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Efficient solubilization and purification of highly insoluble membrane proteins expressed as inclusion bodies using perfluorooctanoic acid

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A B S T R A C T

The purification of membrane proteins can be challenging due to their low solubility in conventional detergents and/or chaotropic solutions. The introduction of fusion systems that promote the formation of inclusion bodies has facilitated the over-expression of membrane proteins. In this protocol, we describe the use of perfluorooctanoic acid (PFOA) as an aid in the purification of highly hydrophobic membrane proteins expressed as inclusion bodies. The advantage of utilizing PFOA is threefold: first, PFOA is able to reliably solubilize inclusion bodies, second, PFOA is compatible with nickel affinity chromatography, and third, PFOA can be efficiently dialyzed away to produce a detergent free sample. To demonstrate the utility of employing PFOA, we expressed and purified a segment of the extremely hydrophobic membrane protein caveolin-1.

1. Introduction

Membrane proteins are major players in cellular biology. They are responsible for a plethora of cellular functions such as signal transduction and transport [1]. Additionally, a large number of drug targets have been identified as membrane proteins, indicating that these proteins are heavily involved in normal cell function [2]. However, the purification and analysis of these membrane proteins can be challenging because of their highly hydrophobic characteristics and strong propensity to aggregate [3]. In addition, recombinant expression of membrane proteins usually results in low yields due to stresses put on the host membrane which results in toxicity. However, the over-expression of membrane proteins into inclusion bodies has emerged as a powerful tool to achieve high levels of protein in *E. coli* cells by eliminating the toxicity issues mentioned above which limit protein production [4]. Inclusion bodies can be isolated through a series of wash treatments that separate them from the soluble and membrane components of the host cell. While this insolubility is an attractive feature in aiding the isolation of the protein, it can be an obstacle when it comes to solubilizing the protein. Typically strong chaotropic solutions such as 8 M urea or 6 M guanadinium hydrochloride are utilized. However, for highly hydrophobic membrane proteins, these solutions are often not powerful enough to completely dissolve the inclusion bodies. Alternatively, strong detergents such as sodium dodecyl sulfate are attractive, but many of these detergents are not compatible with widely used purification techniques such as nickel affinity chromatography. Furthermore, it is often difficult, if not impossible, to remove these harsh detergents from the sample, which can be problematic, as it is often desirable to acquire experimental results in the presence of a native-like detergent and lipid systems.

Perfluorooctanoic acid (PFOA) is a powerful detergent that has been shown to have the ability to solubilize membrane proteins (Fig. 1) [5,6]. In this report, we extend the utility of PFOA by showing that it can dissolve highly hydrophobic membrane proteins expressed as inclusion bodies. Furthermore, it is compatible with nickel affinity chromatography, and can be easily removed by dialysis, providing a detergent-free precipitate that can then be solubilized in a detergent or lipid system of choice. To demonstrate the usefulness of PFOA, we detail the purification of the integral membrane protein caveolin-1 from inclusion bodies. Caveolin-1 is the preeminent protein in membrane invaginations called caveolae, which has been shown to crucial for caveolae formation, signal transduction, mechano-protection, and endocytosis; however studies of this protein have been hindered by its extremely hydrophobic character [7–10]. Because of this, caveolin-1 is an ideal candidate to demonstrate the utility of PFOA.

2. Materials and methods

Perfluorooctanoic acid was purchased from Synquest laboratories (Alachua, FL). PFOA has been shown to exhibit relatively low toxicity

Abbreviations: PFOA, perfluorooctanoic acid; CMC, critical micelle concentration

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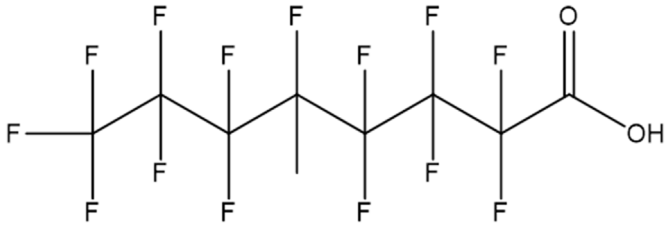

Fig. 1. Structure of perfluorooctanoic acid (PFOA).

effects; therefore, standard chemical hygiene protocols are sufficient [11]. Dialysis tubing was purchased from Spectrum laboratories (Rancho Dominguez, CA). Ni Sepharose 6 Fast Flow resin was purchased from GE Health Sciences (Piscataway, NJ). All other reagents were of standard ACS grade.

2.1. Protein expression

H9_TrpLE_caveolin-1_62–178 was cloned into the pET-24a vector, and transformed into BL21 (DE3) cells. 1 mL of an overnight culture (20 h) in MDG media was used to inoculate 1 L of ZYM-5052 media [12]. The culture was shaken at 250 rpm on an orbital shaker at 37 °C for 12–14 h. Cells were harvested at 8200 × *g* for 15 min at 4 °C, re-suspended in 0.9% (w/v) NaCl, and re-centrifuged at 5000 × *g* for 30 min at 4 °C. Pellets were stored at −80 °C until needed.

2.2. Protein purification

1 L cell pellets were resuspended in 200 mL of a buffer containing 20% (w/v) sucrose, 10 mM Tris pH 8.0, 1 mM EDTA and 50 mM BME. Cells were lysed by sonication in a Branson Sonifier 450 for 15 min (power level 40 and duty cycle 5) with stirring at 4 °C. Next, the lysis was centrifuged for 2 h at 27,500 × *g* at 4 °C. The supernatant was removed, and the pellet was resuspended in 200 mL of a buffer containing 1% (v/v) Triton X-100, 10 mM Tris pH 8.0 followed by sonication for 15 min with stirring at 4 °C. The mixture was centrifuged at 27,500 × *g* for 1 h at 4 °C. The supernatant was discarded, and the remaining pellet contained the isolated inclusion bodies.

2.3. Solubilization of inclusion bodies

Isolated inclusion bodies were dissolved in 40 mL of 8% (w/v) PFOA, 25 mM phosphate pH 8.0 and homogenized using a dounce homogenizer. The solution was then centrifuged at 50,000 × *g* for 30 min at 22 °C. The supernatant contained the solubilized inclusion bodies.

2.4. Ni-NTA purification

After solubilization into 8% (w/v) PFOA, the supernatant was filtered through a 0.2 μm filter and loaded onto a column containing 20 mL of Ni sepharose 6 resin. The column was washed with approximately 5 column volumes of 1% (w/v) PFOA, 25 mM phosphate pH 8.0, or until the absorbance at 280 nm was steady, to remove any unbound protein. Samples were eluted in the presence of 1% (w/v) PFOA, 25 mM phosphate pH 8.0, 250 mM imidazole.

2.5. Dialysis

The most concentrated column fractions were pooled, and placed in 10,000 MWCO dialysis tubing. Samples were dialyzed against 20 L of 50 mM ammonium sulfate for 24 h at room temperature with stirring. Precipitated protein was isolated by centrifugation at 4000 × *g* for 30 min at 22 °C. The pellet contained the purified precipitated protein.

3. Results and discussion

3.1. Inclusion body production and solubilization

Inclusion bodies have emerged as a powerful tool to obtain high levels of membrane proteins expressed in *E. coli* cells. One of the challenges of membrane protein expression, especially of a non-native membrane proteins, is that the over-expressed protein can crowd the membrane and become toxic to the bacterial cell [13]. This leads to low protein expression that can make protein isolation very challenging due to the high background of endogenous host proteins. However, the fusion of a membrane protein to particular proteins will cause the protein of interest to be rapidly expressed in an unfolded state, and incorporated into insoluble cytoplasmic aggregates (i.e. inclusion bodies). One of the common proteins utilized to promote inclusion body formation is trp leader (trpLE) which has been shown to result in significantly enhanced membrane protein expression [14]. Advantageously, the properties of these aggregates can then be exploited to extract the protein of interest. First, the cells are lysed in a buffer containing sucrose which removes soluble cellular components. After centrifugation, this leaves a pellet that contains only the inclusion bodies and other hydrophobic membrane components. These hydrophobic membrane components can be removed via a second lysis step that utilizes a buffer containing a mild detergent (in this case Triton X-100). Since Triton X-100 will not solubilize the inclusion bodies, after centrifugation, the majority of the pellet contains inclusion bodies.

Normally, once the inclusion bodies have been isolated from the whole cell milieu, they are solubilized in either 8 M urea or 6 M guanidinium hydrochloride. However, in the case of highly insoluble transmembrane domains, even solutions of these strong chaotropic agents cannot effectively solubilize the inclusion bodies. For example, when the membrane interacting domain of caveolin-1 is expressed with the fusion protein trp leader, the inclusion body pellet is not soluble in either 8 M urea or 6 M guanidinium hydrochloride (data not shown). The membrane interacting domain of caveolin-1 has a GRAVY score of 0.659 which is an index of the overall hydrophobicity of a protein [15]. Higher GRAVY scores indicate a more hydrophobic protein while lower scores (negative) indicate a more hydrophilic protein. Therefore, the GRAVY score can be used as a benchmark, and based on our data, membrane proteins with GRAVY scores greater than 0.5 are ideal candidates for PFOA solubilization. For this reason, the conventional methodology for processing inclusion bodies was not applicable. It was determined that a solution of 8% (w/v) PFOA was optimal for efficiently and rapidly solubilizing inclusion bodies of all types and hydrophobicities (Fig. 2). However, caution should be taken when employing solutions with lower concentrations of PFOA as they may not completely solubilize the inclusion body pellet. Additionally, we find that in some cases, the addition of 1% (w/v) PFOA to a solution containing 8 M urea can significantly enhance the ability of 8 M urea to solubilize hydrophobic inclusion bodies, thereby decreasing the need for a very high detergent concentration.

The pKa of PFOA has been reported to be between 2.8 and 3. However, there is a study that has determined the pKa to be substantially lower approximately −0.5 [16,17]. Whichever value is most representative, the low pKa of PFOA is an advantage because it will not interfere with typical buffering agents in the 4 to 12 range. It is also important to note that PFOA can form precipitates with potassium counterions, therefore, buffer conditions are limited to the use of sodium or ammonium counter ions. Additionally, it should be noted that due to the exclusionary fluorous nature of PFOA, it is incompatible with certain detergents, such as Empigen BB, so one should test miscibility before working with other detergents. In particular, detergents with positively charged groups are likely to form insoluble precipitates. However, non-ionic detergents such as Triton X-100 are compatible. Similarly, guanadinium hydrochloride is not compatible with PFOA as it forms a precipitate.