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Direct extraction and purification of recombinant membrane proteins from *Pichia pastoris* protoplasts

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Running Head

Extraction of MPs from yeast protoplasts

Abstract

In the past decade, the methylotrophic yeast *Pichia pastoris* has proved to be one of the most efficient systems for mass production of recombinant eukaryotic membrane proteins (MPs), leading to the crystallization and structure determination for a variety of them. The actual overexpression of functional MPs achieved with this system is however often accompanied by the formation of a variable but significant proportion of misfolded and/or aggregated proteins that are co-extracted and co-purified during the purification process. In order to minimize this unwanted phenomenon, we devised a novel procedure in which MPs produced in *Pichia pastoris* are directly solubilized from whole cells instead of crude membrane preparation. This approach aims at favouring the extraction of correctly folded membrane proteins that have been targeted to the plasma membrane, limiting the solubilization of the misfolded proteins and protein aggregates that are stored in

internal membrane compartments. The method described herewith is based on the formation of protoplasts through enzymatic treatment prior to protein solubilization. This chapter details a set of protocols going from yeast cell preparation and protein solubilization to purification using affinity and size exclusion chromatography.

Keywords

Pichia pastoris, yeast, membrane protein, protoplast, detergent solubilization, purification

1. Introduction

When coping with membrane protein (MP) solubilization from any cellular expression system, the most common approach consists in the preliminary preparation of the membrane fraction. This membrane enrichment step can be quite valuable during the downstream extraction and purification phases by reducing the amount of soluble proteases as well as potential soluble contaminants from the sample.

However, membrane fractionation necessarily involves cell lysis procedures that could be more or less drastic. When harsh mechanical conditions are used, undesired and poorly controlled events such as heat and shear forces may occur that are often detrimental to the integrity of membrane proteins.

In addition, standard MP protocols usually recover the whole membrane fractions issuing from the different cellular compartments and containing a mixture of properly folded and misfolded proteins. Even if this phenomenon is still poorly characterized in the context of membrane protein production, it is actually widely recognised that overexpression overwhelms the cell biosynthesis and translocation machineries that often elicits improper MP folding. Such events cause a number of stresses and responses with various outcomes including the retention of misfolded proteins within the secretory pathway and the formation of protein aggregates before their eventual degradation (1, 2). As a result, when preparing membranes for protein purification purposes, the following solubilization step may lead to the co-extraction of various heterogeneous forms of the protein of interest that are further difficult to separate.

The protocol presented here proposes to limit these phenomena by performing a short solubilization step directly on whole cells in order to favour the extraction of

the undamaged, correctly folded MPs that have been targeted to the plasma membrane.

This method has been developed in our lab with the yeast *Pichia pastoris*, a robust and versatile system that we routinely use for the recombinant expression of various MPs (3–7). Because this cellular host possesses a very thick and robust cell wall that is detergent-resistant, a preliminary enzymatic treatment is required to obtain yeast protoplasts (i.e. yeast cells devoid of their cell wall) before the detergent solubilization and purification steps.

The whole procedure has been successfully applied to a variety of eukaryotic (mainly human) MPs recombinantly expressed in *P. pastoris*, encompassing GPCRs, ion channels and other integral MPs. By comparison with the standard procedure where the same proteins were extracted from membrane preparations after cell lysis, the method not only proved to be much more rapid, but, most importantly, allowed to significantly reduce the presence of protein aggregates after purification in several cases (manuscript in preparation).

Here, we illustrate this method with the solubilization and purification of the human adenosine A2A receptor extracted from yeast protoplasts. The starting biological material consists of yeast pellets obtained after the expression of a N-Flag-10His-HUMAN_AA2A-Biotag construct, following the conditions reported in (3). A full description of the generation and selection of *Pichia pastoris* clones, as well as the subsequent procedures for recombinant expression can be found in other recent and comprehensive chapters of the Methods in Molecular Biology series (8, 9).

The present protocol reports the enzymatic treatment of yeast cells expressing recombinant protein to obtain protoplasts, which are then directly subjected to detergent solubilization (Figure 1). A subsequent two-step purification procedure (Ion Metal Affinity Chromatography and Size Exclusion Chromatography) is also

presented to illustrate the yield and purity of the MPs that can be isolated with this method.

2. Materials

2.1. Zymolyase® treatment

1. Recombinant cell pellet.
2. Milli-Q water.
3. SED buffer: 1 M sorbitol, 25 mM ethylenediaminetetraacetic acid (EDTA), 1 M dithiothreitol (DTT) (added extemporaneously).
4. 1 M sorbitol.
5. CG buffer: 20 mM trisodium citrate, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF) (added extemporaneously). Adjust the pH to 5.8 with hydrochloric acid.
6. Zymolyase® 20T (Amsbio). It can be resuspended in water at 200 U / ml and stored at -20°C for a few months.

2.2. Protein solubilization

1. Solubilization buffer: 50 mM Tris HCl pH 7.4, 500 mM NaCl, 10% glycerol, 20 mM imidazole (*see Note 1*), detergent (*see Note 2*), inhibitor protease cocktail (added extemporaneously, e.g. cOmplete™ EDTA-free, Roche).
2. Ultracentrifuge equipped with an appropriate fixed-angle rotor and adapted polycarbonate tubes.

2.3. Purification of solubilized material

1. Automated protein purification system (ÄKTA Purifier, GE Healthcare, or equivalent). A sample pump or a superloop is required for the affinity chromatography step.

2. 1 ml pre-packed nickel affinity chromatography column (e.g. HisTrap HP 1 ml, GE Healthcare).
3. 0.22 μ M syringe filter.
4. Buffer A: 50 mM Tris HCl pH 7.4, 500 mM NaCl, 20 mM imidazole, detergent (*see Note 2*).
5. Buffer B: 50 mM Tris HCl pH 7.4, 500 mM NaCl, 500 mM imidazole, detergent (*see Note 2*).
6. Centrifugal protein concentrator (e.g. Vivaspin 6, Sartorius, cut-off adapted to the protein of interest).
7. Size Exclusion Chromatography (SEC) column (e.g. Superdex 200 Increase 10/300, GE Healthcare).
8. SEC running buffer: 50 mM Tris HCl pH 7.4, 150 mM NaCl, detergent (*see Note 2*).

2.4. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

1. 40% Acrylamide/Bis-acrylamide solution, 19:1.
2. 3 M Tris-HCl pH 8.45, 0.3% (w/v) SDS.
3. 80% (v/v) glycerol.
4. 10% (w/v) ammonium persulfate (APS).
5. Tetramethylethylenediamine (TEMED).
6. Gel casting stand and electrophoresis chamber (e.g. Mini-PROTEAN system, Bio-Rad).
7. Membrane preparation samples.
8. Tris-Tricine-SDS cathode running buffer: 1 M Tris-HCl pH 8.2, 1 M Tricine, 1% (w/v) SDS.
9. Tris anode running buffer: 1 M Tris-HCl pH 8.9.

10. 2X Tricine Sample Buffer (SB 2X): 100 mM Tris-HCl pH 6.8, 25% (v/v) glycerol, 8% (w/v) SDS, 0.02% (w/v) Coomassie blue G250, 200 mM DTT.

2.5. Protein transfer and Western Blot Immunodetection

1. Tris-Glycine transfer buffer: 25 mM Tris, 200 mM glycine, 0.02% (w/v) SDS, 20% (v/v) ethanol.
2. 0.45 μ m nitrocellulose blotting membrane.
3. Whatman paper.
4. Electroblotting system (e.g. Mini Trans-Blot Cell, Bio-Rad).
5. Phosphate-buffered saline (PBS).
6. PBS containing 0.02% (v/v) Tween 80 (PBST).
7. Blocking buffer: PBST with 5% (w/v) non-fat dry milk.
8. Primary anti-tag or anti-protein antibody (e.g. monoclonal anti-FLAG antibody from mouse, Sigma).
9. Secondary anti-mouse IgG antibody linked to a reporter system (traditionally HRP-conjugated antibody, here an IRD800-coupled antibody).
10. Reagents and detection device adapted to the reporter system selected.
11. Orbital shaker.

3. Methods

3.1. Protoplasting: Zymolyase® treatment of recombinant cell paste

Zymolyase® is a mixture of several carbohydrases (i.e., β -1,3 glucan laminaripentaohydrolase, β -1,3 glucanase, protease and mannanase) extracted from *Arthrobacter luteus*. These enzymes work in conjunction to degrade efficiently the cell wall of a large spectrum of yeasts, thus leading to the formation of yeast protoplasts. A number of other enzymes for yeast cell wall degradation are commercially available but Zymolyase® has proved to be the most efficient in our hands for this protocol with *Pichia pastoris*.

3.1.1. Cell paste preparation

The following proportions are given for 3 g of wet cell paste, obtained from about 250 ml of *Pichia pastoris* culture.

1. Resuspend the cell pellet in 50 ml Milli-Q H₂O.
2. Harvest the cells by centrifuging for 5 min at 5,000 g, 4°C.
3. Discard the supernatant and wash the cell pellet with 50 ml SED buffer.
4. Harvest the cells by centrifuging for 5 min at 5,000 g, 4°C.
5. Discard the supernatant and wash the cell pellet with 50 ml 1 M sorbitol.
6. Harvest the cells by centrifuging for 5 min at 5,000 g, 4°C.
7. Discard the supernatant and resuspend the cell pellet in 37.5 ml of CG buffer, in order to reach a cell paste concentration of 80 g/L.

3.1.2. Enzymatic cell wall degradation

1. Add 20 U of Zymolyase® per gram of cell paste initially prepared.
2. Incubate for 15 min (*see Note 3*) at room temperature under gentle agitation (*see Note 4*).

3. Harvest the protoplasts formed by a soft centrifugation for 5 min at 750 g, room temperature. In order to avoid protoplast lysis, reduce the acceleration and deceleration speed of the centrifuge.
4. Carefully discard the supernatant using a pipette.

3.2. Protein solubilization

As for every MP solubilization, the successful selection of the best detergent to use is a fine-tuned combination between the extraction efficiency and the preservation of the protein fold and function. It both depends on the protein of interest and the expression system. The choice of the detergent, here DDM for AA2A, has thus to be adjusted accordingly (*see Note 2*).

1. Gently resuspend the pellet in 37.5 ml of solubilization buffer (no vortexing). The cell paste concentration should be close to 80 g/L.
2. Incubate for 30 min at room temperature under gentle agitation.
3. Separate the solubilized fraction by ultracentrifuging for 30 min at 100,000 g and 4°C. Store 20 µl of the suspension before ultracentrifugation at 4°C for SDS-PAGE analysis.
4. After ultracentrifugation, recover the supernatant containing the solubilized fraction. Store 20 µl at 4°C for SDS-PAGE analysis.
5. The ultracentrifuge pellet can also be analyzed by SDS-PAGE after resuspension in 37.5 ml of solubilization buffer without detergents, imidazole and protease inhibitor cocktail. Use a glass Teflon homogenizer to obtain a homogeneous suspension.

3.3. Purification of solubilized material

3.3.1. Nickel affinity chromatography

The following protocol is designed for automated purification (e.g. using ÄKTA protein purification systems). It can however be easily adapted for batch/gravity flow purification. The whole purification process can be performed at a flow rate of 1 ml/min.

1. Filter the ultracentrifuge supernatant using a 0.22 μm syringe filter.
2. Add imidazole at a final concentration of 20 mM.
3. Equilibrate a 1 ml pre-packed nickel affinity column with buffer A.
4. Inject the filtrated supernatant onto the column. Store 20 μl of the injected sample at 4°C for SDS-PAGE analysis.
5. Wash with 20 ml of buffer A, or until the 280 nm-absorbance signal returns to the baseline.
6. Wash for 10 ml with 20% buffer B (corresponding to about 100 mM imidazole) (*see Note 5*).
7. Elute the protein of interest either with a gradient of buffer B or with a step of 100% buffer B (*see Note 6*). Collect 0.5 ml elution fractions.
8. Pool the fractions representative of one elution peak and concentrate them, using a centrifugal concentrator, to a volume suited for injection onto the size exclusion chromatography column (according to the manufacturer's recommendations, typically a maximum of 500 μl for a 24 ml column). For concentration, choose a cut-off adapted to the size of the protein of interest (typically 30 or 50 kDa MWCO).
9. Store 20 μl of the concentrated pool of interest and all other representative elution fractions (flow through and wash fractions) at 4°C for SDS-PAGE analysis.

3.3.2. Size Exclusion Chromatography (SEC)

The following protocol is designed for automated purification (e.g. using ÄKTA protein purification systems). Superdex 200 10/300 SEC column or equivalent is

generally well suited for small-scale preparative purification of MPs between 10 kDa and 600 kDa.

1. Equilibrate the SEC column with at least 1.5 column-volume (CV) of running buffer.
2. Inject the concentrated sample. Set the flow rate according to the manufacturer's recommendations. Collect 0.5 ml elution fractions.
3. Store 20 μ l of each peak-representative fraction at 4°C for SDS-PAGE analysis.

Figure 2 shows a typical SEC elution profile obtained during the purification of the adenosine A2A receptor using the procedure described above. SEC enables the separation of oligomeric objects and contaminants from monomeric receptor of interest. Oligomeric and/or aggregated objects are eluted close to the void volume of the column (corresponding to peak 1) and correspond to the inactive protein. The monomeric and ligand-binding active receptor is mainly eluted in peak 2. In typical similar experiments performed on proteins extracted from whole membrane fractions after cell lysis (standard procedure), peak 1 has systematically about 3 to 5 times the intensity of peak 2 (data not shown). These data exemplify how this method may be highly beneficial to reduce the amount of aggregated MPs during extraction. In terms of yield, the protocol described here enables to recover about 1.5 mg of monomeric adenosine A2A receptor from 1 liter of yeast culture. However, protein extraction from yeast protoplasts does not significantly improve the final amount of active purified receptors compared to more classical methods.

Fractions representative of each elution peak (respectively 1, 2 and 3) have to be analysed using biochemical methods in order to assess their protein content. In this chapter we describe SDS-PAGE analyses, but functional tests such as radioligand binding assay may be also performed if available for the protein of interest.

3.4. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

3.4.1. 10% SDS tricine polyacrylamide gel preparation

The following proportions are given for casting two gels of 1 mm thickness in a standard Mini-Protean system from Bio-Rad.

1. Prepare the separating gel by mixing 2.5 ml of acrylamide solution, 3.3 ml of Tris-HCl SDS buffer, 1.25 ml of 80% (v/v) glycerol, 2.9 ml H₂O. Add 90 µl of APS and 6 µl of TEMED, mix, and immediately cast the gel. Allow space for the stacking gel.
2. Prepare the stacking gel by mixing 0.6 ml of acrylamide solution, 1.6 ml of Tris-HCl SDS buffer, 4.1 ml H₂O. Add 90 µl of APS and 6 µl of TEMED, mix, and cast carefully over the separating gel. The presence of glycerol in the separating part avoids the need to wait for its complete polymerization before casting the stacking gel. Insert a 10-well gel comb immediately without introducing air bubbles.
3. Let polymerize for about 30 min.

3.4.2. Gel electrophoresis and Coomassie staining

1. Incubate 20 µl of the elution fractions of interest in 20 µl of SB 2X for about 10 min at room temperature (*see Note 7*).
2. Load in parallel two 10% SDS-polyacrylamide gels with respectively 20 µl (first gel) and 10 µl (second gel) of the sample in its sample buffer. The first gel will be used for total protein staining with Coomassie blue, the second one will be transferred on a nitrocellulose membrane for immunodetection of the protein of interest.
3. Proceed to electrophoresis using Tris-Tricine-SDS cathode running buffer and Tris anode running buffer in a tank unit. Run for about 1 h 30 min at 100 V.
4. Stain the first gel with Coomassie blue following the manufacturer's instructions.

Figure 3A presents a typical Commassie blue SDS-PAGE analysis obtained for adenosine A2A receptor purification. A number of contaminant proteins are still present in the IMAC elution pool but SEC enables to obtain highly purified proteins.

3.5. Protein transfer and Western Blot Immunodetection

1. Transfer the proteins from the second gel to a nitrocellulose membrane by electroblotting in Tris-glycine transfer buffer for about 1 h 30 min at 100 V. An ice pack can be added into the electroblotting device to mitigate the heat produced.
2. Incubate the membrane in 50 ml blocking buffer for 1 h at room temperature on an orbital shaker. Alternatively, incubate the membrane overnight at 4 °C.
3. Remove the blocking solution and incubate the membrane with the selected antibody diluted in blocking buffer (for instance a monoclonal anti-flag antibody at a final concentration of 0.1 $\mu\text{g}/\text{ml}$) for 1 h at room temperature on an orbital shaker.
4. Wash the membrane 3 times, each time with 50 ml of PBST on an orbital shaker for 5 min at room temperature.
5. Remove the PBST and incubate the membrane with the adapted ant-IgG antibody diluted in blocking buffer (typical final concentration of 0.1 $\mu\text{g}/\text{ml}$) for 1 h at room temperature on an orbital shaker.
6. Wash the membrane 3 times, each time with 50 ml of PBST on an orbital shaker for 5 min at room temperature.
7. Remove the PBST and wash the membrane with 50 ml of PBS on an orbital shaker for 5 min at room temperature.
8. Store the membrane in PBS until revelation.
9. Proceed to membrane revelation according to the reporter system selected and following the manufacturer's recommendations.

Figure 3B shows the western blot analysis of adenosine A2A receptor purification, using an anti-flag secondary antibody. Several bands corresponding to the receptor of interest can be identified. The major signal, at the apparent molecular weight of 55 kDa, corresponds to the complete form of the receptor. SEC peak 2 thus contains the complete and monomeric form of the adenosine A2A receptor. A radioactive ligand binding assay further confirms the functional state of this fraction of the purified receptor (data not shown).

4. Notes

1. Imidazole can be added to the solubilized sample after the ultracentrifugation step. The use of high quality imidazole (e.g. I0250, Sigma), which shows a very limited absorption signal at 280 nm, is strongly recommended for ion immobilized metal affinity chromatography.
2. The detergent used in the buffers has to be selected according to its solubilization and stabilization properties toward your protein of interest. We recommend a preliminary solubilization screening using different detergents before large-scale purification. In terms of concentration, we recommend using about 10 to 20 times the critical micellar concentration (CMC) during the solubilization step, and at least twice the CMC for subsequent purification steps.
3. The duration of Zymolyase® treatment and/or amount of Zymolyase® used may be critical for protein integrity and can thus be adjusted to the protein of interest.

4. Whereas yeast cells are very resistant to mechanical stress, yeast protoplasts are as fragile as mammalian cells and shall thus be handled very carefully in order to avoid mechanical cell lysis.
5. This first step enables to get rid of unspecific contaminants, and can be adapted according to the protein of interest and the cellular context. In our experience, G protein coupled receptors labeled with a decahistidine tag elute from Ni Sepharose High Performance nickel affinity columns from about 150 mM imidazole.
6. Protein elution using an imidazole gradient (typically 0 to 100% of B buffer spread in 25 ml) can be of great use to separate monomeric from oligomeric or aggregated MPs. Based on our experience, aggregated proteins show a higher affinity for the resin and are thus eluted later than monomeric proteins.
7. It is usually not recommended to boil MP samples prior to electrophoresis. When compact and highly hydrophobic proteins such as GPCRs are boiled, they usually aggregate and stay stuck in the concentrating gel.

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Figure 1: Protoplasting and protein solubilization procedure. Recombinant yeast cell pellet is first submitted to Zymolyase® treatment for cell wall degradation. The protoplasts formed are then directly used for protein solubilization using detergents. An ultracentrifugation step enables to recover the solubilized fraction that will undergo the purification process.

Figure 2: Size exclusion chromatography elution profile of the adenosine A2A receptor. Profile obtained on a Superdex 200 Increase 10/300 GL SEC column with absorbance measured at 280 nm. In this case, SEC enables the separation of 3 main peaks named respectively 1, 2 and 3.

Figure 3: SDS-PAGE analysis of representative fractions from adenosine A2A receptor purification. P: ultracentrifugation pellet; S: solubilized fraction loaded onto nickel affinity chromatography column; FT: IMAC flow through; E: IMAC elution pool; W1 and W2: IMAC washes before elution; 1, 2 and 3: SEC elution peaks. **A:** Analysis of the total protein content of 10 μ l of each sample on a 10% polyacrylamide Tris-Tricine gel stained with Coomassie blue. **B:** Western Blot analysis of the Adenosine A2A receptor content of 5 μ l of each sample. Immunoblotting realized with a M2 anti-flag antibody (0.1 μ g/ml).