BMC	Standard Operation Procedure Refolding by dilution from insoluble fraction of microfluidizer-lysed Ecoli cells

1. Aim:

Efficient refolding by dilution method of Escherichia coli-expressed protein

2. Applicability:

The biological molecules with required re-folding action after bacterial expression

3. Responsibilities:

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4. Required materials:

One Shot™ BL21(DE3) Chemically Competent E. coli (ThermoFisher, USA)

Prestained Protein Ladders (Sigma-Aldrich, USA)

Urea BioXtra, pH 7.5-9.5 (Sigma-Aldrich, USA)

Plasmid Mini Kit (Promega, USA)

Ni-NTA resin (Merck, Germany)

Tryptone, yeast extract, and agar (Difco, USA)

LB Broth, Luria low salt (Sigma-Aldrich, USA)

Isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma-Aldrich, USA)

β-mercaptoethanol (βME) (Sigma-Aldrich, USA)

Vivaspin concentrators (Sigma-Aldrich, USA)

5. Procedure:

A. Protein expression in E.coli cells

Day 1-2

The expression plasmid was used for the transformation of E. coli BL21(DE3) competent cells. The culture was grown at 37 °C in LB medium supplemented with 100 µg/mL of ampicillin until the optical density reached a value of 0.6 at a wavelength of 600 nm (OD600). The culture was cooled to 20 °C, and then induction with the final concentration of 0.5 mM of IPTG was carried out.

Culture samples were withdrawn after 8h and 16 h and analyzed using sodium SDS-PAGE and Western blot analysis. The expression level reach its maximum usually after 16 h of incubation. The cells were pelleted by centrifugation at 6000 g for 10 min (Beckman) and frozen in liquid nitrogen.

B. Protein extraction from microfluidizer-lysed cells

Day 3

Frozen E. coli cells (25 g) were thawed and washed with PBS, so the thawed pellet was resuspended with 1 X PBS buffer and centrifugated (Beckman) at 6000 g for 10 min at 4 ˚C. The pelet was then resuspended with lysis buffer containing 100 mM Tris-HCl pH 7.0, 5 mM EDTA, 5 mM DTT, 0.5 mM PMSF.

Note: The Final volume must be at least 50 ml for using microfludizer LM20.

Microfludize the cell suspension with microfludizer at 20K level for 4-5 times. Centrifuge the suspension 20 minutes at 50,000 g at 4 ˚C.

Discard the supernatant and re-suspend pellet with wash buffer containing 100 mM Tris-HCl pH 7.0, 5 mM EDTA, 5 mM DTT, 2 M urea, 2% w/v Triton X-100, so the volume is 5 ml per each gram of cells (w/w). Centrifuge the suspension 15-20 minutes at 50,000 at 4 ˚C. Repeat this step twice.

Discard the supernatant and re-suspend pellet with wash buffer containing 100 mM Tris-HCl pH 7.0, 5 mM EDTA, 2 mM DTT, so the volume is 5 ml per each gram of cells (w/w). Centrifuge the suspension for 15-20 minutes at 50,000 g at 4 ˚C.

Extract the protein with extraction buffer containing 50 mM Tris-HCl pH 7.0, 5 mM EDTA, 2 mM DTT, 6 M urea, so the volume is max. 1-2 ml per each gram of cells (w/w). Centrifuge 30 min at 50,000 g at 4 ˚C, and analyze the supernatant by SDS-PAGE and Western blot. The supernatant is used for the purification and refolding.

C. Refolding by dilution method

To use efficient method that facilitates in vitro protein renaturation, the supernatant obtained from the previous step was purify using IMAC and refolded using dilution. The final step of the purification process was performed by gel filtration technique.

Day 4: Afinity purification

The supernatant, after solubilization in extraction buffer (plus added 5 mM imidazole), was applied onto the column with Ni-NTA resin and flow-through fractions were applied twice. The resin was washed with 20 CVs of the same binding buffer containing low concentration of imidazole and with 10 CVs of wash buffer containing increased concentration of imidazole (20 mM) and containing descending concentrations of urea (6 M, 4 M, 2 M, 1 M, and 0.5 M).

Gravity elution was performed with 3 CVs of elution buffer (20 mM Tris-HCl pH 7.9, 500 mM NaCl, 1 M imidazole, and 0.5 M urea), and 2-mL fractions were collected. The elution step was repeated twice. Fractions from the first and second elution cycles were analyzed by SDS-PAGE electrophoresis.

Day 5-7: Dilution method

The protein sample was concentrated to 100 µl (Vivaspin concentrator) and subjected to the refolding process by diluting the purified protein in the 80 mL of refolding buffer containing 10 mM Tris-HCl (pH 7.9), 0.2 M arginine, 1 mM EDTA, 20% glycerol, 2 mM GSH, and 1 mM GSSG. The sample was left on a magnetic stirrer for 60 h at 4 °C, and then was concentrated to 250 µL (Vivaspin concentrator).

D. Gel filtration of refolded protein extracted from the insoluble fraction

Day 8

In the final step of purification process, the protein refolded by dialysis was loaded onto Superdex 75 column with an internal diameter of 10 mm and length of 30 cm connected to AKTA Pure25 system (GE Healthcare). Gel filtration was performed using PBS (pH 7.4) at a flow rate of 0.5 mL/min. The protein was eluted and the process was monitored by measuring UV absorbance at 280 nm. The collected fractions (0.5 mL) were analyzed by SDS-PAGE and analytical SEC. The selected fractions were concentrated, and the final protein concentration was determined using NanoDrop spectrophotometer (Thermo Fisher Scientific, USA). The yield of purified protein obtained from the insoluble fraction was usually between 0.5 mg/L – 0.9 mg/L when the refolding procedure was performed by dilution.

Note: Renaturation is a critical step in the recovery process of any biologically active protein from an insoluble fraction. There are several approaches to protein refolding that need to be considered. Refolding can be initiated by reducing or removing the chaotropic solvent. In addition, parameters such as pH, redox potential, buffer additives, and protein concentration need to be empirically optimized for each individual protein. The best strategy is to screen different refolding conditions in parallel to find the appropriate combination that results in the highest recovery of active protein and shows least amount of soluble multimers.

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