Video Article

Method for Efficient Refolding and Purification of Chemoreceptor Ligand Binding Domain

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Abstract

Identification of natural ligands of chemoreceptors and structural studies aimed at elucidation of the molecular basis of the ligand specificity can be greatly facilitated by the production of milligram amounts of pure, folded ligand binding domains. Attempts to heterologously express periplasmic ligand binding domains of bacterial chemoreceptors in *Escherichia coli* (*E. coli*) often result in their targeting into inclusion bodies. Here, a method is presented for protein recovery from inclusion bodies, its refolding and purification, using the periplasmic dCACHE ligand binding domain of *Campylobacter jejuni* (*C. jejuni*) chemoreceptor Tlp3 as an example. The approach involves expression of the protein of interest with a cleavable His_6-tag, isolation and urea-mediated solubilisation of inclusion bodies, protein refolding by urea depletion, and purification by means of affinity chromatography, followed by tag removal and size-exclusion chromatography. The circular dichroism spectroscopy is used to confirm the folded state of the pure protein. It has been demonstrated that this protocol is generally useful for production of milligram amounts of dCACHE periplasmic ligand binding domains of other bacterial chemoreceptors in a soluble and crystallisable form.

Video Link

The video component of this article can be found at https://www.jove.com/video/57092/

Introduction

Chemotaxis and motility have been shown to play important roles in *Campylobacter jejuni* pathogenesis by promoting bacterial colonisation and invasion of the host[1,2,3]. Chemotaxis allows bacteria to move towards an optimal environment for growth, as guided by chemical signals. This process involves recognition of intracellular and environmental chemical cues by a set of proteins termed chemoreceptors, or transducer-like proteins (Tlps). Most chemoreceptors are membrane-embedded proteins with an extracytoplasmic ligand binding domain (LBD), a transmembrane domain and a cytoplasmic signalling domain, the latter of which interacts with the cytosolic signalling proteins that transmit the signal to the flagellar motors[4,5,6,7].

Eleven different chemoreceptors have been identified in the *C. jejuni* genome[4,8]. To date, only some of these chemoreceptors have been characterised and the ligand specificity of Tlp1[9], Tlp3[10,11], Tlp4[11], Tlp7[12], and Tlp11[13] is known. Identification of natural ligands of the remaining chemoreceptors in this species, and numerous chemoreceptors in other bacteria, can be greatly facilitated by the production of folded and highly pure recombinant chemoreceptor LBDs[14,15,16]. However, attempts to heterologously express periplasmic LBDs of bacterial chemoreceptors in *Escherichia coli* often result in their targeting into inclusion bodies[17,18,19]. Nevertheless, this phenomenon can facilitate easy isolation and recovery of the protein in hand. Here, a method is presented for protein recovery from inclusion bodies, its refolding and purification, using the periplasmic LBD of the *C. jejuni* chemoreceptor Tlp3 as an example. This example was chosen because Tlp3-LBD belongs to the dCACHE family[20] of sensing domains which are abundantly found in two-component histidine kinases and chemoreceptors in prokaryotes[20,21,22,23].

In this approach, the expression construct, based on a pET151/D-TOPO vector, has been designed to incorporate an N-terminal His_6-tag followed by a tobacco etch virus (TEV) protease cleavage site, for subsequent tag removal[19]. The protocol describes protein overexpression in *E. coli*, isolation and urea-mediated solubilisation of inclusion bodies, and protein refolding by urea depletion. Following refolding, the sample is purified by affinity chromatography, with optional tag removal and size-exclusion chromatography. The folded state of the purified protein is confirmed using circular dichroism spectroscopy. This is a modified version of the method that has been previously developed to recover and purify the LBD of a different chemoreceptor, *Helicobacter pylori* TlpC[19]. This procedure, summarised in **Figure 1**, yields 10 - 20 mg of pure, untagged Tlp3-LBD per 1 L of bacterial culture, with a protein purity of >90% as estimated by SDS-PAGE.

Protocol

1. Expression of His_6-Tlp3-LBD in *E. coli*

1. Inoculate 150 mL of sterile Luria-Bertani (LB) broth containing 50 µg mL^{-1} ampicillin with BL21-Codon-Plus(DE3)-RIPL cells transformed with the pET151/D-TOPO vector for expression of His_6-Tlp3-LBD (amino acid residues 42-291), and incubate it at 37 °C in a shaker (orbit diameter, 25 mm) with 200 rpm overnight.
2. Prepare six 2 L Erlenmeyer flasks containing 800 mL of sterile LB broth and 50 µg mL^{-1} ampicillin. Inoculate each flask with 20 mL of the overnight culture obtained from step 1.1. Incubate them at 37 °C with continuous shaking at 200 rpm until the optical density at 600 nm reaches 0.6.
3. Take a 1 mL aliquot from one of the flasks (uninduced control sample), pellet using a benchtop centrifuge (13,000 x g, 4 °C, 10 min) and discard the supernatant. Store the bacterial pellet at -20 °C for subsequent SDS-PAGE analysis.
4. Induce protein expression by adding 1 mM of isopropyl β-D-thiogalactoside (IPTG) to each flask with culture. Continue incubating the flasks at 37 °C in a shaker at 200 rpm for an additional 4 h.
5. Harvest the cells by centrifugation at 5,000 x g for 15 min at 4 °C and discard the supernatant.
   NOTE: At this point, the procedure can be paused by placing the cell pellet into a -80 °C freezer for storage.

2. Isolation and Denaturation of Inclusion Bodies

1. Transfer all the cell pellets obtained in step 1.5 to a 250 mL beaker and add 100 mL of buffer A (10 mM Tris-HCl, pH 8.0, 150 mM NaCl). Allow the cells to thaw completely (if frozen), and resuspend the pellet. Keep the sample on ice unless indicated otherwise.
2. Pass the resuspended cells through a high-pressure homogeniser three times to lyse the cells and ensure complete shearing of the genomic DNA.
3. Centrifuge the lysate at 10,000 x g for 15 min at 4 °C. Collect a 1 mL sample of the supernatant (soluble fraction) and store it at -20 °C for subsequent SDS-PAGE analysis. Discard the rest of the supernatant and place the pellet on ice.
   NOTE: This pellet is white in color (easily distinguishable from unbroken cells which are the shade of brown in color) and contains inclusion bodies.
4. Resuspend the inclusion bodies pellet thoroughly in 20 mL of ice-cold buffer B (10 mM Tris-HCl pH 8.0, 0.2 mM phenylmethanesulfonyl fluoride (PMSF), 1% Triton X-100). This facilitates solubilisation of the membrane and membrane proteins. Vortex for 1 - 2 min to aid resuspension.
5. Centrifuge the sample at 10,000 x g for 15 min at 4 °C and discard the supernatant.
6. Resuspend the pellet thoroughly in 20 mL of ice-cold buffer B by vortexing the tube for 1 - 2 min. Ensure that the pellet is broken into small pieces. Pipet the sample up and down to aid pellet resuspension.
   NOTE: It is important to resuspend the pellet thoroughly to obtain inclusion bodies free from impurities.
7. Repeat step 2.5. If the supernatant is cloudy or coloured, repeat steps 2.6 and 2.5 (in that order) until the supernatant is clear and colourless.
8. Resuspend the pellet in 20 mL of ice-cold buffer C (10 mM Tris-HCl pH 8.0, 0.2 mM PMSF) by vortexing the tube for 1 - 2 min.
9. Repeat step 2.5.
10. Add 25 mL of ice-cold denaturing buffer D (10 mM Tris-HCl pH 8.0, 8 M urea, 10 mM dithiothreitol (DTT), 0.2 mM PMSF) to dissolve the inclusion bodies pellet. Resuspend thoroughly by vortexing for 1 - 2 min, or until the pellet is broken into small pieces.
11. Mix the suspension by axial rotation with a rotation rate of 30 rpm for 30 - 120 min at 4 °C.
12. Clarify the denatured protein solution by centrifugation at 30,000 x g, 4 °C for 30 min, place the supernatant on ice and discard the pellet.
13. Measure the protein concentration using the Bradford assay.[24] Take an aliquot for SDS-gel analysis and place it at -20 °C.
    NOTE: At this point, the procedure can be paused by snap-freezing the aliquoted sample in liquid nitrogen and keep it at -80 °C for storage.

3. Protein Refolding

1. Prepare 250 mL of buffer E (3 M urea, 100 mM Tris-HCl pH 8.0, 0.4 M L-arginine monohydrochloride, 20 mM reduced L-glutathione, 2 mM oxidized L-glutathione) in a 500 mL beaker and cool the buffer down to 4 °C.
2. Add 60 mg of denatured protein mix containing His_6-Tlp3-LBD obtained in step 2.13 to 250 mL of buffer E, while stirring the buffer at 500 rpm. Incubate the refolding mix at 4 °C with continuous stirring at 500 rpm for 24 - 48 h. The final protein concentration in this step is 0.2 mg mL^{-1}.
3. Prepare 7 L of buffer A in an 8 - 10 L bucket and cool it down to 4 °C in preparation for the next step (step 3.4) that will take place in 24 - 48 h (see the flowchart in **Figure 1**).
4. Immerse an ~50 cm piece of dialysis tubing of 28 mm inflated diameter, with molecular weight cut-off at 12 - 14 kDa into 200 mL of 10 mM ethylenediaminetetraacetic acid disodium salt (EDTA-Na_2) and heat it over a gas flame until boiling. Rinse the dialysis membrane with 200 mL of ddH_2O.
5. Clamp one end of the dialysis membrane with a dialysis tubing closure and transfer the 250 mL refolding mixture obtained in step 3.2 into the dialysis tube. Close the open end with another clamp. Check the integrity of the membrane to ensure there are no leaks.
6. Place the dialysis tube in a dialysis bucket with the pre-cooled 7 L of buffer A prepared in step 3.3. Place a magnetic stir bar, ensuring that it will not touch the dialysis tubing while stirring.
7. Dialyse the sample at 4 °C with continuing stirring at 500 rpm and change the buffer at least four times over a period of 12 h. After the last buffer change, leave the sample to dialyse overnight.
   NOTE: During dialysis, the excess of urea (~3 M) is gradually removed from the denatured protein solution, which allows the protein to refold. Heavy protein precipitation can be observed at the end of dialysis.
8. Remove the dialysis tube from the bucket and transfer its contents into a 500 mL beaker. Keep the protein solution on ice, unless indicated otherwise.

9. Filter the protein solution through a 0.43 µm pore size membrane into a 500 mL glass bottle to remove any precipitated protein.

4. Purification of His_6-tagged Protein Using Immobilised Metal Ion Affinity Chromatography

1. **Charge and equilibrate a 5 mL prepacked chelating column.**
    1. Connect the column to a peristaltic pump, ensuring that there is no air trapped in the connecting tubing.
    2. Wash the column by passing through 25 - 50 mL of ddH_2O.
    3. Charge the column by loading 3 mL of 0.1 M $NiCl_2$ and then passing through 25 mL of ddH_2O.
    4. Equilibrate the column with 25 mL of buffer F (loading buffer, 20 mM Tris-HCl pH 8.0, 500 mM NaCl, 20 mM imidazole).
2. Adjust the refolded protein sample obtained in step 3.9 to the composition of buffer F by adding 2.5 mL of 1 M Tris-HCl pH 8.0, 25 mL of 5 M NaCl and 2.5 mL of 2 M imidazole stock solutions.
3. Load the sample onto the column at a rate of 5 mL/min or less and discard the flow-through (unbound proteins).
4. Wash the column with 50 - 100 mL of buffer F to remove non-specifically bound proteins and discard the flow-through.
5. Elute His_6-Tlp3-LBD with 25 mL of buffer G (elution buffer, 20 mM Tris-HCl, 500 mM NaCl, 500 mM imidazole) and pool the flow-through fractions containing the protein (as determined by using the Bradford reagent).
6. Measure the protein concentration in the pooled sample using the Bradford assay. Take a small aliquot for SDS-PAGE analysis and place at -20 °C.

5. His_6-tag Removal Using TEV Protease (Optional)

1. Prepare, and cool down to 4 °C, 4 L of buffer H (TEV cleavage buffer, 10 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 % (v/v) glycerol, 2 mM DTT).
2. Cut approximately 5 - 7 cm of dialysis tubing (diameter 2 - 3 cm) and immerse it in 200 mL of ddH_2O.
3. Add His_6-tagged TEV protease to the His_6-tag protein prepared in step 4.5 to a final molar ratio 1 TEV: 8 protein.
4. Clamp one end of the tubing with a dialysis tubing closure and fill it with the protein/TEV protease mixture. Close the other end and ensure there are no leaks.
5. Place the dialysis tube into the pre-cooled 4 L of buffer H (from step 5.1) and incubate it at 4 °C with continuous stirring for 2 h. Change the buffer and continue dialysis overnight to allow the TEV-mediated cleavage reaction to complete.
6. In the meantime, prepare and cool (4 °C) 4 L of buffer I (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 % (v/v) glycerol) in preparation for the next day.
7. After the overnight dialysis, exchange the buffer to buffer I prepared in step 5.6 and dialyse for a further 1 - 2 h at 4 °C.
8. Remove the tube from the dialysis bucket, unclip one end of the tube and transfer the protein solution to a 50 mL Falcon tube. Filter the solution through a 0.43 µm pore size syringe filter and keep it on ice, unless stated otherwise.
9. Measure the volume of the sample and adjust the Tris, NaCl and imidazole concentration to the composition of buffer F (*e.g.* for 25 mL of protein solution in buffer J add 0.25 mL of 1 M Tris-HCl pH 8.0, 1.75 mL of 5 M NaCl, and 0.25 mL of 2 M imidazole).
10. Prepare a 5 mL HiTrap Chelating HP column as described in step 4.1.
11. Load the sample onto the column (flow rate: 5 mL/min) and collect the flow-through, which contains the untagged Tlp3-LBD (residues 42 - 291). Uncleaved protein, the cleaved His_6-tag and His_6-TEV are retained by the column.
12. Wash the column with 5 mL buffer F (loading buffer) to allow all untagged protein to flow through and collect the eluate.
13. Pool the eluate obtained in steps 5.11 and 5.12 and measure the protein concentration. Take a small aliquot and store it at -20 °C for SDS-PAGE analysis.

6. Size-exclusion Chromatography (Gel Filtration) of Tlp3-LBD

1. Prepare 1 L of buffer A (10 mM Tris-HCl pH 8.0, 150 mM NaCl) and filter it using a 0.43 µm membrane.
2. Equilibrate a 26/60 size-exclusion column with buffer A at a flow rate of 4 mL/min.
3. Concentrate the protein sample obtained in step 5.13 to a volume of 3 - 4 mL using 15 mL centrifugal concentrator with a 10 kDa cut-off (4 °C, 4,000 x g).
4. Clarify the protein solution by centrifugation at 13,000 x g, 4 °C for 30 min to remove any precipitated protein.
5. Load the sample onto the pre-equilibrated 26/60 gel filtration column. Perform chromatography in buffer A at a flow rate of 4 mL/min. Monitor the UV trace. Tlp3-LBD elutes at a retention volume of 210 - 220 mL. Pool the peak fractions.
6. Measure the protein concentration. Take a small aliquot for SDS-PAGE analysis.

7. SDS-PAGE Analysis of Samples Collected at Various Stages of Protein Purification

1. Prepare 1 L of SDS-PAGE running buffer (buffer J), containing 25 mM Tris, 250 mM glycine pH 8.3, and 0.1 % (w/v) sodium dodecyl sulfate (SDS).
2. Prepare 10 mL of 5x SDS-PAGE loading buffer (buffer K), containing 0.25 M Tris-HCl pH 6.8, 10% (w/v) SDS, 50% (v/v) glycerol, 1 mM DTT, and 0.05% bromophenol blue.
3. Allow the aliquots collected during the different steps of the purification (steps 1.3, 2.3, 2.13, 4.6, 5.13, 6.6) to thaw.
4. For each sample, mix the volume corresponding to 15 µg of total protein with 2 µL of 5x SDS-PAGE loading buffer, and adjust the volume to 10 µL with ddH_2O.
5. Heat the samples at 95 °C for 5 - 10 min, spin them at 10,000 x g for 30 s and transfer the samples into a clean tube.
6. Prepare an SDS-PAGE gel, using 15% polyacrylamide separating gel (for 5 mL: 2.5 mL 30% (w/v) bis-acrylamide, 1.2 mL 1.5 M Tris-HCl pH 8.8, 1.2 mL ddH_2O, 50 µL 10% (w/v) SDS, 50 µL 20% (w/v) ammonium persulfate (APS), 3 µL tetramethylethylenediamine (TEMED) and 5% polyacrylamide stacking gel (for 2 mL: 340 µL 30% (w/v) bis-acrylamide, 250 µL 1 M Tris pH 6.8, 1.37 mL ddH_2O, 20 µL 10% (w/v) SDS, 20 µL 20% (w/v) APS, 2 µL TEMED).

7. Assemble the electrophoresis apparatus and fill it with 1x SDS page running buffer as per manufacturer's recommendation.
8. Load a protein molecular weight marker and the samples prepared in step 7.5. Perform the electrophoresis at a constant current of 25 mA.
9. Transfer the gel into a container and rinse it with ddH_2O. Add 150 mL of the Coomassie Blue staining solution containing 25% (v/v) isopropanol, 10% (v/v) acetic acid and 0.03% (w/v) Brilliant Blue R-250. Place the container on a horizontal rotation platform for 30 min at room temperature.
10. Rinse the gel with ddH_2O and place into the destaining solution containing 5% (v/v) methanol and 7% (v/v) acetic acid. Destain while mixing using a horizontal rotation platform for 1 h.
11. Image the gel and analyse.

8. Circular Dichroism (CD) Spectroscopy Analysis of Secondary Structure of refolded Pure Protein

1. Transfer 0.5 - 1 mg of refolded protein obtained in step 6.6 into a dialysis tube and place it in a dialysis bucket containing 5 L of buffer L (50 mM sodium phosphate pH 7.4), precooled to 4 °C. Dialyse the sample at 4 °C with continuous stirring and perform at least 3 - 4 buffer changes over 2 - 4 h before leaving the sample to dialyse overnight.
2. Concentrate the dialysed protein solution to 0.06 mg mL^{-1} using a 10 kDa cut-off centrifugal concentrator.
3. Clarify the solution by centrifugation at 13,000 x g, 4 °C for 30 min.
4. Record the far-UV CD spectrum of the sample at 25 °C over a wavelength range of 200 - 260 nm using a spectropolarimeter with a scan rate of 20 nm min^{-1}. Use the dialysis buffer as a blank control. Repeat recording in triplicate and generate the averaged spectrum.
5. Calculate the secondary structure content by deconvoluting the CD spectra using the CDSSTR program from the DichroWeb sever[25] (http://dichroweb.cryst.bbk.ac.uk).

Representative Results

Recombinant expression of His_6-Tlp3-LBD in *E. coli* resulted in protein deposition in inclusion bodies. The expression yield from 1 L of bacterial culture calculated in step 2.13 was approximately 100 mg of His_6-Tlp3-LBD deposited in inclusion bodies. The protein isolation procedure, described here and illustrated in **Figure 1**, consists of the solubilisation of inclusion bodies, protein refolding and purification, by means of affinity chromatography, tag removal and size-exclusion chromatography, and yields 10 - 20 mg of pure, untagged Tlp3-LBD per 1 L of bacterial culture.

The protein eluted from the gel-filtration column as a single, symmetrical peak corresponding to a retention volume of 220 mL (**Figure 2A**). Calculation of the molecular weight (MW) using a calibration plot of log (MW) versus retention volume ($V_{retention}$ (mL) = 549.3-73.9 x log(MW)) available at the EMBL Protein Expression and Purification Core Facility website (http://www.embl.de/pepcore/pepcore_services/protein_purification/chromatography/hiload26-0_superdex200/index.html), yielded the value of 29 kDa. This value was very close to that calculated from the amino acid sequence (28.7 kDa), which suggested that Tlp3-LBD is a monomer in solution.

To evaluate the purification process, samples collected at different steps were evaluated using SDS-PAGE analysis. As shown in **Figure 2B**, inclusion bodies contained predominantly His_6-Tlp3-LBD. Small amounts of this protein were also present in the soluble fraction of the induced culture (IPTG(+)), and in the uninduced culture (IPTG(-)) – apparently as a result of the leaky expression of the T7 polymerase. His_6-Tlp3-LBD migrated on the polyacrylamide gel with an apparent molecular weight of 28 kDa, which is close to the value calculated from the amino acid sequence (31.8 kDa). The His_6-tag removal, affinity chromatography and gel filtration steps yielded highly pure protein (>90% electrophoretic homogeneity).

To confirm that the protein obtained by this procedure was folded, the secondary structure of His_6-Tlp3-LBD was evaluated by CD spectroscopy. Estimation of the α-helix and β-sheet content from the CD spectrum (**Figure 3**) using CDSSTR gave values of 31% α and 23% β. These values were close to those predicted from the sequence analysis using the Jpred3 server (http://www.compbio.dundee.ac.uk/www-jpred/) (37% α and 26% β), indicating that the protein recovered from the urea-denatured inclusion bodies was folded.

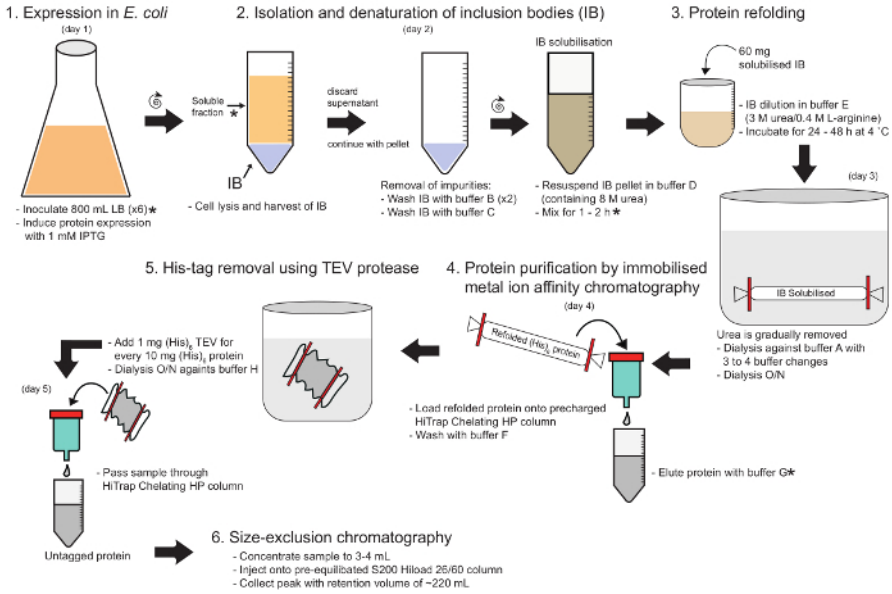

Figure 1: Schematic of presented method. Recombinant His_6-Tlp3-LBD is expressd in *E. coli* upon induction with IPTG (see section 1). After 4 h expression, bacterial cells are harvested and lysed (see section 2). The insoluble fraction containing the inclusion bodies (IB) is washed for removal of membrane and membrane proteins impurities, after which the IB are dissolved in a buffer containing urea at high concentration (see section 2). His_6-Tlp3-LBD is refolded by dilution into buffer E followed by exhaustive dialysis for gradual urea removal (section 3). Refolded His_6-Tlp3-LBD is then purified by immobilized metal ion affinity chromatography as described in section 4. The His_6-tag is removed using TEV protease (section 5). Untagged Tlp3-LBD is concentrated and further purified by gel filtration chromatography (section 6). Points for collection of samples for SDS-PAGE analysis are indicated with *. Please click here to view a larger version of this figure.

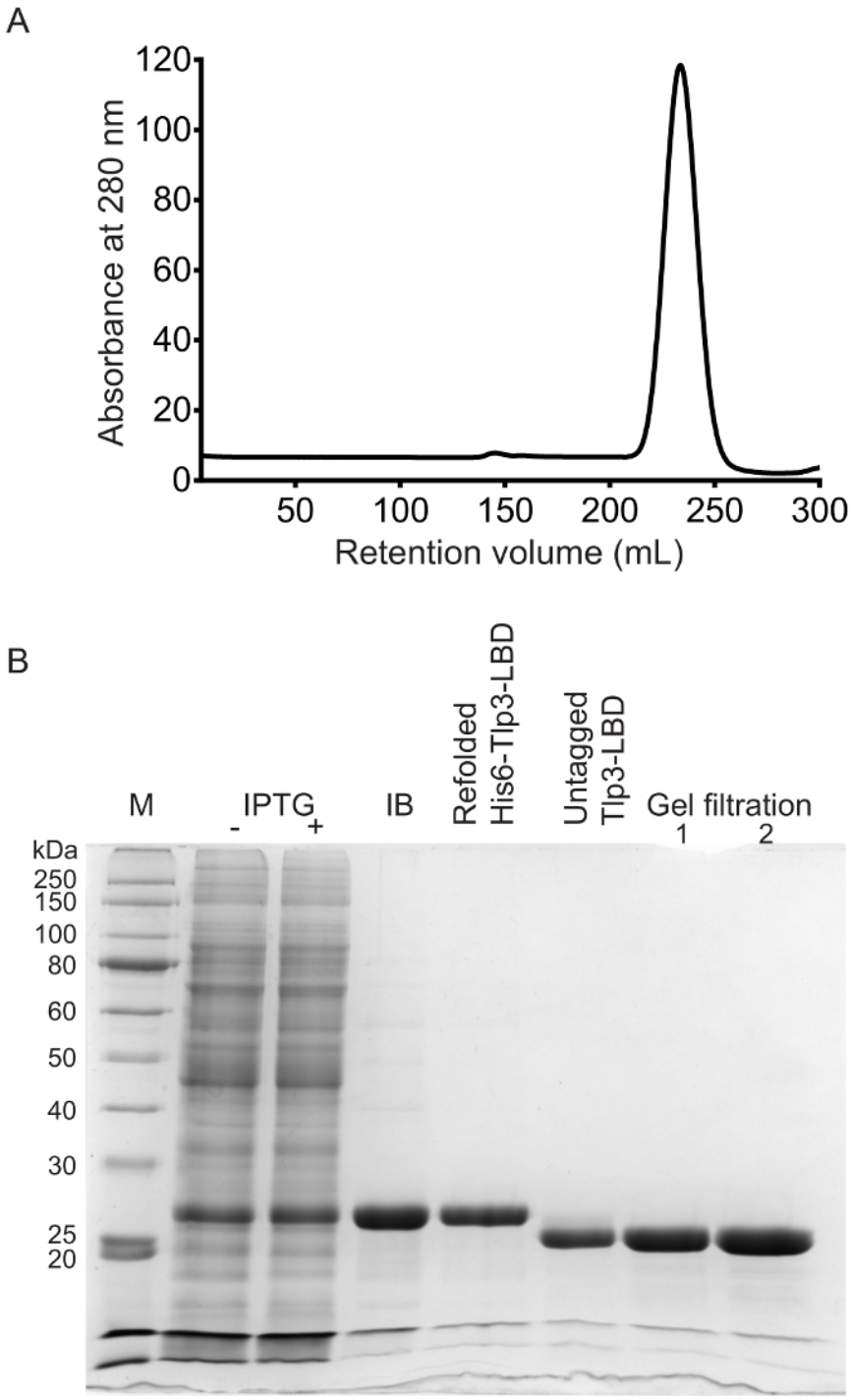

Figure 2: Purification of recombinant Tlp3-LBD. (A) Size-exclusion chromatography trace of untagged Tlp3-LBD on a Superdex 200 HiLoad 26/60 gel filtration column. The protein eluted with a retention volume of 220 mL. **(B)** Reduced 15% SDS-PAGE Coomassie Blue-stained gel. M: protein molecular weight marker; IPTG (-): uninduced control sample collected in step 1.3; IPTG (+): soluble fraction after IPTG induction (collected in step 2.3); IB: isolated inclusion bodies (step 2.13); Refolded His_6-Tlp3-LBD: refolded protein sample from step 4.6; Untagged Tlp3-LBD: protein sample obtained in step 5.13; Gel filtration 1 and 2: two fractions from the protein peak eluted from the gel-filtration column (step 6.6). Please click here to view a larger version of this figure.

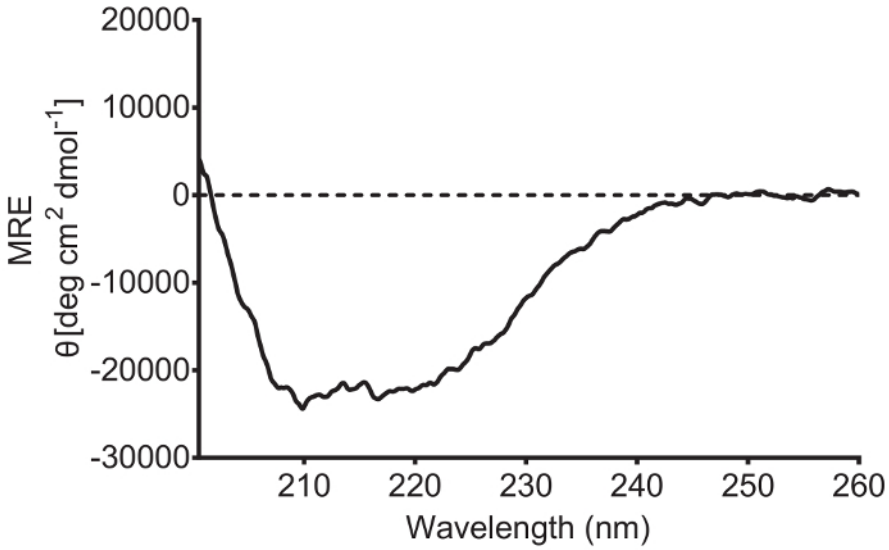

Figure 3: Circular dichroism spectrum of purified recombinant Tlp3-LBD. The spectrum was recorded at 25 °C in 50 mM sodium phosphate pH 7.4. Please click here to view a larger version of this figure.

Discussion

A simple procedure for expression and refolding from inclusion bodies of the periplasmic LBD of the bacterial chemoreceptor Tlp3 is presented. Preparation of the pure protein involves over-expression of the pET-plasmid-encoded gene in *E. coli*, purification and solubilisation of inclusion bodies, refolding of the denatured protein and its purification by the consecutive affinity and size-exclusion chromatography steps. The urea-facilitated denaturation and dilution/dialysis-mediated refolding are the critical steps in the protocol, optimisation of which is often required to ensure the proper renaturation of the protein deposited in inclusion bodies[26].

Refolding of Tlp3-LBD was achieved in a two-step manner, first by diluting the denatured sample into a buffer containing urea, and then by dialysing the sample against a buffer devoid of it. The refolded protein obtained using this protocol was functional and crystallisable[18,23]. However, the presented method has some limitations. It is well known that carbamylation of amino groups often occurs when protein is denatured and refolded in the presence of urea[27,28,29]. This is due to the fact that the dissolved urea decomposes with time and produces cyanate[30] that reacts with the protein amino groups to form a stable carbamylated product and, to a minor extent, with other functional groups[31,32]. The decomposition of urea is accelerated under conditions of alkaline pH and elevated temperature. So, it is recommended to make fresh urea solutions from ultrapure (>99%) solid reagent, and perform the refolding/dialysis steps at low temperature (4 °C)[30,33], to diminish cyanate formation. Moreover, choosing the buffer containing primary amines, such as Tris, glycine or ammonium bicarbonate, for the refolding mix is important to allow scavenging of the produced cyanate[29,33]. Other option is to use guanidine hydrochloride instead of urea, as guanidine hydrochloride has not been reported to chemically modify proteins.

In addition to urea, a reducing agent (DTT or β-mercaptoethanol) is often required to solubilize inclusion bodies and to prevent non-native intra- and intermolecular disulfide bonds formation by maintaining the cysteine residues in their reduced state[26,34]. Tlp3-LBD has one intramolecular disulfide bond, and in this protocol, 10 mM DTT was incorporated into the inclusion bodies denaturation buffer (step 2.10) to aid the resuspension of the insoluble protein. DTT concentration was then reduced by ~100 fold by diluting the sample into the protein refolding buffer, followed by the dialysis step to gradually remove all DTT. It is important to note that the application of this procedure to the refolding of proteins with multiple disulfide bonds may need optimization of the concentration of both oxidizing and reducing agents (*e.g.* oxidized and reduced glutathione (GSSH/GSH), GSSH/DTT, cystamine/cysteamineor cystine/cysteine) to promote proper formation of disulfide bridges[34,35]. Furthermore, if the protein of interest has unpaired cysteines that are not involved in disulfide bond formation, a reducing agent should be added to all purification buffers. Prediction of the potential disulfide bridges from the amino acid sequence of a protein can be performed using several *in silico* tools (*e.g.* cys_rec: http://linux1.softberry.com/berry.phtml?topic=cys_rec&group=programs&subgroup=propt).

The use of the presented protocol for purification of different proteins is also likely to require optimization of the concentration of imidazole during the washing step 4.4. As shown in **Figure 2B** (lane labelled IB), the isolated inclusion bodies contained, in this instance, predominantly the protein of interest. If additional significant bands are visible on SDS PAGE gel of the IB sample, increasing the imidazole concentration in step 4.4 will be required to remove the contaminants, but might reduce protein yield[36,37]. Other option is to apply a linear gradient of imidazole concentration and pool the eluted fractions that contain the protein of interest.

The final step in the purification, size-exclusion chromatography, provides the means to simultaneously estimate the molecular mass of the eluted particles and derive the oligomeric state of the protein. However, estimation of the molecular mass by size-exclusion chromatography is only accurate for spherical molecules, which is not the case for many proteins[38,39]. One can determine the protein molecular mass and oligomeric state with higher accuracy by using size-exclusion chromatography coupled to multi-angle light scattering (SEC-MALS)[40] or analytical ultracentrifugation[41]. We have previously confirmed that Tlp3-LBD is monomeric in solution by using SEC-MALS analysis[23], and this result is consistent with the reports on other dCACHE LBDs[42,43].

The presented protocol, in its original or slightly modified form, has been used to refold and purify several chemoreceptor LBDs from the dCACHE family for X-ray crystallographic studies[17,18,19,23,42,44]. This procedure may be generally useful for production of milligram amounts

of periplasmic LBDs of other bacterial chemoreceptors in a soluble and crystallisable form. In each separate case, protocol optimization is likely needed. In addition to the points discussed above, optimal solubilisation of inclusion bodies and protein refolding may require the use of detergents (*e.g.* SDS or N-acetyl trimethyl ammonium chloride), additives (*e.g.* L-arginine) and adjustment of their concentration and incubation time in individual refolding steps[26,34,45,46]. Furthermore, the protein concentration in the refolding step significantly affects the refolding yield. The range of concentrations from 1 ng mL^{-1} to 10 mg mL^{-1} should generally be tested. For Tlp3-LBD, the optimal concentration of protein in the refolding mix was 0.2 mg mL^{-1}.

Extremely low refolding yield is usually a sign that the trial refolding conditions are far from optimal. One can expect that high yield is consistent with a folded protein, which can be validated by using CD spectroscopy (as outlined in this procedure). Furthermore, crystallisability of the resultant protein can sugguest the protein's folded state. Alternatively, a functional assay (if available) can be used to confirm that the protein is folded. In the case of Tlp3-LBD, for example, the refolded protein was shown to retain its ligand-binding ability, as shown by isothermal calorimetry.[23]

Disclosures

The authors declare no competing financial interests.

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