

OVEREXPRESSION AND PURIFICATION OF INTEGRAL MEMBRANE PROTEINS IN YEAST

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Abstract

The budding yeast *Saccharomyces cerevisiae* is a viable system for the overexpression and functional analysis of eukaryotic integral membrane proteins (IMPs). In this chapter we describe a general protocol for the initial cloning, transformation, overexpression, and subsequent purification of a putative IMP and discuss critical optimization steps and approaches. Since expression and purification are often the two predominant hurdles one will face in studying this difficult class of biological macromolecules the intent is to outline the general workflow while providing insights based upon our collective experience. These insights should facilitate tailoring of the outlined protocol to individual IMPs and expression or purification routines.

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1. INTRODUCTION

Obtaining sufficient quantities of a purified integral membrane protein (IMP) for downstream experiments, such as structural or functional analysis, can be a daunting task. Common hurdles that one may encounter include obtaining sufficient IMP overexpression, extracting the IMP from cellular membranes with a detergent and purifying the IMP in functional form. Advances in addressing these bottlenecks should facilitate efforts by the broader scientific community in pursuing their own particular IMP of interest. One such advance is use of the budding yeast *Saccharomyces cerevisiae* to overexpress IMPs (Bill, 2001; Bonander *et al.*, 2005; Griffith *et al.*, 2003; Hays *et al.*, 2009; Li *et al.*, 2009; White *et al.*, 2007). When combined with a broad range of methods for *in vivo* functional characterization of IMPs in yeast, with its exhaustive genetic toolkit, one can appreciate the inherent power of using *S. cerevisiae* as an expression system. Thus, the objective of this chapter is to provide a general approach for overexpression of IMPs in the yeast *S. cerevisiae*. In addition, we will provide an introduction to purifying the IMP of interest following expression. To accomplish this, we will describe our approach to the task while highlighting critical steps within the protocol that may require heightened attention. It is important to note that overexpression and purification of functional IMPs is still a laborious endeavor fraught with problems. As with most difficult journeys, many small decisions often come together in dictating the outcome.

2. GENERAL CONSIDERATIONS

S. cerevisiae is an intensely studied eukaryotic organism. The approach we have taken with the current chapter is to outline the yeast expression protocol currently deployed within our research efforts. At almost every step throughout this chapter, an alternative method, vector, column, buffer, affinity tag, etc., could be employed with possibly better outcomes for the specific protein being studied. Our intent is to convey a generic strategy and, where possible, highlight alternatives that we feel the reader should be aware of. Since working with IMPs is often an endeavor replete with nuances and hurdles, it is our desire that this chapter will provide a foundation for those not familiar with membrane protein overexpression and purification.

This chapter is organized around the expression and purification of a putative integral membrane protein termed “POI” for “protein of interest.” The intent is that a reader can substitute the membrane protein he/she is interested in for this target. As with most procedures, our approach is not

the only viable strategy. It works very well in many cases though modifications can be customized to suit the system under study. We have made some key choices based upon our prior experience including: (1) yeast strain W303- Δ pep4 (*leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 Δ pep4 MAT α*) is used, (2) pRS423-GAL1-based inducible plasmid to drive expression, (3) a C-terminal [linker]-[3C-protease]-[10 \times His] tag fused to the expressed protein, and (4) solubilization in the detergent *n*-dodecyl- β -D-maltopyranoside (DDM). Each of these is a critical step and should be examined if the described procedure should fail. Finally, previously published protocols may also be of interest to the reader (Hays *et al.*, 2009; Li *et al.*, 2009; Newby *et al.*, 2009).

3. PROTOCOL—MOLECULAR BIOLOGY

Our experience is such that multiple expression plasmids, affinity tags, and fusion constructs should be tried when pursuing a specific protein. Vectors designed to better leverage *S. cerevisiae* as an expression system are often chimeric shuttle vectors with yeast and bacterial derived sequences. The yeast contribution to the vector sequence will determine the location of transformation: extrachromosomal ectopic expression or chromosomal integration in mitotically stable yeast strains (Boer *et al.*, 2007). Episomal expression requires that the cloned gene needs be free of introns for plasmid or genomic expression. If properly implemented, episomal overexpression in yeast can be rapidly deployed and often yields milligram quantities of IMPs (Li *et al.*, 2009; Mumberg *et al.*, 1995). This is accomplished through the autonomously replicating sequence from native yeast 2μ plasmid (Christianson *et al.*, 1992). Thus, for the current example, POI is cloned into a high-copy 2μ episomal expression vector containing a GAL1 promoter (Fig. 29.1). The GAL1 promoter is useful because it is tightly repressed in the presence of glucose and strongly induced by galactose allowing for stringent control of protein expression. Expression levels can be further manipulated by altering the copy number through the origin of replication and by swapping out the GAL1 promoter for constitutive (ADH1, TEF2) or other inducible promoters (e.g., MET25, PHO5) (Mumberg *et al.*, 1994). In our experience, constitutive promoters are not effective for IMP overexpression.

To facilitate the process of shuttling a gene between numerous expression vectors, and even expression systems, we use ligation independent cloning (LIC). We previously described in detail how LIC cloning is performed within our yeast system (Supplementary Information in Li *et al.*, 2009). Our experience has led us to prefer a C-terminal rhinovirus 3C cleavable poly-histidine tag as an initial choice when pursuing novel IMPs. Approximately 30% of IMPs contain an N-terminal signal peptide

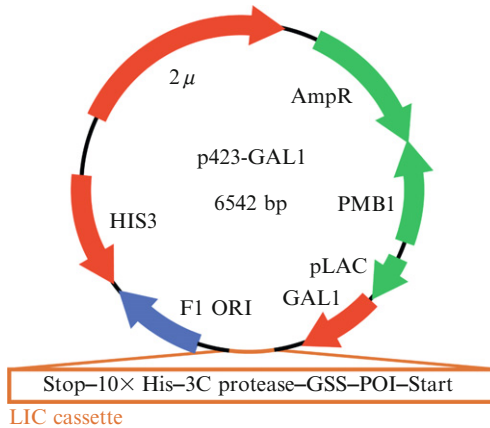


Figure 29.1 Schematic of the p423-GAL1 expression plasmid. Yeast 2μ -based expression plasmid containing a GSS-3C protease-10× His tag for the protein of interest using LIC. Plasmid contains yeast (shown in red), bacterial (shown in green), and phage (shown in blue) elements conducive to molecular cloning and transformation methodologies.

involved in proper protein maturation and targeting to cellular membranes. Since N-terminal tags can interfere with this processing, the preference is to use C-terminal tags when available. In addition, C-terminal tags provide greater assurance that the protein being purified through initial steps is the full-length construct and free of truncation or degradation. If the POI does not contain a signal peptide, which is often difficult to ascertain for eukaryotic genes, then N-terminal tags provide greater flexibility in developing expression constructs. Whatever tag is chosen, care should be taken to ensure that it is either added to the design of synthetic primers during cloning or already present within the selected plasmid. Also, a critical step when including C-terminal tags is to ensure that the native stop codon is removed from the gene of interest. For the current discussion, we will clone POI into our p423-GAL1 expression plasmid containing the following design: Start-[POI]-[linker]-[3C site]-[10× His]-Stop. The choice of using a rhinovirus 3C protease for tag cleavage is described later.

A general protocol for cloning POI into this plasmid is as follows. Refer to [Li *et al.* \(2009\)](#) for a detailed protocol:

1. POI is PCR amplified with primers palindromic to our p423-GAL1 LIC vector.
2. Amplified POI and p423-GAL1 separately undergo T4-polymerase 3'-5' exonuclease digestion in the presence of dATP and dTTP, respectively.
3. The digested gene and plasmid are then combined at room temperature, annealed, and transformed into competent *Escherichia coli* cells.

4. Colony PCR is used to confirm POI insertion into p423-GAL1. Sequencing the plasmid with GAL1 and CYC1 primers validates POI identity.

4. PROTOCOL—CELL GROWTH

The plasmid containing POI destined for transcription, translation, and proper membrane insertion must first be introduced into the yeast host through transformation. Although there are several methods to introduce genetic material into *S. cerevisiae*, including *Agarobacterium tumefaciens*-mediated transformation (Piers *et al.*, 1996), we use a lithium acetate transformation protocol with PEG 3350. The episomal vector p423-GAL1 contains the HIS3 gene needed by our strain, W303- Δ pep4 (*leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 Δ pep4 MAT α*), and must be cultured in synthetic complete media without histidine to maintain selection for the plasmid containing POI. Cultures are grown in 375 ml volumes containing SC-His with 2% glucose in 1L baffled flasks shaking at 220 rpm at 30 °C. Following a growth period of 24 h, the optical density at 600 nm ranges between 15 and 20 for most cultures with glucose concentration generally \ll 0.1%. The culture is induced by adding 125 ml of 4 \times YPG (yeast extract, bactopectone, and galactose) to each flask bringing the final volume to 500 ml. DMSO has previously been shown to improve the expression of certain IMPs and may be tried as a growth additive during induction (Andre *et al.*, 2006). Growths can easily transition from shaker flasks to the zymurgy route of large-scale fermentation as the choice of inducible promoter enables careful regulation and timing of expression. Cells are harvested after 16 h at 6000 \times g and resuspended in 30 ml of lysis buffer containing 50 mM Tris (pH 7.4, RT), 500 mM NaCl, and 20% glycerol (v/v) per half liter of growth. Ideally, we adjust the volume of growth culture to obtain a minimum of 2–3 mg purified protein per growth (200–300 μ l at 10 mg/ml).

5. PROTOCOL—MEMBRANE PREPARATION AND SOLUBILIZATION

Once harvested, cells expressing POI are lysed mechanically using a microfluidizer or by bead beating in the presence of protease inhibitors. For bead beating, we use a 90-ml canister containing approximately 40 ml of resuspended cell pellet. Each canister is then filled to the top with 0.5 mm prechilled glass beads and lysed using four cycles of 1 min “on” and 1 min “off.” We find that aggressive protease inhibition is not always needed and

is target specific. Crude cell lysate is then centrifuged at $6000\times g$ for 15 min. After this centrifugation, qualitative lysis efficiency can be determined by the debris pellet which will contain two layers: a bottom pink (strain dependent) layer of unlysed cells and top lighter layer of organelles and cellular debris from lysed cells. The ratio of the top lysed cells to the bottom unlysed cells is a qualitative indicator of lysis efficiency. We typically have $>90\%$ efficiency at this stage but $>70\%$ is considered acceptable. Collect cell lysate from the supernatant of the previous low-speed spin while being careful not to contaminate supernatant with cell debris, and spin the supernatant at $138,000\times g$ ($42,000$ rpm using a Ti 45 rotor) for 2 h. Discard the supernatant from the high-speed spin. Occasionally, a loose upper layer is obtained following the high-speed spin that should be retained as it often contains a predominant portion of the expressed protein. Resuspend membranes in approximately 5 ml of membrane resuspension buffer (50 mM Tris (pH 7.4, RT), 200 mM NaCl, 10% (v/v) glycerol, and 2 mM fresh PMSF) per liter of culture growth with 10 μ l HALT protease inhibitor cocktail (or your protease inhibitor cocktail of choice). Stir on ice for 30 min and flash freeze membranes in LN2 or use immediately.

We commonly use the following detergents for solubilizing membrane proteins leading to structural work: *n*-octyl- β -D-glucopyranoside (OG), *n*-nonyl- β -D-glucopyranoside (NG), *n*-decyl- β -D-maltopyranoside (DM), *n*-dodecyl- β -D-maltopyranoside (DDM), *n*-dodecyl-*N,N*-dimethylamine-*N*-oxide (LDAO), and *n*-dodecylphosphocholine (FC-12). Detergents are purchased in high-purity form (i.e., "ANAGRADE") from Anatrace. Numerous other detergents are possible depending on the individual experiment being performed. Once the POI-3C-10His has been expressed, it is important to assess how well it can be extracted from the membrane with a detergent. This is generally accomplished through a broad screen of several detergents. The recommended concentrations, for detergents listed above, when solubilizing cellular membranes are 270 mM OG, 140 mM NG, 10 mM DM, 20 mM DDM, 200 mM LDAO, and 20 mM FC-12 (10 \times CMC for other detergents is a recommended starting point). A detailed protocol for performing this step is available in Box 1 of [Newby *et al.* \(2009\)](#). Generally, small aliquots of cellular membranes are mixed with an equal volume of buffer containing detergent at the above concentration and then stirred at 4 $^{\circ}$ C for 12–14 h. Unsolubilized cellular membranes will pellet at $200,000\times g$, so the extent to which a given detergent is able to solubilize POI-3C-10His can be evaluated by the amount of protein left in the supernatant following a high-speed spin. When evaluating initial expression levels via western blots, one may observe several background bands specific to yeast that may be visible in an epitope dependent manner. For anti-His westerns IST2, a 946 amino acid polypeptide containing a stretch of seven histidines near the C-terminus, runs at around 100 kDa. When using anti-FLAG, an unidentified contaminant band often appears

around 60 kDa. An HRP-conjugated Penta-His antibody (Invitrogen) works best for probing C-terminal poly-histidine tags in our experience. If available, functional assays to verify activity following detergent solubilization are highly informative.

6. PROTOCOL—PROTEIN PURIFICATION

Once the POI-3C-10His protein is extracted from cellular membranes in soluble form, it may be purified to obtain a sample that is *Pure* (free of other proteins and contaminants), *Homogenous* (single uniform population), *Stable* (typically over a week in concentrated form at 4 °C), and *Free* of protein-free detergent micelles (this combined state will be referred to as “PHSF”). To accomplish this, we employ a narrow range of techniques including immobilized metal affinity (IMAC), size-exclusion (SEC) and ion-exchange chromatography. These methods are synergistic, iterative, and employed to varying degrees depending on the target protein. For the current discussion, we will detail a standard approach of IMAC followed by cleavage of the expression tag, reverse-IMAC to remove uncleaved protein, and finally SEC to obtain the purified protein in diluted form. This sample will then be concentrated and analyzed prior to use. If this sample is intended for structure determination (i.e., crystallization) then special caution should be taken to avoid a significant concentration of protein-free detergent micelles (Newby *et al.*, 2009).

Thus, we will continue with the theme of purifying our target protein, POI-3C-10His, which was solubilized in the previous section. Recommended detergent concentrations for SEC buffers are as follows: 40 mM OG, 12 mM NG, 4 mM DM, 1 mM DDM, 12 mM LDAO, and 4 mM FC-12 (2× CMC is a good starting point for most detergents). For the current example, we will use 1 mM DDM in all buffers (as determined in Section 5). The initial step to protein purification is a metal-affinity purification of the solubilized membranes; we generally use 125 μ l of Ni-NTA agarose resin (Qiagen) per mg of expected protein yield. The selected IMAC resin should be prepared according to manufacturer's specifications and optimized as needed. The solubilized membranes should be incubated with IMAC resin at 4 °C with nutation for at least 1 h though generally not longer than 3 h. We have found that the degree of target protein binding to Ni-NTA resin does not increase substantially past 3 h though increased proteolysis and binding of contaminant proteins may occur. Following incubation, the Ni-NTA resin containing bound POI-3C-10 \times His protein should be transferred to a gravity flow column and washed with 20 column volumes of Buffer A (20 mM Tris (pH 7.4, RT), 200 mM NaCl, 10% (v/v) glycerol, 4 mM β -ME, 1 mM PMSF, and 1 mM DDM) containing 10 mM

imidazole. If following the wash by absorbance, it is beneficial to wash until A_{280} nm returns to baseline. It is important at this point to obtain about 10 μ l of initial flow-through for SDS-PAGE analysis. The above steps are repeated with Wash 2 (Buffer A with 25 mM imidazole) and Wash 3 (Buffer A with 40 mM imidazole) buffers. Finally, POI-3C-10 \times His is eluted from the column using the IMAC elution buffer (Buffer A with 300 mM imidazole). If possible, reduce the flow rate prior to elution to ensure the target protein elutes in a minimal volume. Be careful to observe the eluted sample for turbidity, especially over the ensuing several minutes as the protein may be unstable in the prescribed buffer and thus precipitate out of solution at this point. If precipitation occurs, one can make appropriate changes to the IMAC buffers (e.g., changing salt concentration or pH) to increase protein stability. It is also advisable to perform a buffer exchange immediately following elution into 20 mM HEPES (pH 7.4), 150 mM NaCl, 10% (v/v) glycerol, 4 mM β -ME, 1 mM PMSF, and 1 mM DDM (SEC buffer). This can be accomplished with a small desalting column such as the Econo-Pac 10 DG disposable chromatography column from Bio-Rad (cat. no. 732-2010). Following IMAC and buffer exchange, the POI-3C-His protein is ready for cleavage of the linker-3C-10 \times His expression tag.

There are a broad number of site-specific proteases for cleaving affinity tags, though care should be taken to ensure they are active in the prescribed detergent (Mohanty *et al.*, 2003). The human rhinovirus 3C protease and thrombin are both robust and efficient proteases that have worked very well for cleaving affinity tags attached to detergent solubilized membrane proteins. We have had great success with an MBP-3C fusion construct described previously (Alexandrov *et al.*, 2001). To cleave the POI-3C-His affinity tag, the protein should be incubated overnight at 4 °C with approximately a 1:5 ratio of protease to target protein in whatever volume of buffer is obtained in the desalting step above. Retain pre- and postcleavage 10 μ l samples for SDS-PAGE gel analysis to evaluate cleavage. Following cleavage a reverse-IMAC purification (i.e., the flow-through is retained) is performed using metal-affinity resin to separate cleaved 3C-His tag and protease (which is also His tagged) from the target protein. This step entails a 1h incubation with IMAC resin, such as "Talon" metal-affinity resin, in batch at 4 °C. Following incubation, the flow-through should be retained—this contains the cleaved POI protein that will be purified in the next step. Elute resin bound protein from the column using the IMAC elution buffer and collect a 10- μ l sample for analysis on a gel to ascertain if nonspecific binding of the target protein is occurring. Following completion of this step, the Ni-purified, 3C-cleaved POI protein is now ready for further purification.

Ion-exchange chromatography is a powerful technique that separates macromolecules based upon charge state at a given pH. Though not discussed within this chapter, we have often used this technique to purify

difficult targets, concentrate protein, reduce protein-free detergent micelles, perform detergent exchanges, or obtain a pH stability profile. We generally use 1 or 5 ml disposable HiTrap sepharose Q or SP ion-exchange columns from GE Healthcare. Though not performed on every membrane protein, ion-exchange chromatography has proven to be a valuable technique and should be leveraged when needed.

The collective experience from the numerous IMP purifications that we have performed is that SEC is an essential step in the process of obtaining a PHSF sample. SEC allows one to rapidly evaluate the quality of the purified protein by analyzing the retention time, shape, and number of eluted peaks from the sample. Elution in the void volume of a properly sized SEC column (i.e., the void volume is significantly higher than the expected molecular weight of the target protein-micelle complex) is indicative of protein that is not stable under the prescribed solution conditions. Often this means a new solubilization buffer should be used with optimized parameters for detergent selection, pH, and salt concentration. If the target is present within the included volume then careful analysis of the peaks should be performed. Is the POI resident within a single, Gaussian shaped, peak or multiple peaks indicative of several oligomeric states? If the latter, it may shift to the void over time and, either way, is often indicative of stability issues within the specified buffer. Ideally, one will see a single well-defined peak within the included volume corresponding to (and verified by gels/blots) the POI. For a detailed discussion of membrane protein SEC characteristics refer to Figure 3 of [Newby *et al.* \(2009\)](#). Coupling fluorescence with SEC, termed fluorescence-detection SEC, is another approach that requires very small amounts of expressed protein and is therefore conducive for broad screens ([Kawate and Gouaux, 2006](#)). Troubleshooting is often required during the SEC purification step to ascertain the correct buffer conditions for stabilizing the protein in solution within a monodisperse peak. A standard approach to this process is varying pH (e.g., 5.5 in MES, 7.0 in HEPES, and 8.0 in Tris), salt concentration (e.g., 25 mM, 250 mM, and 500 mM NaCl), presence or absence of osmolytes (e.g., adding varying concentrations of glycerol or sucrose), and addition of putative or known ligands. It is important to note that when approaching this step one should be systematic and linear to clearly differentiate effects on protein stability and homogeneity.

In continuing with our example of expressing and purifying POI, we now have a Ni-purified and 3C-cleaved protein sample that has been purified away from cleaved affinity tag and protease. Next, we describe a general SEC purification step for this protein sample. There are a number of chromatography columns available and care should be taken to ensure that the column is appropriate for the desired task and will not interact with the detergent (e.g., TSK columns may interact with the detergent LDAO) or POI. We generally use a Superdex 200 10/300 GL column from GE

Healthcare (cat. no. 17-5175-01). This column has a separation molecular weight range of 10,000–600,000 that is ideally suited for most membrane proteins. The POI protein is now in the SEC buffer described above. It is important that the SEC column be equilibrated for a minimum of 3 h at 0.5 ml/min or overnight at 0.1 ml/min to ensure complete equilibration with the detergent (DDM in our example). Once equilibrated, the POI sample can be run in iterative rounds with a peak height of approximately one absorbance unit at 280 nm. The amount of loaded sample will vary depending on the presence of contaminating or oligomeric peaks. Generally, our approach is to use a chromatography station equipped with an auto-injector and fraction collector to enable automated runs, often overnight. Care should be taken that the column is not overloaded with sample, as this may mask secondary peaks and lead to incomplete purification. A common approach is to inject 0.5 ml of two OD A_{280} /ml sample per run, though the optimal injection amount is ultimately sample dependent.

Some general considerations regarding the purification step should be highlighted. In particular, when working with solubilized membrane proteins, the actual identity of a sample is a membrane protein with a detergent micelle surrounding it. This micelle will often contain endogenous lipids from the expression host. First, the protein–detergent–lipid complex (PDLC) will likely have a shorter retention time relative to a soluble protein of the same mass. Thus, it can be hard to ascertain the oligomeric state of a PDLC based upon SEC retention time alone. This holds true when comparing it to molecular weight standards because these standards are usually composed of small molecules and soluble proteins. In addition, IMPs tend to migrate slightly faster than expected on SDS–PAGE gels, giving the impression that your target is of a smaller mass than expected. Another common hurdle is that detergent micelles can occlude the protease recognition site when trying to remove an expression tag resulting in no, or attenuated, cleavage. Two common ways to avoid this potential problem are to add a short linker, often three additional amino acids, between the target protein and protease recognition site, or to move the expression tag to the other protein terminus. Moving the tag may lead to additional problems since approximately 30% of IMPs contain a signal peptide at the N-terminus. N-terminal tags can interfere with the processing of this signal peptide by the signal peptidase leading to retention of the membrane protein intracellularly and, as a result, decreased expression levels. Finally, when concentrating the purified protein, it should be remembered that the sample contains protein-free detergent micelles (since the detergent concentration is above the detergent CMC). Since these micelles can impact biophysical properties of the sample, such as crystallization, it is important that detailed notes be maintained regarding the concentration factor (i.e., starting volume relative to final volume postconcentration) for the

sample. If possible, one should generally work to minimize the concentration factor and thereby minimize the protein-free detergent micelles.

7. PROTOCOL—PROTEIN CHARACTERIZATION

Separating the protein from detergent micelles: The lack of absorbance at 280 nm by detergent micelles means that to separate the protein containing micelles from those that do not requires other detection strategies. We have found that an in-line four-way detection scheme is useful in differentiating these species and separating them from each other. These detectors consist of UV absorbance and refractive index (RI) detectors for measuring concentration, a differential pressure or intrinsic viscosity detector that indicates properties of size and shape, and a right angle light scattering detector that indicates molecular mass. In concert, these allow one to (1) optimize detergent micelle concentration while maintaining PDLC homogeneity of concentrated protein and (2) measure the PDLC oligomeric state (mass), size (R_h), shape (IV), detergent: protein ratio, and rate of change of RI (dn/dc). For common detergents, we have measured size-exclusion retention volume (SERV), dn/dc, micelle molecular weight, and retention behavior on different molecular weight cut-off filters for empty micelles in various systems. These micelle parameters are dependent on buffer composition, column type, detergent concentration, and the presence of PDLCs. The goal is to minimize the detergent micelle concentration during purification and concentration. The micelle SERV relative to the PDLC SERV dictates whether the PDLC can be concentrated before SEC (as they often have different SERVs), and if SEC can be used to remove excess micelles after protein concentration. Detergent dn/dc is used to quantify excess [detergent micelle] after protein concentration, and the amount of detergent bound in the PDLC. To accurately measure the PDLC physical parameters, the PDLC peaks must be baseline-resolved, of adequate intensity, and Gaussian with no comigrating excess micelles or other buffer contaminants (i.e., single SEC peaks for all four detectors). A simpler approach is to include an in-line RI detector to measure solution viscosity. These detectors can be added to existing chromatography stations with minimal alterations and will identify SERV values for the specific solubilization detergent and buffer combination being used. Overall, characterization of the PDLC within the prescribed detergent using the above methods facilitates the development of a robust purification and protein concentration scheme conducive to downstream endeavors. One should view PDLC and empty detergent micelles as separate entities during purification and work to identify the latter early within purification to minimize as needed.

8. CONCLUSION

S. cerevisiae is a viable and powerful system for overexpressing IMPs as yeast is a genetically tractable and inexpensive expression system that can be easily manipulated experimentally and is conducive for high-, medium-, or low-throughput methodologies. Furthermore, being a eukaryotic organism it contains the necessary posttranslational modification and membrane targeting machinery to facilitate expression of many higher eukaryotic IMPs (Li *et al.*, 2009). The methods described in this chapter are focused on the overexpression and purification of a nominated IMP within yeast. Subsequent purification of these proteins can be accomplished if one takes appropriate caution and is aware of common hurdles. Whenever possible, functional assays should be incorporated into the purification protocol to ensure the POI being purified is in functional form. As the collective knowledge and experience in working with IMPs increases so have the rewards and novel biological insights. Indeed, the outlook is very positive (White, 2009). With so little known about the vast majority of IMPs, we are undoubtedly entering a period of dramatic growth in our understanding.

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