Solubilization, Folding, and Purification of a Recombinant Peptidoglycan-Associated Lipoprotein (PAL) Expressed in *Escherichia coli*

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Studies aiming at heterologous expression of highly hydrophobic proteins, such as outer membrane proteins in general and peptidoglycan-associated lipoprotein (PAL) in particular, are not trivial due to difficulties in obtaining recombinant protein in a soluble state, which is desired because it allows purification by traditional chromatographic methods. PAL is associated with the integrity of the cellular envelope in Gram-negative bacteria and interacts strongly with the peptidoglycan layer. However, it is incorporated into inclusion bodies in studies focusing on its heterologous production. This protocol describes an efficient protein refolding method to solubilize and purify a recombinant PAL. Initially, recombinant PAL–enriched inclusion bodies obtained after the induction of PAL expression in *Escherichia coli* are treated with 8 M urea and then undergo buffer exchange via dialysis. Afterward, the soluble, recombinant PAL is purified using standard chromatographic methods. © 2018 by John Wiley & Sons, Inc.

Keywords: inclusion bodies • outer membrane protein • PAL • protein refolding • protein solubilization • protein purification • recombinant protein

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INTRODUCTION

Heterologous expression and purification of membrane proteins or proteins rich in hydrophobic regions is challenging. Despite the most recent advances in protein engineering, the formation of inclusion bodies, especially when employing an *Escherichia coli*-based protein expression system, is common. Optimizing the conditions for protein expression by using different bacterial strains, expression vectors, and induction conditions (temperature, inducing agent, and time) is generally the first approach attempted to successfully obtain a target protein. Although the soluble expression of a protein aids its purification by chromatographic methods, the conditions that permit soluble expression are not always found. Consequently, alternative methodologies for protein solubilization and purification, i.e., so-called protein refolding, are excellent options for obtaining proteins that are not easily produced by standard methods (Basu, Li, & Leong, 2011; Su et al., 2011; UNIT 6.5; Wingfield, Palmer, & Liang, 2014).

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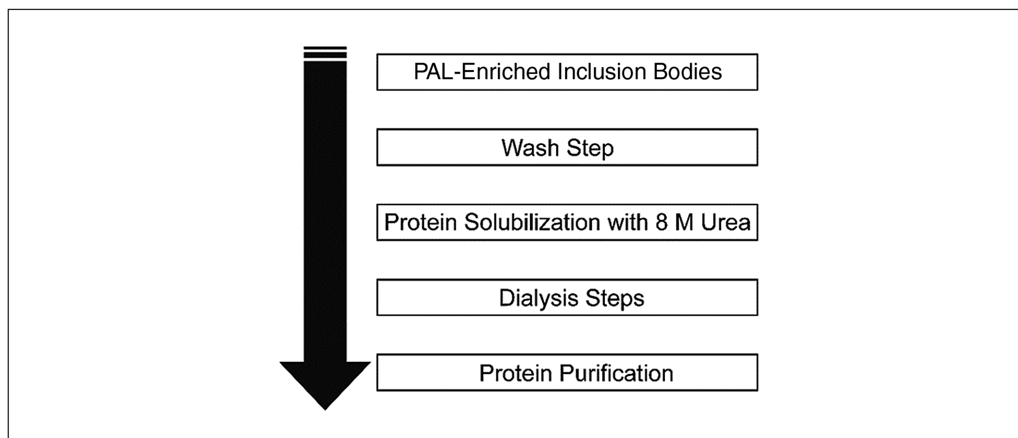

Figure 6.17.1 Schematic sequence of the steps for solubilization and purification of PAL over-expressed in *E. coli*, starting from PAL-enriched inclusion bodies.

Peptidoglycan-associated lipoprotein (PAL) is a challenging protein due to its highly hydrophobic character (Santos et al., 2012). PAL is anchored in the outer membrane of Gram-negative bacteria via an N-terminal helix, whereas its C-terminus interacts with the peptidoglycan layer (Godlewska, Wiśniewska, Pietras, & Jagusztyn-Krynicka, 2009; Parsons, Lin, & Orban, 2006). Of the various roles of PAL, maintaining the integrity of the bacterial cell envelope appears to be its major role (Cascales, Bernadac, Gavioli, Lazzaroni, & Lloubes, 2002); however, this function is poorly understood, particularly due to difficulties in recombinant PAL production.

The basic protocol below describes an efficient method to solubilize and purify PAL from PAL-enriched inclusion bodies (Fig. 6.17.1). First, pellets are washed and then denatured using an 8 M urea solution. Second, the protein is refolded during buffer exchange via dialysis, and the soluble fraction is purified using chromatographic methods. The additional support protocol describes the preparation of the PAL-enriched inclusion bodies.

BASIC PROTOCOL

SOLUBILIZATION, FOLDING, AND PURIFICATION OF A RECOMBINANT PEPTIDOGLYCAN-ASSOCIATED LIPOPROTEIN EXPRESSED IN *ESCHERICHIA COLI*

This protocol involves three stages: (a) preparing pellets of washed PAL-enriched inclusion bodies, (b) solubilizing and refolding recombinant protein from the washed inclusion bodies, and (c) purifying the protein solubilized from the inclusion bodies. The protocol specifically describes how to extract, fold, and purify recombinant PAL from inclusion bodies at a high yield using 8 M urea solution.

Materials

Pellets of PAL-enriched inclusion bodies (see Support Protocol)
Wash buffer with and without urea (recipe), 4°C
8 M urea solution (see recipe)
Refolding buffer (see recipe), 4°C

50-, 100-, 1000-, and 4000-ml beakers
Cole-Parmer 4710 ultrasonic homogenizer (or equivalent)
Sorvall RC-5C Plus Superspeed Centrifuge set to 4°C and SS-34 and GSA rotors (or equivalents)
Sorvall SS-34 and GSA rotor tubes (or equivalents)
Pasteur pipet

Biometra WT 16 orbital shaker (or equivalent)
Spectra/Por® 3 dialysis tubing, MWCO 3500 (or equivalent)

Additional reagents and equipment for standard chromatographic techniques [e.g., *UNIT 9.4* (Petty, 2001) and *UNIT 8.3* (Hagel, 2001)] and SDS-PAGE (*UNIT 10.1*; Gallagher, 2012)

Prepare pellets of washed PAL-enriched inclusion bodies

1. Thaw pellets of PAL-enriched inclusion bodies on ice for ~10 min and then resuspend pellets in cold wash buffer with urea, using ~10 ml buffer per gram wet weight of the inclusion bodies. Place each resuspended pellet in a 50-ml beaker and keep suspensions cool by placing the beakers on ice. Incubate beakers on ice for 5 min.

2. Sonicate resuspended pellets four times for 50 sec each at 70% of the maximum power of an ultrasonic homogenizer. Keep suspensions cool on ice and allow a 2-min rest interval between sonication steps.

   Carefully homogenize the pellets during the wash step. The complete removal of soluble proteins and cellular components increases the protein yield and purity at the end of the process.

3. Transfer suspensions to SS-34 rotor tubes and centrifuge 40 min at 27,000 × g, 4°C. Discard supernatants and, using the homogenizer, resuspend pellets in cold wash buffer with urea using the same ratio of buffer volume to gram wet weight of the inclusion bodies as in step 1.

4. Repeat steps 2 and 3 two more times.

5. Resuspend washed pellets in cold wash buffer without urea, using ~3.33 ml wash buffer per gram wet weight of the inclusion bodies as in step 1.

   This protocol was developed using inclusion bodies derived from 1 L bacterial culture and produced as described in the support protocol. Thus, the washed pallets were resuspended in a total of 10 ml wash buffer because ~3 g wet weight of PAL-enriched inclusion bodies was used in step 1.

Solubilize and refold recombinant protein from washed inclusion bodies

6. Divide 10 ml washed and resuspended pellets into two 100-ml beakers. Place beakers on an orbital shaker at 30 rpm at room temperature.

7. Using a Pasteur pipet, slowly add 8 M urea solution dropwise to suspensions.

   The freshly prepared 8 M urea should be added until a transparent and homogeneous solution is obtained. Approximately 40 ml of 8 M urea will be added to each beaker.

8. Dialyze denatured pellet samples against cold refolding buffer using a two-step dialysis procedure:

   a. Dialyze at a 1:10 sample/buffer ratio at 4°C for 4 hr in a 1000-ml beaker.
   b. Dialyze at a 1:100 sample/buffer ratio at 4°C for 16 hr in a 4000-ml beaker.

   Follow the instructions provided by the dialysis membrane manufacturer before proceeding with the dialysis step. Dialysis membranes are usually sold embedded in glycerol, which needs to be removed before use to ensure efficient buffer exchange.

9. Collect dialyzed solutions into SS-34 rotor tubes and recover solubilized protein by centrifugation for 40 min at 27,000 × g, 4°C.

   It is also recommended that the solution be filtered through a 0.22-μm filter after the centrifugation step.

Purify protein solubilized from inclusion bodies

10. Purify recovered soluble protein using standard chromatographic techniques and evaluate results by SDS-PAGE.

    *The purification of the target protein will depend on the type of tag that was initially chosen. Xylella fastidiosa PAL, which was solubilized and purified using this protocol, was originally cloned into a pET-type vector that adds a C-terminal His tag to the protein sequence; thus, nickel affinity chromatography (*UNIT 9.4*; Petty, 2001) was the most appropriate technique for this protein's purification. However, because this process features a refolding procedure that generates aberrant folded states (unfolding, misfolding, oligomerization, etc.), an additional purification step using size-exclusion chromatography (*UNIT 8.3*; Hagel, 2001) was necessary to further refine the protein product.*

SUPPORT PROTOCOL

PREPARATION OF INCLUSION BODIES ENRICHED IN PEPTIDOGLYCAN-ASSOCIATED LIPOPROTEIN

Three steps are described here: (a) overexpressing recombinant PAL in *E. coli*; (b) lysing the PAL-overexpressing *E. coli* cells; and (c) recovering the pellets of PAL-enriched inclusion bodies, which are used in the basic protocol.

Additional Materials *(also see Basic Protocol)*

E. coli BL21 (DE3) strain carrying PAL gene cloned into pET29a(+) (Novagen) expression vector (in petri dish or as frozen stock culture)
LB broth (see recipe)
30 mg/ml kanamycin stock solution (see recipe)
0.5 M isopropyl-β-D-thiogalactopyranoside (IPTG) stock solution (see recipe)
Lysis buffer (see recipe), 4°C

Sterile 15-ml culture tubes
Innova 4430 incubator shaker (or equivalent) set to 37°C
2000-ml Erlenmeyer flasks
Ultrospec 1100 pro UV/visible spectrophotometer (or equivalent)

Overexpress recombinant PAL in E. coli

1. In the late afternoon, prepare two sterile 15-ml culture tubes, each containing 3 ml LB broth supplemented with 30 µg/ml kanamycin. Inoculate each culture with a single colony from a petri dish of *E. coli* cells carrying the PAL gene cloned into the pET29a(+) expression vector or a 3-µl aliquot of a frozen stock culture. Incubate cultures overnight with shaking at 300 rpm and 37°C.

2. The next morning, transfer each pre-inoculum to a 2000-ml Erlenmeyer flask with 500 ml LB broth containing 30 µg/ml kanamycin and grow cultures at 300 rpm and 37°C using the shaker until an OD_{600} of 0.6 to 0.8 is achieved. Use a spectrophotometer to monitor bacterial growth by measuring the OD_{600} every 30 min.

3. Induce PAL expression by adding 0.4 M IPTG to each of the two 500-ml bacterial cultures and cultivate cultures for 4 hr at 300 rpm and 37°C.

4. Harvest two 500-ml bacterial cultures by transfer to GSA rotor tubes and centrifugation for 15 min at 3000 × *g*, 4°C.

   At the time of harvest, PAL expression is expected to have been induced.

Lyse PAL-overexpressing E. coli cells

5. Resuspend obtained cell pellets in 50 ml cold lysis buffer.

   CAUTION: *The lysis step employs the protease inhibitor phenylmethylsulfonyl fluoride (PMSF). PMSF is a very hazardous substance that can cause severe damage to the eyes*

and is toxic if swallowed, as it liberates toxic gas and causes burns. Wear gloves and personal protective equipment when handling it.

6. Incubate resuspensions on ice for 30 min.

7. Disrupt cells by sonication with an ultrasonic homogenizer eight times for 50 sec each at 70% of the maximum power and use intervals of 2 min between sonication steps. Keep samples on ice at all times.

Recover pellets of PAL-enriched inclusion bodies

8. Collect pellets of PAL-enriched inclusion bodies by centrifuging for 40 min at 27,000 × *g*, 4°C, and then discarding the supernatant fraction.

9. Store pellets of PAL-enriched inclusion bodies by freezing them directly at –20°C until use.

REAGENTS AND SOLUTIONS

Use Milli-Q-purified water or its equivalent in all recipes and protocol steps. For common stock solutions, see APPENDIX 2E.

Isopropyl-β-D-thiogalactopyranoside (IPTG) stock solution, 0.5 M

Dissolve 1.8 g IPTG in 15 ml sterilized water. Pass through a 0.22-µm filter and aliquot. Store at –20°C for ≤2 months.

Kanamycin stock solution, 30 mg/ml

Dissolve 0.3 g kanamycin in 10 ml sterilized water. Pass through a 0.22-µm filter and aliquot. Store at –20°C for ≤1 year.

LB broth

10 g tryptone
5 g yeast extract
10 g NaCl
Dissolve in 800 ml H_2O
Adjust to 1 L with H_2O
Sterilize by autoclaving at 121°C for 20 min
Store at room temperature for ≤1 year

Lysis buffer

50 mM Tris·Cl, pH 8.0 (*APPENDIX 2E*)
300 mM NaCl
1 mg/ml lysozyme
1 mM PMSF
Store at 4°C for ≤1 week without lysozyme or PMSF
Add lysozyme and PMSF to other components immediately before use

Refolding buffer

50 mM Tris·Cl, pH 8.0 (*APPENDIX 2E*)
300 mM NaCl
0.1 mM EDTA
10% (v/v) glycerol
Store at 4°C for ≤1 week

Urea solution, 8 M

Dissolve 48 g urea in 100 ml sterilized water. Prepare fresh immediately before use.

The dissolution of urea in water is an endothermic reaction, so the solution will initially become cold to the touch. A 30°C water bath and periodic mixing will aid in complete dissolution of the powdered urea.

CAUTION: *Do not raise the temperature of the solution to >30°C because cyanates can form. Cyanates and their thiol derivatives, besides being cytotoxic, can cause protein modifications. Therefore, urea solutions should always be freshly prepared for use.*

Wash buffer with and without urea

50 mM Tris·Cl, pH 8.0 (*APPENDIX 2E*)
300 mM NaCl
1 M urea (60 g/L)
Store at 4°C for ≤1 week without urea
If required, add urea to other components immediately before use

COMMENTARY

Background Information

The search for and optimization of new methodologies and protocols to solubilize and purify recombinant proteins that are usually incorporated into inclusion bodies are especially challenging when the target protein has a history of being difficult to produce as a soluble fraction, e.g., membrane proteins and highly hydrophobic proteins (Su, Lu, & Liu, 2011; *UNIT 6.5*; Wingfield et al., 2014).

A protein refolding protocol is successful when it produces sufficient protein to support the execution of functional and structural experiments. Thus, the efficiency of a refolding protocol is directly proportional to the yield of active, refolded protein.

PAL and other outer membrane proteins are commonly incorporated into inclusion bodies after their heterologous overexpression in *E. coli* (Santos et al., 2012). Not surprisingly, only studies in which solely the periplasmic portion of PAL was expressed have succeeded in obtaining the protein as a soluble fraction (Bonsor, Grishkovskaya, Dodson, & Kleanthous, 2007; Loftus et al., 2006).

A well-known protocol for the solubilization and purification of a bacterial wild-type PAL was first developed in 1988 (Zlotnick et al., 1988). However, although that protocol uses several ultracentrifugation steps, heat treatment, and detergents, it results in a low protein yield and is not appropriate for recombinant PAL–enriched inclusion bodies that are produced in *E. coli*. When using a heterologous protein expression system, the yield of recombinant PAL produced is much greater than the production of native PAL expressed by the host; this fact motivated us to develop a new methodology to produce recombinant PAL at a high yield. In this way, the protocol described herein is powerful because it produces a high yield of purified recombinant PAL per liter of bacterial culture and can support functional and structural characterization of PAL. This protocol is also recommended to be applied to the production of other outer membrane proteins that are challenging to obtain.

Critical Parameters and Troubleshooting

Preparing washed pellets of inclusion bodies

Washing of the PAL-enriched inclusion bodies is a key step toward complete protein solubilization. First, ensure that the pellets of inclusion bodies are completely dissolved in the wash buffer, which contains 1 M urea. Incubation of the pellets with the wash buffer at room temperature for 10 to 15 min may aid in homogenization, especially if the pellets were stored frozen; regardless, place the pellets on ice for a few minutes before beginning the sonication step.

Care must be taken with suspension of the pellets every time that they are resuspended in the wash buffer. After each sonication and centrifugation cycle, the pellets become more resistant to resuspension. The sonication step may also aid in dissolution of the pellets; however, do not proceed with centrifugation if the pellets appear grumous in the resuspension solution. An additional sonication step (or several) is recommended to ensure complete dissolution of the pellets of PAL-enriched inclusion bodies.

During the wash step, almost all soluble protein and cellular materials are removed from the pellets of inclusion bodies. Thus, if this step is not effectively performed, problems may occur during the subsequent steps,

including solubilization with 8 M urea and chromatography, resulting in a low yield and low purity of the refolded protein.

Although the storage of pellets of PAL-enriched inclusion bodies may be appropriate (see Support Protocol, step 9), use of the pellets directly after their production generally eases the dissolution process compared with starting with frozen inclusion bodies.

Solubilizing protein from urea-washed inclusion bodies

The successful solubilization of PAL from the washed and resuspended inclusion bodies is a slow process and must be performed by dropwise addition of 8 M urea solution using a Pasteur pipet while keeping the pellets resuspended by orbital shaking. If the 8 M urea solution is rapidly added to the suspension, the hydrophobic portions of the protein may be exposed to the aqueous solution prematurely, leading to precipitation, depending on the electrostatic interactions that stabilize the protein (Monera, Kay, & Hodges, 1994). The problems resulting from an inefficient solubilization step can be recognized after complete addition of the urea solution; a non-transparent and homogeneous solution should be evident after urea addition, and after dialysis, a large amount of precipitated protein should be observed inside the dialysis tubing. Accordingly, do not rush the dropwise addition of the 8 M urea solution.

A second critical point in protein solubilization is the dialysis-based buffer exchange, which removes excess urea. Two aspects of this topic must be addressed. First, the urea must be removed slowly; thus, we strongly recommend a two-step dialysis procedure, as described in step 8 of the basic protocol. If only the 1:100 dialysis step is used, the rapid removal of urea will not allow the denatured protein enough time to fold properly, which will compromise the final yield of the process because a large amount of the protein that is solubilized will precipitate again. The second aspect to consider is the choice of the correct dialysis (refolding) buffer. After extensive practical investigation, we have observed that the ideal buffer for PAL solubilization must contain an agent capable of stabilizing the hydrophobic regions [10% (v/v) glycerol] and an agent capable of preventing degradation by proteases (EDTA); however, the choice of buffer also depends on the intrinsic characteristics of the target protein (Basu et al., 2011). If a large amount of aggregated protein is observed in the post-dialysis centrifugation

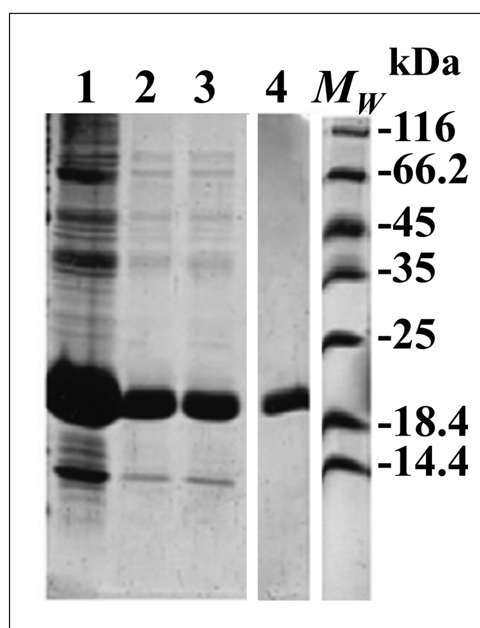

Figure 6.17.2 Expected SDS-PAGE results following solubilization, folding, and purification of PAL using the basic protocol. A 13.5% polyacrylamide gel stained with Coomassie blue is shown. Lane 1, PAL-enriched inclusion bodies; lane 2, washed PAL-enriched inclusion bodies; lane 3, denatured washed PAL-enriched inclusion bodies; lane 4, purified recombinant PAL; M_W: molecular-weight markers (kDa).

sample, the dialysis buffer must be optimized. Varying the ionic strength and pH or supplementing the solution with other additives may be useful (Basu et al., 2011). The use of online programs to evaluate the amino-acid content and the degree of hydrophobicity of the protein can be of great value when defining the dialysis buffer.

We choose not to use detergents or reducing agents at any stage of the protocol because such components, and especially reducing agents, may interfere with PAL activity. However, detergents can be used for membrane proteins. For the solubilization and purification of recombinant PAL using this protocol, whether detergent is used does not lead to significant differences in protein yield, so we choose not to use it.

Anticipated Results

The yield and purity of the refolded PAL obtained using the basic protocol will depend on correct execution of the refolding steps. Nearly 20 mg purified recombinant PAL can be obtained per liter of bacterial culture. The purity of the recombinant PAL after the purification steps can reach 98%, as reflected by SDS-PAGE analysis (Fig. 6.17.2). Taken

together, these results are more than enough to support many biochemical and structural studies.

The stability of the refolded purified protein can vary depending on the protein characteristics. Usually, PAL has several cysteine residues, which, because of the oxidization-reduction process, can accelerate protein degradation. In general, aliquots of the protein can be stored at –80°C in the dialysis buffer for ≤6 months. To prevent degradation due to freeze-thaw cycles, the samples should be divided into small aliquots for freezing and should not be refrozen.

Time Considerations

When the pellets of PAL-enriched inclusion bodies have already been prepared, the time required to produce washed PAL-enriched pellets and to solubilize and purify PAL (see Basic Protocol) will be ~2 days. The time required to only produce the pellets of PAL-enriched inclusion bodies (see Support Protocol) is ~1 day. These estimates of time assume that all requirements for the purification steps, including the preparation of the chromatographic columns and the buffers to be used in the purification, were previously completed. Adding time (1 day) may be required if a second chromatography step is necessary, as another round of analysis by SDS-PAGE would also be incorporated (see Basic Protocol). If the dialysis buffer (see Basic Protocol) requires optimization, at least 1 month should be dedicated exclusively to this step. Buffer optimization is undoubtedly a procedure that helps to guarantee a successful refolding procedure.

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Conflicts of Interest

The authors declare that they have no competing interests.

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Key References

Santos et al. (2012). See above.

Original publication on which the basic protocol is based.

Zlotnick et al. (1988). See above.

Source of the protocol for the solubilization and purification of a bacterial wild-type PAL.

Internet Resources

https://www.expasy.org/proteomics.

Source of scientific databases and software tools in proteomics.