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Two-step purification of outer membrane proteins

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Abstract

Here, we describe a simple and efficient method for the purification of *Escherichia coli* outer membrane proteins. We have tested this protocol for the purification of Wza and Osmoporin C (OmpC) proteins. Both proteins were purified to homogeneity, in two steps, by anion exchange and size exclusion chromatography with a final yield of 92.5 mg for the Wza protein and 291.5 mg for the OmpC protein. The purity of the samples was judged by electrophoretic analysis, mass spectrometry, single particle analysis, three-dimensional (3D) crystallisation and X-ray diffraction.

Keywords

Membrane protein purification; Wza; OmpC; OmpA; MALDI-TOF; Electron microscopy; 3D crystals; X-ray diffraction

1. Introduction

Approximately 40% of the sequenced genes encode for membrane associated proteins. Many membrane proteins are related to diseases. To understand their function, it is important to elucidate their structure, which will provide the basis for designing better drugs. The expression and purification of high levels of active protein is the bottleneck for membrane protein X-ray crystallography. About 50% of the outer membrane mass of Gram-negative bacteria consists of protein, either in the form of integral membrane proteins or as lipoproteins that are anchored to the membrane by means of N-terminally attached lipids.

The first important step in purifying membrane proteins from any membrane system is to solubilise them from their environment surrounded by lipids. The success of the purification relies greatly on the choice of detergents and their concentrations, especially when one wants to purify the membrane protein complexes in their intact (native) form.

The outer membrane protects Gram-negative bacteria against a harsh environment. At the same time, the embedded proteins fulfil a number of tasks that are crucial to the bacterial cell, such as solute and protein translocation, as well as signal transduction. Unlike membrane proteins from all other sources, integral outer membrane proteins do not consist of transmembrane α-helices, but instead fold into antiparallel β-barrels.

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The two outer membrane proteins, Wza and Osmoporin C (OmpC) that were studied differ structurally and functionally from each other. Wza is a highly conserved multimeric outer membrane protein complex required for the surface expression of the serotype K30 group 1 capsular polysaccharide in *Escherichia coli*. The group 1 K30 antigen from *E. coli* (O9a:K30) is present on the cell surface and the genes involved in synthesis and transport are encoded at a locus called *cps* [1]. The group 1 capsule clusters are characterised by the presence of four genes: *orfx*, *wza*, *wzb* and *wzc* [2]. Initial biochemical characterisation by Drummelsmith and Whitfield [3] reported that the *wza* gene encodes an outer integral membrane lipoprotein termed Wza. The apparent molecular weight of the Wza protein in solution is 40,232 Da. This protein has been identified as being required for the export of polysaccharides to the cell surface [4]. Electron microscopy of the purified protein showed the presence of ring-like structures with a large pore in the middle [3]. Towards the goal of obtaining detailed structural information, we have recently succeeded in obtaining a three-dimensional (3D) reconstruction model [5] and crystallising Wza from *E. coli* [6].

On the other hand, porins are pore proteins that form outer membrane channels in Gram-negative bacteria, mitochondria and chloroplasts [7]. They mediate the exchange of small molecules between the outer and inner environment of bacterial cells or the organelles. Bacterial porins have been classified into specific and unspecific pore proteins and their expression is modulated by the living environment of the bacteria [8]. Osmoporin C is one of the major outer membrane proteins of *E. coli*. The protein forms three large water-filled channels per trimer, allowing the diffusion of small hydrophilic molecules such as nutrients and waste products across the outer bacterial membrane. Several antibiotics, including β-lactams, use the porin pathway to cross the outer membrane and to find their targets [9]. The pore properties of porins can be modified drastically by mutations at the pore constriction. Each subunit contains 16 beta-strands forming a transmembrane β-barrel whose pore is constricted by the third extracellular loop (L3).

Here, we present the development of a two-step purification protocol for bacterial outer membrane proteins. This protocol was used for the purification and structural studies of two very different outer membrane proteins, Wza and OmpC, from *E. coli*.

2. Materials and methods

2.1. Protein extraction

The extraction procedure was the same for both the Wza and OmpC proteins. Bacterial cells were disrupted using a French press (110 MPa). The crude extract was fractionated by ultracen-trifugation. The supernatant solution was discarded and the pellet containing cell envelopes was resuspended in 160 ml 20 mM sodium phosphate pH 7 and 2% *N*-lauroylsarcosine (Sigma) to solubilise the inner membrane [10]. The pellet was solubilised at room temperature with rolling for 1 h. The extract was centrifuged again for 1 h at 289 K. The supernatant solution was then discarded and the pellet, now enriched in outer membranes, was resuspended in 160 ml 20 mM sodium phosphate, 50 mM NaCl and 0.5% *N*-tetradecyl-*N*,*N*-dimethyl-3-ammonio-1-propanesulfonate (SB3-14; Sigma). The membranes were solubilised overnight at room temperature with rolling. The insoluble materials were then removed by centrifugation at 85,000 *g* for 1 h at 289 K.

2.2. Wza purification

Purification of Wza was performed using anion exchange chromatography. A Biorad Q (Bio-Rad) column was used and equilibrated with 20 mM sodium phosphate, 50 mM NaCl and 0.05% SB3-14. The protein was eluted either with a linear gradient or a step segment of 12, 22 and 100% using a solution consisting of 20 mM sodium phosphate, 1 M NaCl and

0.05% SB3-14. The fractions containing Wza (12% of 1 M NaCl) were pooled and dialysed overnight against 20 mM sodium phosphate, 50 mM NaCl. The Biorad Q column was also used to exchange the detergent to *n*-dodecyl-β-D-maltoside (DDM; Anatrace, USA). The protein was washed with 100 ml 20 mM Tris pH 8.5, 50 mM NaCl and 0.008% DDM and eluted with 20 mM Tris–HCl pH 8.5, 1 M NaCl and 0.008% DDM. The sample was dialysed overnight in 20 mM Tris base pH 8, 150 mM NaCl and 0.008% DDM and loaded onto a Superdex 200 size exclusion column (GE Healthcare). The protein was then eluted using 1.2 × column volumes of the same buffer with one major peak at ~400 kDa. The sample was dialysed overnight in 20 mM Tris base pH 7.5, 80 mM NaCl and 0.008% DDM. Purity was checked at all stages with SDS-PAGE using Coomassie Blue staining (Fig. 1a) and MALDI-TOF (Fig. 2). Table 1 summarizes the yield of pure protein after each purification step.

2.3. OmpC purification

Purification of OmpC was performed following the Wza protocol. The detergent was exchanged to 1% *n*-octyl-β-D-glucopyranoside (bOG; Anatrace). Purity was checked at all stages with SDS-PAGE using Coomassie Blue staining (Fig. 3a). Table 1 summarizes the yield of pure protein after each purification step.

2.4. Electron microscopy

Carbon-coated copper grids (number 400) were inverted on the surface of a 10 μl droplet of Wza (5–10 μg/ml in 25 mM Tris, pH 7.5, 80 mM NaCl, 0.008% (w/v) DDM for several minutes and then blotted sequentially for 5 s on a Whatman filter paper until dry. Grids then were placed on a 20 μl droplet of freshly prepared 2% (w/v) uranyl acetate for several seconds and blotted briefly (Fig. 1b and d).

2.5. Crystallisation

The crystallisation conditions for the Wza protein have been previously reported [6] (Fig. 1c and e).

Crystallisation trials for the OmpC protein were performed using the hanging-drop vapour-diffusion system at 293 K. The protein solution contained 20 mM Tris base pH 8, 1% bOG and 12 mg/ml protein. Each drop was prepared by mixing 1 μl protein solution with the same volume of reservoir solution. Initial crystallisation conditions were screened using the hanging-drop method and a screen developed by Professor Iwata [11]. Crystals appeared after 1 week in several conditions (data not shown) (Fig. 3b).

2.6. X-ray diffraction studies

Crystal quality was checked using an in-house source (Rigaku RAXIS 007) and beamlines at the European Synchrotron Radiation Facility.

3. Results and discussion

The purification of Wza protein has been previously reported from our group [4]. In this manuscript we have modified the purification protocol in order to obtain higher yield of the purified protein. Purifying the Wza protein, we encountered a problem that seriously affected the crystal quality. The protein was initially purified using a linear gradient of 0–100% 1 M NaCl elution buffer and size exclusion chromatography. This resulted in the production of a very pure Wza sample as judged by Coomassie Blue staining. MALDI-TOF revealed that this batch actually contained a 35 kDa contaminant (not detectable by silver staining) (Fig. 2b). The protein was identified by trypsin digest to be another outer membrane protein, OmpA, from *E. coli*. Size exclusion chromatography failed to separate

the Wza (multimer is ~50 kDa) from the OmpA. The sample was further analysed by electron microscopy, and it revealed that the Wza–OmpA solution was aggregated (Fig. 1d). Trying to remove this contaminant did not prove to be trivial. Attempts to isolate OmpA by either cation chromatography or precipitation with ammonium sulfate failed. In our previous paper [4] we reported that the minor contaminants could be removed with hydroxyapatite chromatography, but this step resulted in 70% loss of the total protein due to precipitation from the high sodium phosphate salt during elution from the column. Since we required high amounts of the protein for crystal studies, we investigated other purification approaches.

Further analysis of the biophysical properties of OmpA revealed that its p*I* (5.74) was very close to that of Wza (5.31). This would explain the co-elution with the Wza protein on the anion exchange chromatography step. To avoid this, the elution protocol was changed to a step gradient instead of a linear elution. We found that Wza could be eluted with 12% and OmpA with 22% of 1 M NaCl elution buffer, respectively. The MALDI-TOF showed only the presence of a single peak at ~40 kDa for the Wza monomer (Fig. 2a), and the electron microscopy analysis showed that the sample was monodisperse (Fig. 1b).

Both pure Wza and Wza–OmpA samples crystallise in an orthorhombic crystal form. The crystals look different but in some cases they had the same shape. X-ray diffraction studies of the crystals revealed that the two protein batches behave very differently. The crystals from the pure Wza diffracted X-rays to 3.0Å using a synchrotron radiation source, whereas the Wza–OmpA crystals only diffracted to 10 Å, despite the fact that they had the same unit cells. This was observed for both the small needles and the similar to the pure Wza crystals.

Furthermore, utilising the monodisperse sample (Fig. 1b), we have obtained the three-dimensional structure of Wza at a 15.5Å resolution using single particle averaging on a dataset of cryo-negatively stained protein [5]. Symmetry analysis of the three-dimensional structure suggests that Wza is an octameric complex with a C4 quasi-rotational symmetry and is organized as a tetramer of dimeric subunits. Wza is best described as a stack of two 4Å high rings with differing diameters providing a mushroom-like aspect from the side. The larger ring has a distinctive square shape with a diameter of 115 Å, whereas the smaller is almost circular with a diameter of 90 Å. In the center of the complex and enclosed by the four symmetrical arms is a small elliptical cage-like cavity of ~40 Å in diameter. The central cavity is effectively sealed at the top and bottom of the complex but has small inter-arm holes when viewed from the side.

On the other hand, the purification of the OmpC protein was straight forward. We originally purified this protein by following the well-characterised protocol by Garavito and Rosenbusch [12]. The purification and crystallisation of OmpC from *E. coli* have already been reported by Kim [13]. Both protocols are time consuming, require precipitation of the protein with ethanol and expensive detergents. Therefore, we adopted the same purification protocol as for the Wza. Extraction of the protein was achieved by using the inexpensive swittergent SB3-14, and only exchanged to the expensive and more suitable detergent bOG prior to crystallisation. Again, using a linear gradient resulted in the elution of OmpC with contaminants (they could be visualised by Coomassie Blue staining). The step elution approach resulted in the production of highly pure OmpC (Fig. 3a). The contaminated sample did not result in the production of crystals whereas the pure sample gave crystals (Fig. 3b) that diffracted X-rays to 3.3Å using an in-house source. Kim [13] has reported the production of crystals that were diffracting X-rays to 4Å and showed intense non-Bragg scattering. This was probably due to non-compact or not well-ordered crystal packing. In our case, we have obtained and refined the crystal structure of OmpC from *E. coli* to 3.3Å (data not shown, manuscript under preparation). The structure solution shows a very tight crystal packing and crystal contacts between the hydrophilic portion of the protein.

These experiments show that protein impurity alters the nucleation and/or the growth of the Wza and OmpC crystals. The impurity was either incorporated in the crystal lattice or inhibited the formation of a well-packed crystal lattice.

These results share similarities with observations reported for other proteins. Traces of protein impurities have been shown to cause heterogeneous and secondary nucleation, lattice defects, including vacancies, strain, stress in selected growth sectors [14], alteration of morphology and of unit-cell parameters [15], modification of crystal habit, or termination of growth [16]. Depending on the degree of relationship and on the affinity, adsorption, attachment and subsequent incorporation of impurities can be detectable either at a macroscopic or at a microscopic scale.

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Abbreviations

bOG	*n*-octyl-β-D-glucopyranoside
DDM	*n*-dodecyl-β-D-maltoside
E. coli	Escherichia coli
OmpC	Osmoporin C
SB3-14	*N*-tetradecyl-*N*,*N*-dimethyl-3-ammonio-1-propanesulfonate

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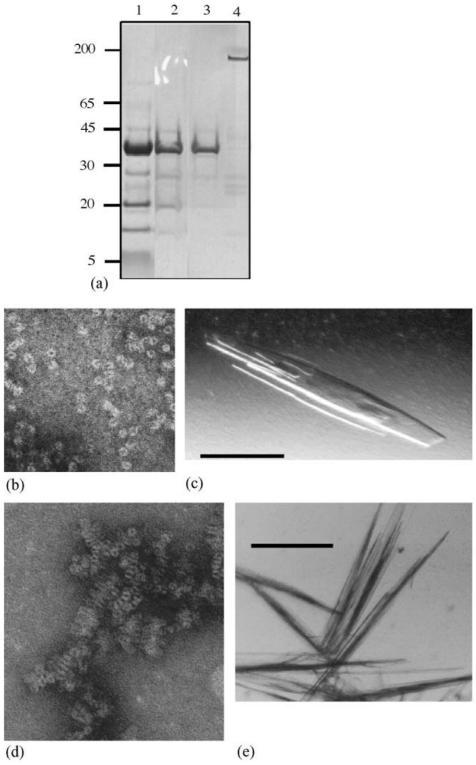

Fig. 1.

Characterisation of protein impurities and their effects on Wza crystallisation. (a) Electrophoretic analysis of Wza. Lane 1 shows the extracted protein, lane 2 Wza after anion exchange, lane 3 the pure Wza after gel filtration and lane 4 shows the Wza without heating to show the multimeric complex. Molecular weight markers are shown on the left side of the SDS-PAGE in kDa. Electron microscopy analysis of Wza (b) and Wza–OmpA (d) samples. (c and e) Effect of purification on the growth of well-ordered crystals. Scale bar 100 mm.

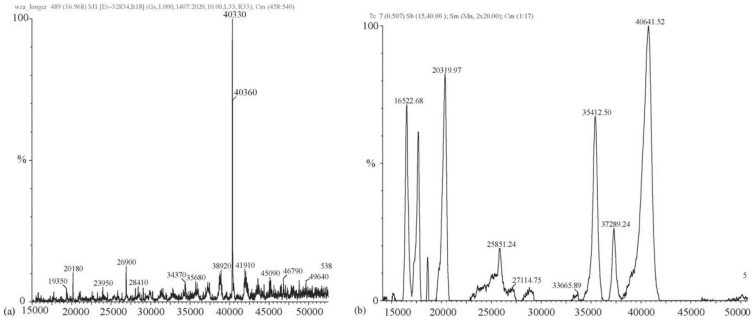

Fig. 2.

MALDI-TOF analysis of the Wza samples. (a) On a step elution protocol, Wza appears as a single peak at ~40 kDa. (b) Using a linear gradient, Wza co-elutes with the OmpA (~35 kDa). MALDI-TOF values are shown in Da.

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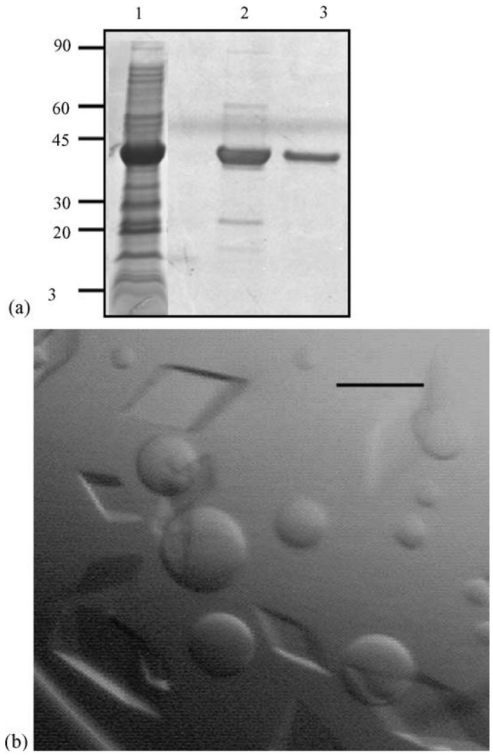

Fig. 3.
(a) SDS-PAGE of OmpC protein. Lane 1 shows the extraction of OmpC from outer membranes, lane 2 the OmpC after the anion exchange chromatography step and lane 3 pure OmpC after size exclusion chromatography. Molecular weight markers are shown on the left side of the SDS-PAGE in kDa. (b) 3D crystals of OmpC. Scale bar 100 mm.

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Table 1

Purification yield (%) and mg of total protein obtained with different purification procedures for Wza and OmpC proteins

Protein	Wza		OmpC	
	(%)	mg	(%)	mg
Extract	100	319	100	454
Anion exchange	67	213.73	72	326.88
Size exclusion	30.17	92.5	65	291.5