Chapter 22

Isolation of Bacteria Envelope Proteins

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Abstract

Proteomic analysis on cell envelope proteins from Gram-negative bacteria requires specific isolation techniques. We found that conventional extraction methods such as osmotic shock cause extracts to be heavily contaminated with soluble cytoplasmic proteins. These cytoplasmic protein contaminants constitute the major signal in proteomic analysis and can overwhelm the signals coming from genuine envelope components. After extensive testing of various protocols for the preparation of envelope contents, we found that a modified version of the method of Oliver and Beckwith (1) consistently produces the cleanest extract of periplasmic and outer membrane proteins (2). We have designated this very simple method TSE extraction because it uses a Tris-sucrose solution supplemented with EDTA. Cytoplasmic and inner membrane protein contaminants are not evident on 1D SDS polyacrylamide gels and contribute to less than 6% of total signal in very sensitive mass spectrometry analysis. This straightforward method is therefore ideal for analyzing specific proteomic changes in the cell envelope.

Key words: Gram-negative bacteria, Periplasmic extraction, Outer membrane extraction, Envelope proteins

1. Introduction

Bacterial cells are protected by a complex, multilayered structure called the cell envelope. In Gram-negative bacteria, the cell envelope is composed of an inner cytoplasmic membrane, a thin peptidoglycan cell wall, and a lipopolysaccharide-containing outer membrane that surrounds the peptidoglycan layer; the inner and outer membranes delimit an aqueous space termed the "periplasm" (3). The periplasm has gained increasing attention as a compartment for recombinant protein expression, in part because it favors disulfide bond formation. Additionally, the periplasm only contains a small proportion of the total cellular protein; therefore, expression into this compartment results in a substantial purification upon periplasmic extraction. Many methods have been established to mechanically or chemically disrupt the outer membrane and cell wall in order

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to release periplasmic contents, either for purification of recombinant proteins or for proteomic studies. Some of these methods, such as osmotic shock, lysozyme-EDTA treatment, polymyxin digestion, and chloroform extraction, showed high enrichment of recombinant proteins in the extracts and up to 90% recovery of the target proteins (4–6). However, we found that these periplasm extraction methods, presumably optimized for maximal recovery of recombinant proteins, also release a substantial amount of cytoplasmic protein. Periplasmic proteins are estimated to constitute only ~4–16% of the total cellular protein in *Escherichia coli* (7, 8). Thus, cytoplasmic proteins outnumber and out-mass periplasmic contents by approximately ten-fold. As a consequence, relatively minor release of cytosolic contents will result in substantial contamination of a periplasmic extract. This release can be due to cell lysis during the repetitive pelleting and resuspension steps of the osmotic shock procedure, or it can result from destabilization of the inner membrane by chemicals such as chloroform and polymyxin, which are utilized in other extraction procedures (9, 10).

During a study examining substrate specificity of the periplasmic oxidoreductases in *E. coli* (2), we found that the osmotic shock and polymyxin procedures failed to produce a clean periplasmic fraction suitable for proteomic analysis. Indeed, when the most abundant "periplasmic" spots on two-dimensional polyacrylamide gels were analyzed by mass spectrometry, we found that many actually corresponded to cytoplasmic proteins. In addition, we noticed that the amount of cytoplasmic proteins released by these procedures varied substantially from experiment to experiment, making the accurate quantification of periplasmic contents extremely difficult.

To circumvent these problems, we tested a large number of periplasmic extraction procedures. We found that modification of a periplasmic extraction method described by Oliver and Beckwith (1), which we renamed TSE extraction, gave the cleanest periplasmic preparation by far. We note that this methodology contains many outer membrane proteins in addition to periplasmic proteins but has very few contaminating cytosolic or inner membrane proteins (11). As outlined below, outer membrane proteins can be removed by an additional ultracentrifugation step. Mass spectrometry identification of a typical extract prepared from *E. coli* by this method (omitting the ultracentrifugation step) contains detectable quantities of ~130 periplasmic proteins (34% of total), 50 outer membrane proteins (13% of total), 2 extracellular proteins, ~180 cytoplasmic proteins, and ~20 inner membrane proteins (altogether 53% of total) (Table 1 and Fig. 1). Although the number of cytoplasmic and inner membrane proteins detected seems substantial, the average signal coming from cytoplasmic and inner membrane proteins is only two spectra counts, 13-fold lower than the average signal of outer membrane proteins and 23-fold lower than that of

Table 1
Classification of protein identified in *E. coli* MC4100 envelope extract prepared by the TSE method according to their subcellular localization and relative intensity determined by mass spectrometry

Subcellular localization	Relative intensity (spectral counts)			
	Total no. of proteins	High (>20)	Medium (3–20)	Low (1–2)
Periplasmic	129	36	63	30
Outer membrane	50	9	24	17
Extracellular	2	1	1	0
Inner membrane	21	0	2	19
Cytoplasmic	183	0	31	152
Total	385	46	121	218

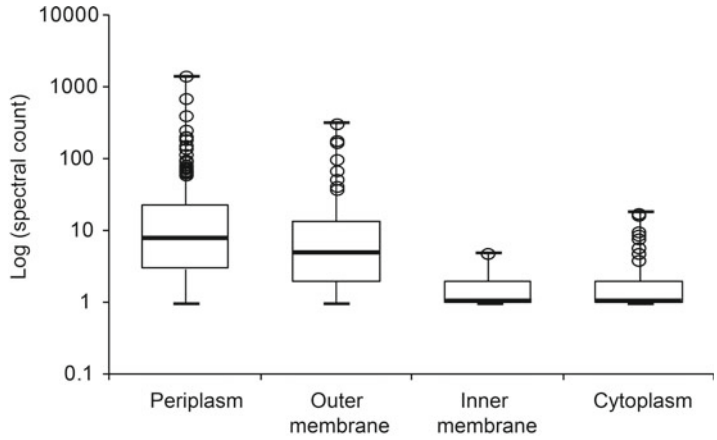

Fig. 1. Intensity distribution of proteins identified by mass spectrometry analysis on a typical extract prepared from *E. coli* using the TSE method as measured by spectral count (SC). The number of SC for a protein is the total number of MS/MS spectra taken on peptides from this protein in a given 2D-LC-MS/MS analysis. This value is linearly correlated with the protein abundance. Proteins are grouped according to their subcellular localization. The *lower boxes* represent the first quartile (25%) and the *upper boxes* represent the third quartile (75%). The *thick lines* across each of the boxes show the medians. Note that the first quartiles in inner membrane and cytoplasmic samples equal the medians in these samples. *Short horizontal lines* show the maximal and minimal values, and *circles* represent the outliers, which are three times larger than the likely range of variation.

periplasmic proteins. Among the 100 most abundant proteins, only eight are cytoplasmic; the rest are either outer membrane proteins (21%) or periplasmic proteins (71%). These eight cytoplasmic protein contaminants are among the most abundant *E. coli* proteins, with copy numbers higher than 1,000 per cell (12), and include thioredoxin and EF-Tu, which are known to be very easily extracted in standard periplasmic extraction procedures (13). Assuming that

overall spectral signal corresponds to protein abundance, the TSE extract contains 78% periplasmic proteins, 16% outer membrane proteins, 0.4% inner membrane proteins, and 5% cytosolic proteins. We note that mass spectrometry analysis is extremely sensitive, and the majority of the cytoplasmic proteins are unlikely to be visible using techniques with lower sensitivity, such as SDS-PAGE or 2D gel electrophoresis. A recent periplasmic proteome study in *Pseudomonas aeruginosa* compared extractions prepared by three methods, one of which is similar to our method except for their use of lysozyme in the extraction buffer and a higher concentration of EDTA (14). This similar method resulted in the highest number of unique spots on 2D gels corresponding to periplasmic proteins and the lowest level of cytoplasmic contaminations.

In this simple TSE extraction procedure, cells are first pelleted to remove the media, resuspended in a concentrated solution of sucrose in Tris buffer supplemented with EDTA, incubated for 30 min on ice, and then recentrifuged. The supernatant is the TSE periplasm/outer membrane extract. Sucrose in the extraction buffer increases the extracellular osmolality, causing the cells to shrink and release water and periplasmic contents into the surrounding medium. Sucrose can go through the outer membrane to enter the periplasmic space but is unable to penetrate the inner membrane. The presence of sucrose at the water–inner membrane interface presumably helps to stabilize the membrane (15) and therefore prevents cell lysis. Sucrose is also a well-known protein stabilizer (16), protecting the released proteins. EDTA facilitates periplasmic extraction by chelating divalent ions, which normally stabilize the lipopolysaccharide (LPS) in the outer membrane, resulting in LPS release and increased permeability of the outer membrane (17). After 30 min of incubation in the extracting buffer, the centrifugation step acts to efficiently separate the soluble envelope proteins from other cellular components. If desired, an additional ultracentrifugation step at 100,000 × *g* for 1 h can be performed to separate outer membrane proteins (which pellet at 100,000 × *g*) from periplasmic proteins, which remain soluble (11). This method is an effective way to extract soluble envelope proteins and additionally is less labor intensive than many other methods. We named it TSE, standing for Tris, Sucrose, and EDTA, the components of the extraction buffer.

We compared extracts purified by TSE extraction with those prepared by either polymyxin extraction or a modified osmotic shock procedure (Fig. 2). Polymyxin and modified osmotic shock were chosen as the gold standards of periplasmic purification because we found that they produce much purer periplasmic extracts than other methods (lysozyme-EDTA, traditional osmotic shock, and chloroform extractions). The purity of subcellular extracts in *E. coli* has traditionally been estimated by analyzing for protein markers of known subcellular localization.

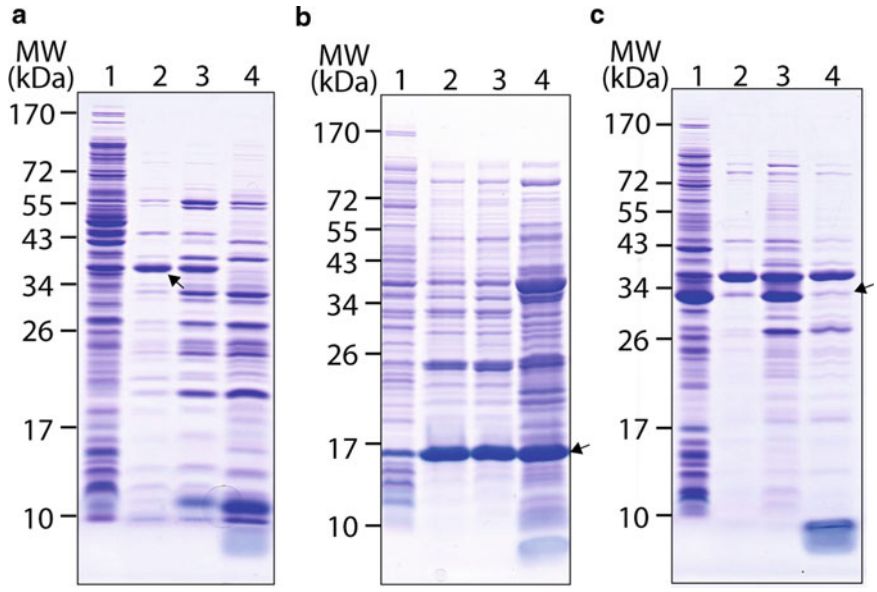

Fig. 2. Comparison of the envelope extracts prepared using the TSE procedure and two other methods. Periplasmic proteins were separated via SDS-PAGE using 14% Tris-glycine gels. For all gels, *lane 1* whole cell lysate, *lane 2* TSE extract, *lane 3* modified osmotic shock extract, *lane 4* polymyxin extract. *Panel* (**a**) shows the extraction of the *E. coli* K12 strain MG1655. The *arrow* indicates enrichment of the outer membrane proteins OmpC and OmpF. *Panel* (**b**) shows the extraction of the *E. coli* K12 strain MG1655 over-expressing a 16 kDa periplasmic protein, Spy (marked by an *arrow*). Over-expression of Spy makes the cells vulnerable to lysis during the periplasmic extraction procedures. However, the TSE method still produces a cleaner periplasm than the polymyxin method (compare *lanes* 2 and 4). *Panel* (**c**) shows the extraction of the *E. coli* BL21 strain over-expressing a 33 kDa cytoplasmic protein, Hsp33 (marked by an *arrow*). Although the TSE (*lane* 2) and polymyxin methods (*lane* 4) resulted in a slight release of Hsp33 into the periplasm, the modified osmotic shock method (*lane* 3) released a much larger fraction of Hsp33 into the periplasm. In conclusion, the TSE method produces the cleanest periplasmic extract under all the conditions we have tested.

Often, β-galactosidase serves as a cytoplasmic marker and plasmid-encoded β-lactamase serves as a periplasmic marker (18). However, we speculate that this method may overestimate the purity of the preparations since β-galactosidase, which is over 100 kDa, is likely to be much harder to "shock out" than smaller cytosolic proteins like thioredoxin. A simpler and more realistic way to estimate the periplasmic purity is to compare the band pattern of periplasmic extracts on 1D SDS-PAGE gels with the band pattern of whole cell extracts (see Fig. 2). Since envelope proteins constitute only about 4–16% of the proteome, a clean extract is expected to produce a distinctive band pattern with very little overlap with that generated by whole cell extraction. Substantial contamination with cytosolic proteins can be demonstrated by the appearance of dominant bands from the total cell extract appearing as major components of the so-called periplasm. We found that for various strain backgrounds (*E. coli* K12 and B strains) including those that over-express various proteins, the TSE method generates the cleanest envelope pattern of all methods tested (Fig. 2).

2. Materials

Prepare all solutions using distilled deionized water and reagent grade chemicals. Filter all solutions and store at 4°C.

1. Tris-sucrose-EDTA (TSE) buffer: 200 mM Tris–HCl, pH 8.0, 500 mM sucrose, 1 mM EDTA. Aliquot an appropriate amount and add protease inhibitor cocktail before use.
2. Complete Mini, EDTA-free Protease Inhibitor Cocktail Tablets (Roche Applied Science, Indianapolis, IN, USA). Use 1 tablet per 10 mL of TSE buffer. Alternative protease inhibitor cocktails can be purchased from various vendors. Use the dosage specified by the manufacturer.
3. Centrifuge and associated rotor.
4. Microcentrifuge.
5. High speed ultracentrifuge and associated rotor.
6. Polymyxin buffer: 50 mM Tris–HCl, pH 7.5, 5 mM EDTA, 150 mM NaCl, 1 mg/mL polymyxin B sulfate.
7. Ice-cold water.
8. Reducing SDS sample buffer: 60 mM Tris–HCl, pH 7.0, 2% SDS, 1.32 M glycerol, 0.01% bromophenol blue, 1% 2-mercaptoethanol.

3. Methods

The protocols described below are to be performed on a 100 mL of a fresh bacteria culture at an optical density of 1.0 at 600 nm. For cell cultures of different volumes or different optical densities, adjust the volume of reagents proportionately (see Note 1). Carry out all procedures on ice unless otherwise specified.

3.1. TSE Periplasmic Extraction

1. Harvest bacterial cells by centrifugation at 3,000 × *g* for 20 min at 4°C (see Note 2).
2. Discard the supernatant and carefully remove the last few drops of liquid with a pipette (see Note 3).
3. Gently resuspend the pellet in 1 mL of TSE buffer using a wire loop (see Notes 4–7).
4. Incubate the cells in TSE buffer on ice for 30 min (see Note 8).
5. Transfer the cell suspension to a microcentrifuge tube and centrifuge at 16,000 × *g* (maximal speed) for 30 min at 4°C.
6. Transfer the supernatant to a new microcentrifuge tube; this supernatant constitutes the envelope extract.

7. (Optional) If separation of outer membrane proteins from periplasmic proteins is desired, centrifuge the supernatant from step 6 at 100,000 × *g* for 1 h at 4°C. The pellet from this step contains the outer membrane proteins; the supernatant contains the periplasmic proteins.

3.2. Polymyxin Treatment Control (see Fig. 2)

This procedure is adapted from Jonda et al. (see ref. 19).

1. Follow steps 1 and 2 in Subheading 3.1 to pellet cells.
2. Gently resuspend the pellet in 1 mL of 1 mg/mL polymyxin buffer and incubate cells on ice for 1 h.
3. Continue with steps 5 and 6 in Subheading 3.1.

3.3. Modified Osmotic Shock Control (see Fig. 2)

This procedure is adapted from the protocol for PeriPreps™ Periplasting Kit (Epicenter Biotechnologies, Madison, WI, USA).

1. Follow steps 1 and 2 in Subheading 3.1 to pellet cells.
2. Gently resuspend the pellet in 0.5 mL TSE buffer and incubate on ice for 15 min.
3. Add 0.5 mL of ice-cold water, mix, and incubate on ice for an additional 15 min.
4. Continue with steps 5 and 6 in Subheading 3.1.

3.4. Whole Cell Lysate Control (see Fig. 2)

1. Follow steps 1 and 2 in Subheading 3.1 to pellet 1 mL of cells.
2. Resuspend the pellet in 0.1 mL reducing SDS sample buffer and heat at 99°C for 10 min.

4. Notes

1. We have routinely used cell amounts as low as 6×10^8–2×10^9 cells (the equivalent of 2 mL at an optical density of 1.0 at 600 nm). We do not recommend using fewer cells because resuspension of cells in less than 20 µL of TSE buffer is difficult.
2. Centrifugation at higher *g* forces may produce tight pellets, which are more difficult to resuspend, thus increasing the risk of cell lysis.
3. Any remaining liquid will dilute the TSE buffer and therefore decrease the efficiency of extraction.
4. Cell pellets must be freshly prepared. Old or frozen pellets should not be used as they are prone to cell lysis, particularly during resuspension.
5. Avoid vigorous pipetting because this may cause cell lysis resulting in contamination of the extract with cytoplasmic proteins.

6. If cells are difficult to resuspend, increase the amount of TSE buffer to 2.5 mL.
7. If the concentration of target protein is low, decrease the amount of TSE to 0.5 mL to produce a more concentrated periplasmic extract. Extracts can also be concentrated with centrifugal filter devices or precipitated by 10% TCA. TSE buffer shows no interference with TCA precipitation.
8. The release of envelope proteins in the presence of sucrose and EDTA is solely dependent on diffusion. Therefore, we use prolonged incubation and centrifugation steps to ensure substantial release of envelope proteins.

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