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FhuA deletion variant Δ1-159 overexpression in inclusion bodies and refolding with Polyethylene-Poly(ethylene glycol) diblock copolymer

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ABSTRACT

Membrane protein isolation is a challenging problem. In fact especially their extraction from the respective membrane is difficult and often goes along with losses in yield. Usually expensive detergents are needed to extract the target protein from the membrane. Therefore finding an efficient overexpression and extraction method and an alternative to detergents is desirable.

In this study we describe a new and fast method to express, extract and purify an engineered variant of the FhuA protein (FhuA Δ1–159) that acts as passive diffusion channel, using a diblock copolymer as an alternative to detergents like octyl-POE (*n*-octylpolyoxyethylene). The N-terminal leader sequence, facilitating the protein's transport to the outer membrane was deleted (FhuA Δ1-159 Δsignal), resulting in protein accumulation in easy to isolate inclusion bodies. Urea was used to solubilise the unfolded protein and dialysis against phosphate-buffer containing the commercially available diblock copolymer PE–PEG[Polyethylene-Poly(ethyleneglycol)] lead to protein refolding.

Circular dichroism spectroscopy revealed a high β-sheet percentage within the refolded protein secondary structure indicating the successful reconstitution of FhuA Δ1-159 Δsignal native state. Furthermore the channel functionality of FhuA Δ1-159 Δsignal was verified by measuring the in and out-flux through the protein when inserted into liposome membrane, using the HRP/TMB (HRP = Horse Radish Peroxidase, TMB = 3,3′,5,5′-tetramethylbenzidine) assay system.

Introduction

The common procedure to obtain bacterial membrane proteins includes their expression into the membrane, culture harvesting by centrifugation and cell disruption. The membrane fractions with the desired protein are isolated by differential centrifugation and the target membrane protein is solubilised by the addition of a suitable detergent [1]. However protein yields gained by known procedures to extract membrane proteins from the bacterial outer membrane are low and the protein purity is comparatively poor [2] and further purification steps are necessary to obtain adequate purity. If the target protein is a channel protein, overexpression might lead to increased osmotic stress, decreasing the obtainable cell density.

In contrast the expression of membrane proteins without their signal sequence for transport and integration into the lipid membrane, usually results in high concentrations of unfolded protein, which accumulates in inclusion bodies that can be isolated easily [3]. However proteins present in inclusion bodies are highly insoluble due to the formation of aggregates [4]. A solubilisation step leading to monomolecular dispersion and a minimum of non-native intra- or interchain interactions is needed [3]. After solubilisation the obtained protein has to be refolded to regain the native state. To refold the protein, the solubilisation agent has to be removed and an environment promoting correct protein folding needs to be provided. By extensive dialysis against buffer containing a suitable detergent, both removal of the solubilisation agent and membrane protein refolding, can be achieved. However the large volumes of buffer and thus detergent necessary for dialysis, often lead to high costs. As an alternative to detergents amphipols can be used to refold membrane proteins from inclusion bodies by dialysis [5]. These mild surfactants, composed of a hydrophilic backbone grafted with alkyl chains [6], are well suited to recover a membrane proteins native state. However amphipols have to be synthesised by rather complex many-step synthesis or if commercially obtained are often very expensive.

In the present study a new method to express and isolate an engineered variant of the Ferric hydroxamate uptake protein component A (FhuA) from inclusion bodies is described.

FhuA is a monomeric β-barrel outer membrane protein of *Escherichia coli* with a MW of 78.9 kDa. It consists of 22 antiparallel β-strands and an N-terminal cork-domain harbouring four-stranded β-sheets and four short α-helices [7]. The lack of the

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cork-domain (AA 1–159) in the engineered variant FhuA Δ1-159 (MW = 62 kDa) results in a passive diffusion channel with application in nanocontainer technology [8]. Up to now FhuA Δ1-159 has been expressed natively and has been isolated and solubilised from the *E. coli* outer membrane [8]. The commercial detergents octyl-POE [9], OG (Octylglucoside) [10], LDAO (Lauryldimethylamine-oxide) [11] were used to solubilise FhuA deletion variants in a rather time consuming and expensive procedure. Expression of native FhuA Δ1-159 leads to elevated osmotic stress, limiting the obtainable cell number to ~2–2.5 x 10^9 cells/ ml, furthermore making it necessary to freshly transform the *E. coli* expression host prior to each expression [9]. Therefore the development and establishment of a new, cost and time effective method to obtain high yields of FhuA deletion variants was desirable.

To express the FhuA deletion variant into inclusion bodies the N-terminal signal sequence has been deleted from the protein (FhuA Δ1-159 Δsignal). Protein obtained from the inclusion bodies was refolded (after solubilisation in urea) by using the commercially available and water soluble diblock copolymer PE–PEG[Poly-ethylene-Poly(ethyleneglycol)] (average M_n 2250), as a cheap alternative to detergents. PE–PEGwas chosen due to structural similarities to octyl-POE [9] (structure of PE–PEG see Supp. Mat.: Figure S1).

The secondary structure integrity of refolded FhuA Δ1-159 Δsignal was analysed by circular dichroism (CD) spectroscopy. Furthermore the FhuA Δ1-159 Δsignal channel functionality was detemined after protein reconstitution into liposomes and kinetic flux measurement based on the HRP/TMB (HRP = Horse Radish Peroxidase, TMB = 3,3′,5,5′-tetramethylbenzidine) assay system. This assay system was chosen due to its good reproducibility in combination with the FhuA deletion variants reconstituted into liposomes [9].

Materials and methods

All chemicals were of analytical grade or higher and obtained from Applichem (Darmstadt, Germany) or Sigma Aldrich (Taufkirchen, Germany). Protein concentrations were determined using the standard BCA kit (Pierce Chemical Co, Rockford, USA).

Deletion of FhuA Δ1-159 N-terminal signal sequence and cloning of resulting construct

The FhuA Δ1-159 signal sequence (coded by base pairs 4–99) was deleted by PCR mutagenesis as reported in Papworth et al. [12]. As a template for mutagenesis vector pPR-IBA (IBA GmbH, Göttingen, Germany) harbouring ORF (open reading frame) FhuA Δ1-159 was used. The parental plasmid was digested by *Dpn*I. The resulting plasmid was transformed into *E. coli* BL21 [13]. Presence of the desired deletion (FhuA Δ1-159 Δsignal) was verified by DNA-sequencing (carried out by GATC, Konstanz, Germany). Primer sequences are reported in Table 1.

Expression of FhuA Δ1-159 Δsignal

E. coli BL21 containing pPR-IBA FhuA Δ1-159 Δsignal was grown and protein expression was carried out in the Biostat ED fermenter from Sartorius (Göttingen, Germany) with a working volume of 10 L based on a protocol described previously [8]. The culture broth was harvested by centrifugation and pellets were kept at −20 °C until inclusion bodies were isolated.

Table 1
Oligo nucleotide primers used to delete signal sequence of fhuA Δ1–159.

Sequence Name	Sequence (5' → 3')
FhuA Δ1-159 Δsignal-fwd	GGT CCC GAA TTC ATG CTG AAA GAA GTT CAG
FhuA Δ1-159 Δsignal-rev	CTG AAC TTC TTT CAG CAT GAA TTC GGG ACC

Isolation of inclusion bodies harbouring FhuA Δ1-159 Δsignal

Inclusion bodies were isolated and purified using the method described by O'Callaghan et al. [14] with slight modifications. The cell pellet obtained from approximately 0.5 L culture broth was resuspended in 50 mL potassium phosphate buffer 0.1 M (pH 8, 0.2 M Na_2HPO_4, 0.2 M NaH_2PO_4), containing 5 mM EDTA, 0.1 M NaCl, 0.5% Triton 100 (v/v), 0.1 mM PMSF and 1 mM DTT and homogenised by sonication. Then 0.1 mg/mL lysozyme, 0.01 mg/mL DNaseI and 10 mM $MgSO_4$ were added and the sample was incubated for 1 h at 37 °C with gentle shaking. The suspension was centrifuged for 15 min at 4 °C and 5000 rpm and the supernatant was discarded. The pellet was washed twice with the same buffer lacking lysozyme, DNaseI and DTT. A final washing step was carried out using 0.1 M potassium phosphate buffer at pH 8.0 containing 1 mM EDTA and 0.1 mM PMSF.

Solubilisation and refolding of FhuA Δ1-159 Δsignal

The method for protein solubilisation and refolding via dialysis is based on the protocol of Steinle et al. [15]. The pure inclusion body pellet was resuspended in 5 mL 0.1 M potassium phosphate buffer (pH 8), containing 50 mM Glycine, 0.25 mM PE–PEGand 0.1 mM PMSF. The sample was centrifuged for 15 min at 4 °C and 5000 rpm and the supernatant was discarded. Afterwards the pellet was dissolved in 20 mL of the same buffer supplemented with 8 M urea, while stirring vigorously. The pellet was solubilised completely over night. The clear solution was transferred to a dialysis tube (Spectra/Por®4 MWCO 12–14,000) and dialysed against buffer containing 0.1 M potassium phosphate buffer (pH 8) containing 4 M urea, 1 mM EDTA and 0.25 mM PE–PEG(Sigma Aldrich Cat 435473), followed by two additional dialysis steps against the same buffer with step-wise decreased urea concentration, (1 M, 0 M). Each dialysis step took 24 h. All steps were carried out at 4 °C. Afterwards the retentate was collected from the tube and centrifuged at 5000 rpm, 4 °C for 30 min. The supernatant containing the refolded FhuA Δ1-159 Δsignal was decanted into a fresh centrifugal tube and stored at 4 °C.

A sample of refolded FhuA Δ1-159 Δsignal was furthermore dialysed three times against 0.1 M potassium phosphate buffer (pH 7.4, 0.2 M Na_2HPO_4, 0.2 M NaH_2PO_4) containing octyl-POE to exchange PE–PEGagainst the detergent.

SDS–PAGE of FhuA Δ1-159 Δsignal obtained from inclusion bodies

Purity of protein samples was determined using 10% acrylamide gels containing 0.1% SDS running in a Biorad Mini-PROTEAN (Hercules, California, USA) system. Bands were visualised by Coomassie staining [16].

Expression and membrane isolation of FhuA Δ1–159

Expression and isolation from the membrane of FhuA Δ1-159 was carried out, as descibed in [8]. The protein was solubilized in 0.1 M potassium phosphate buffer (pH 8) containing 3% octyl-POE.

Secondary structure determination of FhuA Δ1-159 Δsignal by circular dichroism spectroscopy

Dichroic spectra of refolded FhuA Δ1-159 Δsignal, refolded FhuA Δ1-159 Δsignal that was further dialysed against octyl-POE and FhuA Δ1-159 solubilised from the membrane (using