Coupling of MBP fusion protein cleavage with sparse matrix crystallization screens to overcome problematic protein solubility

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BioTechniques 39:476-480 (October 2005)
doi 10.2144/000112033

Nuclear magnetic resonance (NMR) and crystallographic techniques require large quantities of pure, soluble protein. However, numerous target proteins exhibit limited solubility resulting in low recovery and reduced concentrations following purification. Molecular biological techniques have been developed to assist in this area including the use of a fusion protein attached to the N or C terminus. For example, maltose binding protein (MBP) can increase solubility (1), although its proteolytic removal often results in precipitation of the target protein. Furthermore, efforts to prepare diffraction quality crystals of MBP fusion proteins have required laborious optimization of linkers, ultimately yielding crystals of limited diffraction quality (2). Mutations of surface residues can improve solubility, but this often requires knowledge of the protein structure. More comprehensive approaches, such as sparse matrix screening (3), have yielded greater success. Examples include cell lysis in the presence of specific solubilizing agents to optimize yield. Button dialysis (4) or microdrops (5) can screen a wide range of pH and additives, and these methods have been successful in improving solubility and crystal formation (6). The latter techniques, however, are often limited by methodologies requiring low protein concentrations that cannot be increased significantly throughout the course of study. Here we report a novel strategy to rapidly survey a diverse number of solubilization reagents while simultaneously removing the MBP tag. This “fusion protein cleavage screen”

combines the enhanced expression and solubility properties of MBP with the efficiency of sparse matrix screening. As a case study, we report results for the 42-kDa catalytic domain of human type II inosine monophosphate dehydrogenase (IMPDH2Δ), which is being studied by X-ray crystallography in our laboratory.

Initial attempts to purify the catalytic domain IMPDH2Δ met with low expression and poor solubility. Use of the H-MBP-3C vector (7) dramatically increased expression. Cells were grown

in LB broth at 37°C to an absorbance (A) at 600 nm of 0.6 and induced with isopropyl β-D-thiogalactopyranoside (IPTG; Sigma, St. Louis, MO, USA) for 4 h. The cells were harvested by centrifugation and suspended in buffer A [10 mM NaH₂PO₄/Na₂HPO₄ buffer, pH 7.5, with 0.25 M NaCl, and 0.1 mM dithiothreitol (DTT)] and then lysed at 4°C by sonication, clarified, and the supernatant passed over Ni-NTA® resin (Qiagen, Valencia, CA, USA). However, the protein precipitated during 3C protease cleavage on the resin. Therefore, the protein was eluted with buffer A containing 0.50 M imidazole (Sigma), and trials were conducted to cleave the eluted fusion protein, although these were unsuccessful.

Subsequently, an efficient method was developed for determination of solubilization conditions. We again employed the viral 3C protease of type H-3Cpro because it is highly specific, pH insensitive, and tolerant of a diverse number of ionic conditions (7,8). Similarly, MBP is soluble at >25 mg/mL in 10 mM Tris, pH 7.5 (9) and is very soluble from pH 5.0 to 9.0. Before

Figure 1. Fusion protein cleavage screen and fusion protein cleavage of H-MBP-3C-IMPDH2Δ. (A) Fusion protein cleavage screen. A 96-well plate with sitting drop platforms containing the cleavage reactions after overnight incubation at 20°C with a well solution of water. (Inset) Expanded views of initial (0 h) and 8 h time points are shown in rows. Comparing the two time points for the protein-only control (left) demonstrated no independent precipitation of the fusion protein. Two representative conditions are illustrated by comparison of the two time points. Both unsuccessful (middle, precipitated) and successful (right, unprecipitated) examples are depicted. An additional plate of drops with protein and additive was run to confirm the solubility in the absence of 3C protease (data not shown). (B) Fusion protein cleavage of H-MBP-3C-IMPDH2Δ. The Coomassie® Blue-stained sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel demonstrated the fusion protein before cleavage (lane a) and after overnight cleavage with 3C protease (lane b). MBP, maltose binding protein; IMPDH2Δ, the 42-kDa catalytic domain of human type II inosine monophosphate dehydrogenase.

cleavage of H-MBP-3C-IMPDH2Δ, the fusion protein was soluble at more than 10 mg/mL in 10 mM Tris, pH 7.5. Therefore, samples were prepared with 10 mM Tris buffer, pH 7.5, 1 mM DTT, at 2.5, 5.0, and 10 mg/mL protein concentrations.

For sparse matrix screens, the Crystal Screen™, Crystal Screen 2, Additive Screen, and Detergent Screen (all from Hampton Research, Aliso Viejo, CA, USA) were utilized, as well as additional solutions of amino acids (10), sugars (11), substrates of IMPDH or analogs thereof, and stocks of glycerol or urea at high concentration. These reagents were each screened separately at a variety of different concentrations over a wide pH range. Aside from crystal screening reagents, conditions designed to enhance solubility, such as the JBS Solubility Kit (Jena Bioscience GmbH, Jena, Germany) (12) or other commercial additive and detergent screens may be suitable, although these were not tested

here. Matrix screening solutions were dispensed onto the drop platform of a 96-well sitting drop plate (Corning, Corning NY, USA) or an IMPACT® plate (Hampton Research) (Figure 1A). The 2-μL drops of protein solution were mixed with 1 or 2 μL additive and 1 μL protease, resulting in a 1:50 (w/w) ratio of protease to target protein. For the IMPACT plates, the drop was covered with paraffin oil to prevent dehydration. Plates were sealed and incubated for 8 h at 4° or 20°C. Clear drops were run on a PhastGel™ (Amersham Biosciences, Piscataway, NJ, USA) 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel to determine whether cleavage had occurred (Figure 1B).

For H-MBP-3C-IMPDH2Δ, several drops remained clear after incubation. Cleavage was nearly complete for most trials except those containing pH > 9.0 or < 4.0, ZnCl₂, or imidazole, which appeared to inhibit cleavage, respectively. Conditions that successfully solubilized IMPDH2Δ included increasing the pH to 8.5, chaotropic salts (2 M urea), osmolytes (20% w/v glycerol, 3% w/v polyethylene glycol 400, or 5% w/v Jeffamine M600), and substrate (20 mM inosine monophosphate). These conditions were optimized further, yielding solubility levels of 8 mg/mL. The established conditions were used to maintain solubility during bulk cleavage and after removal of MBP and 3C protease by anion exchange. The solubilization reagents were retained in crystallization trials that produced diffraction quality crystals (Figure 2).

By combining the increased expression and ease of purification of a 6His-MBP fusion protein with the microdrop method of sparse matrix screening, we have developed an efficient method for rapid determination of conditions that maximize solubility. Though commercial screens were optimized for crystallization, they proved useful in surveying solubility. For minimal concentration changes, IMPACT plates can be utilized with the drop containing fusion protein, additive, and protease covered by paraffin oil. The upper limits of solubility can be tested by vapor diffusion for reaction

mixtures in sitting drops placed over wells containing only the additive solutions. If initial conditions are too concentrated and/or precipitation is reversible, more dilute conditions can be found by placing reaction drops over wells containing additional water. Notably, supersaturation can be achieved in these vapor diffusion experiments, resulting in crystals. However, these were not suitable for X-ray diffraction due to their fragility and lack of a defined habit.

Despite the wide range of pH, salts, osmolytes, detergents, and amino acids, 3C protease robustly cleaved the fusion protein, and several solubilizing conditions were identified. Like standard microdrop methods, a lower quantity of sample was required per condition than with dialysis. This permitted a large sampling of additives and rapid detection of solubility enhancement. The H-MBP-3C vector utilized in the work and the GST fusion vector pGEX-6P (Amersham Biosciences) should be amenable to this technique, although MBP generally provides greater solubility. This method may not be suited to thrombin or Factor Xa, due to their less robust activities (see www4.amershambiosciences.com/APTRIX/upp01077.nsf/Content/lsn_online_article_190402_c). Notably, the method should be amenable to nanoliter-scale drop sizes (13) with the caveat that sufficient protein is needed to confirm protease cleavage. Perhaps this could be accomplished with a more sensitive stain for SDS-PAGE. Overall, the microdrop fusion protein cleavage screen may be of general utility for proteins with poor solubility that can only be expressed as fusion constructs.

ACKNOWLEDGMENTS

The authors thank Dr. L. Hedstrom for the type II IMPDH construct, Drs. S. Pascal and A. Alexandrov for the H-MBP-3C and H-3Cpro vectors, and Dr. R. Basavappa, E. Petri, and B. Tolbert for helpful discussions. This research was funded by National Institutes of Health (NIH) grant nos. GM63162 (J.E.W.) and GM525991 (B.M.G.).

Figure 2. Crystals obtained from optimized conditions. (A) Crystal optimized from 2 M urea. (B) Crystal optimized from 20% glycerol.

COMPETING INTERESTS
STATEMENT

The authors declare no competing interests.

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Received 13 July 2005; accepted
14 August 2005.

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