2% milk PBS). Wells were washed 4 times with 0.1% Tween/PBS followed by 4 times with PBS. Bound scFv or MBP-scFv fusion were revealed by addition of rabbit anti-Fab serum followed by detection with a peroxidase-conjugated anti-rabbit antibody. After incubation with ABTS solution, the absorbance was measured at 405 nm. Samples isolated by amylose chromatography were also tested by ELISA. In this case, active fusion protein was revealed by addition of anti-MBP serum, followed by detection with a peroxidase-conjugated anti-rabbit antibody.

Fusion protein affinity to cortisol was measured by equilibrium dialysis at 4°C, essentially as described [17], but the sample volume was reduced, using 200 μl compartments. ^{125}I-labelled-cortisol was diluted with different amounts of non-radioactive cortisol and dialyzed against the concentrated proteins from the culture medium for 20 h through a dialysis membrane (Spectra/por MWCO: 6000–8000). At equilibrium, the culture medium compartment contains the free steroid plus the bound steroid and the opposite compartment contains the free steroid at the same concentration. The K_d was determined graphically by Scatchard representation.

3. Results

3.1. Production of the scFv or MBP-scFv fusion in a wild-type E. coli strain

We have previously shown that the 6D6 scFv fused to a prokaryotic signal sequence accumulates within the wild-type cells as insoluble protein aggregates (inclusion bodies) ([15] and Fig. 1A, left side). To obtain a higher yield of functional protein in the soluble fraction, we chose to use the MBP fusion technique. For this purpose, we cloned the 6D6 scFv gene into the pMalp vector to make pMalscFv. After IPTG induction of the wild-type GM1 bacterial strain harboring pMalscFv, the localisation of the fusion protein was studied by Western blot visualized by an anti-MBP serum. Unfortunately, although the fusion protein was produced in large amounts, it was localised mostly in the cellular insoluble fraction and was not detected in the culture medium as for the scFv (Fig. 1B, left side). Moreover, the protein was partially degraded, as already observed with other fusion proteins [18]. Therefore even when fused to a periplasmic protein, most of the 6D6 scFv remained insoluble. Previous studies show that recombinant antibody fragments tend to aggregate upon production in the periplasm of *E. coli* [6,19,20]. To avoid aggregation of scFv, we used *E. coli* tol mutants, that release the periplasmic proteins in the culture medium [12,13].

3.2. Secretion of MBP in wild-type and tolR E. coli strain

The TPS300 strain (*tolR*) was chosen because its growth rate is almost identical to that of the GM1 wild-type strain, unlike all the other *tol* mutants. The extracellular localisation of MBP in this strain was verified by immunoblotting with rabbit anti-MBP serum. The *mal* operon was induced with maltose in both *tolR* and wild-type strains. The MBP produced by the wild-type strain was localised only in the cellular fraction whereas in the case of the *tolR* mutant, MBP was found in both cellular and in culture medium fractions (Fig. 1C).

Thus, the *tol* mutant allowed the periplasmic MBP to leak into the medium. Since the leakiness did not prevent bacterial growth (data not shown), we investigated if the product of a

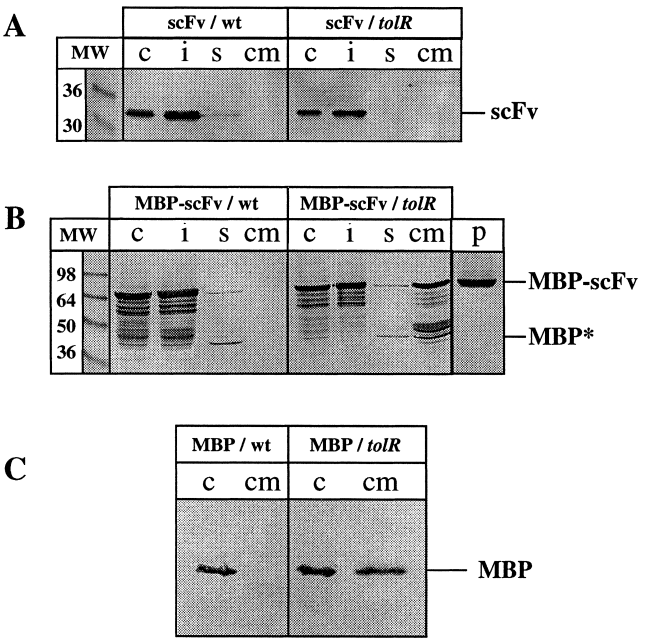

Fig. 1. Localisation of scFv, MBP-scFv and MBP in the wild-type (wt) and *tolR* strains. Bacteria harboring the pscFv (A) or pMalscFv (B) plasmid were induced 2 h with IPTG. Bacteria without plasmid (C) were induced 2 h with maltose. Proteins were transferred onto nitrocellulose and immunoblot analysis were carried out using anti-Fab (A) or anti-MBP (B,C) serum. Lane c: whole cells; lane i: insoluble cellular fraction; lane s: soluble cellular fraction; lane cm: concentrated culture medium (all samples corresponded to 2×10^8 cells). Lane p: fractions eluted from the affinity column of cortisol. MBP* corresponds to the MBP part of the degraded MBP-scFv fusion.

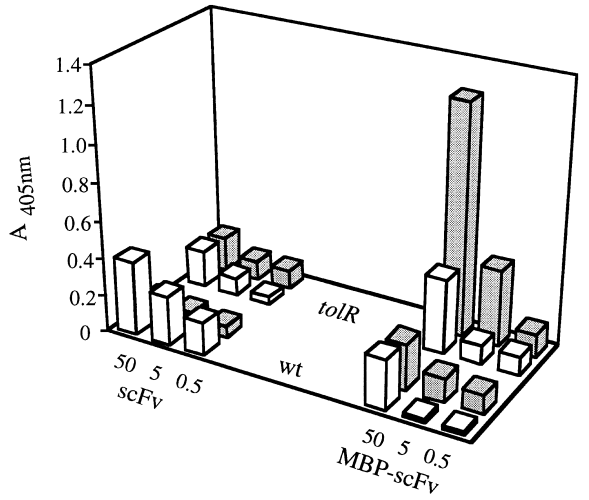

Fig. 2. Cortisol binding activity. The activities of 50, 5 or 0.5 μl of soluble fractions (white bars) or concentrated culture medium (grey bars) of both wild-type (wt) and *tolR* strains producing the scFv or MBP-scFv were determined by ELISA. Bound proteins were revealed using the anti-Fab serum.

fusion between the MBP and the scFv could be released into the culture medium in the same way.

3.3. Production of scFv and MBP-scFv fusion in the tolR E. coli strain

3.3.1. Localisation. After induction, the localisation of the MBP-scFv fusion in *tolR* and wild-type *E. coli* strains was compared by Western blotting. To serve as a control, scFv protein (not as a fusion protein) was produced in the same way. In both strains, a large amount of MBP-scFv as well as scFv protein were insoluble and only traces of MBP-scFv were detected in the soluble fractions (Fig. 1B). Furthermore, neither scFv protein nor MBP-scFv were detected in the culture medium of the wild-type strain. However, the fusion protein was present in large amounts in the culture medium of the *tolR* strain (Fig. 1B). Under similar condition no scFv was detected in the culture medium (Fig. 1A). The MBP part of the degraded MBP-scFv fusion (MBP*) was also released and could be detected by Western blot analysis with anti-MBP serum as faster migrating protein. Thus, we showed that in the *tolR* mutant strain, soluble MBP-scFv could be released from the periplasm to the culture medium, as is the case for wild-type MBP.

3.3.2. Activity of scFv protein and MBP-scFv. The activity of scFv and MBP-scFv proteins localised in soluble fractions or in culture medium of both strains were analyzed by ELISA (Fig. 2). Bound proteins were revealed with anti-Fab serum in all cases. For scFv, a very weak signal was obtained with the wild-type soluble fraction. The other signals were considered as being not significant. In the case of the MBP-scFv, a strong signal (1.3 OD_{405}) was obtained with the culture medium of the *tolR* strain. Signals obtained with other MBP-scFv fractions (wild-type or *tolR* strain) were not significant. These results suggested that a part of the MBP-scFv fusion released by the *tolR* strain was active.

3.4. Purification of soluble MBP-scFv

The fusion protein was purified from culture medium by affinity chromatography on BSA-cortisol-coated resin. SDS-PAGE analysis of fractions eluted from the column revealed one protein which corresponded to the complete fusion protein (Fig. 1B, lane p).

We also attempted to purify the fusion protein from culture medium by amylose affinity column chromatography. As shown in Fig. 3, elution of the column with maltose released MBP-scFv and the MBP*. In fraction 4 (Fig. 3), the full-length fusion protein was almost pure, although some degraded fragments were detected in other fractions. Analysis of anti-cortisol activity by ELISA revealed that high activity co-eluted with the full-length fusion protein.

3.5. Affinity of the MBP-scFv fusion

The dissociation constant of the MBP-scFv cortisol complex was determined by equilibrium dialysis. The value ob-

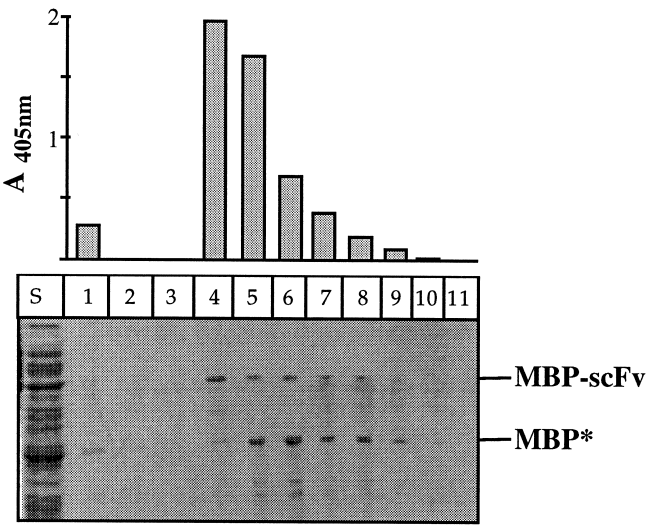

Fig. 3. Purification of the MBP-scFv fusion protein. Sample corresponding to 50 ml of the culture medium was loaded onto an amylose column (lane S). Ten microlitres of each fraction eluted with 10 mM maltose (lanes 1–11) was analysed by SDS-PAGE and revealed by Coomassie-blue staining (bottom) or was analysed by ELISA (top) using the anti-MBP serum. MBP* corresponds to the MBP part of the degraded MBP-scFv fusion.

tained, 2×10^{-8} M, was similar to those determined for the scFv alone (5×10^{-8} M) and for the 6D6 monoclonal antibody (10^{-8} M) [15].

By this procedure we routinely purified 1 mg of soluble, active protein from 1 l of culture medium. Under similar conditions using the GM1 wild-type strain, less than 50 μg of pure scFv, could be isolated.

4. Discussion

We describe a procedure to increase the solubility of a periplasmic MBP-scFv fusion. Many heterologous proteins that contain disulphide bonds are inactive when produced in *E. coli* because they cannot fold properly. This problem is often encountered with the expression of scFv [15]. The lack of eukaryotic folding catalysts (chaperones) may be responsible for abnormal hydrophobic contacts between molecules, which may lead to aggregation. However, coexpression of cytoplasmic chaperon proteins (GroES/L or DnaK/J) did not improve the recovery of anti-cortisol scFv and attempts to renature in vitro insoluble scFv were unsuccessful (data not shown). To circumvent this problem, we produced scFv as a fusion protein with the MBP, a well-characterised and strictly periplasmic protein, targeted by the chaperon protein SecB to the export machinery [21–23]. Despite these modifications, the recombinant protein produced in a wild-type *E. coli* strain remained almost completely insoluble.

It has been shown that periplasmic proteins from *tol E. coli* strains leak into the culture medium [13,14]. The chromosome-coded MBP can be recovered from the culture medium of these mutants. We thus attempted to produce scFv or MBP-scFv fusion in a *tolR* strain that, unlike the other *tol* mutants, grows without cellular lysis even after induction. In the *tolR* mutant, a large amount of the MBP-scFv was detected in solution in the culture medium whereas no scFv could be detected. As expected, only the culture medium of *tolR* strain producing the fusion protein showed a high anti-cortisol activity by ELISA.

The fusion protein was partially degraded in both wild-type and *tolR* strains. This degradation might be due to protease activity in the periplasm or in the outer membrane [24,25]. When the culture medium of *tolR* strain was loaded onto a cortisol column, the complete fusion protein specifically bound whereas other proteins or degraded fusion protein fragments did not bind. The same fraction was loaded onto amylose, a saccharide that binds to native MBP. The complete fusion protein bound to amylose. However, degradation fragments of the fusion protein with full-length MBP, also bound. This indicated that the degradation occurred in the scFv part of the fusion protein. Moreover, if the MBP-scFv fusion was cleaved by cytoplasmic protease in the MBP part of the molecule, it would lack the signal sequence and therefore would not be secreted [26]. We did not attempt to use protease deficient bacterial strains because the production of MBP-scFv fusion in these strains does not improve the recovery of full-length protein [27]. We concluded that only the complete MBP-scFv fusion is able to bind both cortisol and amylose.

The K_d of the MBP-scFv fusion from the culture medium (2×10^{-8}M) was almost identical to the K_d of the original 6D6 monoclonal antibody (10^{-8} M). Furthermore, the affinity was stronger for the scFv-MBP fusion than for the scFv protein alone (5×10^{-8} M). This is consistent with previously reported results in which it has been proposed that MBP might stabilise scFv [18].

The TolR protein is localised in the inner membrane and implicated in outer membrane integrity [28]. As a result, periplasmic proteins are released into the culture medium. The initial hypothesis was that periplasmic aggregation could be reduced if the recombinant product was released into the culture medium by preventing the accumulation of periplasmic folding intermediates. Consistent with this view we found that MBP-scFv was aggregated in wild-type cells whereas 50% of MBP-scFv fusion was released into the culture medium of *tolR* strain. This suggested that the fusion protein was probably exported and folded correctly into the periplasm. The remaining fusion protein was found almost completely aggregated in the cells. Surprisingly, scFv was not detected in the culture medium of *tolR* strain although its signal peptide was correctly processed. The signal peptide was shown as being cleaved only after translocation was complete; presumably it was cleaved at an early stage during translocation implying that at least the N-terminus of the precursor was translocated (see [29] for review). It is possible that remaining polypeptide chain could be blocked by incorrect folding at an earlier stage. The efficient secretion of proteins through the cytoplasmic membrane of *E. coli* requires chaperon proteins, such as SecB, to maintain the precursor in a translocation-competent conformation [21–23]. It was shown that the antifolding activity of SecB promotes the export of MBP [21] and that the mature part of MBP determines the dependence of the protein on SecB for export [30]. Therefore the fusion of scFv with MBP might be responsible for engaging SecB, and allows the translocation of the MBP-scFv into the periplasm. In contrast, without MBP, scFv is poorly translocated.

We developed a method to produce active recombinant protein that would be strictly insoluble in wild-type *E. coli* strain. This was achieved by using MBP fusion and a leaky *E. coli* strain, *tolR*.

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