Profinity eXact™ Fusion-Tag System Performs On-Column Cleavage and Yields Pure Native Protein From Lysate in Less Than an Hour

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Introduction

Improved purification methods are required for protein structure and function studies, necessitated by growing demands from proteomics, drug development, and biotechnology programs. To simplify purification of recombinant proteins, including many with unknown biochemical properties, several genetically engineered affinity tags, or purification tags, are used. Commonly used tags are polyhistidine (His), glutathione-S-transferase (GST), and the antibody peptide epitope, FLAG (Arnau et al. 2006). The tag is fused to the N- or C-terminus of the protein of interest, allowing the fusion protein to be purified to near homogeneity in a single-step procedure using a resin with strong binding avidity and selectivity to the tag.

Once the fusion protein has been purified, it is often necessary to remove the tag before subsequent use in downstream applications (Arnau et al. 2006), because the tag may alter protein conformation (Chant et al. 2005), affect biologically important functions (Araújo et al. 2000, Fonda et al. 2002, Goel et al. 2000), or interfere with protein crystallization (Bucher et al. 2002, Kim et al. 2001, Smyth et al. 2003). The most popular method to remove the tag involves building a protease cleavage site between the tag and the target protein within the expression vector, and cleaving the resultant fusion protein using purified preparations of the cognate protease specific to the engineered site. The most frequently used processing proteases for this purpose are the tobacco etch virus (TEV) protease, thrombin, factor Xa, and enterokinase. Although these tag-removal systems alleviate problems associated with presence of the tag in the final purified protein, they have several principal drawbacks: 1) the high enzyme-to-substrate ratios, the elevated temperatures required for optimal or efficient processing, and the duration of the reaction may affect cleavage specificity as well as stability of the target protein (Arnau et al. 2006, Jenny et al. 2003); 2) the extended length of purification protocols due to additional cleavage and protease-removal steps may hamper high-throughput purification approaches and result in loss of target protein; 3) the nature of protease cleavage mechanisms often result in generation of protein products that still contain extra residues on their N-termini.

These complications can be easily avoided by using the Profinity eXact fusion-tag purification system. The Profinity eXact system offers a novel alternative to existing purification and cleavage tools, providing a truly one-step purification-cleavage protocol for bacterial recombinant protein production. The system is comprised of Profinity eXact purification resin and the Profinity eXact tag, which is a small 8 kD polypeptide expressed as a fusion to the N-terminus of the target protein. The ligand coupled to the resin matrix is based on the bacterial protease subtilisin BPN', which has been extensively engineered to increase stability and to isolate the substrate-binding and proteolytic functions of the enzyme (Abdulaev et al. 2005, Ruan et al. 2004). The incorporated modifications allow for conventional affinity binding with high selectivity, as well as specific and controlled triggering of the highly active cleavage reaction. Cleavage is achieved upon the addition of low concentrations of small anions, such as fluoride or azide. The native recombinant protein is released without any residual amino acids at the N-terminus, and the 8 kD Profinity eXact tag remains bound to the modified subtilisin ligand linked to the resin. Purification of fusion proteins is performed under native conditions, with tag cleavage and elution of purified protein from the column completed in about an hour.

To demonstrate the advantages of the Profinity eXact system one-step protocol, we compared the purification process of maltose-binding protein (MBP) fused with either GST or the Profinity eXact tag. To mimic the tag-removal capabilities of the Profinity eXact system, the GST-MBP fusions were also engineered with intervening thrombin or TEV cleavage sites. Performance parameters tested in this study include the time required for obtaining tag-free MBP and final yield and purity of the purified protein.

Methods

Vectors and Purification Resins

pGEX2T vector, thrombin protease, and GSTrap HP, HiTrap benzamidine FF, and HisTrap FF columns were purchased from GE Healthcare. AcTEV protease was purchased from Invitrogen Corporation. Profinity eXact pPAL7 expression vector and Bio-Scale™ Mini Profinity eXact™ cartridges (1 ml) were from Bio-Rad Laboratories, Inc.

Expression Vector Construction

The gene encoding MBP was amplified from pMAL vector (Invitrogen) using iProof™ high-fidelity polymerase (Bio-Rad). After digestion with the corresponding restriction enzymes (BamHI and EcoRI), the fragment containing MBP was cloned into pGEX2T vector to obtain a fusion with a thrombin cleavage site (GST-Th-MBP). To obtain the GST-TEV-MBP fusion with AcTEV cleavage site, the sequence encoding the thrombin cleavage site (LVPR^GS) in the vector containing the GST-Th-MBP fusion was replaced by the sequence ENLYFQ^G, using a QuikChange II mutagenesis kit (Stratagene Corporation) according to manufacturer instructions. To obtain Profinity eXact tag-MBP fusion, an MBP-encoded PCR fragment was cloned into the Profinity eXact pPAL7 vector using restriction-independent cloning as instructed in the Profinity eXact system manual.

Protein Expression and Purification

The resulting constructs were transformed into *Escherichia coli* BL21(DE3) chemi-competent expression cells (Bio-Rad), and a single clone was grown in autoinduction media overnight at 37°C to allow for induction and expression of the tag-MBP fusion proteins (Studier et al. 2005). Cell lysate was prepared by sonication of the resuspended cell pellet in the purification binding buffer corresponding to each resin matrix: 1x PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2PO_4, 1.8 mM KH_2PO_4) for GSTrap columns and 0.1 M potassium phosphate buffer, 0.1 mM EDTA, pH 7.2 for Bio-Scale Mini Profinity eXact cartridges. A total of 5 ml of lysate was used for each purification. Fusion protein purification was performed according to manufacturer instructions in a syringe format. Sample and buffer were applied using a syringe attached to the column. In case of GST-MBP fusions, a slow flow rate was maintained during loading and washing (~1 ml/min or 20 drops/min). Elution fractions, 1 ml each, were collected in 1.5 ml tubes. Elution buffer used for the GST gene fusion system (GE Healthcare) was 50 mM Tris-HCl, 10 mM reduced glutathione, pH 8.0. Elution buffer for the Profinity eXact fusion-tag system was 0.1 M potassium phosphate buffer, 0.1 M potassium fluoride, 0.1 mM EDTA, pH 7.2.

Before proceeding to large-scale proteolytic cleavage of the eluted GST-Th-MBP and GST-TEV-MBP fusion proteins, small-scale cleavage reactions were conducted to optimize the enzyme-to-substrate ratio for each of the two proteolytic enymes — thrombin and AcTEV. Thrombin cleavage was carried out on-column. The eluate was immediately passed through an inline HiTrap benzamidine FF column to trap the thrombin protease, and the purified MBP was collected in the effluent. Removal of the GST tag from the GST-TEV-MBP fusion was achieved concurrently with buffer exchange by including a His-tagged AcTEV protease during dialysis of the eluted fusion protein in glutathione-free buffer (20 mM Tris-HCl, 0.5 mM EDTA, 5 mM DTT). Tag-free MBP was then obtained in the flow-through fraction after passing the TEV cleavage reaction over a GSTrap column to remove the released GST, immediately followed by a HisTrap column to remove the AcTEV protease.

Preparation of tag-free MBP using the Profinity eXact system was performed according to the standard protocol. After binding and washing of the column, the proteolytic activity of the affinity matrix was activated by applying 2 column volumes (CV) of room temperature 0.1 M sodium phosphate buffer, pH 7.2, containing 0.1 M sodium fluoride, to the column and then incubating for 30 min to allow cleavage of the tag from the fusion protein. Purified, tag-free MBP with a native N-terminus was released from the column once flow resumed.

Purity and Yield Determinations

Yield of the tag-free, purified MBP was estimated from each purification using A_{280} absorbance and an extinction coefficient of 1.61 mg/ml per one A_{280} unit. Purity was determined by SDS-PAGE analysis using Criterion™ 4–20% Tris-HCl gels, followed by staining with Bio-Safe™ Coomassie stain and image acquisition and analysis using a Molecular Imager® GS-800™ calibrated densitometer and Quantity One® 1-D analysis software (all from Bio-Rad). A standard gel loading quantity of 3 µg of purified MBP was used to compare the three different purification procedures.

Results and Discussion

We purified MBP proteins using the GST gene fusion and Profinity eXact fusion-tag systems, monitoring the duration of the purification, yield, and purity of the tag-free protein. The GE Healthcare protocol for manual purification was chosen as the most comparable method to purify milligram quantities of MBP across the different systems studied.

MBP Purification Using GST-Tag and Enzymatic Tag Removal

We first performed cleavage time-course studies of each enzyme to optimize the digest conditions. A total of 0.1 mg of GST-Th-MBP and GST-TEV-MBP was incubated with 1 U of thrombin or 33, 16, and 8 U of TEV protease. Samples were removed from the digest mixture at various time points and analyzed by SDS-PAGE to estimate the yield, and extent of digestion. Figure 1 shows the progression of the thrombin digest, with nearly complete processing of the fusion protein in 16 hr (Figure 1, lane 8). In case of TEV cleavage, three different ratios of enzyme to fusion protein were tested: 0.33 U/µg, as recommended by the vendor, and 2-fold and 4-fold less enzyme per fusion protein (0.16 U/µg and 0.08 U/µg). SDS-PAGE analysis of the cleavage using enzyme to fusion protein ratios of 0.16 U/µg and 0.08 U/µg is presented in Figure 2. Although higher amounts of the protease can process the fusion protein more quickly, the same extent of GST-TEV-MBP cleavage can be achieved

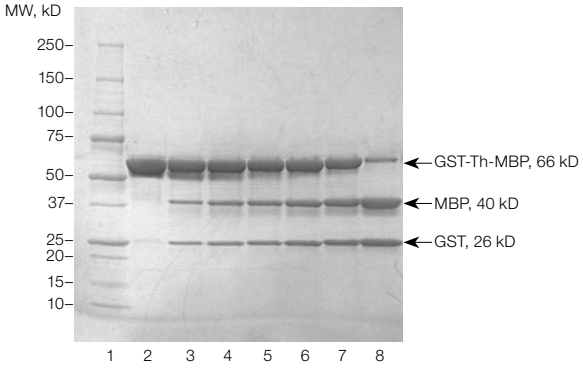

Fig. 1. Time course of thrombin cleavage of GST-Th-MBP fusion protein. One unit of thrombin was added to 0.1 mg of GST-Th-MBP fusion protein in 1x PBS buffer. The reaction was incubated at room temperature, and aliquots were taken once every hour for 5 hr; the last point was taken after overnight incubation (16 hr). Lane 1, Precision Plus Protein™ unstained standards; lanes 2–8 contain samples collected at 0, 1, 2, 3, 4, 5, or 16 hr of incubation.

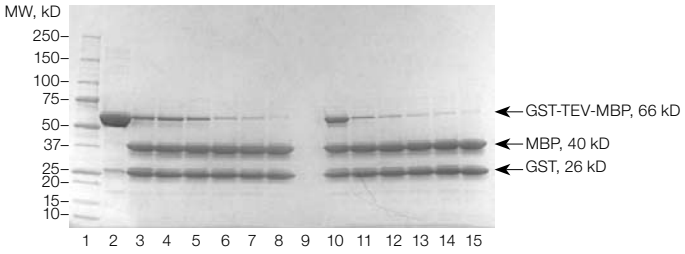

Fig. 2. Time course of TEV cleavage of GST-TEV-MBP fusion protein. Lane 1, Precision Plus Protein unstained standards; lanes 2–8, samples treated with 0.08 U/µg of enzyme and collected at 0, 1, 2, 3, 4, 5, or 16 hr of incubation; lane 9, blank; lanes 10–15, samples treated with 0.16 U/µg of enzyme and collected at 1, 2, 3, 4, 5, or 16 hr of incubation.

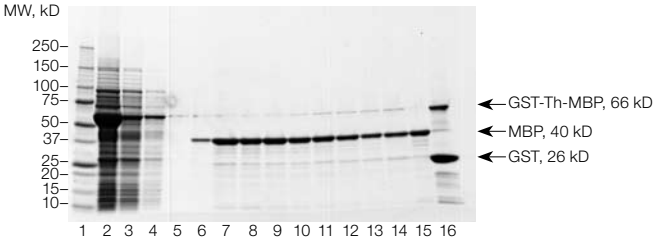

Fig. 3. GST-Th-MBP fusion purification and on-column cleavage with thrombin. Lane 1, Precision Plus Protein unstained standards; lane 2, lysate; lane 3, flowthrough; lane 4, wash; lanes 5–14, flow-through fractions from GSTrap and HiTrap benzamidine FF columns containing tag-free MBP; lane 15, pooled fractions (lanes 5–14); lane 16, bound components from GSTrap column.

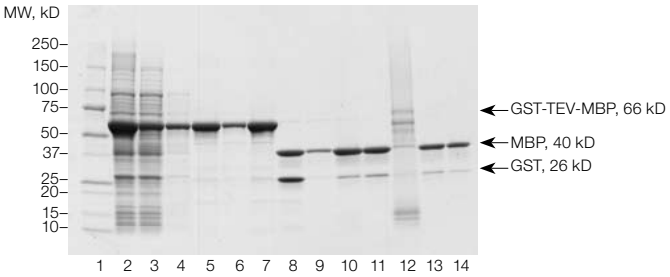

Fig. 4. GST-TEV-MBP fusion purification and cleavage with TEV protease. After cleavage, GST and MBP mixture was passed through a GSTrap column to bind cleaved GST. Collected flowthrough with tag-free MBP was loaded onto a HisTrap FF column to remove His-tagged AcTEV; MBP was collected in the flow-through fraction. Lane 1, Precision Plus Protein unstained standards; lane 2, lysate; lane 3, flowthrough; lane 4, wash; lanes 5–6, fractions containing GST-TEV-MBP fusion protein; lane 7, pooled fractions (lanes 5–6); lane 8, cleaved GST-TEV-MBP fusion; lanes 9–12, purified MBP, flow-through fractions from GSTrap column; lane 13, pooled fractions (lanes 9–12); lane 14, MBP from flowthrough of HisTrap FF column.

with longer digestion periods and less enzyme, without compromising product purity. Using optimized cleavage conditions, preparative amounts (5 ml of lysate containing approximately 20 mg of fusion protein) of each of the GST-MBP fusions were processed. Fractions from each step in the two protocols were resolved using SDS-PAGE analysis, and results are shown in Figures 3 and 4 for thrombin and TEV cleavage, respectively. In both cases, the final tag-free MBP protein was found to be contaminated with GST.

Optimization steps like estimation of the enzyme-to-substrate ratio and duration of cleavage, and ruling out whether any nonspecific proteolysis had occurred, added an extra day in the purfication process in studies using GST-MBP fusion proteins. These exploratory and optimization steps can be omitted for Profinity eXact MBP fusion proteins, because the cleavage site is already positioned at the active site of the ligand bound to the matrix, awaiting the signal for cleavage.

MBP Purification Using the Profinity eXact Fusion-Tag System

Purification of MBP using the Profinity eXact system was a one-step process. After loading 5 ml of the lysate (~20 mg of fusion protein) onto the 1 ml Bio-Scale Mini Profinity eXact cartridge, the column was washed with 1 ml of 1 M sodium acetate in binding buffer (0.1 M potassium phosphate buffer, pH 7.2, 0.1 mM EDTA) and then with 15 ml of binding buffer. Washed resin was saturated with 1 ml of the cleavage buffer (binding buffer containing 0.1 M sodium fluoride) and the column was incubated for 30 min at room temperature. Tag-free MBP was eluted by applying 5 ml of cleavage buffer in 1 ml aliquots (Figure 5). The column was regenerated by stripping the tightly bound Profinity eXact tag (kD <100 pM) from the resin, by decreasing the pH to below 2.0 using 3 CV of 0.1 M phosphoric acid.

Table 1 summarizes data for the purification experiments. For all of the parameters used to gauge the success of purification, the Profinity eXact system performed better than the GST system coupled to either thrombin or TEV cleavage. The use of the Profinity eXact tag and purification resin resulted in nearly 2-fold higher MBP protein yields when starting from a fixed amount of fusion protein and carrying it through the process to a tag-free form. The lower yields with protocols using GST tags are presumably due to the additional purification steps and possible system sensitivities to the flow rate, which was hard to control in the syringe format. Purity of MBP proteins using the Profinity eXact system was higher than the GST-based purifications, with no visible contaminants in SDS-PAGE analysis using a 3 µg sample load. The product was not appreciably contaminated with the affinity tag or bacterial proteins even at a 10 µg load, as illustrated in Figure 6. With the Profinity eXact fusion-tag system, fewer steps are required to reach the tag-free form of the target protein, and the duration of the purification process is considerably reduced

Table 1. Summary of MBP purification and cleavage.

Fusion Construct	Cleared Lysate, Starting Material	Purification Steps	Duration of Purification	Yield (Cleaved MBP), mg	Purity, %	Concentration of Final Purified Protein, mg/ml
GST-MBP, thrombin	5 ml, 20 mg fusion protein	5	19 hr	2.0	96.4	0.16
GST-MBP, TEV	5 ml, 20 mg fusion protein	8	20 hr	2.7	96.6	0.39
Profinity eXact MBP	5 ml, 20 mg fusion protein	4	50 min	5.0	98.0	0.90

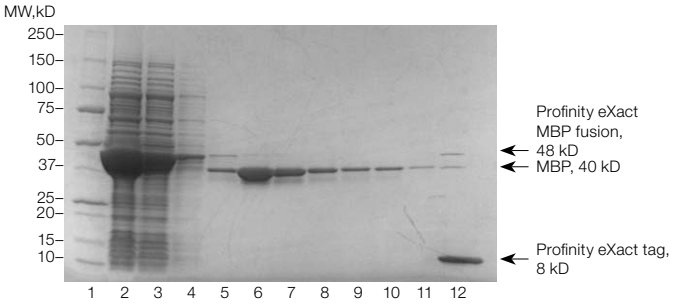

Fig. 5. MBP purification using Profinity eXact tag. Lane 1, Precision Plus Protein unstained standards; lane 2, lysate; lane 3, flowthrough; lane 4, wash; lanes 5–11, tag-free MBP in elution fractions; lane 12, Profinity eXact tag (~8 kD), stripped from the column using 0.1 M phosphoric acid.

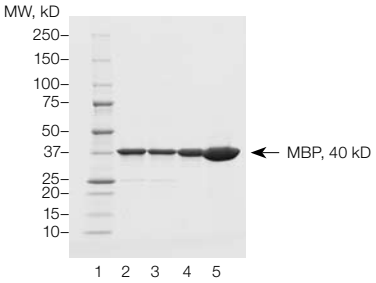

Fig. 6. Purity analysis of isolated MBP using GST fusion and enzymatic tag removal or eXact tag fusion and one-step on-column tag removal. Lane 1, Precision Plus Protein unstained standards; lanes 2–5, MBP protein purified using different methods; lane 2, purified as GST-fusion, tag cleaved with thrombin; lane 3, purified as GST-fusion, tag cleaved with AcTEV; lanes 4–5, purified as eXact-tag fusion; lanes 2–4 contained 3 µg sample protein per lane; lane 5 contained 10 µg sample protein per lane.

from nearly a day to less than one hour. The use of the Profinity eXact system also results in the eluted tag-free protein in a more concentrated form. Unlike the thrombin and TEV cleavage systems that leave terminal GS and G residues, respectively, MBP purified with the Profinity eXact system is in its native form and is amenable to direct use in downstream applications.

Conclusions

Affinity tagging has enabled the expression and purification of large numbers of proteins using single-step purification methods. All tags have the potential to interfere with the biological activity of a protein and to influence its behavior and so need to be removed. Using MBP as a model protein and the GST system for comparison, we have shown that the Profinity eXact fusion-tag system can become the method of choice for rapid production of pure, tag-free target proteins.

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