Chapter 15

Purification of Proteins Fused to Maltose-Binding Protein

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Abstract

Maltose-binding protein (MBP) is one of the most popular fusion partners being used for producing recombinant proteins in bacterial cells. MBP allows one to use a simple capture affinity step on amylose–agarose columns, resulting in a protein that is often 70–90% pure. In addition to protein-isolation applications, MBP provides a high degree of translation and facilitates the proper folding and solubility of the target protein. This chapter describes efficient procedures for isolating highly purified MBP-target proteins. Special attention is given to considerations for downstream applications such as structural determination studies, protein activity assays, and assessing the chemical characteristics of the target protein.

Key words: Maltose-binding protein, Protein expression and purification, Protein solubility, Protein aggregation and soluble aggregates, Fusing protein tags, Folding, Purification techniques, Amylose–agarose, TEV protease

1. Introduction

MBP is one of the oldest and most popular fusion partners being used for producing recombinant proteins in bacterial cells. It is the product of the *malE* gene in *Escherichia coli*, part of the maltose/maltodextrin system of that organism, and it acts as a receptor for chemotaxis and gene regulation (1). An advantage of MBP is that it can be expressed in bacterial cells in both secreted and nonsecreted forms. Expression levels are higher when the protein is produced in the cytoplasm, however fusing the target protein to the secreted form of MBP delivers the complex into the periplasm, and this can facilitate the folding of proteins with disulfide bonds (2) (see Note 1).

MBP enhances both the production and solubility of its fusion partner by a mechanism that is still not completely understood. Studies have suggested that MBP functions as a "chaperone magnet"

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by recruiting chaperones that normally associate with MBP to the vicinity of the target protein (3) or that form large micelle-like aggregates with incompletely folded passenger proteins held inside (4, 5). Recent studies also show that the MBP plays a passive role in the folding of its target fusion partner and works similarly to the solubility-enhancing protein NusA (6).

Despite the high-metabolic burden on the host cell, owing to the considerable size of the protein (approximately 42 kDa), MBP is still considered to be one of the best choices for circumventing heterologous expression problems. The isolation and purification of a protein tagged with MBP can be achieved by using a cheap and convenient affinity column that can yield tagged protein that is 70–90% pure following a single-capture step. In order to achieve a higher degree of purification, which is often required for downstream applications such as structural studies, one should add additional purification steps such as ion exchange, hydrophobic exchange, and size exclusion chromatography.

Ion exchange chromatography is essential as an intermediate step for separating target proteins from protein contaminants such as chaperons and other host cell proteins. It also allows one to separate the target protein from heterogeneously folded forms that are a consequence of the expression and purification conditions used and from heterogeneity in posttranslational modifications. Sometimes purification techniques that separate proteins according to their charge are insufficient, and other approaches based on different principles, such as hydrophobic exchange chromatography or hydroxyapatite, should be used. As a final polishing step, it is often recommended to use size-exclusion chromatography, not only to eliminate protein contaminants and low molecular weight molecules but also to obtain a homogeneous oligomeric form. An added value of the gel filtration step is that the protein will elute in the final desired buffer.

Following purification, the MBP tag can be removed from the target protein by a specific protease (see Notes 2 and 3). However, structural studies of the proteins, and crystallography in particular, may gain a huge advantage from using the uncleaved protein because the structure of MBP has already been solved, and the rather straightforward procedure of molecular replacement phasing can be employed, instead of the exhaustive time-consuming procedure of heavy atom derivative phasing (see Note 4).

Advantages of the MBP fusion system include enhanced expression, improved solubility, ease of purification, and mild elution conditions. MBP purification procedures are highly efficient and compatible with most downstream applications, making MBP one of the most desirable choices of fusion partner for recombinant protein expression. Here, efficient procedures for isolating highly purified MBP-target proteins are described.

2. Materials

All materials may be sourced from Sigma-Aldrich unless otherwise stated.

2.1. Protein Expression Conditions

1. Vectors: the *Rack1* expression plasmid lpMAL-c2MBP-Rack1 was a kind gift from Prof. Daria Mochly-Rosen.
2. *E. coli* BL21(DE3) strain (Novagen).
3. Luria-Bertani (LB) medium: prepare 1 L using 10 g bactotryptone, 5 g bacto-yeast extract, 10 g NaCl, 166 µL NaOH (10 N), and 10 mL MgSO4 (1 M).
4. Isopropyl-β-d-thiogalactopyranoside.
5. Incubator-Shaker, e.g., Innova 43 (New Brunswick Scientific).

2.2. Amylose–Agarose Column

Homemade amylose–agarose column.

1. Sepharose 6B.
2. Vinyl sulfonic acid.
3. Amylose (Type III from potato, Sigma, St. Louis, MO).
4. 1 M sodium carbonate pH 11.
5. 0.9% NaCl.
6. 20% Ethanol.
7. Sinter glass.

Commercial amylose–agarose column: amylose resin high flow (New England Biolabs).

2.3. Protein Purification

2.3.1. Lysis and Clarification

1. Micro-fluidizer (M-110 EHIS; Microfluidics Corp., Newton, MA).
2. Buffer A (20 mM Tris–Cl pH 7.4, 200 mM NaCl, 1 mM EDTA, 0.02% NaN_3).
3. Protease inhibitor cocktail.
4. DNase 1.
5. Lysozyme (Thermo Scientific).
6. Filter GF/D (Whatman) and 0.45 µm filter (Whatman).

2.3.2. Capture: Affinity Chromatography: Amylose Resin

1. ÄKTAexplorer system (GE Healthcare).
2. Maltose.
3. A 12% sodium dodecyl sulfate–polyacrylamide (SDS–PAGE) gel.

2.3.3. Intermediate Purification: Ion Exchange Chromatography

1. Resource 30Q column (GE Healthcare) 7× 1.6 cm.
2. Buffer A1: 20 mM Tris–Cl pH 8.0, 0.02% NaN_3.

3. Buffer B: buffer A1 + 1 M NaCl.
4. Centriplus cut-off 30 kDa (Amicon, Millipore).

2.3.4. Final Polishing: Size Exclusion Chromatography

1. Sephacryl S100 FF column (GE Healthcare) 92 × 2.6 cm.
2. Buffer A2 (20 mM Tris–Cl pH 8.0, 0.1 M NaCl, 0.02% NaN_3).

2.3.5. Column Regeneration and Storage

1. 0.1% SDS.
2. 0.5 M NaOH.
3. 20% Ethanol.

3. Methods

3.1. Expression Conditions

1. *E. coli* BL21(DE3) competent cells are transformed with 10 ng of pMAL-c2MBP-Rack1 and plated on LB agar plates containing 100 μg/mL ampicilin and incubated for 16 h at 37°C, (see Note 1).
2. A single colony is used to inoculate a tube containing 10 mL LB with 100 μg/mL ampicilin.
3. The cells are grown in a shaker incubator for 16 h, transferred into 1 L of LB medium at an inoculum to medium ratio of 1:100 and placed in a 37°C incubator shaker.
4. IPTG is added to a final concentration of 0.3 mM when the OD600 reaches 0.6.
5. The cells are harvested after 6 h of incubation at 30°C.
6. Pellets are kept at –80°C until further processing.

3.2. Preparation of an Amylose–Agarose Column

As an alternative to commercially available columns, home-made amylose–agarose columns may be prepared using a procedure similar to that used to prepare lactose–sepharose beads (7).

1. Twenty-five milliliters of Sepharose 6B is washed with water in a Sinter glass and with 1 M sodium carbonate pH 11.
2. The resin is resuspended in 25 mL of 1 M sodium carbonate pH 11 and allowed to react by mixing for 70 min at room temperature with 5 mL vinyl sulfonic acid.
3. After washing with 500 mL of water, the resin is resuspended in a 25 mL solution of 2.6 g amylose in 1 M sodium carbonate pH 11, with continuous stirring overnight.
4. After washing again with water, 0.9% NaCl, and water again, the resin is maintained in a solution of 20% ethanol/80% water at 4°C. The Amylose-agarose column can be purchased from New England Biolab (Amylose Resin High Flow #E8022L) or it can be prepared at home (See Note 5).

3.3. Protein Purification

3.3.1. Lysis and Clarification

All procedures should be performed at 4°C.

1. The frozen cell pellet from a 1 L culture is thawed on ice and resuspended in 70 mL of buffer A supplemented with protease inhibitor cocktail 1:200, 50 µg/mL DNase I, and 0.2 mg/mL lysozyme.
2. The cells are lysed mechanically using a Micro-fluidizer at 21,000 psi.
3. Insoluble cell debris is removed from the cell lysate by centrifugation at 4°C for 20 min (15,000 × *g*); subsequently, the cleared lysate is first filtered through a GF/D filter and then a 0.45 µm filter.

3.3.2. Capture: Affinity Chromatography: Amylose Resin (See Fig. 1)

1. An amylose–agarose column 9.2 × 2.6 cm (49 mL) is equilibrated, prior to the lysis steps, with buffer A using an ÄKTAexplorer system at 4°C. Equilibration is confirmed by measuring pH and conductivity. Pressure limit: 0.5 MPa.
2. The column is loaded with filtered lysate at 1.7 mL/min and washed with buffer A at 2.5 mL/min up to low optical density (~5 cv, column volume).
3. Protein is eluted with elution buffer (buffer A + 20 mM maltose) at 1.5 mL/min, collecting fractions of 9 mL during 4 cv.

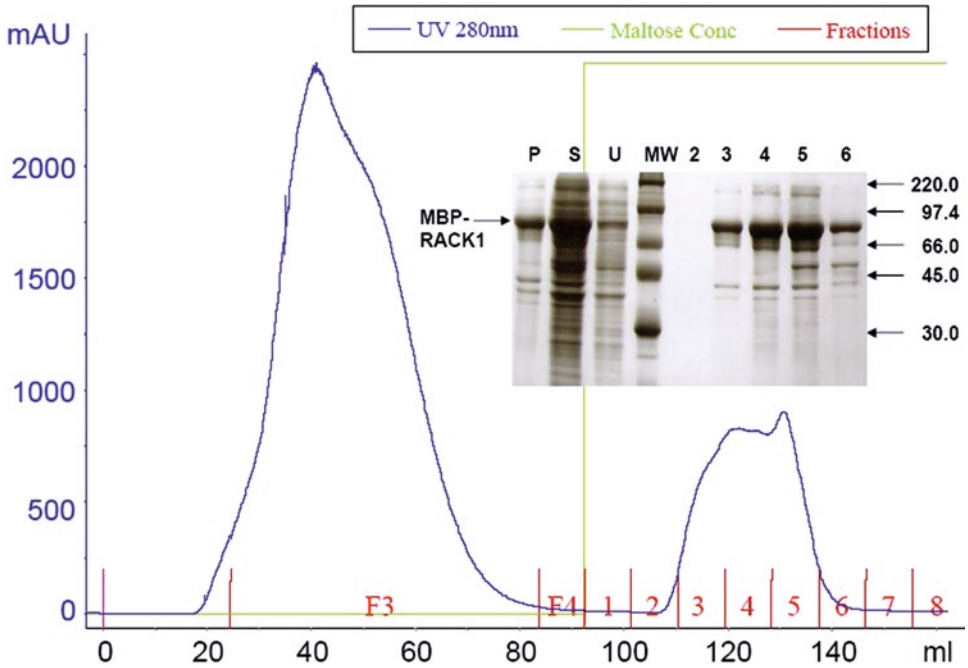

Fig. 1. Capture – affinity chromatography – amylose resin. A cell pellet from a 1 L culture was lysed, clarified, and purified on a homemade amylose resin column, as described in Subheading 3.3.2. Fractions were analyzed by SDS–PAGE. *P* pellet, *S* supernatant after lysis and centrifugation, *U* unbound to amylose resin, *MW* molecular weight markers.

4. Samples from each fraction are analyzed for protein content by SDS–PAGE. Protein-containing fractions are then pooled according to the profile obtained.

3.3.3. Intermediate Purification: Ion Exchange Chromatography (See Fig. 2)

1. A Resource 30Q column (7 × 1.6 cm, 14 mL) is equilibrated with buffer A1, and equilibration is confirmed by measuring pH and conductivity as before.
2. Pooled protein from the affinity step is diluted 1:4 with buffer A1 to reduce conductivity, filtered with a 0.45 μm filter and loaded at 6 mL/min.
3. The column is washed with 4% buffer B at 4 mL/min up to low optical density (~3 cv).
4. Protein is eluted with a 15 cv gradient (4–15% buffer B at 4 mL/min) collecting fractions of 4 mL, and then 4 cv 15–30% buffer B, and 4 cv 30–100% buffer B at 6 mL/min, collecting fractions of 9 mL.
5. Samples from each fraction are analyzed for protein content by SDS–PAGE. Protein-containing fractions are then pooled according to the profile obtained. The main peak elutes at around 11.5% buffer B.

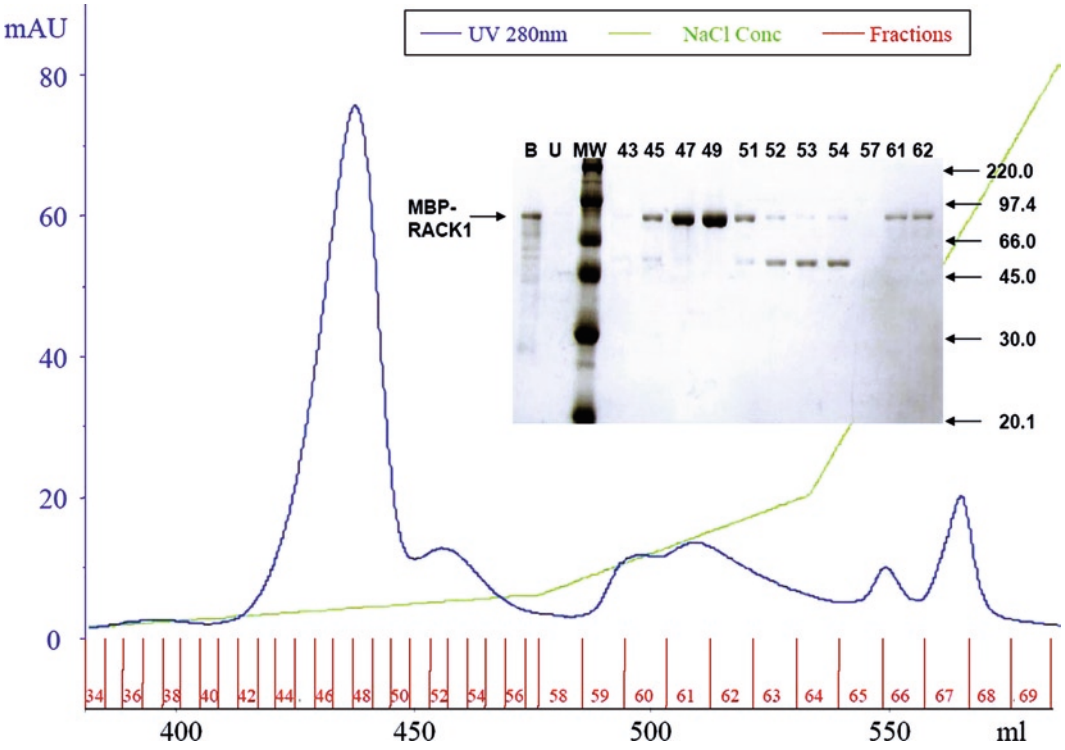

Fig. 2. Intermediate purification: ion exchange chromatography. Affinity purified protein was further purified by anion exchange chromatography, as described in Subheading 3.3.3. Fractions were analyzed by SDS–PAGE. *B* before binding, *U* unbound to anion exchange resin, *MW* molecular weight markers.

6. The pooled sample is concentrated to 10 mL with a Centriplus unit (cut-off 30 kDa).

3.3.4. Final Polishing: Size Exclusion Chromatography (See Fig. 3)

1. A Sephacryl S100 FF column (92 × 2.6 cm, 489 mL) is equilibrated with buffer A2. Equilibration is confirmed by measuring pH and conductivity as before.
2. The concentrated peak from Subheading 3.3.3, step 6 is loaded and run (isocratic elution) at 2 mL/min, collecting fractions of 4 mL.
3. Samples from each fraction are analyzed for protein content by SDS–PAGE. The main peak elutes at around 0.47 cv (see Note 6).

3.3.5. Column Regeneration and Storage

1. Amylose–agarose columns are regenerated with 0.1% SDS at room temperature, then water, and maintained in 20% ethanol at 4°C (see Note 7) (consult manufacturer's instructions if using purchased columns).
2. Resource 30Q columns are regenerated with 0.5 M NaOH (up-flow direction), then circulate buffer until the pH is neutral, then circulate water and finally 20% ethanol. Maintain the columns in 20% ethanol at 4°C.
3. Sephacryl S100 FF columns are regenerated with 0.5 M NaOH (up-flow direction), then circulate buffer until the pH is neutral, and maintain the columns in buffer containing 0.02% NaN_3 at room temperature.

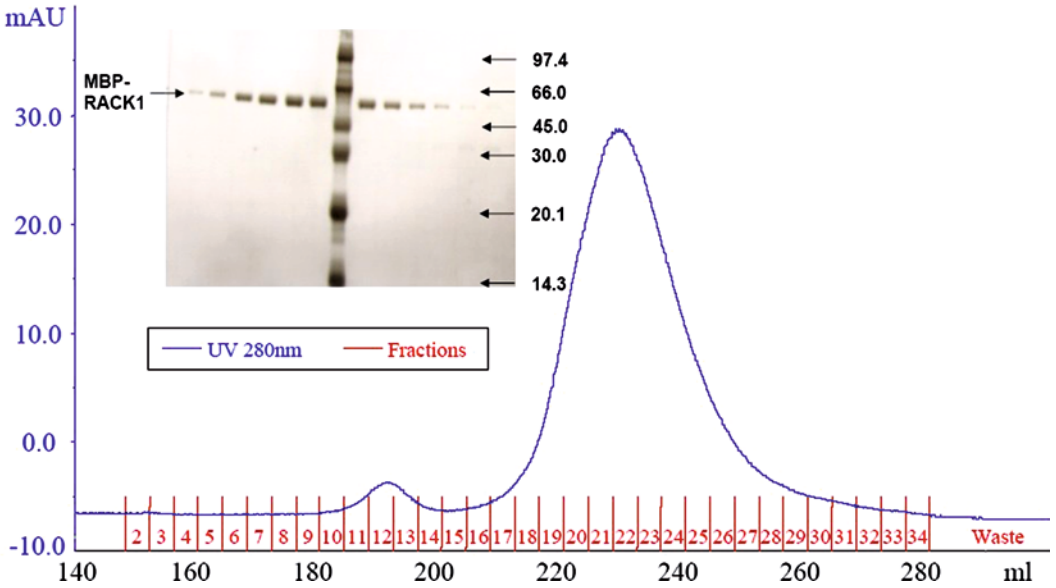

Fig. 3. Final polishing: size exclusion chromatography. The pooled eluate obtained following anion exchange was further purified by gel filtration chromatography as described in Subheading 3.3.4. Fractions were analyzed by SDS–PAGE (*MW* molecular weight markers).

4. Notes

1. The most popular commercial vectors are the pMAL vectors, available from NEB. These vectors allow the expression of a secreted or cytosolic form of MBP, fused to a target protein, under the regulation of an IPTG-induced *tac* promoter. The use of this promoter allows pMAL vectors to be used in a wide variety of bacterial hosts, since the *tac* promoter utilizes the bacterial RNA polymerase for transcription. Removal of the MBP fusion protein is also an option in many vectors, where protease recognition sites such as factor Xa and enterokinase are inserted between the MBP and the target gene multiple cloning site (see Chapter 19 for more details on the removal of affinity tags). The more recent pMAL vectors from NEB contain mutated MBP that allows improved affinity binding to amylose resins. There are also several options for noncommercial vectors, available from the Addgene repository (www.addgene.org). In some noncommercial vectors, the MBP was cloned under the control of a T7 promoter, suitable for tighter regulation of expression using *E. coli* DE3 strains.
2. A noteworthy system was developed by Waugh's group to assess the solubility of the target protein after the removal of the MBP. This system requires the coexpression of the MBP-target protein with a compatible vector containing the TEV protease gene. Both plasmids are cotransformed into the same cell and induced by IPTG and anhydrotetracycline, respectively. The target protein is then analyzed by SDS–PAGE to determine solubility. This approach will predict whether the fusion protein will be cleaved efficiently by TEV protease and whether the cleaved protein will remain soluble after cleavage (8, 9).
3. Early MBP-containing vectors were designed with a proteolytic cleavage site for factor Xa or Thrombin at the junction between the MBP and the target protein, allowing the removal of the MBP from the chimera. Cleavage with these proteases may sometimes result in nonspecific digestion of the target protein. This problem can be solved by using more specific proteases such as Enterokinase, Rhinovirus 3C protease (Precision™/GE Healthcare) or TEV protease. The advantage of using the TEV protease is that it is active at 4°C, whereas the other proteases usually require higher temperatures and a long period of incubation, which may cause enhanced aggregation and inactivation of the cleaved protein. Another advantage of using this protease is its resistance to detergents that are often essential in the preparation of membrane proteins and other hydrophobic proteins (10). There are

several commercial and noncommercial vectors that express a TEV protease that enables one to remove the MBP from the target protein (11–13). TEV protease does not have to be purchased and can be produced using a simple expression and purification procedure in a bacterial system developed by David Waugh's laboratory (14). This option considerably reduces the cost of preparation for downstream applications especially in large-scale production.

4. Large-affinity tags such as MBP may offer some advantages for structural biology applications, since they can facilitate the crystallization of problematic proteins. For this purpose, the target protein must be rigidly fused to the MBP by a short spacer, such as three to five alanines, to reduce the conformational heterogeneity introduced by a flexible linker. Moreover, fusion of membrane proteins to MBP can increase the size of the hydrophilic domain, and eventually facilitate crystallization (15).
5. Tris–HCl, MOPS, HEPES, and phosphate buffers at pH values between 6.5 and 8.5 are all compatible buffers for MBP binding. Since MBP binds to amylose primarily via hydrogen bonds, high ionic strengths such as 1 M NaCl can be used in order to reduce nonspecific adsorption of proteins to the resin (13). Optional additives that can be added are 0.02% sodium azide to avoid bacterial contamination in the medium and reducing agents such as 10 mM β-mercaptoethanol or 1 mM DTT that serve to maintain reduced cysteines and to avoid the formation of nonspecific disulfide bridges that can cause aggregation.
6. Troubleshooting tips

   Inadequate binding of MBP-tagged proteins to the amylose resin:

   (a) The presence of endogenous amylases during bacterial growth may competitively inhibit binding to the amylose column. This problem can be partially overcome by using 0.2% glucose in the growth medium, in order to repress the endogenous amylase expression (see Instruction Manual from pMAL™ Protein Fusion and Purification System, NEB).

   (b) The presence of nonionic detergents such as Triton X-100 and Tween-20 can interfere with binding. If detergents are essential to the target protein, use less than 0.05% in order to solubilize the extract (13). However, if this concentration is too low, you might need to consider improving binding by screening alternative detergents.

   (c) The oligomeric state of the molecule (soluble aggregates) can affect its binding to affinity columns. The presence of soluble aggregates can be analyzed by gel filtration.

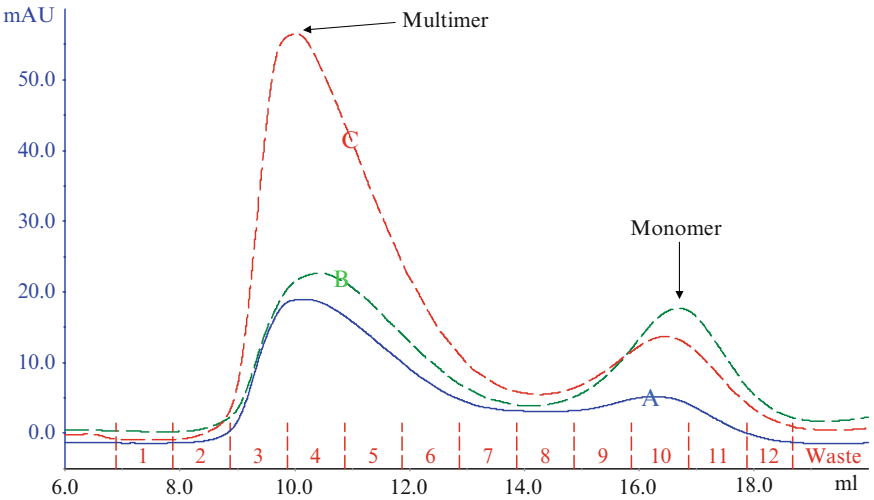

Fig. 4. Gel filtration analysis of the oligomeric state of an MBP fusion protein. A 30 kDa cytosolic protein, prone to aggregation, was fused to MBP in order to increase its solubility. The construct was transformed into *E. coli* BL21 cells. Upon reaching an OD600 of 0.7, the sample was induced with 0.4 mM IPTG and incubated for 4 h at 37°C (**a**). Other transformed cells were grown at 37°C until the OD600 reached 0.3. At this point, 0.1% glycerol and 0.1 mM potassium glutamate were added directly to the medium (we used SIGMA G-1501 L-Glutamic acid monopotassium salt). Next, the sample was subjected to a 20 min heat-shock treatment at 42°C, after which the temperature was reduced to 37°C, and the sample was induced with 0.4 mM IPTG at an OD of 0.7. One sample was harvested at 4 h postinduction at 37°C (**b**), and another was harvested at 16 h postinduction at 37°C (**c**). All samples were lysed, purified on an amylose–agarose column, and loaded on an analytical Superdex 200 gel filtration column to check the oligomeric state of the protein.

The formation of oligomers can be reduced by changing the expression conditions or the purification procedure and by screening different buffers and additives. In some cases, heat-shock treatment can greatly enhance the monomeric fraction of the expressed protein. In Fig. 4, MBP-fused protein was expressed under three different conditions: 37°C for 4 h, heat shock treatment followed by 4 h incubation, and heat shock treatment followed by 16 h induction. These results indicate that when the samples were subjected to heat shock treatment, a larger fraction of the protein shifted to the monomeric state. Although overnight induction resulted in an increased yield of the total protein, the yield of the monomeric form decreased (see Fig. 4).

(d) In some cases, however, the efficiency of binding and purification using amylose resin is not satisfactory. Here, one might consider adding a polyhistidine tag (His6) to the N terminus of MBP. This addition does not interfere with the ability of MBP to promote the solubility and proper folding of its fusion partners, and it can be used for binding to the more commonly used immobilized metal affinity chromatography systems (IMAC) (9, 14,

16, 17) (see also Chapter 17). Alternatively, NEB has recently developed improved MBP mutants with higher abilities to bind amylose resin (NEB pMAL-p4 and c4 series).

Protein is not eluted efficiently from the column.

(e) If the kinetics of the elution is too low, the protein is not completely eluted from the resin or is not eluted in a sharply concentrated peak. The following parameters should be considered as a means of improving the situation: (i) decreasing the elution flow rate, (ii) overnight incubation in the elution buffer, when performing batch purification, and (iii) increasing the concentration of maltose in the elution buffer by using from 20 to 100 mM maltose.

(f) The oligomeric state of the protein can change as a result of the high protein concentration in the column. Here, changes in the buffer can prevent aggregation and the following options should be considered: (i) increasing ionic strength up to 1 M NaCl or KCl, (ii) adding detergents or additives such as glycerol to the buffers, and (iii) performing batch binding instead of column binding.

(g) If multiple protein bands are present after elution, then protein degradation is to be suspected. Western blot analysis can be performed to verify if proteolysis is occurring. Conducting all purification steps at 4°C, reducing the overall time taken to carry out the procedure, and using protease inhibitors during the cell disruption process, can all help to reduce proteolysis.

(h) If the additional bands visible on SDS–PAGE are not the result of target protein degradation, there are two main reasons that usually explain the presence of cellular protein contaminants: (i) contaminating proteins are binding nonspecifically to the resin, (ii) contaminants are sticking to the target protein. If contaminants are bound nonspecifically to the resin, consider decreasing the resin volume to increase competition, or increasing the ionic strength of the buffers (up to 1 M NaCl or KCl), to reduce hydrophobic interactions with the resin. If contaminants stick to the target protein, increasing the washing step is the first option that should be considered. If this does not work, consider increasing the ionic strength of the buffers (up to 1 M NaCl or KCl), adding additives such as glycerol, adding reducing agents in order to disrupt nonspecific intermolecular disulfide bonds, or adding detergents that might reduce hydrophobic

interactions. If taking these options does not reduce the presence of contaminants, additional purification steps should be performed before or after affinity purification.

(i) Maltose should be removed completely after the two last purification steps (ion exchange and gel filtration). When MBP fusion proteins are purified by affinity chromatography without further purification columns, dialysis after affinity purification is not enough to eliminate maltose from the protein solution. Maltose can be completely removed by binding the fusion protein to hydroxyapatite, or by ion exchange, or hydrophobic exchange, or any other resin that can bind the fusion protein and not the sugar. The resin is then washed extensively before protein elution (see pMAL™ Protein Fusion and Purification System manual from NEB).

(7) Amylose–agarose columns can be regenerated with 0.1% SDS at room temperature, water (according to New England Biolabs) or 0.1 M NaOH for a very short time and then neutralized. Alternatively, they can be regenerated with 50 mM HEPES pH 7.4, 4 M urea, 0.5% w/v SDS and then 50 mM HEPES pH 7.4, 150 mM $(NH4)_2SO_4$, 2 mM EDTA, 2 mM EGTA and water (18), and kept in 20% ethanol at 4°C. GE Healthcare developed MBPTrap™ HP, a ready to use and very successful new column for purifying recombinant proteins tagged with maltose-binding protein (MBP). MBPTrap™ HP can be easily regenerated using 0.5 M NaOH, so columns can be used for repeated runs, with reproducible results (GE Healthcare Date file 28-9136-33 AA MBPTrap HP 1 ml and 5 ml).

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