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Heat Modifiability of Outer Membrane Proteins from Gram-Negative Bacteria

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Summary

β -barrel membrane proteins are somewhat unique in that their folding states can be monitored using semi-native SDS-PAGE methods to determine if they are folded properly or not. This property, which is commonly referred to as heat modifiability, has been used for many years on both purified protein and on whole cells to monitor folded states of proteins of interest. Additionally, heat modifiability assays have proven indispensable in studying the BAM complex and its role in folding and inserting β -barrel membrane proteins into the outer membrane. Here, we describe the protocol our lab uses for performing the heat modifiability assay in our studies on outer membrane proteins.

Keywords

BamA; Heat modifiability; OMP; Outer membrane protein; β -barrel membrane protein; BAM complex; Protein folding

1. Introduction

β -barrel outer membrane proteins (OMPs) from Gram-negative bacteria often exhibit a useful characteristic when analyzed by semi-native SDS-PAGE methods (1-5). Unboiled protein samples will run differently than samples that have been boiled. This behavior is due to the extensive hydrogen bonding network holding the beta strands together into the barrel shape, a structural feature that is often resistant to denaturation by SDS alone. As a result, an OMP that has been solubilized with a mild detergent like DDM retains a relatively compact globular shape on semi-native SDS-PAGE which allows it to migrate further along the gel than the same sample that has been denatured (unfolded) by being boiled (heat). This migration difference between the folded and unfolded states of an OMP is the basis of the heat modifiability assay. Here we present a protocol our lab uses when determining whether OMPs are folded properly or not, either (i) after purification from recombinant expression or (ii) after refolding from inclusion bodies, for structure determination. We also show some

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real examples of OMPs that we have recently worked on in our lab and determined their crystal structures.

2. Materials

Reagents and Equipment

1. Protein sample(s): *EcBamA*, *HdBamA*³, ProtX, *EcTamA*, and *NmTbpA* are samples from our lab used here.
2. UV-vis spectrometer for determining protein concentration.
3. SDS sample loading buffer (2×): 20% glycerol, 120 mM Tris-HCl, pH 6.8, 2% SDS, 0.02% bromophenol blue (*see* Note 1). The concentration of SDS is varied from 0% - 1% in our assays.
4. Microcentrifuge tubes (1.5 mL).
5. Microliter pipettes and tips.
6. Benchtop microcentrifuge for 1.5 mL microcentrifuge tubes.
7. Native gels (*see* Note 2).
8. MES-SDS Running Buffer (20×): 1 M MES, 1 M Tris base, 2% (w/v) SDS, 20 mM EDTA, pH 7.3.
9. Polyacrylamide gel electrophoresis (PAGE) system.
10. Heat block set to 95°C.
11. Instant Blue protein stain (Expedeon).
12. Gel staining box.
13. Ice bucket and ice.

3. Methods

Heat Modifiability of OMPs to Assess Folding

1. Determine the concentration of all protein samples using the absorbance at $\lambda = 280$ nm, the calculated protein extinction coefficient, and the path length (Beer's law).
2. Adjust the concentration of the protein samples to ~2 mg/mL by either concentrating or diluting the samples (*see* Note 3).

¹For the dye, we advise against using coomassie G250 here since we have experienced odd results when substituted for bromophenol blue. We hypothesize that the coomassie G250, which is in fact used for blue native PAGE methods, interacts with the samples due to its properties while that is not the case for the bromophenol blue.

²Most native gels, either fixed or gradient, should work here. For the examples shown here, we used precast NativePAGE Novex 4-12% Bis-Tris protein gels (Life Technologies).

³For convenience, it is easier to maintain all samples at the same sample concentration. However, it is acceptable to also use your protein samples at varying concentrations as long as you ensure ~2 μ g of protein is being loaded and you must ensure you adjust the volume of buffer required to maintain constant volume of 10 μ L. Also, 2 μ g is a starting point but you may find that you need to add more or less protein for best visualization.

3. Prior to preparing the samples for analysis, assemble the gel running apparatus so that the gel may be loaded immediately upon sample preparation. Place the gel apparatus tank into a bed of ice within an ice bucket. Insert the gel and then fill the tank completely with cold 1× MES running buffer. It is important to keep the buffer within the tank cold during the entire experiment (*see* Note 4). Once assembled, you can move to the next step.
4. Place 1 µL of the protein sample into two 1.5 mL microcentrifuge tubes, labeling one as ‘boiled’ and the other as ‘unboiled’. Repeat for each sample.
5. Add 9 µL of sample buffer (i.e. the buffer the protein sample is in) to each tube.
6. Add 10 µL of 2× SDS loading buffer to each tube and mix by gently pipetting up and down a few times (*see* Note 5).
7. Place the ‘boiled’ samples in a heating block set to 95°C for 5 min while leaving the ‘unboiled’ samples at room temperature (*see* Note 6).
8. Centrifuge the boiled samples using a microcentrifuge at full speed for 30 s and then load the samples onto the native gel assembled in **step 3** (*see* Note 7).
9. Run the gel for 60 min at constant 150 volts.
10. Remove the gel and place it into a gel staining box. Add enough Instant Blue gel stain to cover the gel and place on a rotating or rocking platform for 5 min (*see* Note 8).
11. Visualize the gel to determine if the samples show heat modifiability (Fig. 1) (*see* Notes 9 and 10).

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⁴Since we keep our electrophoresis equipment at room temperature, it was easier for us to do everything at room temperature and put the gel apparatus in ice to keep it cool. However, a gel apparatus set up in a cold room would be just as good and would not require the ice bucket or ice.

⁵Here, you can also use other concentrations of SDS. As you see in our examples, *Ec*TamA appeared to be quite sensitive to this variable (Fig. 2).

⁶When boiling samples, sometimes the tops on the microcentrifuge tubes can pop up due to the pressure. To prevent this, poke a single small hole into the tops of microcentrifuge tubes to relieve the pressure buildup.

⁷If desired, protein standards can also be loaded to help indicate the first lane. The protein standards can also be used to align results from several gels run separately.

⁸If no protein standards are used to indicate the first lane, be sure to mark the gel to indicate the first lane. One method is to just cut off the corner on the side of the gel next to the first lane.

⁹While 5 min will allow you to view the bands in the gel, one should leave the gel to stain overnight and then wash in water for 1 h at least 3 times and then leave in water overnight. If desired, the gel can then be imaged and densitometry performed to determine the percentage of folded vs unfolded states.

¹⁰While the folded state of most OMPs will migrate faster than the unfolded state, it is also common for the folded state to migrate slower than the unfolded state. This is often observed for OMPs that are found as oligomers in their fully folded states.

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Fig. 1. Heat modifiability assay of outer membrane proteins from Gram-negative bacteria

Shown is a stained semi-native SDS-PAGE gel for various OMPs that were either boiled (+) or left at room temperature for 5 min (–) prior to loading onto the gel for analysis. Lanes 1 and 2 are of *HdBamA* Δ3, lanes 3 and 4 are of *EcTamA*, lanes 5 and 6 are of a sample we are referring to as ProtX, lanes 7 and 8 are of *EcBamA*, and lanes 9 and 10 are of *NmTbpA*. All samples were ‘heat modifiable’ (i.e. they showed a gel-shift in this assay) except for *EcTamA* in these assays. However, knowing that *EcTamA* was indeed folded properly, further investigations revealed that *EcTamA* is indeed heat modifiable as well after a few optimization of the assay conditions (see Fig. 2).

Fig. 2. Optimizing the heat modifiability assay for SDS-sensitive *EcTamaA*

EcTamaA did not show heat modifiability in our standard assays, however, knowing it was folded properly prompted further investigation to quell our curiosity. Here, we found that *EcTamaA* was more sensitive to SDS than the other OMPs and that reducing the SDS in our sample loading buffer and incubation time (IncT) was enough to detect predominantly the folded form in our assays. Gel A shows a preliminary stained semi-native SDS-PAGE gel varying the percent of SDS in our sample loading buffer. While we could observe a smear from the folded (F) to unfolded (U) states for all unboiled (–) samples (lanes 1, 3, and 5), at least 0.5% SDS was required to allow any migration of the unfolded/boiled sample (lanes 2, 4, and 6). Gel B shows another gel where the incubation time (IncT) was varied as well, yet no difference was observed (lanes 7, 8, and 9) indicating the incubation time was contributing minimally. However, the percentage of SDS was increased and we found that 1% was sufficient to fully unfold the protein even with no heat or incubation time (lanes 10 and 11). Following up on this in Gel C, the concentration of SDS in the sample loading buffer was varied. It was found that no SDS in the sample loading buffer yielded the largest band for the folded state and that an increase in the percent of SDS in the sample loading buffer was accompanied by an increase in the percentage of unfolded protein (lanes 12–15). As shown in Gel A, boiled samples do not migrate into the gel in the absence of at least 0.5% SDS in the sample loading buffer. However, folded proteins do not need the SDS to migrate in the gel (compare lanes 2 and 4 of Gel A to lanes 12 and 13 of Gel C).