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Generation of monoclonal antibodies for the assessment of protein purification by recombinant ribosomal coupling

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Abstract

We recently described a conceptually novel method for the purification of recombinant proteins with a propensity to form inclusion bodies in the cytoplasm of *Escherichia coli*. Recombinant proteins were covalently coupled to the *E. coli* ribosome by fusing them to ribosomal protein 23 (rpL23) followed by expression in an rpL23 deficient strain of *E. coli*. This allowed for the isolation of ribsomes with covalently coupled target proteins which could be efficiently purified by centrifugation after in vitro proteolysis at a specific site incorporated between rpL23 and the target protein. rpL23-GFP-His is among the fusion proteins used in our previous study for ribosomal coupling of C-terminally His-tagged green fluorescent protein. To assess the efficiency of separation of target protein from ribosomes, by site-specific proteolysis, we required monoclonal antibodies directed against rpL23 and GFP. We therefore purified rpL23-GFP-His, rpL23-His and GFP from *E. coli* recombinants using affinity, ion exchange and hydrophobic interaction chromatography. These proteins could be purified with yields of 150, 150 and 1500 μg per gram cellular wet weight, respectively. However, rpL23-GFP-His could only be expressed in a soluble form and subsequently purified, when cells were cultivated at reduced temperatures. The purified rpL23-GFP-His fusion protein was used to immunize balb/c mice and the hybridoma cell lines resulting from in vitro cell fusion were screened by ELISA using rpL23-His and GFP to select for monoclonal antibodies specific for each protein. This resulted in 20 antibodies directed against rpL23 and 3 antibodies directed against GFP. Antibodies were screened for isotypes and their efficiency in western immunoblots. The most efficient antibody against rpL23 and GFP were purified by Protein G Sepharose affinity chromatography. The purified antibodies were used to evaluate the separation of ribosomes from GFP, streptavidin, murine interleukin-6, a phagedisplay antibody and yeast elongation factor 1A by centrifugation, when ribosomes with covalently coupled target protein were cleaved at specific proteolytic cleavage sites. We conclude that the generated antibodies can be used to evaluate ribosomal coupling of recombinant target proteins as well as the efficiency of their separation from the ribosome.

Keywords: Ribosome; Protein purification; Recombinant *E. coli*

1. Introduction

Recombinant protein technology has become a discipline of extreme importance to fields spanning from scientific research to the pharmaceutical industry. Among the most well-documented systems used in various scales are the enterobacterium *Escherichia coli* [1]. Recombinant proteins from bacteria, archaeabacteria and eukaryotes are in many cases efficiently expressed and accumulated in *E. coli* and purification by chromatographic procedures is simplified as a direct consequence. Cytoplasmic expression of recombinant proteins at high levels induces a non-natural situation in bacterial cells and changes in the de novo folding pathways are a frequently observed result. Inclusion bodies containing aggregated and misfolded proteins are the most commonly observed phenomenon. A number of methods are used either to refold proteins from inclusion bodies to the native state or to avoid the in vivo inclusion body formation by modification of the expression strategy employed [2].

A popular approach used to increase the in vivo solubility of recombinant proteins is fusion technology. Several different proteins have been demonstrated to either actively or indirectly increase the solubility of targets to which they have been fused [3,4]. Fusion technology is however limited by the degree of solubility promoted by the tag. In an attempt to develop an alternative and more standardized system we have used the *E. coli*

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ribosome as a handle for recombinant proteins [5]. Target proteins are rescued from in vivo aggregation by fusing them to ribosomal protein L23 (rpL23). The fusion protein is expressed in a strain of *E. coli* deficient in the essential gene *rplW*, encoding rpL23. This allows for the complete covalent coupling of target proteins to the highly soluble ribosomal particles. Ribosomes with coupled target protein can subsequently be isolated by centrifugation methods and the target protein released by site-specific protease cleavage. Target proteins are finally separated from the high molecular weight ribosomes by centrifugation. The method proved effective in the preparation of green fluorescent protein (GFP), streptavidin and murine interleukin-6 in yields of approximately 500 μg recombinant protein per gram cellular wet weight. Other proteins were expressed at similar yields but failed to be efficiently separated from the purified ribosomes by specific protease cleavage.

Here we use GFP as a model target protein in the ribosomal coupling system. We show that monoclonal antibodies with specific affinities towards rpL23 and GFP can be used to access and potentially optimize separation of target proteins from the ribosome.

2. Materials and methods

2.1. Strains and plasmids

Bl21 were from Novagen. Plasmids pGLO (GFP), pBAD-rpL23 (rpL23-His) and pBAD-rpL23-GFP (rpL23-GFP-His) are described in Ref. [5].

2.2. Protein expression and purification

rpL23-His, GFP and rpL23-GFP-His were expressed in BL21. Cells were cultivated in 2xTY media (15 g/l tryptone, 10 g/l yeast extract and 5 g/l NaCl) containing 100 μg/ml ampicillin. Overexpression from the ara*BAD* promoter was induced by addition of 0.2% L-arabinose when the optical density at 600 nm (OD_{600}) reached 0.8. Cultivation was continued for 3 h at 37 °C. The cultivation temperature was however adjusted to 30 °C in cultures expressing rpL23-GFP-His prior to induction and cultivation was continued for 5 h at 30 °C. Small scale tests of solubility were done at 37 °C for each of the three constructs as described previously [6].

The expression yields for rpL23-GFP-His reached approximately 5 mg protein per gram cellular wet weight with approximately 50% accumulating into inclusion bodies. Cells expressing rpL23-GFP-His were lysed by sonication in buffer A containing 50 mM Tris–HCl, pH 7.6, 10 mM $MgCl_2$, 0.1 mM phenylmethylsulfonylfluoride (PMSF), 1 mM 1,4-dithioreitol (DTT). All columns used in this work were from Amersham Biosciences. The centrifuged lysate (250,000 × *g* for 1.15 h) was allowed to flow through a Q-Sepharose-FF (no binding of rpL23-GFP-His) and a SP-Sepharose-FF column (binding of rpL23-GFP-His). The rpL23-GFP-His was eluted at approximately 400 mM NaCl in buffer A. The eluate from SP-Sepharose FF was subjected to immobilized metal affinity chromatography (IMAC) as described in Ref. [6]. The IMAC eluate was dialysed against buffer A containing 100 mM NaCl and 50% glycerol. The procedure yielded approximately 150 μg of protein per gram cellular wet weight.

rpL23-His and GFP was expressed to approximately 10 mg protein per gram cellular wet weight with most of the protein in a soluble form. rpL23-His was purified from a supernatant of lysed cells prepared as for rpL23-GFP-His in buffer A containing 500 mM NaCl and 5 mM imidazol. The sample was subjected to IMAC as described [6] and the eluate containing rpL23-His was allowed to flow through a Q-Sepharose HP (no binding of rpL23-His) column. rpL23-His was diluted in buffer A, bound to a SP-Sepharose-HP column and eluted at approximately 500 mM NaCl in buffer A. The procedure yielded approximately 150 μg rpL23-His per gram cellular wet weight.

A lysed supernatant of cells expressing GFP was prepared in buffer B (50 mM Hepes pH 7.6, 10 mM $MgCl_2$,15 mM NaN_3, 1 mM PMSF, 0.1 mM DTT. The protein was bound to a Q-Sepharose FF column and eluted at approximately 150 mM NaCl in buffer B. The concentration of $(NH_4)_2SO_4$ in the eluate was adjusted to 2 M and the sample was bound to Phe-Sepharose-HP. GFP was eluted at 300 mM $(NH_4)_2SO_4$ in buffer B and dialysed against buffer B. The sample was allowed to flow through SP-Sepharose-HP (no GFP binding) and bound to Source 30Q. GFP elutes at 150 mM NaCl in buffer B. The procedure yielded approximately 1.25 mg protein per gram cellular wet weight.

2.3. Generation and screening of monoclonal antibodies

Purified rpL23-GFP-His was used to immunize balb/c mice and monoclonal antibodies were produced by the hybridoma technique, purified on Protein G sepharose 4B and screened for isotype as described previously [7]. Clones were screened by ELISA for their specificity to either rpL23-His or GFP using proteins purified as described above. Western immunoblots were prepared as described [6] using the purified antibodies as primary antibodies and rabbit anti-mouse antibodies (DAKO, Denmark) as secondary antibodies.

2.4. Assay for separation of target protein from ribosomes

Ribosomes with coupled target proteins were produced, cleaved and assayed on coomassie blue stained gels and western immunoblots essentially as described [5].

3. Results and discussion

The ribosomal coupling system uses rpL23 as an anchor for the target protein to the ribosome (Fig. 1A). Our model construct is optimized for efficient coupling of target protein and separation from the ribosome [5]. Ribosomal protein rpL23 is fused to GFP via a linker region containing two consecutive factor Xa cleavage sites. The C-terminal of GFP is fused to six consecutive histidine residues for further purification purposes. Since the assessment and optimization of the separation of target protein from the ribosome has proven difficult we decided to produce antibodies specific towards rpL23. We decided to use the rpL23-GFP-His model construct for immunization and