Application of the Profinity eXact™ Fusion-Tag System to Eukaryotic Expression Systems

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Introduction

Protein overexpression and affinity purification are used in various biological research fields and play an important role in the postgenomic era. While expression of recombinant proteins in bacteria is often the preferred method, eukaryotic expression systems, especially those using insect and mammalian cells, are indispensable for producing proteins that are difficult to express in bacteria or that have specific required posttranslational modifications. Currently, different purification tags, like 6xHis and GST, are used with eukaryotic expression systems, but they have serious drawbacks. For example, insect and mammalian cells contain a higher percentage of His residues in their proteins than *E. coli*, which can lead to significant background binding to immobilized metal ions (Kimple and Sondek 2004). Furthermore, current affinity purification systems require a tag-removal step, which is necessary if the tag interferes with the function of the protein.

The Profinity eXact protein purification system offers a unique approach to affinity purification. It utilizes an immobilized, engineered protease that specifically recognizes and binds with subnanomolar affinity to an 8 kD Profinity eXact affinity tag fused to the N-terminus of the recombinant protein (Bryan 2000, Ruan et al. 2004). After column washing to remove impurities, a specific, controlled cleavage and removal of the tag from the target protein is rapidly performed directly on the column, resulting in the release of highly purified recombinant protein with a native or desired N-terminus. Since the tag remains firmly attached to the resin, the result of this simple process is a true, single-step affinity purification and tag removal procedure.

The Profinity eXact tag is derived from the Pro region of a protease isolated from *Bacillus subtilis*. Further, the Profinity eXact system was developed for bacterial expression, allowing single-step purification of tag-free proteins with high purity and high yields compared to other protein-based affinity resins. Potential concerns for utilization of this expression and purification system in eukaryotic cells include: (1) poor expression of the tag, (2) potential degradation of the tag by eukaryotic proteases due to its prokaryotic origin, (3) potential posttranslational modification or tertiary structure misfolding of the tag affecting binding and proper cleavage, and (4) excessive background contamination in the eluate because of high nonspecific binding of eukaryotic proteins to the immobilized protease.

In this report, we investigated these concerns using small-scale transfection of insect Sf9 cells and mammalian HeLa cells followed by expression and purification analysis. The results indicate that the Profinity eXact system can be used in eukaryotic cells for single-step purification of tag-free proteins with low background and without compromising the obvious function of the system. We tested expression and purification using GFP, MBP, and AKT protein kinases. The AKT kinases are key signal transducers in different biological processes including cell survival, cell growth, gene expression, and oncogenesis (Crowell et al. 2007). Our results show that all proteins tested can be expressed and purified well using the Profinity eXact system.

Methods

Cell Line, Vectors, Affinity Resin, and Assay

Vector pIEx6, Sf9 insect cells, BacVector insect cell medium, and Insect GeneJuice transfection reagent were from EMD Biosciences. Polyclonal primary antibody to green fluorescent protein (GFP) was from MBL International and monoclonal antibody to maltose binding protein (MBP) was from New England BioLabs. Secondary antibodies conjugated with HRP and Immun-Star™ WesternC™ chemiluminescence assay kit were from Bio-Rad Laboratories, Inc. pcDNA3.1 vector was from Life Technologies Corporation. The pPAL-MBP and pPAL-GFP vectors and the Profinity eXact resin were also from Bio-Rad.

Expression Vector Construction

The DNA fragment encoding the Profinity eXact tag was amplified by PCR using pPAL7 plasmid as a template and inserted into the pIEx6 plasmid digested with NcoI and NotI. The multiple cloning site downstream of the Profinity eXact tag was carried over to the pIEx6 vector for use in subcloning. The GFP gene was amplified by PCR using pPAL-GFP plasmid as a template and inserted into either pIEx6 vector or pIEx vector containing the Profinity eXact tag in order

to obtain N-terminal-6xHis or Profinity eXact fusion–tagged GFP. The AKT1 cDNA clone was purchased from OriGene Technologies, Inc., amplified by PCR, and inserted in the tag-containing pIEx vector, downstream of the Profinity eXact tag. Both GFP and AKT1 protein sequences are immediately downstream of the Profinity eXact tag cleavage site FKAL.

The MBP gene was amplified by PCR using the pPAL-MBP plasmid as a template and inserted into the pcDNA3.1 vector to make the nontagged MBP construct pcDNA-MBP. The fragment from pPAL-MBP containing the Profinity eXact fusion tag and MBP was digested and inserted into the pcDNA3.1 vector to make the Profinity eXact fusion–tagged MBP gene, pcDNA–Profinity eXact tag–MBP.

Insect Sf9 Cells Expression System

Insect Sf9 cell cultures were maintained routinely in BacVector insect cell medium in a shaking flask at 28°C and 150 rpm. Endotoxin-free pIEx–Profinity eXact tag–GFP and pIEx-GFP plasmids (20 µg) were transfected into a 10 ml suspension of Sf9 insect cells with 100 µl of Insect GeneJuice transfection reagent according to the manufacturer's instructions. After 3 days' incubation, the cells were harvested, resuspended, and sonicated in Profinity eXact bind/wash buffer (0.1 M sodium phosphate, pH 7.2) or in lysis buffer (50 mM Tris-acetate, 100 mM NaOAc, 5% glycerol, 5 mM β-mercaptoethanol, pH 7.2). The supernatant was then applied to 20 µl of equilibrated Profinity eXact resin in a Mini Bio-Spin™ spin column (Bio-Rad) and incubated at room temperature for 10 min. The flow-through fraction was collected and the resin was washed with Profinity eXact bind/wash buffer or the above lysis buffer three times with ten column volumes (CV) each time. The column was then incubated in 40 µl of the above binding buffer or lysis buffer containing 10 mM sodium azide on a rotator. Following a 30 min room temperature cleavage incubation, the tag-free protein was eluted from the resin by a simple spin, and the elution process was repeated once more. Posttransfection expression was monitored on a 4–20% Criterion Stain Free™ gel followed by image acquisition and analysis using a Criterion Stain Free gel imaging system. The purification process was monitored by loading 10 µl of each fraction onto a 4–20% Criterion Tris-HCl gel followed by Bio-Safe™ Coomassie staining. Image acquisition and analysis were performed using the Molecular Imager® GS-800™ calibrated densitometer and Quantity One® 1-D analysis software.

HeLa Cell Expression System

Mammalian HeLa cell cultures were maintained routinely in medium containing 90% DMEM, 10% FBS, 1 mM NEAA, and 1 mM sodium pyruvate in culture dishes at 37°C with 5% CO_2. pcDNA-MBP or pcDNA–Profinity eXact tag–MBP plasmids (2 µg) were transfected using TransFectin™ lipid reagent (Bio-Rad) into HeLa cells cultured in 6-well plates following manufacturer's instructions. The transfected cells were harvested after a 21 hr incubation at 37°C. The cells were resuspended and lysed in Profinity eXact bind/wash buffer containing 0.5% NP-40. The supernatant was then applied to 20 µl of equilibrated Profinity eXact resin. The flow-through fraction was collected and the resin was washed in the Profinity eXact bind/wash buffer 2 to 3 times with 10 CV each time. The tag-free target protein was cleaved for 30–60 min and eluted from the resin using the Profinity eXact elution buffer with 0.1 M sodium fluoride as the cleavage trigger. The purification process was monitored by loading 10 µl of each fraction on a 4–20% Criterion Tris-HCl gel followed by Bio-Safe Coomassie stain. Image acquisition and analysis were performed using the GS-800 densitometer and Quantity One software.

Western Blot Analysis

Protein samples separated on SDS-PAGE gels were transferred onto PVDF membranes, and the standard protocol was followed for western blot analysis. The membranes were incubated for at least 1 hr in blocking buffer (TBS with 0.5% Tween 20 and 3% BSA) before incubation with either 1:10,000 or 1:500 diluted anti-MBP or anti-GFP antibodies, respectively. After three washes, the membranes were incubated with 1:10,000 diluted HRP conjugated goat–anti-mouse or goat–anti-rabbit secondary antibodies, respectively. The signal was developed using the Immun-Star WesternC kit and imaged using the Molecular Imager® ChemiDoc™ XRS imaging system (Bio-Rad).

Molecular Weight Determination of the Profinity eXact Tag by Mass Spectrometry

Following the standard resin cleaning procedure using 0.1 M H_3PO_4, the Profinity eXact tag was stripped from the resin used for the purification of Profinity eXact fusion–tagged MBP from HeLa cell and bacterial lysates, vacuum-dried, and resuspended in 2.5% TFA. The solutions were desalted using ZipTip pipet tips with C_{18} resin (Millipore) and eluted with 50% acetonitrile containing 0.1% TFA. A small aliquot of each eluate was loaded separately or combined onto a ProteinChip® NP20 array with SPA matrix, and the mass spectra obtained using the ProteinChip SELDI system (all from Bio-Rad).

Results and Discussion

Expression and Purification of Profinity eXact Fusion–Tagged Proteins in Insect Sf9 Cells

To examine the expression and purification of Profinity eXact fusion–tagged proteins in insect cells, genes encoding GFP and AKT1 were fused immediately downstream of the Profinity eXact tag sequence in the plasmid-based insect cell expression vector, pIEx6. The transcription of the chimeric genes is driven by the IE1 promoter, which together with the hr5 enhancer recruits the endogenous insect cell transcription machinery, thereby avoiding baculovirus infection and associated cytotoxic effects. Small-scale transfection was performed and the expression of the Profinity eXact fusion–tagged GFP was analyzed by western blot using anti-GFP antibody. The expression of the protein was about 2-fold lower than the expression of the 6xHis fusion–tagged GFP in the cell lysate (Figure 1), which is not surprising because the

Profinity eXact tag is much bigger than the 6xHis tag, and the Profinity eXact tag used here is not codon-optimized specifically for insect cells.

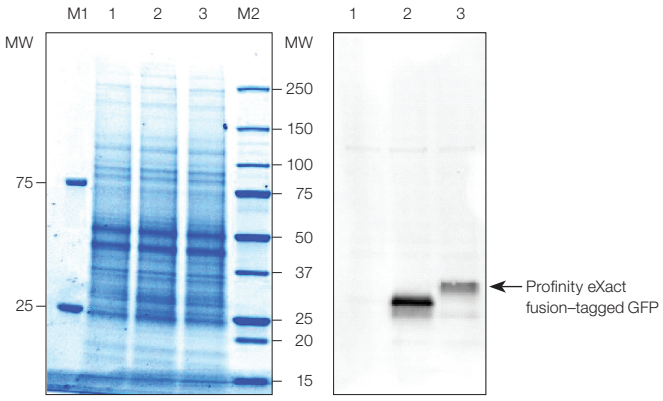

Fig. 1. Expression analysis of Profinity eXact fusion–tagged GFP by western blot. Small-scale transfection of insect Sf9 cells was conducted using the constructs containing genes that encode GFP tagged with His or the Profinity eXact tag. The total cell lysate was separated on a 4–20% Criterion Stain Free gel **(left panel)** and imaged before transferring onto a PVDF membrane. Western blot analysis using polyclonal anti-GFP antibody is shown **(right panel)**. **M1**, Precision Plus Protein™ Dual Color standards; **1**, insect cell control; **2**, His-GFP; **3**, Profinity eXact tag–GFP; **M2**, Precision Plus Protein Unstained standards.

The cell lysate was then loaded onto Profinity eXact resin, and the tag-free GFP was obtained in high purity (Figure 2). Another protein, human AKT1 kinase, was also tested and the results confirmed expression of the protein by western blot analysis (data not shown). The purity of the eluted tag-free AKT1 protein was lower than that of GFP mainly due to lower overall expression. The major contaminant band is likely undigested Profinity eXact fusion–tagged AKT1, which can easily be removed by incubating with the elution buffer for a prolonged period. The results indicate that the Profinity eXact system can be used in insect cells for protein expression and single-step purification to obtain tag-free proteins.

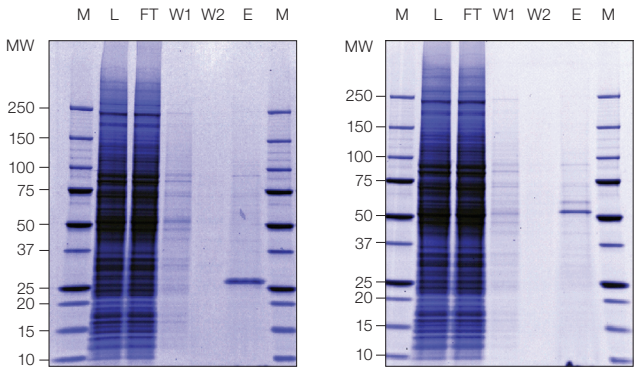

Fig. 2. Small-scale purification of tag-free GFP (left) and AKT1 (right) from the Sf9 cell transfectants. L, lysate; **FT**, flowthrough; **W1** and **W2**, washes; **E**, elution. **M**, Precision Plus Protein Unstained standards.

Expression and Purification of Profinity eXact Fusion–Tagged Proteins in HeLa Cells

Protein expression in mammalian cells is an alternative means of expressing and purifying target proteins, especially those that do not express well in bacteria, or that need posttranslational modifications for their activity. To analyze the applicability of the Profinity eXact system to mammalian cell expression, chimeric genes encoding Profinity eXact fusion–tagged or non-tagged MBP were constructed in the CMV promoter–based mammalian expression vector pcDNA3.1. The constructs were transfected and transiently expressed in rapidly growing HeLa cells. After a 21 hr incubation at 37°C, cell lysates were analyzed by western blot for the expression of the target proteins. The results indicate that Profinity eXact fusion–tagged MBP can be expressed well in HeLa cells, and that the expression level is comparable to the MBP without any tag, suggesting that the tag does not affect the expression or the stability of the proteins (Figure 3).

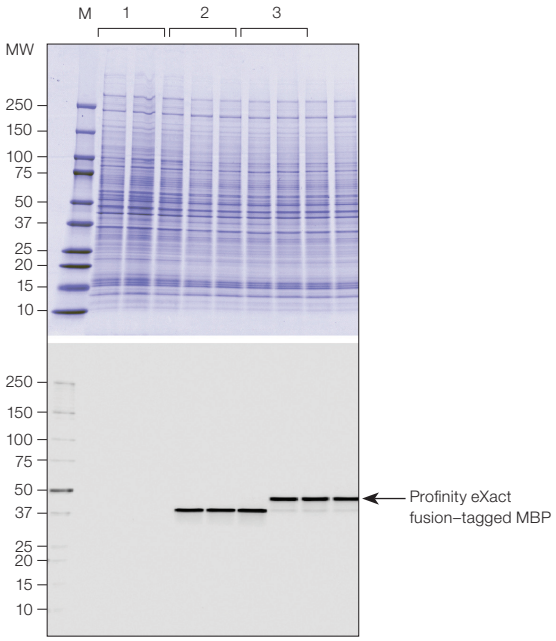

Fig. 3. Expression of Profinity eXact fusion–tagged MBP in HeLa cells. Protein extracts from each HeLa cell transfectant were loaded in triplicate. M, Precision Plus Protein Dual Color standards; **1**, HeLa cells without plasmid; **2**, HeLa cells transfected with MBP gene construct; **3**, HeLa cells transfected with the chimeric gene construct of the Profinity eXact fusion tag and MBP. Monoclonal anti-MBP antibody was used as the primary antibody.

Furthermore, small-scale purification of the tag-free MBP was conducted using the cell lysate from the HeLa cell transfectant (Figure 4). The tag-free MBP was obtained in the elution fraction with high homogeneity, indicating the Profinity eXact system can be used to express Profinity eXact fusion–tagged proteins in mammalian cells and purify tag-free proteins.

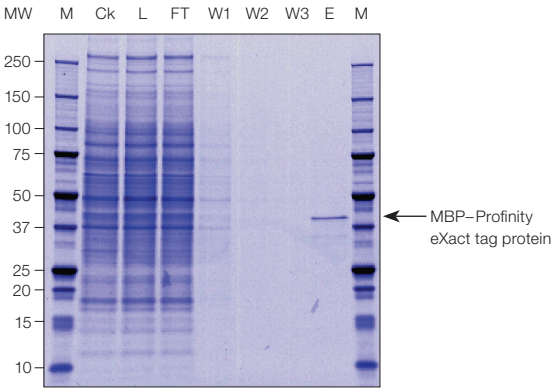

Fig. 4. Small-scale purification of tag-free MBP from HeLa cells. The purification of the MBP was analyzed by SDS-PAGE by separating all the purification fractions on 4–20% Criterion gel. **Ck**, HeLa cells without plasmid transfection; **L**, cell lysate; **FT**, flowthrough; **W1–W3**, washes; **E**, elution. **M**, Precision Plus Protein Dual Color standards.

Proteins or peptides expressed in mammalian cells often undergo posttranslational modifications, which may affect their function. To determine whether the Profinity eXact tag, which is of prokaryotic origin, is modified in HeLa cells, the Profinity eXact tags stripped from the column after purification from either bacterial or HeLa cell lysates were analyzed for their molecular mass using a SELDI mass spectrometer. The results (Figure 5) showed that the Profinity eXact tag expressed in mammalian cells has the same molecular weight as the tag expressed in *E. coli*. This indicates that the Profinity eXact tag is not modified after translation, thus its function as a purification tag and its cleavability are most likely not impacted in mammalian cells. The successful purification of the tag-free MBP (Figure 4) confirms that the Profinity eXact system is fully functional in HeLa cells.

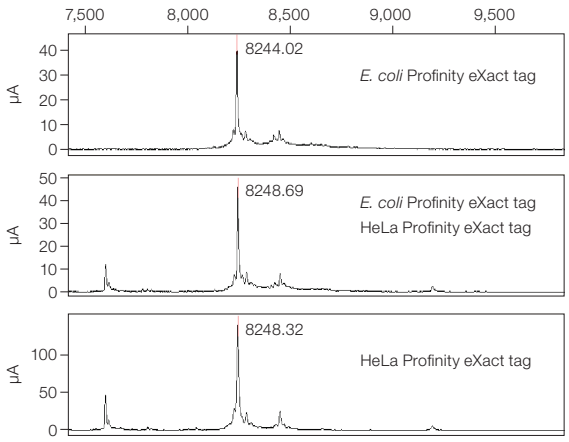

Fig. 5. Mass analysis of the Profinity eXact tags expressed in bacteria and HeLa cells. The Profinity eXact tags stripped from the column after purification from bacterial or HeLa cell lysates were spotted on uncoated ProteinChip arrays and their molecular mass was determined by mass spectrometry using the ProteinChip SELDI system.

Conclusions

This work demonstrates that the Profinity eXact purification system is adaptable for use in mammalian and insect cells. Both expression of the Profinity eXact fusion–tagged proteins and single-step purification of the tag-free proteins with high purity can be achieved: (1) with equal or slightly poor expression of the tag in HeLa and insect cells, (2) without obvious degradation of the tag by eukaryotic proteases due to its prokaryotic origin, (3) without potential posttranslational modification or tertiary structure misfolding of the tag in HeLa cells, and (4) with very low background contamination in the eluate from nonspecific binding of eukaryotic proteins to the immobilized protease. Enhanced expression in either the insect or mammalian cell systems may be observed by utilizing a host-appropriate, codon-optimized version of the Profinity eXact tag and target proteins.

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