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Chapter 3

Cell fractionation

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Key words. *Spheroplast, peptidoglycan, osmotic shock, freeze and thaw, protein solubilisation, membrane, subcellular localization.*

Summary

Protein function is generally dependent on its subcellular localisation. In gram-negative bacteria such as *Escherichia coli,* a protein can be targeted to five different compartments: the cytoplasm, the inner membrane, the periplasm, the outer membrane and the extracellular medium. Different approaches can be used to determine the protein localisation within cell such as *in silico* identification of protein signal sequences and motifs, electron microscopy and immunogold labelling, optical fluorescence microscopy, and biochemical technics. In this chapter, we describe a simple and efficient method to isolate the different compartments of *Escherichia coli* by a fractionation method and to determine the presence of the protein of interest. For inner membrane proteins we propose a method to discriminate between integral and peripheral membrane proteins.

1. Introduction

Many gram-negative bacteria secrete extracellular proteins such as hydrolytic enzymes or toxins. Secretion can occur through specific macrocomplex systems composed of a more or less large number of proteins located in the cell envelope. Identifying the localization of these proteins is therefore an important task to address the assembly and the molecular mechanism of these secretion systems.

Four subcellular compartments compose Gram-negative bacteria, even five if we consider the extracellular medium in which effectors are delivered. These different compartments are: the

cytoplasm, the inner membrane (IM), the periplasm, in which the peptidoglycan layer extends, and the outer membrane (OM) (***1, 2***). Isolation and characterization of effectors from the extracellular medium will be described in the chapter [**33]** of this issue. In this chapter, we will first describe a simple and efficient method to recover proteins from the periplasm and to generate spheroplasts from *Escherichia coli* cells. Then we will present a method to recover the cytoplasmic and the membrane fractions from the spheroplasts by several cycles of freezing and thawing. Finally, we present how treatments with specific buffers can give insight into protein-membrane associations.

2. Materials

2.1. Cell fractionation

1. TES buffer: 200 mM Tris-HCl, pH 8.0, 0.5 mM EDTA (ethylenediaminetetraacetic acid), 0.5 M Sucrose
2. Lysosyme 10 mg/mL (freshly prepared solution).
3. DNase 1 10 mg/mL.
4. $MgCl_2$ 1M (stock solution).
5. 100× Phénylméthylsulfonyl (PMSF) 0.1 M in absolute Ethanol. Store at -20°C.
6. Beckman coulter Optima TLX ultracentrifuge with TLA 55K rotor or equivalent.

2.2. Proteins solubilisation

3. Urea 2 M
4. NaCl 0.5 M
5. Triton X-100 1% (v/v)
6. Sodium Carbonate 100 mM pH 11.5, ice cold.
7. Trichloroacetic acid (TCA) 10% (v/v). The stock solution [TCA 100% (w/v)] is stored at 4°C in a brown bottle.
8. Acetone 90% (v/v) in ultrapure water stored at -20°C in a brown bottle.

3. Methods

3.1. Cell fractionation / Spheroplasts formation

In this section, we detail step by step spheroplasts preparation from *Escherichia coli* cells (*see* **Note 1**) using a method based on lysozyme/EDTA treatment (***3***) and a mild osmotic shock (***4-6***) (*see* **Note 2).**

1. Grow a 3 mL starter culture overnight in LB medium at 37°C with required antibiotics.
2. Inoculate a 20 mL culture at OD_{600} = 0.05 and incubate at 37°C until the optical density of the culture is around 0.8. If necessary, induce protein production under the required conditions (*see* **Note 3**)
3. Take 1 mL of culture and centrifuge for 5 minutes at 5,000 × g to pellet the cells. Discard the supernatant. Resuspend the pellet in an appropriate volume of SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) loading buffer. This fraction will be referred as the total cell fraction (T).

The next steps will be performed at 4°C and all the buffers must previously be cooled on ice before use.

4. Centrifuge the remaining culture for 5 minutes at 5,000 × g at 4°C (*see* **Note 4**). Discard the supernatant.
5. Gently resuspend the cell pellet in 200 µL TES buffer (described in materials section, *see* **Note 5**). Do not vortex and do not pipette, only resuspend the cell pellet by inverting the tube.
6. Add 8 µL of a freshly prepared solution of lysozyme (10 mg/mL in TES buffer) and mix gently by shaking the tube.
7. Add 720 µL of TES buffer diluted 2× in water (v/v) and incubate for 30 minutes on ice. Gently mix the suspension to perform the osmotic shock by gently inverting and rolling the tube (*see* **Note 6**).
8. Centrifuge at 5,000 × g for 5 minutes at 4°C. Keep the pellet as the spheroplast fraction (IM + cytoplasm (+OM)) and the supernatant as the periplasmic fraction (P).
9. Resuspend the spheroplast fraction in 1 mL TES buffer diluted 2× in water (v/v) containing 2 mM of PMSF, 2 mM $MgCl_2$ and 10 µg/mL DNase 1 (*see* **Note 7, Note 8**).
10. Lyse the spheroplasts by performing 4 cycles of freezing and thawing, from -273 °C (liquid azote) to 37°C (*see* **Note 9**).
11. Remove unbroken cells and cell debris by centrifugation at 2,000 × g for 5 min. Keep the supernatant as cytoplasmic and membrane fraction.

12. Centrifuge the supernatant at 120,000 × g, 4°C for 45 minutes. Use a Beckman coulter Optima TLX ultracentrifuge and a TLA55 fixed-angle rotor for small volume ultracentrifugation or similar. The pellet is kept as the membrane fraction and the supernatant is conserved as the cytoplasmic fraction.
13. The membrane fraction is suspended in 1 mL TES buffer diluted 2× in water (v/v) or in the desired buffer (*see* **Note 10**). Separation of inner membrane and outer membrane is described in the chapter [**7**] of this issue.
14. At this step, fractions (OD_{600} = 0.2-0.4) could be tested for the presence of the protein of interest by SDS-PAGE and western-blot analysis with required antibodies. As a control, the same fractions can be tested for the presence of specific IM, OM, cytoplasm or periplasm markers.

3.2. Protein solubilisation (*see* Note 11).

1. Prepare 1mL of membrane fractions as previously described.
2. Aliquot the membrane fractions into five samples, 200 µL each.
3. Centrifuge at 120,000 × g for 45 min at 4°C to pellet the membranes as described previously (see section 3.1, step 12).
4. Resuspend each pellet in either 200 µL of 0.5 M NaCl, 2 M urea, 100 mM sodium carbonate, pH 11.5 ice cold or 1% (v/v) Triton X-100 in order to compare the five extraction conditions.
5. Incubated at least 1 hour at 4°C with agitation.
6. Centrifuge the suspensions at 120,000 × *g* for 45 minutes at 4°C. Carefully collect the different supernatants and transfer into new tubes.
7. Resuspend each pellets in SDS-PAGE loading buffer and kept as the membrane associated proteins fractions.
8. Add 10% TCA (final concentration) to supernatant samples and incubate at least for 1 hour at 4°C to allow protein precipitation (*see* **Note 12, Note 13**).
9. Centrifuge at 18,000 × g for 30 minutes at 4°C.
10. Wash the pellets with 200 µL acetone 90% (pre-chilled solution).
11. Centrifuge at 18,000 × g for 10 minutes at 4°C.
12. Discard carefully the supernatant and air-dry the pellet containing the extracted membrane proteins at RT for 5-10 min (*see* **Note 14**).
13. Resuspend pellets in SDS-PAGE loading buffer and keep as extracted membrane protein fractions.

14. Perform a western blot analysis to identify the extraction condition that is suitable to the protein of interest.

4. Notes :

1. Spheroplasts are cells resulting from a loss of the bacterial cell wall. Outer membrane has been altered but the cytoplasm remains delimited by the inner membrane (***7***).
2. *Escherichia coli* cells are first incubated in a concentrated sucrose solution containing EDTA. Sucrose makes the medium hypertonic while EDTA chelates divalent cations and destabilises the outer membrane. Then, lysozyme is added to cleave the periplasmic peptidoglycan layer. However, peptidoglycan hydrolysis is not total and a mild osmotic shock is required to maximize the procedure. Then the periplasmic content of the cell is separated from the spheroplasts by centrifugation.
3. The volume of the cell culture can be adapted according to the downstream application
4. Pre-cool centrifuge before use.
5. The TES buffer is responsible for outer-membrane destabilization. 0.5 M Sucrose makes the medium hypertonic, 0.5 mM EDTA and 200 mM Tris-HCl, pH 8 affect the membrane structure by removing the lipopolysaccharide coat from the cells (***8***).
6. This mild osmotic shock provokes a sudden influx of water in the periplasmic space and increases the distance between polysaccharide chains of the peptidoglycan. This facilitates the lysozyme binding and the degradation of the peptidoglycan (***8***).
7. Phenylmethylslfonyl (PMSF) is a serine protease inhibitor. However, it has been shown that addition of trypsin inhibitor after proteolysis is not required to prevent further digestion, as trypsin digestion is very specific.
8. During spheroplasts lysis, DNA is released in the medium, adheres to membranes and makes the preparation difficult to handle. To circumvent this issue, DNase 1 is added to lysates. As DNase 1 activity requires magnesium, Mg^{2+} is added in excess in order to overtake chelation by the EDTA present in the TES buffer.
9. Three to five cycles of freezing and thawing are an efficient and a simple method to disrupt spheroplasts (***9***). However, spheroplasts can also be disrupted by sonication. In this case, sonicate spheroplast suspension twice for 30 s. Keep the suspension cold during sonication. A Branson Microtip Sonifier 450 can be used with a microtip probe.

10. Membrane fraction resuspension can be difficult. Passing the sample through the needle of a syringe several times can optimize this step.
11. When studying a poorly characterized protein, it is important to compare different extraction conditions to optimize protein solubilisation. The use of appropriate solubilisation buffer can provide information about protein localisation into the cell, and even to differentiate between integral and peripheral membrane proteins. Thus, a high salt buffer allows the extraction of peripheral proteins associated with membrane by electrostatic interactions. Urea 2 M is commonly used to extract peripheral proteins that associate with the membrane by hydrophobic bonds (***10***). Triton-X100 is the most commonly used detergent for the solubilisation of integral inner membrane proteins (***11***). It is worthy of note that, during preparation of membrane fractions, membranes tend to re-anneal by an unknown mechanism, resulting in the formation of closed membrane vesicles that might trap some of the proteins of interest. In this case, alkaline carbonate buffer can be added to convert membrane vesicles to open membrane sheets and therefore to release trapped proteins into the supernatant (***12***). Membranes can subsequently be cleared from the sample by centrifugation.
12. This step can be done overnight in a cold room under rotary agitation.
13. Be careful, after centrifugation pellets are not always visible. Carefully place the tubes in the centrifuge to locate the location of the future pellet.
14. Never let the pellet air-dry completely, as this will dramatically impede resuspension.

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