Chapter 16

Enhancing the Solubility of Recombinant Proteins in *Escherichia coli* by Using Hexahistidine-Tagged Maltose-Binding Protein as a Fusion Partner

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Abstract

In the field of biotechnology, fusing recombinant proteins to highly soluble partners is a common practice for overcoming aggregation in *Escherichia coli*. *E. coli* maltose-binding protein (MBP) has been recognized as one of the most effective solubilizing agents, having frequently been observed to improve the yield, enhance the solubility, and promote the proper folding of its fusion partners. The use of a dual hexahistidine–maltose-binding protein affinity tag (His_6–MBP) has the additional advantage of allowing the fusion protein to be purified by immobilized metal affinity chromatography (IMAC) instead of or in addition to amylose affinity chromatography. This chapter describes a generic method for the overproduction of combinatorially tagged His_6–MBP fusion proteins in *E. coli*, with particular emphasis on the use of recombinational cloning to construct expression vectors. In addition, simple methods for evaluating the solubility of the fusion protein and the passenger protein after it is cleaved from the dual His_6–MBP tag are presented.

Key words: Maltose-binding protein, MBP, solubility enhancer, His tag, His_6–MBP, Gateway cloning, TEV protease, tobacco etch virus protease.

1. Introduction

For functional and structural studies, recombinant proteins usually need to be purified on a relatively large scale and under native (nondenaturing) conditions. Originally developed to facilitate the detection and purification of recombinant proteins, affinity tags and other types of genetically engineered fusion partners are widely used in the biotechnology industry (1). However,

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gradually it has become evident that certain tags have additional benefits, such as a positive impact on the yield of recombinant protein, the enhancement of solubility, and even the promotion of the proper folding of their fusion partners (2). *Escherichia coli* maltose-binding protein (MBP) is one of the most effective solubility enhancers (3, 4). Moreover, MBP is the only solubility-enhancing protein that is also a natural affinity tag. Unfortunately, however, amylose affinity chromatography has proven to be problematic at times, not only because of the relatively low binding capacity of amylose resin for MBP but also because of persistent contaminants that require additional chromatographic steps to remove (5–7). We have demonstrated that a hexahistidine tag (His_6) can be added to the N-terminus of MBP without interfering with its ability to promote the solubility of its fusion partners (8). Therefore, IMAC can be used to circumvent the problems associated with amylose affinity chromatography.

In this chapter, we illustrate the utility of a dual His_6–MBP tag as a solubility-enhancing fusion partner. In our laboratory, we routinely construct expression vectors by recombinational cloning, as described here. However, alternative methods for constructing MBP and His_6–MBP fusion vectors by conventional cloning techniques (i.e., using restriction enzymes and DNA ligase) or by ligation independent cloning (LIC) have been described elsewhere (9–13), as have methods for the large-scale production and purification of MBP and His_6–MBP fusion proteins (10, 14–16).

2. Materials

2.1. Recombinational Vector Construction

1. The Gateway destination vector pDEST–HisMBP, which can be obtained from AddGene (http://www.addgene.org) or the authors.
2. The Gateway donor vector pDONR221 (Invitrogen, Carlsbad, CA, USA).
3. Chemically competent DB3.1 or one shot CcdB survival competent cells (Invitrogen, Carlsbad, CA, USA) for propagating pDEST–HisMBP and pDONR221 plasmids.
4. Competent *gyrA*+ cells (e.g., DH5α, MC1061, HB101) (*see* **Note 1**).
5. Gateway BP Clonase II (Invitrogen, Carlsbad, CA, USA) (*see* **Note 2**).
6. Gateway LR Clonase II (Invitrogen, Carlsbad, CA, USA) (*see* **Note 2**).

7. Reagents and thermostable DNA polymerase for PCR amplification (*see* **Note 3**).
8. Synthetic oligodeoxyribonucleotide primers for PCR amplification (*see* **Fig. 16.1**).
9. TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA).
10. 1000-Fold antibiotic stock solutions: Kanamycin, 35 mg/ml in H_2O and filter sterilize. Ampicillin, 100 mg/ml in H_2O and filter sterilize. Store at –20°C.
11. LB medium and LB agar plates containing ampicillin (100 μg/ml). LB medium: dissolve 10 g bacto tryptone, 5 g bacto yeast extract, and 5 g NaCl in 1 l of H_2O and sterilize by autoclaving. For LB agar, also add 12 g of bactoagar before autoclaving. To prepare plates, allow medium to cool until flask or bottle can be held in hands without burning (~50°C), then add 1 ml 1000-fold ampicillin stock solution, mix by gentle swirling, and pour or pipet 30 ml into each sterile petri dish (100 mm diameter).
12. E-gels and an E-gel base (Qiagen, Valencia, CA, USA) for submarine gel electrophoresis of DNA (*see* **Note 4**).
13. QIAquick gel extraction kit (Qiagen, Valencia, CA, USA) for the extraction of DNA from agarose gels.
14. QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA, USA) for small-scale plasmid DNA isolation (*see* **Note 5**).
15. A microcentrifuge capable of operating at 14,000 rpm.
16. An incubator set at 37°C.

2.2. Protein Expression

1. Competent BL21-Pro cells (B&D Clontech, Palo Alto, CA, USA) containing the TEV protease expression vector pRK603 (17). pRK603 plasmid can be obtained from AddGene (http://www.addgene.org) (*see* **Notes 6** and **7**).
2. Competent BL21(DE3) CodonPlus-RIL cells (Stratagene, La Jolla, CA) (*see* **Note 7**).
3. A derivative of pDEST–HisMBP that produces a His_6–MBP fusion protein with a TEV protease recognition site in the linker between MBP and the passenger protein (*see* **Section 3.1**).
4. LB agar plates and broth containing both ampicillin (100 μg/ml) and kanamycin (35 μg/ml). *See* **Section 2.1**, **Step 11** for LB broth and LB agar recipes.
5. 1000-Fold chloramphenicol stock solution: 30 mg/ml in ethanol and filter sterilize, store at –20°C.
6. Isopropyl-β-D-thiogalactopyranoside (IPTG), analytical grade. Prepare a 1000-fold stock solution of 1 M in H_2O and filter sterilize. Store at –20°C.

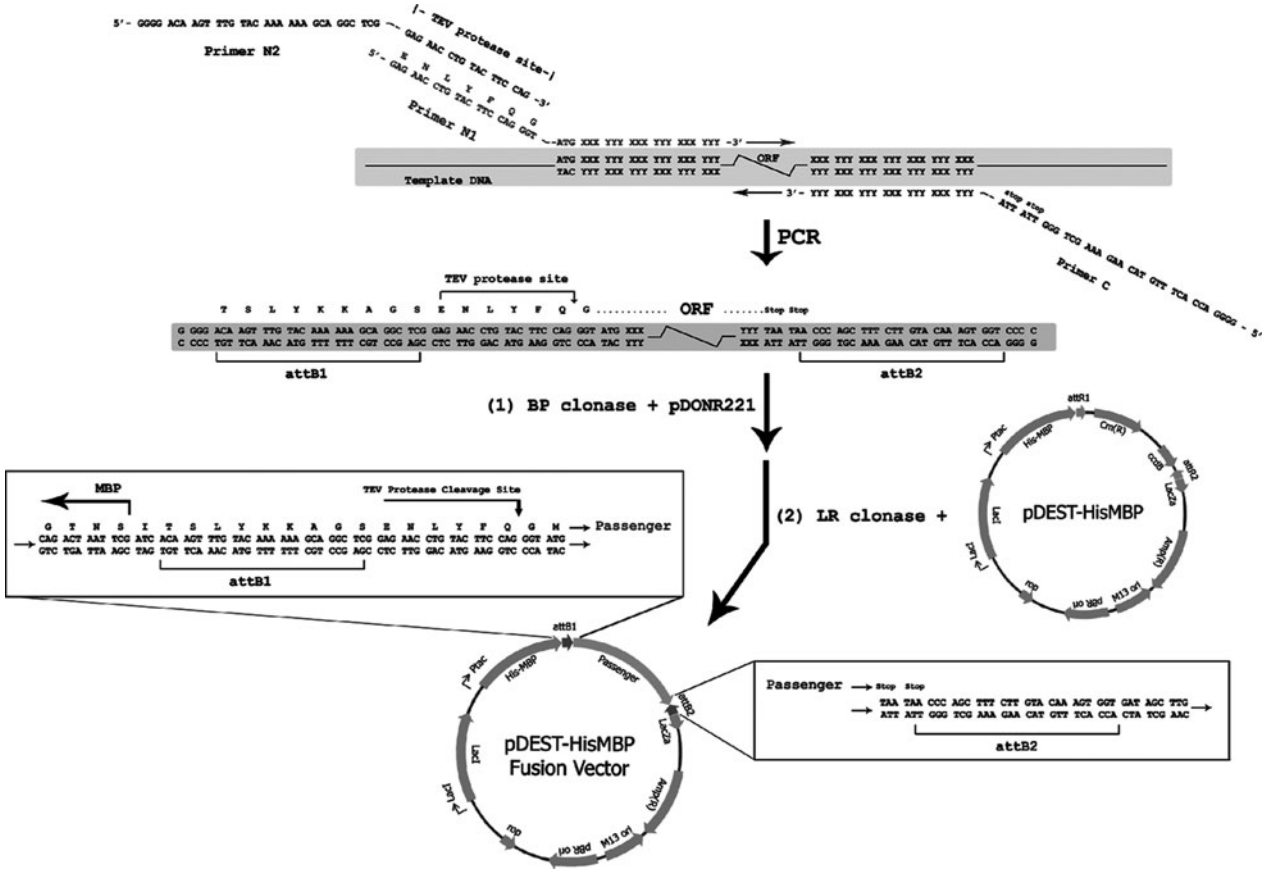

Fig 16.1. Construction of a pDEST–HisMBP fusion protein expression vector using PCR and Gateway cloning technology. The ORF of interest is amplified from the template DNA by PCR, using primers N1, N2, and C. Primers N1 and C are designed to base pair to the 5′- and 3′-ends of the coding region, respectively, and contain unpaired 5′-extensions as shown. Primer N2 base pairs with the sequence that is complementary to the unpaired extension of primer N1. The final PCR product is recombined with the pDONR221 vector to generate an entry clone, via the BP reaction. This entry clone is subsequently recombined with pDEST–HisMBP using LR Clonase to yield the final pDEST–HisMBP fusion vector. Abbreviations: Ptac, tac promoter; attR1/attR2 and attB1/attB2, recombination sites for Gateway cloning; Cm(R), chloramphenicol acetyl transferase (chloramphenicol resistance) gene; ccdB, gene encoding DNA gyrase poison CcdB; LacZα, gene encoding β-galactosidase; Amp(R), β-lactamase (ampicillin resistance) gene; M13 ori, origin of replication from bacteriophage M13; pBR ori, ColE1 origin of replication; rop, repressor of primer gene; lacI, gene encoding lactose repressor.

7. Anhydrotetracycline (ACROS Organics/Fisher Scientific, Springfield, NJ, USA). Prepare a 1000-fold stock solution by dissolving in 50% ethanol at 100 μg /ml. Store in a foil-covered tube at –20°C.
8. Temperature-controlled shaking incubator.
9. Sterile baffle-bottom flasks (Bellco Inc., Vineland, NJ, USA).
10. Cell lysis buffer: 20 mM Tris-HCl (pH 8.0), 1 mM EDTA.
11. Sonicator (with microtip).
12. 2x SDS-PAGE sample buffer (Invitrogen, Carlsbad, CA, USA) and 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO, USA).
13. SDS-PAGE gel, electrophoresis apparatus, and running buffer (*see* **Note 8**).
14. Gel stain (e.g., Gelcode® Blue from Pierce, Rockford, IL, USA, or PhastGel™ Blue R from GE Healthcare, Piscataway, NJ, USA).
15. Spectrophotometer.
16. 1.5 ml microcentrifuge tubes.

3. Methods

3.1. Construction of His_6–MBP Fusion Vectors by Recombinational Cloning

The Gateway recombinational cloning system is based on the site-specific recombination reactions that mediate the integration and excision of bacteriophage lambda into and from the *E. coli* chromosome, respectively. Please refer to the following manual by Invitrogen for detailed information: "Gateway Technology: A universal technology to clone DNA sequences for functional analysis and expression in multiple systems." Complete information about the Gateway system can be found at the following web site: http://www.invitrogen.com/site/us/en/home/Products-and-Services/Applications/Cloning/Gateway-Cloning.html.

3.1.1. pDEST–HisMBP

Currently there are no commercially available Gateway destination vectors for the production of His_6–MBP fusion proteins. However, pDEST–HisMBP can be obtained from the non-profit distributor of biological reagents AddGene, Inc. (http://www.addgene.org). A schematic diagram of pDEST–HisMBP is shown in **Fig. 16.1**. This plasmid was constructed by inserting an in-frame hexahistidine coding sequence between codons 3 and 4 of MBP in pKM596 (11).

The Gateway cloning cassette in pDEST–HisMBP carries a gene encoding the DNA gyrase poison CcdB, which provides a negative selection against non-recombined destination and donor vectors so that only the desired recombinant is obtained when the end products of the recombinational cloning reaction are transformed into *E. coli* and grown in the presence of ampicillin or kanamycin, respectively. pDEST–HisMBP and other vectors that carry the *ccdB* gene must be propagated in a host strain with a *gyrA* mutation (e.g., *E. coli* DB3.1) or in "CcdB Survival cells" that render the cells immune to the action of CcdB.

3.1.2. Gateway Cloning Protocol

To construct a His_6–MBP fusion protein expression vector, three primers (two forward primers and one reverse primer) are employed: Primer N1 (containing the TEV protease cleavage site as a 5′-unpaired extension), primer N2 (consisting of the TEV protease recognition site and an attB1 recombination site), and primer C (containing an attB2 recombination site as a 5′-unpaired extension) (**Fig. 16.1**). For the 3′-end of primer N1 and primer C, approximately 20–25 nucleotides that are complimentary to the ends of the open reading frame are required. The open reading frame of interest first is amplified using primers N1 and C. The first PCR amplicon then becomes the template for PCR with primers N2 and C. The PCR reaction is be performed in one step containing all three primers (*see* **Note 9**). However, to favor the accumulation of the desired product, the attB-containing primers are used at typical concentrations for PCR but the concentration of the gene-specific N-terminal primer N1 is 10- to 20-fold lower. The final PCR amplicon is inserted first into the donor vector pDONR221 by recombinational cloning with BP clonase and then into the destination vector pDEST–HisMBP in a second recombinational cloning reaction with LR clonase.

1. The PCR mix is prepared as follows (*see* **Note 10**): 1 μl template DNA (~10 ng/μl), 10 μl thermostable DNA polymerase 10X reaction buffer, 1.5 μl dNTP solution (10 mM each), 1.0 μl primer N1 (~30 ng), 3 μl primer N2 (~300 ng), 3.0 μl primer C (~300 ng), 1 μl thermostable DNA polymerase, 79.5 μl H_2O (to 100 μl total volume).
2. The reaction is placed in the PCR thermal cycler with the following program: initial melt for 5 min at 95°C; 35 cycles of 95°C for 30 s, 55°C for 30 s, and 68°C for 60 s (*see* **Notes 10** and **11**); hold at 4°C.
3. Purification of the PCR amplicon by agarose gel electrophoresis (*see* **Note 4**) is recommended to remove attB primer dimers.
4. To create the His_6–MBP fusion vector, the PCR product is recombined first into pDONR221 to yield an entry clone

intermediate (BP reaction), and then into pDEST–HisMBP (LR reaction; *see* **Note 12**).

a. Add to a microcentrifuge tube on ice: 50–100 ng of the PCR product in TE or H_2O, 200 ng of pDONR221 DNA, and enough TE to bring the total volume to 8 μl. Mix well.
b. Thaw BP Clonase II enzyme mix on ice (2 min) and then vortex briefly (2 s) twice.
c. Add 2 μl of BP Clonase II enzyme mix to the components in (a.) and vortex briefly twice.
d. Incubate the reaction at 25°C for at least 4 h (*see* **Note 13**).
e. Add to the reaction: 2 μl of the destination vector (pDEST–HisMBP) at a concentration of 50 ng/μl and 3 μl of LR Clonase II enzyme mix. The final reaction volume is 15 μl. Mix by vortexing briefly.
f. Incubate the reaction at room temperature for 3–4 h.
g. Add 2.5 μl of the proteinase K solution and incubate for 10 min at 37°C.
h. Transform 2 μl of the reaction into 50 μl of electrocompetent DH5α cells (*see* **Note 1**).
i. Pellet the cells by centrifugation, gently resuspend pellet in 100–200 μl of LB broth, and spread on an LB agar plate containing ampicillin (100 μg/ml), the selective marker for pDEST–HisMBP (*see* **Fig. 16.1**). Incubate the plate at 37°C overnight (*see* **Note 14**).

5. Plasmid DNA is isolated from saturated cultures started from individual ampicillin-resistant colonies and screened by PCR, using the gene-specific primers N1 and C, to confirm that the clones contain the expected gene. Alternatively, plasmids can be purified and screened by conventional restriction digests using appropriate enzymes. We routinely sequence clones that screen positive by either PCR or restriction digest to ensure that there are no PCR-induced mutations.

3.2. Protein Expression

To assess the yield and solubility of the fusion protein, the amount of total fusion protein produced in the crude cell extract is directly compared to the soluble fraction by visual inspection of a Coomassie blue-stained gel. A parallel in vivo cleavage experiment with TEV protease is run to determine if the fusion protein is a good substrate for the protease and whether or not the cleaved target protein remains soluble after it is released from His_6–MBP.

3.2.1. Selecting a Host Strain of E. coli

To achieve regulated expression of TEV protease, the in vivo processing experiment must be performed in a strain of *E. coli*

that produces the Tet repressor, such as BL21-Pro or DH5α-Pro (B&D Clontech, Palo Alto, CA, USA). The Tet repressor blocks the synthesis of TEV protease mRNA and allows the enzyme to be regulated independently of the IPTG-inducible fusion protein. We have observed that delaying the induction of TEV protease until the fusion protein substrate has had time to accumulate in the cells often results in greater solubility of the passenger protein after cleavage (11, 17). Independent production of TEV protease from the expression vector pRK603 (17) is initiated by adding anhydrotetracycline to the cell culture, usually 2 h after induction of the fusion protein with IPTG. We prefer using BL21-Pro because of its robust growth characteristics and the fact that it lacks two proteases (Lon and OmpT) that are present in many *E. coli* K12 strains such as DH5α-Pro.

For the large-scale production of His_6–MBP fusion proteins, we prefer BL21(DE3) CodonPlus-RIL (Stratagene, La Jolla, CA), or Rosetta (DE3) (EMD, Madison, WI, USA) as host strains (*see* **Note 7**).

3.2.2. Protein Expression

1. Transform competent BL21-Pro or DH5a-Pro cells that already contain pRK603 with the His_6–MBP fusion protein expression vector and spread them on LB agar plates containing ampicillin (100 μg/ml) and kanamycin (35 μg/ml). Incubate the plate overnight at 37°C.
2. Inoculate 2–5 ml of LB medium containing ampicillin (100 μg/ml) and kanamycin (35 μg/ml) in a culture tube or shake flask with a single colony from the plate. Grow to saturation overnight at 37°C with shaking at 250 rpm.
3. The next morning, inoculate 50 ml of the same medium in a 250 ml baffled-bottom flask with 0.5 ml of the saturated overnight culture.
4. Grow the cells at 37°C with shaking to mid-log phase ($OD_{600\,nm} \sim 0.5$).
5. Add IPTG (1 mM final concentration) and adjust the temperature to 30°C (*see* **Note 15**).
6. After 2 h, divide the culture into two separate flasks (20 ml in each). Label one flask "+" and the other "–".
7. Add anhydrotetracycline to the "+" flask (100 ng/ml final concentration).
8. After 2 h, measure the $OD_{600\,nm}$ of the cultures (dilute cells 1:10 in LB to obtain an accurate reading). An $OD_{600\,nm}$ of about 3–3.5 is normal, although lower densities are possible. If the density of either culture is much lower than this, it may be necessary to adjust the volume of the samples that are analyzed by SDS-PAGE.

Transfer 10 ml of each culture to a 15 ml conical centrifuge tube and pellet the cells by centrifugation (4000×*g*) at 4°C.

9. Resuspend the cell pellets in 1 ml of lysis buffer and then transfer the suspensions to a 1.5 ml microcentrifuge tube.
10. Store the cell suspensions at –80°C overnight. Alternatively, the cells can be disrupted immediately by sonication (without freezing and thawing) and the procedure continued without interruption, as described below.

3.2.3. Sonication and Sample Preparation

1. Thaw the cell suspensions at room temperature, then place them on ice.
2. Lyse the cells by sonication (*see* **Note 16**).
3. Prepare samples of the total intracellular protein from the "+" and "–" cultures (T+ and T–, respectively) for SDS-PAGE by mixing 50 µl of each sonicated cell suspension from step 2 with 50 µl of 2x SDS-PAGE sample buffer containing 10% (v/v) 2-mercaptoethanol.
4. Pellet the insoluble cell debris (and proteins) by centrifuging the sonicated cell suspension from each culture at maximum speed in a microcentrifuge for 10 min at 4°C.
5. Prepare samples of the soluble intracellular protein from the "+" and "–" cultures (S+ and S–, respectively) for SDS-PAGE by mixing 50 µl of each supernatant from step 4 with 50 µl of 2x SDS-PAGE sample buffer containing 20% (v/v) 2-mercaptoethanol.

3.2.4. SDS-PAGE

We typically use pre-cast Tris-glycine or NuPAGE gradient gels for SDS-PAGE to assess the yield and solubility of MBP fusion proteins (*see* **Note 8**). Of course, the investigator is free to choose any appropriate SDS-PAGE formulation, depending on the protein size and laboratory preference.

1. Heat the T–, T+, S–, and S+ protein samples at 90°C for about 5 min and then spin them at maximum speed in a microcentrifuge for 5 min.
2. Assemble the gel in the electrophoresis apparatus, fill it with SDS-PAGE running buffer, load the samples (10 µl each), and carry out the electrophoretic separation according to standard lab practices. T and S samples from each culture ("+" and "–") are loaded in adjacent lanes to allow easy assessment of solubility. Molecular weight standards may also be loaded on the gel, if desired.
3. Stain the proteins in the gel with GelCode® Blue reagent, PhastGel™ Blue R, or a suitable alternative.

3.2.5. Interpreting the Results

The MBP fusion protein should be readily identifiable in the T– sample after the gel is stained since it will normally be the most abundant protein in the cells. Molecular weight standards can also be used to corroborate the identity of the fusion protein band. If the S– sample contains a similar amount of the fusion protein, this indicates that it is highly soluble in *E. coli*. If little or no fusion protein is observed in the S– sample, then this is an indicator of poor solubility. Of course, a range of intermediate states is also possible.

If the fusion protein is an efficient substrate for TEV protease, then little of it will be present in the T+ and S+ samples. Instead, one should observe a prominent band at ca. 42 kDa that corresponds to the His_6–MBP moiety and another prominent band migrating with the expected mobility of the passenger protein. If the fusion protein is a poor substrate for the protease, then the "+" samples will look similar to the "–" samples. If the passenger protein is soluble after it is released from His_6–MBP, then a similar amount will be present in the T+ and S+ lanes. At this point, some or all of the passenger protein may precipitate. If a substantial fraction of the passenger protein is insoluble, then troubleshooting may be necessary. Alternatively, an acceptable yield might still be obtained by scaling up cell production.

Two examples are illustrated in **Fig. 16.2**. In panel A, the second pair of lanes represent the total (T) and soluble (S) intracellular protein fractions from cells overproducing His_6–MBP fused to residues 2–191 of human dual specificity phosphatase 14 (DUSP14). Roughly equal amounts of the fusion protein are readily visible in these two lanes, indicating that it is highly soluble. In the third pair of lanes, the His_6–DUSP14(2–191) fusion protein was cleaved in vivo with TEV protease 2 h after its expression was induced by IPTG. In this case, most if not all of the cleaved DUSP14(2–191) is absent from the soluble fraction (S). Hence, this is an example of a protein that is rendered only temporarily soluble while it is fused to MBP and may not be properly folded. The first pair of lanes in panel A demonstrate that when DUSP14(2–191) is fused to a His tag alone (with no MBP), it is completely insoluble in *E. coli*. Panel B in **Fig. 16.2** shows exactly the same experiment, except that in this case the DUSP14 has been further truncated at its N-terminus (residues 18–191). As before, the His-tagged DUSP14(18–191) is totally insoluble whereas the His_6–MBP–DUSP14(18–191) fusion protein is highly soluble. The difference is that in this case nearly all of the DUSP14(18–191) is soluble after it has been cleaved from the His_6–MBP tag, suggesting that it has become properly folded as a result being fused to His_6–MBP. Indeed, DUSP14(18–191) produced in this manner readily hydrolyzes the phosphotyrosine mimetic *para*-nitrophenyl phosphate (data not shown).

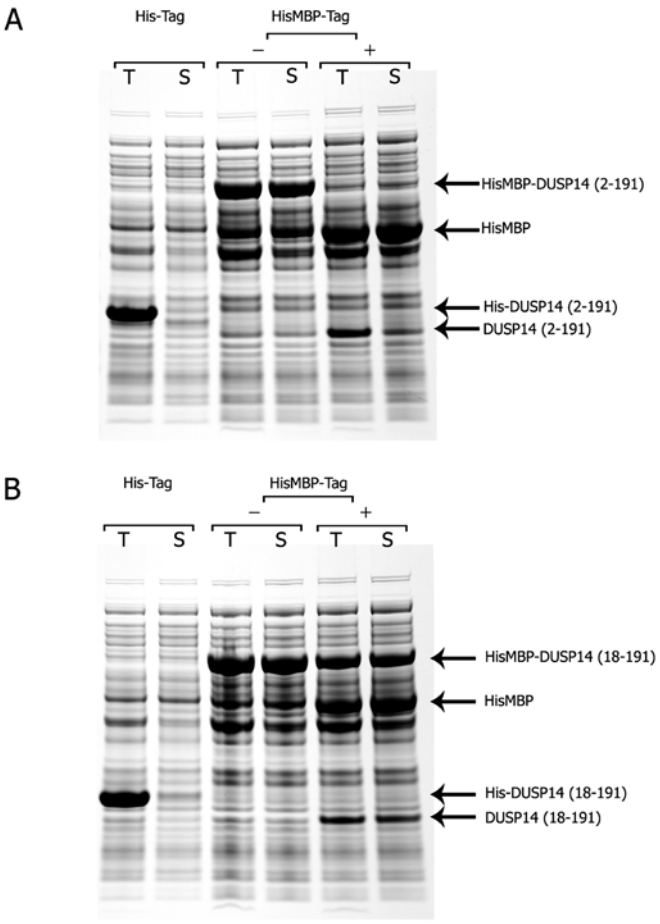

Fig 16.2. Overproduction and intracellular processing of pDEST–HisMBP fusion proteins by TEV protease. DUSP14(2–191) and DUSP14(18–191) were expressed from derivatives of pDEST–HisMBP in BL21-Pro cells that also contained the TEV protease expression vector pRK603 as described (*see* **Section 3.2**). "T" and "S" refer to the total and soluble fractions of the intracellular protein, respectively. All cultures were induced with IPTG to initiate the production of the His_6–MBP fusion proteins. Samples marked "+" were induced with anhydrotetracycline to initiate the production of TEV protease 2 h after the addition of IPTG, whereas samples marked "–" were not induced with anhydrotetracycline. The results of this experiment are discussed in **Section 3.2.5**.

Normally, once it has been established that an aggregation-prone passenger protein can be rendered soluble by fusing it to His_6–MBP, that the fusion protein can be cleaved by TEV protease, and that the passenger protein remains soluble after it is released from His_6–MBP, then the passenger protein is ready to be purified on a large scale. Detailed instructions for purification using a generic IMAC-based protocol have been described elsewhere (15). However, one should be aware that occasionally a passenger protein may accumulate in a soluble but inactive form. Exactly how and why this occurs is unclear. Fusion

to MBP may enable certain proteins to evolve into kinetically trapped folding intermediates that are no longer susceptible to aggregation. Therefore, it is recommended that a biological activity assay (preferably) or biophysical techniques be used at an early stage to confirm the native conformation of the passenger protein before starting a large-scale purification.

4. Notes

1. Any gyrA+ strain of *E. coli* can be used. We prefer competent DH5α cells (Invitrogen, Carlsbad, CA, USA) because they are easy to use and have high transformation efficiencies.
2. Clonase enzyme mixes should be thawed quickly on ice and then returned to the –80°C freezer as soon as possible. It is advisable to prepare multiple aliquots of the enzyme mixes the first time they are thawed in order to avoid repeated freeze–thaw cycles.
3. We recommend a proofreading polymerase such as Pfu Turbo (Stratagene, La Jolla, CA, USA), Platinum Pfx (Invitrogen, Carlsbad, CA, USA), or Deep Vent (New England Biolabs, Beverly, MA, USA) to minimize the occurrence of mutations during PCR.
4. We typically purify fragments by electrophoresis using precast E-gels purchased from Invitrogen. However, suitable equipment and reagents for horizontal agarose gel electrophoresis can be purchased from a wide variety of scientific supply companies. DNA fragments are extracted from slices of the ethidium bromide-stained gel using a QIAquick gel extraction kit (Qiagen, Valencia, CA, USA) in accordance with the instructions supplied with the product.
5. We prefer the QIAprep Spin miniprep kit (Qiagen, Valencia, CA, USA), but similar kits can be obtained from a wide variety of vendors.
6. We prefer to use electrocompetent cells because of the high transformation efficiency that can be achieved (18). Detailed protocols for the preparation of electrocompetent cells and electrotransformation procedures can be obtained from the electroporator manufacturers (e.g., Bio-Rad, BTX, Eppendorf). Briefly, the cells are grown in 1l of LB medium (with antibiotics, if appropriate) to mid-log phase ($OD_{600\ nm}$ ~0.5) and then chilled on ice. The cells

are pelleted at 4°C, resuspended in 1 l of ice-cold H_2O, and then pelleted again. After several such washes with H_2O, the cells are resuspended in 3–4 ml of 10% glycerol, divided into 50 μl aliquots, and then immediately frozen in a dry ice/ethanol bath. The electrocompetent cells are stored at –80°C. Immediately prior to electrotransformation, the cells are thawed on ice and mixed with 10–100 ng of DNA (e.g., a plasmid vector or a Gateway reaction). The mixture is placed into an ice-cold electroporation cuvette and electroporated according to the manufacturer's recommendations (e.g., a 1.5 kV pulse in a cuvette with a 1 mm gap). Immediately add 0.450 ml of SOC medium (18) to the cells and allow them to grow at 37°C with shaking (ca. 250 rpm) for 1 h. Then spread 5–200 μl of the cells on an LB agar plate containing the appropriate antibiotic(s).

7. If the open reading frame encoding the passenger protein contains codons that are rarely used in *E. coli* (http://www.doe-mbi.ucla.edu/cgi/cam/racc.html), this can adversely affect the yield of an MBP fusion protein. In such cases, it is advisable to introduce an additional plasmid into the host cells that carry the cognate tRNA genes for rare codons. The pRIL plasmid (Stratagene, La Jolla, CA, USA) is a derivative of the p15A replicon that carries the *E. coli argU*, *ileY*, and *leuW* genes, which encode the cognate tRNAs for AGG/AGA, AUA, and CUA codons, respectively. pRIL is selected for by resistance to chloramphenicol. In addition to the tRNA genes for AGG/AGA, AUA, and CUA codons, the pRARE accessory plasmid in the Rosetta™ host strain (EMD, Madison, WI, USA) also includes tRNAs for the rarely used CCC and GGA codons. Like pRIL, the pRARE plasmid is a chloramphenicol-resistant derivative of the p15A replicon. Both of these tRNA accessory plasmids are compatible with derivatives of pDEST–HisMBP. On the other hand, they are incompatible with the vector pRK603 that we use for intracellular processing experiments (*see* **Section 3.2.1**). Nevertheless, because pRK603 and the tRNA accessory plasmids have different antibiotic resistance markers, it is possible to force cells to maintain both plasmids by simultaneously selecting for kanamycin and chloramphenicol resistance. Alternatively, the kanamycin-resistant TEV protease expression vector pKM586, a pRK603 derivative with the replication machinery of a pSC101 replicon, which can be obtained from the authors, can be stably maintained in conjunction with p15A-type tRNA plasmids.

8. We find it convenient to use pre-cast gels for SDS-PAGE gels (e.g., 1.0 mm × 10 well, 10–20% Tris-glycine

gradient), running buffer, and electrophoresis supplies from Invitrogen (Carlsbad, CA, USA).

9. Alternatively, the PCR reaction can be performed in two separate steps, using primers N1 and C in the first step and primers N2 and C in the second step. The PCR amplicon from the first step is used as the template for the second PCR. All primers are used at the typical concentrations for PCR in the two-step protocol.
10. The PCR reaction can be modified in numerous ways to optimize results, depending on the nature of the template and primers. *See* (18) (Vol. 2, Chapter 8) for more information.
11. PCR cycle conditions can also be varied. For example, the extension time should be increased for especially long genes. A typical rule of thumb is to extend for 60 s/kb of DNA.
12. This "one-tube" Gateway protocol bypasses the isolation of an "entry clone" intermediate. However, the entry clone may be useful if the investigator intends to experiment with additional Gateway destination vectors, in which case the BP and LR reactions can be performed sequentially in separate steps; detailed instructions are included with the Gateway PCR kit. Alternatively, entry clones can easily be regenerated from expression clones via the BP reaction, as described in the instruction manual.
13. At this point, we remove a 5 μl aliquot from the reaction and add it to 0.5 μl of proteinase K solution. After 10 min at 37°C, we transform 2 μl into 50 μl of competent DH5α cells (*see* **Note 1**) and spread 100–200 μl on an LB agar plate containing kanamycin (35 μg/ml), the selective marker for pDONR221. From the number of colonies obtained, it is possible to gauge the success of the BP reaction. Additionally, entry clones can be recovered from these colonies in the event that no transformants are obtained after the subsequent LR reaction.
14. If very few or no ampicillin-resistant transformants are obtained after the LR reaction, the efficiency of the process can be improved by incubating the BP reaction overnight.
15. The optimum temperature for TEV protease activity is 30°C. At 37°C, the protease does not fold properly in *E. coli* and little processing will occur. Reducing the temperature also improves the solubility of some MBP fusion proteins.
16. We routinely break cells in a 1.5 ml microcentrifuge tube on ice with two or three 30 s pulses using a VCX600

sonicator (Sonics & Materials, Newtown, CT, USA) with a microtip at 38% power. The cells are cooled on ice between pulses.

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