Chapter 1

Protein Antigen Expression in *Escherichia coli* for Antibody Production

David M. Rancour, Steven K. Backues, and Sebastian Y. Bednarek

Abstract

Escherichia coli is a frequently used expression system for the generation of protein encoded by genes from diverse kingdoms and, thus, it is well suited for the production of protein antigens for antibody generation. It is a system of choice for many due to factors such as (1) the commercial availability of a vast array of reagents and materials needed for cloning, expression, and purification and (2) the potential high protein yields that can be acquired in a timely and cost-effective manner. This chapter will focus on (1) the general principles to keep in mind when choosing an antigen to express and (2) the use of a modified pGEX vector system (Rancour et al., *J. Biol. Chem.* 279:54264–54274, 2004) to use in its expression. Simplified protocols are provided for (1) assessing the expression of your protein, (2) testing whether your protein is or is not expressed as a soluble product, (3) performing bulk purifications of soluble or insoluble *E. coli*-expressed protein to acquire enough to be used for a complete immunization protocol, and (4) an optional procedure for epitope tag removal from your expressed protein of interest in order to avoid the unnecessary and sometimes unwanted production of antibodies against the fusion protein affinity chromatography tag. These four procedures have been used extensively and successfully in our lab as a basis for the production of recombinant protein and subsequent antibody production.

Key words: *Escherichia coli*, protein, expression, purification, glutathione-*S*-transferase, GST, epitope tag, antigen, tobacco etch virus (TEV) protease.

1. Introduction

Protein expression in *Escherichia coli* is a frequently used tool for the generation of protein encoded by genes from diverse kingdoms and, thus, it is well suited for the production of protein antigens for antibody generation. To facilitate the generation of useful antibodies against a target protein, there are several characteristics of the protein that need to be assessed prior to commencing with

S.D. Schwartzbach, T. Osafune (eds.), *Immunoelectron Microscopy*, Methods in Molecular Biology 657,
DOI 10.1007/978-1-60761-783-9_1,

expression. Taking the following details into consideration while determining the best antigen to express will greatly increase the chance that quality antibodies will be generated.

The first consideration is whether the protein of interest is known or predicted to be a soluble, a peripheral membrane, or an integral membrane protein. Soluble proteins or domains are much easier to work with in terms of expression, purification, and, in some case, immunization protocols. For peripheral and integral membrane proteins, select cDNA fragments predicted to encode hydrophilic soluble domains of the protein of interest (i.e., domains lacking long contiguous hydrophobic amino acid stretches or transmembrane segments as predicted by hydropathicity analysis using the Kyte–Doolittle (1) and/or Hopp–Woods (2) algorithms or utilizing newer methods of soluble protein prediction such as the method of Smialowski et al. (3)). Insoluble proteins can also be expressed and enriched as inclusion bodies in *E. coli* and then subsequently used for immunization. However, soluble proteins or protein domains most likely best represent the native folding organization of the protein in its cellular context and thus would be the optimal antigen to produce antibodies that would be used for immunocytochemistry. Additional drawbacks to using insoluble proteins include the purity of the protein not being high enough to generate specific anti-sera against the antigen, and affinity purification of specific antibodies becomes problematic (but not impossible) using the original antigen. Insoluble proteins can limit the use of standard liquid chromatography purification methods sans attempts at denaturation and renaturation procedures. Therefore, soluble proteins or domains should be the first choice for easy antigen production and purification to ensure a clean antigen to induce a specific immunological response.

The second consideration is whether the protein of interest is post-translationally modified. If so, does this modification influence its functionality and/or localization? Proteins from eukaryotes are commonly post-translationally modified. For example, in cases where these proteins are extensively decorated with carbohydrates, these modifications may adversely influence the reactivity of antibodies raised against an unmodified protein expressed in *E. coli*. Conversely, if you are interested in antibodies against antigens with specific modifications, you will need to keep in mind that most eukaryotic cell modifications do not take place in *E. coli* and thus you will need to seek other methods for antigen production.

The third consideration is whether the protein of interest is either a homo- or hetero-oligomer. Oligomerization may influence the ability to express the protein of interest as a soluble protein. A self-assembling soluble homo-oligomer [i.e., AtCDC48A (4)] is much easier to produce than a subunit of a multimeric,

membrane-associated complex that needs specific chaperones for assembly. In the latter case, choosing a soluble domain fragment of the protein may be a better antigen choice.

The fourth consideration is whether the antigen is a product of a conserved gene family or relatively unique. To generate isoform-specific antibodies, expression of sequence divergent domains will be required. In cases where the divergent amino acid sequence is limited to short stretches (12–15 aa), synthetic peptides coupled to a carrier protein may be a better antigen choice. Alternatively, choosing a fragment of the protein that does not contain a highly conserve protein domain (e.g., Walker ATPase) could aid in minimizing cross-reactivity of your anti-serum.

Protein expression vectors for *E. coli* typically differ in the promoter/repressor system used for gene expression regulation and the type/position of epitope tags translationally fused to the protein product and are available from various commercial and academic sources. A modified pGEX4T plasmid expression system (4) encoding N-terminally fused glutathione-*S*-transferase (~26-kDa soluble protein from *Schistosoma japonicum*) is used in these protocols. The GST epitope tag has been shown to aid in increasing protein solubility, facilitating purification (5, 6), and allowing for higher yields (7). The Rosetta™ (DE3) pLysS *E. coli* strain (Novagen-EMD Biosciences) is used for protein expression in these protocols because the strain is protease deficient and contains a plasmid encoding six tRNAs underutilized by *E. coli* to alleviate codon bias which sometimes reduces protein expression of genes from divergent organismal origins.

The epitope tag is normally removed from an antigen to minimize the incidental production of antibodies to the tag in addition to the desired antigen. Removal of the epitope tag prior to use as an immunogen also facilitates subsequent affinity purification of antibodies to the protein of interest by allowing the original fusion protein to be used for affinity purification. A very useful feature of the modified pGEX4T-TEV vector system (4) used in these protocols is the inclusion of both the original thrombin and an added tobacco etch virus (TEV) sequence-specific cleavage sites between the GST tag and the protein insert. The specificity of TEV for its recognition sequence is more stringent than that of thrombin (8), thereby reducing unwanted cleavage of your protein of interest. In addition to its utility for tag removal, the use of TEV is quite cost effective because it can be easily produced in-house (9) from publicly available expression strains (10). TEV may also be purchased from several commercial sources including Promega, Eton Biosciences, and Invitrogen.

After the gene encoding the antigen is cloned into the modified pGEX4T-TEV vector, it should be first be transformed into the Rosetta™ (DE3) pLysS or other protein expression compatible *E. coli* strains. To produce a purified tag-free soluble bacterially

expressed protein for use as an immunogen, the resulting clones must first be tested via small-scale induction to verify expression, and then subsequently by small-scale fractionation to determine the solubility of the protein product. Once the expression and solubility of the protein has been verified, large-scale expression and purification of the soluble protein by affinity chromatography using glutathione resin can be initiated. GST tag removal from purified soluble fusion proteins using sequence-specific proteases followed by protease removal by affinity chromatography is the final step in preparing a purified soluble bacterially expressed protein for use as an immunogen. For insoluble proteins, inclusion bodies are isolated and used for immunization. In this chapter, we present protocols for the preparation of tag-free soluble and insoluble bacterially expressed proteins for use as immunogens.

2. Materials

2.1. SDS-PAGE (Sodium Dodecylsulfate-Polyacrylamide Gel Electrophoresis)

1. Precast Ready Gel Tris–HCl SDS-PAGE gels, 4–15% acrylamide, Tris/glycine/SDS buffer (Bio-Rad Laboratories, Inc., Hercules, CA).
2. Small vertical electrophoresis unit (Hoefer, Inc., San Francisco, CA).
3. Power Supply (Bio-Rad Laboratories, Inc., Hercules, CA).
4. 10× SDS-PAGE running buffer: Mix 30.3 g Tris base, 144 g glycine, 10 g SDS, and distilled water (dH_2O) to a final volume of 1 L; dilute with dH_2O to 1× strength for use.
5. 5× *S*DS-PAGE *s*ample *b*uffer (5× SSB): Mix 3.9 mL 2 M Tris–HCl pH 6.8, 2.5 g SDS, 12.5 mL glycerol, 8 mg bromophenol blue, 6.25 mL β-mercaptoethanol, and double distilled water (ddH_2O) to a final volume of 25 mL. Use at 2× strength (2× SSB): 4 mL 5× SSB diluted with ddH_2O to final volume of 10 mL.
6. Protein Molecular Weight Markers: SDS-PAGE broad range standards (Bio-Rad Laboratories, Inc., Hercules, CA) diluted 1:40 in 2× SSB.
7. Coomassie stain for protein gels: 0.1% (w/v) Coomassie R-250 dissolved in fixative [40% (v/v) methanol, 10% (v/v) glacial acetic acid]; store capped at room temperature.
8. Coomassie destain solution for protein gels: 40% (v/v) methanol, 10% (v/v) glacial acetic acid; store capped at room temperature.
9. Kimwipes (Kimberly-Clark Corporation, Irving, TX).
10. Gel drying kit (Promega Corp., Madison, WI).

2.2. Analytical Scale Test of GST-Fusion Protein Expression

1. The modified pGEX4T plasmid engineered with a TEV protease cleavage site between endogenous Thrombin cleavage site and MCS (*see* **Note 1**) or pGEX4T plasmid (GE Healthcare Bio-Sciences Corp., Piscataway, NJ) containing the coding sequence for the protein/domain of interest in Rosetta™ (DE3) pLysS *E. coli* protein expression strain (Novagen-EMD Biosciences, Madison, WI).
2. LB broth: Mix 10.0 g Tryptone (BD Biosciences, San Jose, CA), 5.0 g yeast extract (BD Biosciences, San Jose, CA), 10.0 g sodium chloride, and ddH_2O to a final volume of 1 L; autoclave 20 min at 121°C; cool to 55°C before adding antibiotics.
3. Solid LB plates: Add 15.0 g/L Bactoagar (BD Biosciences, San Jose, CA) to freshly prepared LB broth prior to autoclaving. After autoclaving, cool broth to 55°C in a water bath, add antibiotic, mix by swirling, and aseptically pour a thin layer into 100-mm × 15-mm sterile plastic Petri dishes. Allow plates to cool to RT and store plates inverted in a plastic sleeve at 4°C.
4. Antibiotics: Carbenicillin (1,000×): 50.0 mg/mL in 50% (v/v) ethanol. Chloramphenicol (1,000×): 34.0 mg/mL in 95% (v/v) ethanol. Store both at −20°C.
5. Isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma-Aldrich, St. Louis, MO): 500 mM stock solution in ddH_2O, sterile filter, and store at −20°C.
6. Tris-buffered saline pH 7.4 (TBS pH 7.4; 10× strength): Mix 80.0 g NaCl, 0.2 g KCl, 30.0 g Tris base, and 800 mL of ddH_2O. With concentrated HCl, adjust pH to 7.4 and then bring to a final volume of 1 L with ddH_2O. Use at 1× strength and store at room temperature.
7. Spectrophotometer (600 nm capability) (GE Healthcare Bio-Sciences Corp., Piscataway, NJ).
8. Micro-tip sonicator (Branson Ultrasonics, Danbury, CT).

2.3. Protein Expression Test for Solubility and Capacity to Bind Glutathione–Sepharose

Items in **Section 2.2** are also included:

1. β-Mercaptoethanol (β-ME) (Sigma-Aldrich, St. Louis, MO).
2. NP-40 (Calbiochem-EMD Biosciences, San Diego, CA); stock solution of 20% (v/v) in dH_2O; store at room temperature.
3. Glutathione–Sepharose™ 4 Fast-Flow (GE Life-Sciences, Inc., Piscataway, NJ); working stock of 25% (v/v) in 1× TBS pH 7.4, supplemented with 0.1% (v/v) NP-40 and 0.02% (w/v) sodium azide; store at 4°C.

2.4. Large-Scale Expression and Purification

Items in **Section 2.3** are also included:

1. Phenylmethanesulfonyl fluoride (PMSF) (Sigma-Aldrich, St. Louis, MO) 200 mM stock in dry isopropanol (*see* **Note 2**).
2. Poly-Prep Chromatography Column (Bio-Rad Laboratories, Inc., Hercules, CA).
3. Adenosine 5'-triphosphate, disodium salt (Sigma-Aldrich, St. Louis, MO) made into 100 mM stock solution with dH_2O, stored at −20°C in small (~200 μL) aliquots to avoid freeze-thaw cycles.
4. $MgCl_2$: 1 M stock solution in dH_2O, autoclave, and store at room temperature.
5. L-Glutathione, reduced (Sigma-Aldrich, St. Louis, MO).
6. Tris-buffered saline pH 8.0 (TBS pH 8.0; 10× strength): Mix 8.0 g NaCl, 0.02 g KCl, 3.0 g Tris base, and dH_2O for a final volume of 100 mL adjusting the pH to 8.0 with concentrated HCl; use at 1× strength, store at room temperature.
7. Elution solution: 1× TBS pH 8.0 + 15 mM L-glutathione (reduced) (46 mg/10.0 mL). Prepare immediately before use.
8. Pierce 660 nm Protein Assay Kit (Thermo Scientific Pierce Protein Research Products, Rockford, IL).
9. Microplate spectrophotometer (660 nm capability) (Bio-Tek Instruments, Winooski, VT).
10. 96-Well, flat bottom microtiter plates (Greiner Bio-One North America, Monroe, NC).
11. Dialysis tubing (MWCO 12–14,000, 1 cm flat width, 0.3 mL/cm; Spectra/Por; Spectrum Laboratories, Inc., Rancho Dominguez, CA) and clips.
12. Glycerol.
13. Buffer A (need 50 mL): 25 mM HEPES–NaOH pH 7.5, 1 mM EDTA-NaOH pH 7.5.
14. Lysis buffer: To 50.0 mL buffer A add 50.0 μL NP-40 (100% stock), 5.0 mg lysozyme, 0.5 mg RNAse-A, 2.5 mg DNAse I, 100.0 μL $MgCl_2$ (1 M stock).
15. First wash buffer (need 150.0 mL): 50 mM HEPES–NaOH pH 7.5, 0.3 M NaCl, 1 mM EDTA–NaOH pH 7.5, 0.1% (v/v) NP-40.
16. Second wash buffer (need 25.0 mL): 50 mM HEPES–NaOH pH 7.5, 0.3 M NaCl, 1 mM EDTA–NaOH pH 7.5, 0.1% (v/v) NP-40, 10 mM β-ME.

17. Avanti J-E centrifuge with JA-20 and JA-14 rotors (Beckman-Coulter, Fullerton, CA).
18. 250-mL Polycarbonate centrifuge bottles and 50-mL polycarbonate centrifuge tubes.
19. Tabletop swinging bucket clinical centrifuge (Sorvall-Thermo Fisher Scientific, Waltham, MA).

2.5. Protease Removal of GST from Purified GST-Fusion Proteins

1. Purified GST-fusion protein (from **Section 3.4.2**).
2. Glutathione–Sepharose™ 4 Fast-Flow in TBS pH 7.4 at 25% (v/v).
3. Phosphate-buffered saline (PBS; 10× strength): Mix 80.0 g NaCl, 2.0 g KCl, 21.6 g Na_2HPO_4-$7H_2O$, 2.0 g KH_2PO_4, and dH_2O for a final volume of 1 L, sterile filter; use at 1× strength, sterile filter, and store at room temperature.
4. ProTEV Protease (Promega Corp., Madison, WI) (*see* **Note 3**).
5. Ni–Sepharose™ 6 Fast-Flow (GE Life-Sciences, Inc, Piscataway, NJ); washed and equilibrated in PBS.
6. Thrombin (Novagen-EMD Biosciences, Madison, WI).
7. *p*-Aminobenzamidine–agarose (Sigma-Aldrich, St. Louis, MO).

3. Methods

3.1. SDS-PAGE

1. Attach precast gel to electrophoresis apparatus. Fill upper and lower reservoirs with 1× strength running buffer.
2. Heat samples at 65°C for 15 min. Collect solution by centrifugation at 16,000×*g* for 5 min at room temperature.
3. Load 10 μL of each sample and protein molecular weight markers into individual wells.
4. Run gel according to manufacturer's suggested current/voltage.
5. Disassemble gel apparatus and transfer gel carefully to Coomassie stain in a clean plastic/glass dish and cover. Incubate gel for 30 min (minimum) with gentle orbital shaking.
6. Decant stain and save for later use. Add both destain solution and several wadded kimwipes to the gel containing dish and cover. Incubate with gentle orbital shaking until sufficient contrast is observed between protein bands and gel background. Frequently exchange used kimwipes for new ones to speed destaining.

7. Decant destain solution and save for later use. Rinse gel with dH_2O and then incubate in dH_2O for 10 min.
8. Setup gel in drying frame and dry according to manufacturer's instructions.

3.2. Analytical Scale Test of GST-Fusion Protein Expression

1. *Day 1*: Start individual 2.0 mL overnight cultures in LB plus appropriate antibiotic selection for each construct in an *E. coli* strain compatible with protein expression. Can stab individual colonies from primary transformation plate with sterile disposable pipette tip, streak on new solid LB plate with antibiotics (clone master plate), and eject tip into 2.0 mL culture tube with liquid LB supplemented with antibiotic. Grow overnight (16 h) at 37°C with vigorous orbital shaking (250 rpm).
2. *Day 2*: Prepare sufficient fresh LB supplemented with appropriate antibiotics for an appropriate number of 2.0 mL cultures and a single 1.0 mL spectrophotometer blank.
3. Use 0.1 mL saturated overnight culture to inoculate 2.0 mL cultures.
4. Grow new cultures 1.5 h at 37°C with vigorous orbital shaking (250 rpm).
5. Add IPTG to final concentration of 0.25 mM (add 1.0 μL of 500 mM IPTG stock solution) to all positive induction samples. Add sterile water for negative induction control.
6. Grow all cultures at 37°C with vigorous orbital shaking for 2 h.
7. Remove 1.0 mL from culture and collect cells by centrifugation in microcentrifuge at room temperature at maximum speed (~16,000×*g*) for 1 min. Remove medium by aspiration. Wash cells once with 0.5 mL ice-cold TBS pH 7.4. Recollect cells by centrifugation as before. Place cell pellets on ice.
8. With remaining culture perform $OD_{600\ nm}$ measurements and document.
9. To washed cell pellets from Step 7, add 2× SSB for a final concentration of 0.004 $OD_{600\ nm}$ per mL culture bacterial equivalents/1.0 μL 2× SSB and suspend by vortexing and sonication (*see* **Note 4**). Collect sample solution to tube bottom by pulse centrifugation in microfuge (*see* **Note 5**).
10. Run and stain SDS-PAGE gel (*see* **Section 3.1**).
11. Select the clone or clones that show the highest levels of induced protein (a dark band of the expected molecular weight in the induced but absent in the negative induction control sample) to proceed with the solubility test

(*see* **Section 3.3**). If no induced protein is observed, try (1) validation that IPTG and induction works with expression of GST from an empty vector, (2) changing the expression tag, or (3) choosing a different expression domain.

3.3. Protein Expression Test for Solubility and Capacity to Bind Glutathione–Sepharose

1. *Day 1:* Start individual 2.0 mL overnight cultures from the clone master plate (*see* **Section 3.2**, Step 1) in LB plus appropriate antibiotic selection for each construct/*E. coli* strain. Grow overnight (16 h) at 37°C.
2. *Day 2*: Prepare fresh LB supplemented with appropriate antibiotics for 2.5 mL cultures of each *E. coli* strain clone.
3. Use saturated overnight culture to inoculate new cultures at a 1:20 dilution (for 2.5 mL culture, use 0.125 mL of overnight culture).
4. Grow 1.5 h at 37°C with vigorous shaking.
5. Add IPTG to final concentration of 0.25 mM. Grow at 37°C with vigorous shaking for 2 h (*see* **Note 6**).
6. Collect cells by centrifugation in microfuge (room temp, full speed, 1 min) and remove cell medium completely by aspiration. In one 1.5 mL microfuge tube collect cells from 2.0 mL (2× 1.0 mL) of culture (i.e., collect cells from 1 mL culture by centrifugation, discard supernatant, add second 1 mL of culture to the same centrifuge tube). In a second tube generate a cell pellet for the "total induced protein sample" from 100 μL of culture.
7. Wash cells once with 0.5 mL of TBS pH 7.4 + 2 mM β-mercaptoethanol. Wash includes suspension of cells in solution with vortexing, recollection by centrifugation as above, and removal of wash solution by aspiration.
8. Suspend cells from 100 μL of culture ("second tube"; *see* Steps 6 and 7) in 30 μL 2× SSB (*see* **Note 5**). This is the total induced protein sample.
9. Suspend washed cells from 2.0 mL of induced culture (*see* Step 6–7) in 0.3 mL of TBS pH 7.4 + 2 mM β-mercaptoethanol and place on ice. Disrupt cold cells by sonication with micro-tip probe, a 5–10-s pulse two to three times at low power. Make sure to have cells on ice and prevent heating of sample (*see* **Note 7**).
10. Add 1.5 μL NP-40 detergent from a 20% stock to a final concentration of 0.1%. Mix by brief vortex and incubate on ice for 5 min.
11. Centrifuge samples 10 min at 10,000×*g* at 4°C.
12. Transfer the total supernatant to a new 1.5-mL microfuge tube. This is the soluble protein fraction.

13. To pellets, add 50 μL 2× SSB, suspend by vortexing (*see* **Note 5**). This is the insoluble protein fraction containing inclusion bodies and unbroken cells.
14. To the soluble protein fraction, add 25 μL of 25% (v/v) glutathione–Sepharose 4 Fast-Flow in TBS pH 7.4 + 0.1% NP-40 + 0.02% sodium azide (*see* **Note 8**). Mix by rotating at room temp for 10 min.
15. Collect beads gently by centrifugation (3,000×*g*, 1 min, room temp). Transfer supernatant to new 1.5-mL microfuge tube and store at −20°C for later analysis if needed. This is the unbound soluble protein fraction.
16. Wash beads three times with 1.0 mL TBS pH 7.4 + 0.1% NP-40 + 2 mM β-ME. Wash includes suspending beads gently by inverting tube by hand, collecting by centrifugation (3,000×*g*, 1 min, room temperature), and removal of wash solution by aspiration.
17. Wash beads once with 1 mL TBS pH 7.4 + 2 mM β-ME. Remove all wash solution using a 27-gauge needle on a 1.0-mL syringe by placing the beveled needle tip opening against the side of the microfuge tube.
18. Add 25 μL of 2× SSB to the beads (*see* **Note 5**). This solution contains the soluble protein bound to resin.
19. Analyze samples by SDS-PAGE (*see* **Section 3.1**). Samples to load on gel include (a) protein molecular weight markers, (b) total induced protein (*see* Step 8), (c) insoluble protein (*see* Step 13), (d) soluble protein bound to resin (*see* Step 18), and (e) optional BSA (0.5, 1, 2, and 5 μg) standards.
20. Verify the presence of the expressed protein bound to the glutathione–Sepharose beads and proceed with the large-scale expression and purification (*see* **Sections 3.4.1** and **3.4.2**). If no expressed protein is detected, try a different expression clone (*see* **Note 9**). If the expressed protein is seen only in the insoluble protein fraction, proceed instead with purification of *E. coli* inclusion bodies (*see* **Sections 3.4.1** and **3.4.3**), or attempt to regain solubility by lowering the induction temperature (*see* **Note 10**), changing the expression tag or choosing a different expression domain. If the expressed protein is observed in the total but not in either the soluble protein bound to resin or the insoluble fraction, analyze the unbound soluble protein (*see* Step 15) by protein precipitation (11) and SDS-PAGE (*see* **Section 3.1**). If the protein is expressed and soluble but does not bind to the resin, first increase the incubation time for bead binding. If protein still does not bind either (a) subtly

change the domain to be express through either addition or subtraction of residues to be expressed, (b) change the position of the epitope tag, or (c) choose a different expression tag.

3.4. Large-Scale Expression and Purification

3.4.1. Culture Growth and Protein Induction

1. *Day 1*: Start individual 50.0 mL overnight cultures in LB plus appropriate antibiotic selection for each construct/*E. coli* strain. Grow overnight at 37°C.
2. *Day 2*: Supplement with appropriate antibiotics 1.0 L autoclaved LB in 2.8 L Fernbach flask. Remove 1.0 mL for spectrophotometer measurement blank.
3. Use saturated overnight culture to make a freezer stock of the expression strain for future use. Either mix (a) 850 μL saturated culture with 150 μL sterile autoclaved glycerol or (b) 930 μL saturated culture with 70 μL DMSO in a cryovial, snap freeze in liquid nitrogen, and store at −80°C.
4. Use remaining saturated overnight culture to inoculate 1.0 L culture. Mix by swirling and check $OD_{600\ nm}$ of 1.0 mL.
5. Grow at 37°C with vigorous shaking to $OD_{600\ nm} = 0.4–0.6$ (~1.5 h).
6. Induce culture by adding IPTG to a final concentration of 0.25 mM. Grow at 37°C with vigorous shaking for 2 h. Check $OD_{600\ nm}$.
7. Transfer induced cell culture to four 250 mL centrifuge bottles. Collect cells by centrifugation at 5,000×*g* for 10 min at 4°C. Decant used medium into bleach and then discard.
8. Suspend cell pellets in 20.0 mL/bottle of cold TBS pH 7.4. Pool into one bottle. Rinse empty bottles with TBS pH 7.4 and pool rinse into bottle with cells. Collect cells by centrifugation and discard wash solution after treating with bleach.
9. Suspend cell pellet in 20.0 mL of cold TBS pH 7.4. Transfer cell suspension to 50 mL centrifuge tube. Rinse bottles with 5.0 mL TBS pH 7.4 and pool rinse with cells. Collect cells by centrifugation at 5,000×*g* for 10 min at 4°C. Remove and discard wash solution after treating with bleach.
10. Snap freeze cell pellet in liquid nitrogen. Thaw for protein purification or store at −80°C for later purification.

3.4.2. Soluble Protein Purification

1. *Day 1*: Thaw cells in room temp water bath in the presence of 20 mL of cold TBS pH 7.4, 2 mM β-ME, and 1 mM PMSF (additional protease inhibitor cocktails can be added here if needed). Mix periodically until thawed. When thawed, place cell suspension on ice. Some cell breakages occurs during thawing and therefore solution should be highly viscous due to the release of bacterial chromosomal DNA.
2. Disrupt cold cells by sonication (25–30 s, 3–4 times on ice). Adequately broken cell suspension should become clear [less opaque = disrupted cells] and free-flowing [decreased viscosity = sheared DNA]) (*see* **Note 11**).
3. Add NP-40 detergent to a final concentration of 0.1% (add 110 μL of a 20% (v/v) stock). Vortex briefly and incubate on ice for 5 min. Remove 10 μL and add 40 μL 2× SSB for homogenate fraction (*see* **Note 5**).
4. Centrifuge samples for 10 min at 10,000×*g* at 4°C.
5. Transfer total supernatant to clean 50.0-mL conical tube. Remove 10 μL sample and add 40 μL 2× SSB for soluble fraction (*see* **Note 5**).
6. Suspend pellet from Step 4 with 20.0 mL TBS pH 7.4, take a 10 μL sample, and add 40 μL 2× SSB for insoluble inclusion bodies and unbroken cell fraction (*see* **Note 5**). To supernatant from Step 5, add 4.0 mL of 25% (v/v) glutathione–Sepharose 4 Fast-Flow in TBS pH 7.4 + 0.1% NP-40 + 0.02% sodium azide. Rotate mix at 4°C for 30 min.
7. Collect beads gently by centrifugation in a tabletop swinging-bucket rotor clinical centrifuge at 500×*g* for 5 min at 4°C. Transfer supernatant to clean 50.0 mL conical centrifuge tube. From supernatant, remove a 10 μL sample and add 40 μL 2× SSB for unbound soluble protein sample (*see* **Note 5**). Snap freeze remaining unbound fraction in liquid nitrogen and store at −80°C for later analysis if needed.
8. Wash beads three times in bulk with 50 mL TBS pH 7.4 + 0.1% NP-40 + 2 mM β-ME. Wash includes resuspending beads gently by inverting tube by hand, collecting by centrifugation (3,000×*g*, 1 min, room temperature), and removal of wash solution by aspiration. Transfer beads to pre-rinsed Bio-Rad Poly-Prep column. Allow the beads to settle then wash with 10 bed volumes TBS pH 7.4 + 0.1% NP-40 + 2 mM β-ME discarding the wash solution.
9. Wash column with 10.0 mL of TBS pH 7.4 + 0.1% NP40 + 2 mM β-ME + 1 mM ATP + 1 mM $MgCl_2$ to

remove any bacterial heat shock chaperone proteins associated with the GST-fusion protein.

10. Wash column with a minimum of 50 bed volumes (50.0 mL) of TBS pH 7.4 + 2 mM β-ME (no detergent).
11. Prepare 10.0 mL of fresh elution solution per column.
12. Perform a stepwise elution with elution solution: Fraction 1: 0.5 mL; Fractions 2–8: 1.0 mL each. Collect into individual 1.5 mL microfuge tubes and then place on ice (*see* **Note 12**).
13. Protein peak determination: In a microtiter plate, pipette 10 μL of each protein fraction into a well. To each protein sample add 150 μL Pierce 660 nm Protein Assay reagent, mix by pipetting, incubate 5 min at room temp, and measure $OD_{660\ nm}$ with a microplate reader. Remember to include a "no-protein" control sample.
14. Hydrate dialysis tubing by removing appropriate length from spool, place in beaker of dH_2O, and heat in microwave until hot. Incubate on bench until hydrated and pliable (~1 min). Keep wet once hydrated.
15. Pool fractions, usually fractions 2–4, containing protein peak ($OD_{660\ nm}$>0.05) and transfer to dialysis tubing with clip sealing one end. Seal other end with clip and ensure that clips are correctly fastened.
16. Dialyze against 2× 4 L of TBS pH 7.4 + 2 mM β-ME + 10% (v/v) glycerol at 4°C. Change first dialysis solution after 3–4 h then dialyze overnight.
17. Wash the used glutathione–Sepharose 4 Fast-Flow column with five column volumes of TBS pH 7.4. Store resin in TBS pH 7.4 + 0.02% NaN_3 at 4°C for future use.
18. *Day 2*: Transfer contents of dialysis tube to clean microfuge tube on ice.
19. Perform quantitative protein assay using BSA as a standard. Take 1–4 μg of pure GST-fusion protein and add 2× SSB keeping volume less than 15 μL. Snap freeze purified protein in aliquots and store at −80°C.
20. Perform SDS-PAGE analysis (*see* **Section 3.1**) of the following samples: (a) protein molecular weight markers, (b) homogenate (*see* Step 3), (c) soluble protein fraction (*see* Step 5), (d) insoluble inclusion bodies (*see* Step 6), (e) unbound protein (*see* Step 7), (f) residual resin bound (*see* **Note 13**), and (g) pure GST-fusion protein sample (*see* Step 19). Optional but recommended: run BSA standards (0.5, 1, 2, and 5 μg) on gel to provide a standard curve to quantify purified protein on gel.

21. Verify purity and yield. If overall yield is low, use more starting culture or adjust induction conditions to generate more soluble protein. If the purified protein shows a large amount of breakdown, try using more or different protease inhibitors, or performing the entire protocol at 4°C in a cold room.

3.4.3. Purification of E. coli Inclusion Bodies

1. Suspend/thaw cells in 50.0 mL lysis buffer; incubate thawed mixture 10 min on ice. Split evenly between two 50-mL centrifuge tubes.
2. Centrifuge for 10 min at 4°C and 17,400×*g*. Discard resulting supernatant.
3. Resuspend each cell pellet by vortexing in 25.0 mL first wash buffer. Incubate 5 min on ice.
4. Repeat centrifugation and washing twice.
5. Resuspend each pellet in 10.0 mL second wash buffer. Pool samples into one 50 mL centrifuge tube. Incubate 10 min on ice.
6. Centrifuge: 10 min at 4°C and 17,400×*g*. Discard resulting supernatant.
7. Resuspend pellet in 4.0 mL 2nd wash buffer supplemented with 5% (v/v) glycerol.
8. Take a 10 μL sample and add 40 μL 2× SSB. Make serial dilutions of protein in 2× SSB and analyze on SDS-PAGE gel (*see* **Section 3.1**) containing BSA standards.
9. Snap freeze remaining protein in liquid nitrogen and store at −80°C.

3.5. Protease Removal of GST from Purified GST-Fusion Proteins

1. In a rinsed poly-prep chromatography column (column 1), add 5.0 mg purified GST-fusion protein to 2.0 mL of 25% (v/v) glutathione–Sepharose™ 4 Fast-Flow in TBS pH 7.4 (*see* **Note 14**). Seal the column at both ends and incubate with end-over-end mixing at room temperature for 30 min.
2. Drain unbound material and wash resin with 10 bed volumes of PBS. Add PBS to a total solution/resin volume of 2.0 mL. If using thrombin, be sure to include 2.5 mM $CaCl_2$.
3. Add either ProTEV (200 units) or thrombin (20 units) to the column (column 1), seal, and incubate at room temperature with end-over-end mixing for 3 h at room temperature (*see* **Note 15**).
4. Drain column 1 solution directly into a second rinsed poly-prep chromatography column (column 2) (capped at the bottom) containing either (a) 50 μL of Ni–Sepahrose 6 Fast-Flow resin pre-washed with PBS (for the ProTEV) or (b) 50 μL of *p*-aminobenzamidine–agarose resin pre-washed with

PBS for the thrombin. Rinse column 1 resin with 1.0 mL of PBS and drain directly into column 2 (*see* **Note 16**).

5. Seal column 2 at both ends and incubate with end-over-end mixing for 30 min at room temperature.
6. Drain column 2 into a 5.0-mL polypropylene tube and transfer to ice.
7. Perform a quantitative protein assay on the solution drained from column 2 (*see* **Section 3.4.2**, Step 13) using BSA to generate a standard curve. This will allow determination of total protein content of the sample. Remove 1–4 μg of protein and analyze by SDS-PAGE (*see* **Section 3.1**) to verify the removal of the protease.
8. Make appropriate aliquot volumes (*see* **Note 17**), snap freeze in liquid nitrogen, and store at −80°C. You now have purified antigen, ready to use!

4. Notes

1. pGEX4T-TEV vector construction has been described (4). A straightforward method for its construction is to perform opposite direction "round-the-world" PCR of the desired pGEX4T vector resulting in a linearized vector with in-frame blunt ends directly between the sequence encoding the thrombin cleavage site and the *Bam*HI restriction site. Complementary 5′-phosphorylated hybridized oligos encoding the TEV recognition sequence can be ligated resulting in a circular plasmid for use in protein expression. Alternatively, the original plasmid from GE Healthcare can be used for expression with the GST removal accomplished through thrombin digestion.
2. PMSF is a toxic protease inhibitor so use caution when handling. In addition, PMSF has a short half-life in the presence of water (1 h at pH 7.5) so dissolving in a dry organic solvent and adding immediately before use to buffers is important for its efficacy.
3. A cost-effective alternative to commercially available TEV protease is to obtain an expression strain from the Center for Eukaryotic Structural Genomics-University of Wisconsin-Madison (10) and purify the recombinant His_6-tagged enzyme (i.e., according to (9)).
4. For calculating the volume of 2× SSB to add to induced cell pellets, take $OD_{600\,nm}$ value divide by 0.004. Resulting figure should be the μL volume of 2× SSB you add.

Processing the samples this way will normalize for culture growth rates. In most cases, the cellular DNA will make removal of sample difficult due to high viscosity, therefore pulse sonicate each sample (~5 s, low power) with a microtip sonicator to shear DNA.

5. Once protein sample buffer is added to protein samples, store samples at −20°C until ready to perform SDS-PAGE (*see* **Section 3.1**).
6. To find optimal expression conditions to maximize protein expression, try an induction time course (1–4 h) or an IPTG titration (0.1–1 mM).
7. Upon cell lysis by sonication, the cell suspension should become clear (less opaque = disrupted cells) and free flowing (decreased viscosity = sheared DNA).
8. Wear gloves when handling sodium azide since it is toxic!
9. Unfortunately, it is not uncommon for an expression clone to loose its ability to express the recombinant protein upon restreaking or storage. The frequency with which this happens varies greatly with different expression constructs. If a clone whose expression was verified no longer seems to give any expressed protein in a subsequent test (in a culture started from a restreaked plate), you must pick new colonies off of the original transformation plate. It may also be beneficial to perform a fresh transformation of the construct into the expression strain. If your construct is prone to loss of expression, we suggest making a freezer stock of each expression clone from the saturated culture (*see* **Section 3.2**, Step 1) instead of restreaking a plate. If even this freezer stock is later found to have lost its ability to express the recombinant protein, it may be necessary to use a colony directly from a fresh transformation for large-scale expression.
10. To facilitate solubility of the expressed protein during induction, the temperature at which the induction takes place can be lowered. For example, we use a temperature series of 28°C, room temperature (~20–22°C), and then, finally, 15°C to increase solubility. Do note that by decreasing the induction temperature, you also slow the growth of the culture and the protein expression rate and thus must account for this.
11. When using sonication for cell disruption, there are several points to keep in mind. First, try to prevent frothing of the solution. Frothing promotes protein denaturation. In addition, make sure that the sample does not heat up by keeping the sample on ice and allowing for time breaks in-between sonic applications. Very useful alternatives to cell

disruption by sonication are to (1) use a French Press at 20,000 psi or (2) use commercially available bacterial protein extraction reagents [i.e., BugBuster® Protein Extraction Reagent from Novagen (Novagen-EMD Biosciences, Madison, WI)].

12. Stepwise elution involves gently pipetting a specific volume of elution solution onto the resin, allowing the solution to flow into the bed while simultaneously collecting the eluate until the solvent meniscus reaches the top of the resin bed. Successive volumes of eluant are applied until no more protein elutes from the matrix. This procedure ensures that the eluted protein remains as concentrated as possible.
13. In some cases the GST-fusion protein does not elute efficiently and remains bound to the resin. Resin can be analyzed for the presence of residual protein by transferring 25 μL of a 25% (v/v) slurry of used beads to a new microfuge tube, removing the liquid and adding 30 μL of 2× SSB to solubilize protein for SDS-PAGE analysis. If inefficient elution is a problem, try (1) longer incubation times with the elution solution, (2) a higher pH for the elution solution, and/or (3) a higher glutathione concentration in the elution solution.
14. The starting mass amount of GST-fusion protein mixed with the beads may vary depending on your needs. If your protein of interest is smaller than GST, then the amount of pure, GST-free protein recovered from 5 mg GST-fusion protein will be less than 2.5 mg (assuming 100% recovery). You will need to keep in mind that a typical target protein amount for immunization of a single rabbit is approximately 1 mg.
15. The efficiency of GST removal from a GST-fusion protein with TEV or thrombin depends on several parameters and should be determined empirically on an analytical scale. Each GST-fusion behaves differently and therefore tag removal will need to be optimized for each. Common procedural factors to change include (1) the mass ratio of protease to target (more protease will result in more cleavage), (2) the time of the digestion reaction (1 h to overnight), and (3) the temperature at which the digestion reaction takes place (room temperature or 4°C). Keep in mind that you will need to optimize the digestion efficiency while considering the stability of your protein of interest. In addition, do not forget that thrombin needs Ca^{2+} ions for activity.
16. Verify that the resin has sufficient binding capacity to bind all of the protease.

17. You may need to stabilize your protein for other uses beyond use as an immunogen by adding glycerol to your solution to prevent precipitation.

Acknowledgments

This work was supported in part by the Department of Energy, Division of Energy Biosciences (Grant no. DE–FG02–ER20332 to S. Y. B.), and the DOE Great Lakes Bioenergy Research Center (www.greatlakesbioenergy.org), which is supported by the US Department of Energy, Office of Science, Office of Biological and Environmental Research, through Cooperative Agreement DE-FC02-07ER64494 between The Board of Regents of the University of Wisconsin System and the US Department of Energy.

References

1. Kyte, J. and Doolittle, R. F. (1982) A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.* **157**, 105–132.
2. Hopp, T. P. and Woods, K. R. (1981) Prediction of protein antigenic determinants from amino acid sequences. *Proc. Natl. Acad. Sci. USA* **78**, 3824–3828.
3. Smialowski, P., Martin-Galiano, A. J., Mikolajka, A., Girschick, T., Holak, T. A., and Frishman, D. (2007) Protein solubility: sequence based prediction and experimental verification. *Bioinformatics* **23**, 2536–2542.
4. Rancour, D. M., Park, S., Knight, S. D., and Bednarek, S. Y. (2004) Plant UBX domain-containing protein 1, PUX1, regulates the oligomeric structure and activity of arabidopsis CDC48. *J. Biol. Chem.* **279**, 54264–54274.
5. Dyson, M. R., Shadbolt, S. P., Vincent, K. J., Perera, R. L., and McCafferty, J. (2004) Production of soluble mammalian proteins in *Escherichia coli*: identification of protein features that correlate with successful expression. *BMC Biotechnol.* **4**, 32–49.
6. Shih, Y. P., Kung, W. M., Chen, J. C., Yeh, C. H., Wang, A. H., and Wang, T. F. (2002) High-throughput screening of soluble recombinant proteins. *Protein Sci.* **11**, 1714–1719.
7. Braun, P., Hu, Y., Shen, B., et al. (2002) Proteome-scale purification of human proteins from bacteria. *Proc. Natl. Acad. Sci. USA* **99**, 2654–2659.
8. Kapust, R. B., Tozser, J., Fox, J. D., et al. (2001) Tobacco etch virus protease: mechanism of autolysis and rational design of stable mutants with wild-type catalytic proficiency. *Protein Eng.***14**, 993–1000.
9. Blommel, P. G. and Fox, B. G. (2007) A combined approach to improving large-scale production of tobacco etch virus protease. *Protein Expr. Purif.* **55**, 53–68.
10. Blommel, P. G. and Fox, B. G. (2007) Structural genomics methods applied to production of TEV protease. In: Center for Eukaryotic Structural Genomics-Technology Dissemination Report 15: Center for Eukaryotic Structural Genomics, University of Wisconsin-Madison.
11. Wessel, D. and Flugge, U. I. (1984) A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. *Anal. Biochem.* **138**, 141–143.