Purification of Tag-Free Recombinant Proteins Using the Profinity eXact™ Fusion-Tag System

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Introduction

Affinity tagging has become a popular strategy for purifying recombinant proteins or functional domains in the post-genomic era. Fusing recombinant proteins with an amino acid sequence that selectively interacts with ligands on chromatographic matrices allows purification of virtually any protein without prior knowledge of its physical properties or physiological activities. This is a generic approach that differs from traditional chromatography, where specific materials and conditions have to be developed for individual proteins. A large number of recombinant proteins have been successfully purified from crude extracts in a single affinity chromatographic step with >90% purity, using conditions gentle enough to preserve their structural and functional integrity (Arnau et al. 2006).

However, for some structural studies and therapeutic or diagnostic applications, it is desirable to produce a native, or tag-free, protein in order to avoid potential interference from the affinity tag (Arnau et al. 2006, Bucher et al. 2002). In these cases, a protease is needed to process the purified fusion protein, and a secondary affinity purification is necessary to eliminate this protease and the cleaved fusion tag from the final protein preparation. Tailoring such a working protocol for a specific fusion protein can be challenging. Alternative systems using intein-mediated self-processing of fusion proteins have been designed to generate recombinant proteins with native N-termini. However, this autocatalytic proteolysis process is slow and largely dependent on the primary sequence at the intein-target protein junction (Waugh 2005), limiting its application in high-throughput purification of recombinant proteins.

Profinity eXact purification resin is an integral part of the Profinity eXact fusion-tag system for the expression and purification of recombinant proteins overproduced in *Escherichia coli* cells. It essentially incorporates affinity purification and tag removal in a single step (Ruan et al. 2004) to alleviate bottlenecks in the generation of native recombinant proteins from parent fusion proteins following affinity chromatography purification. The encoding sequence of a target protein is cloned into the Profinity eXact pPAL7 expression vector downsteam from the Profinity eXact tag, resulting in an N-terminally tagged fusion protein (Figure 1). The mutant serine protease immobilized onto Profinity eXact purification resin selectively interacts with the cognate Profinity eXact tag (Kd < 100 pM) (Ruan et al. 2004). Once contaminants from the expression host cells are removed by a postbinding wash, proteolysis is triggered precisely at the junction between the affinity tag and target protein by the addition of fluoride or azide anion. With the Profinity eXact tag retained by the immobilized protease, only the desired native recombinant protein is eluted from the column and is available for downstream applications, often without further manipulation.

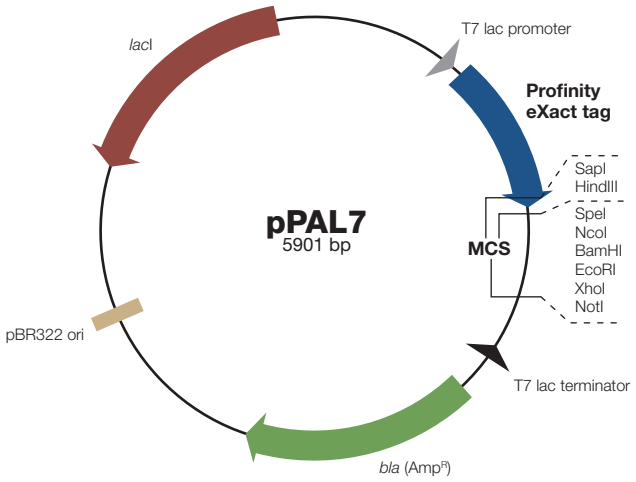

Fig. 1. Profinity eXact pPAL7 vector.

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We employed the Profinity eXact fusion-tag system for the purification of proteins of different molecular mass and tested its performance using various parameters. The data clearly demonstrate the benefits of this novel purification system, including the effective and selective capturing of tagged proteins, the precise on-column proteolytic cleavage of the affinity tag, and the overall efficiency and simplicity of the purification protocol. Performance of Profinity eXact purification resin, included in Profinity eXact mini spin columns and Bio-Scale™ cartridges, was evaluated using parameters such as resin stability through multiple purification and regeneration cycles and resin functionality in the presence of a denaturing reagent such as urea.

Methods

Purification of Recombinant Protein Under Native Conditions

All Profinity eXact fusion-tagged proteins used in this study were overproduced in *E. coli* and purified with Profinity eXact purification resin using either Profinity eXact mini spin columns or Bio-Scale Mini Profinity eXact cartridges, following protocols provided in the Profinity eXact system manual.

Testing Stability of Profinity eXact Purification Resin During Regeneration Cycles

A 1 ml Bio-Scale Mini Profinity eXact purification cartridge was used for the purification of recombinant maltose binding protein (MBP) on a BioLogic DuoFlow™ system for five consecutive cycles. In each purification cycle, the Profinity eXact cartridge was preequilibrated with 5 column volumes (CV) of Profinity eXact bind/wash buffer (0.1 M sodium phosphate buffer, pH 7.2). A total of 9 ml of *E. coli* lysate containing Profinity eXact fusion-tagged MBP was loaded through an injection loop onto the cartridge at 1 ml/min. Host protein contaminants were removed by washing the column with 10 CV of the same buffer. The elution of MBP was triggered by the application of Profinity eXact elution buffer (0.1 M sodium phosphate, 0.1 M sodium fluoride, pH 7.2) at 0.1 ml/min for 30 min at room temperature. The regeneration of this cartridge was achieved by stripping Profinity eXact purification resin with 6 CV of 0.1 M phosphoric acid at 2 ml/min, followed by reequilibration with 15 CV of the Profinity eXact bind/wash buffer. The target protein elution profile of each purification cycle was monitored by measuring absorbance at 280 nm (A_{280}) using a spectrophotometer, and a series of 2 ml fractions were collected with a BioFrac™ fraction collector. The resulting chromatograms were compared using the trace comparison feature of BioLogic DuoFlow software. The quantity of MBP obtained from each purification cycle was calculated from the A_{280} values of the combined elution fractions using an extinction coefficient of 1.61 A_{280} = 1 mg/ml. Purity was determined by SDS-PAGE and the Experion™ automated electrophoresis system as described below.

Purification of MBP in the Presence of Urea

Lyophilized *E. coli* Profinity eXact fusion-tagged MBP control lysate was resuspended in 5 ml of Profinity eXact bind/wash buffer containing 0, 2, or 4 M urea. The resuspended lysate was then loaded onto a 1 ml Profinity eXact cartridge pre-equilibrated with the respective buffer. After protein contaminants from the expression host were removed by washing with 10 CV of the same buffer, the MBP target protein was eluted using Profinity eXact elution buffer containing the respective concentration of urea at 0.1 ml/min for 30 min at room temperature. The purity of MBP was determined by SDS-PAGE and the Experion automated electrophoresis system as described below.

SDS-PAGE Analysis

Samples of purified recombinant proteins were mixed with the respective sample buffer, followed by denaturation at 95°C for 5 min prior to being loaded onto gels. SDS-PAGE was performed in a Criterion™ cell with Criterion™ XT 4–12% Bis-Tris gels using the XT MES running buffer, or Criterion 4–20% Tris-HCl gels using 1x Tris-glycine-SDS running buffer, at 200 V for 60 min. Following electrophoresis, gels were fixed and then stained for 1 hr at room temperature with Bio-Safe™ Coomassie G-250 stain. Gel destaining was performed in water at room temperature for 16–24 hr. The destained gels were imaged with a Molecular Imager® GS-800™ calibrated densitometer and analyzed for purity using the band tools of Quantity One® 1-D analysis software.

Experion Pro260 Analysis

Samples were prepared and analyzed on the Experion automated electrophoresis system according to the directions provided in the Experion Pro260 analysis kit instruction manual for protein separation under reducing conditions. Protein purity and relative quantitation values were automatically calculated by Experion software.

Results and Discussion

Purification of Tag-Free Recombinant Proteins Under Native Conditions

We cloned a variety of gene sequences from both prokaryotic and eukaryotic species for the expression and purification of their protein products using the Profinity eXact fusion-tag system. These proteins also differ in terms of their polypeptide molecular mass and oligomeric state. Some of these proteins have been well-characterized, while the structural properties and biological functions of others have yet to be determined or confirmed.

The tag-free recombinant proteins listed in Table 1 were obtained at satisfactory purity with a generic protocol outlined in the Profinity eXact fusion-tag system manual, with no substantial optimization performed for each individual protein. For very large (>100 kD) proteins, such as the tetrameric β-galactosidase, binding time was extended to improve target protein yield. Subsaturating amounts of

some oligomeric proteins were applied to the resin to ensure complete processing of the fusion proteins according to the guidelines described in the manual. Based on the wide variety of proteins purified using the Profinity eXact system, the binding of fusion proteins to the resin using the Profinity eXact tag appeared to be very selective, independent of fusion protein sequence or structure. Contaminants from expression host cells were easily removed by washing the loaded resin with Profinity eXact bind/wash buffer, and augmentation of binding/washing stringency was generally not necessary. The most commonly seen impurities were truncated fragments of target proteins, which could have been the result of incomplete transcription or translation and are frequently encountered during the purification of N-terminally tagged fusion proteins.

The on-column cleavage of bound fusion proteins was found to be efficient and consistent, even though the primary sequences and tertiary structures of proteins are dramatically different. Unlike the reported promiscuous cleavage behavior exhibited by some commercially available endoproteases, such as recombinant enterokinase and factor Xa (Choi et al. 2001, Jenny et al. 2003, Waugh 2005), the Profiniy eXact tag is thought to preclude nonspecific cleavage of the desired proteins because of the extensive additional interactions between other Profinity eXact tag amino acids outside of the core cleavage recognition motif and the subtilisin protease. In fact, our data provide evidence supporting this notion. The purified polyADP-ribose polymerase mutant and apiose synthase exhibit their expected biological activities (data not shown), which indicate that the physical and biological properties of these proteins have been maintained under the mild chromatographic condition in this one-step purification and tag-removal process.

Table 1. Recombinant proteins and putative functional domains purified using Profinity eXact mini spin columns.

Protein	Source	Molecular Mass, kD	Monomer Purity,* %
β-Galactosidase	Bacteria	116	88
PolyADP-ribose polymerase	Human	83	99
YGR211W**	Yeast	55	91
51 kD protein (unknown function)	Bacteria	51	88
Apiose synthase	Plant	44	92
Chimeric G protein α-subunits***	Bovine	40	99
Maltose binding protein (MBP)		40	
YGL221C†	Yeast	32	79
YDR428C††	Yeast	30	81
Green Fluorescent Protein (GFP)	Jellyfish	25	97
25 kD protein (unknown function)	Bacteria	25	99
Dihydrofolate reductase	Mouse	22	95
IL-1β	Human	17	99

* Determined by SDS-PAGE; ** gb/EDN61799.1; *** courtesy of Kevin Ridge, University of Texas Health Science Center; † gb/AAT93235.1; †† gb/AAB64885.1.

The purification of a tag-free GFP mutant with a 1 ml Bio-Scale Mini Profinity eXact cartridge on a Biologic DuoFlow system resulted in about 4 mg of protein with purity of 98% (Figure 2). Moreover, other than total yield, we have found virtually no difference in performance between cartridge and mini spin column formats, as is evident in the preparation of native MBP (Figure 3); this is an important consideration when progressing from small-scale to more preparative-scale purifications.

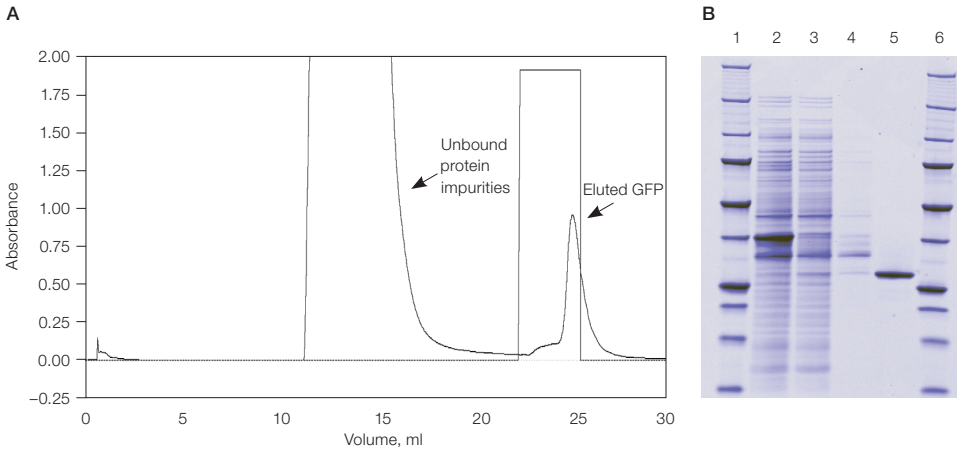

Fig. 2. Purification of a tag-free GFP mutant protein. A, chromatograms from the purification of GFP mutant protein from *E. coli* lysate, performed on a BioLogic DuoFlow system using a Bio-Scale Mini Profinity eXact cartridge; **B**, SDS-PAGE analysis of fractions from the purification of GFP. Lane 1, Precision Plus Protein™ standards; lane 2, crude *E. coli* extract; lane 3, host protein contaminants present in the flow-through fraction; lane 4, host protein contaminants washed from the cartridge by Profinity eXact bind/wash buffer; lane 5, eluted GFP; lane 6, Precision Plus Protein standards. The purity of eluted GFP was 98%, as determined by SDS-PAGE using 3 µg of purified protein.

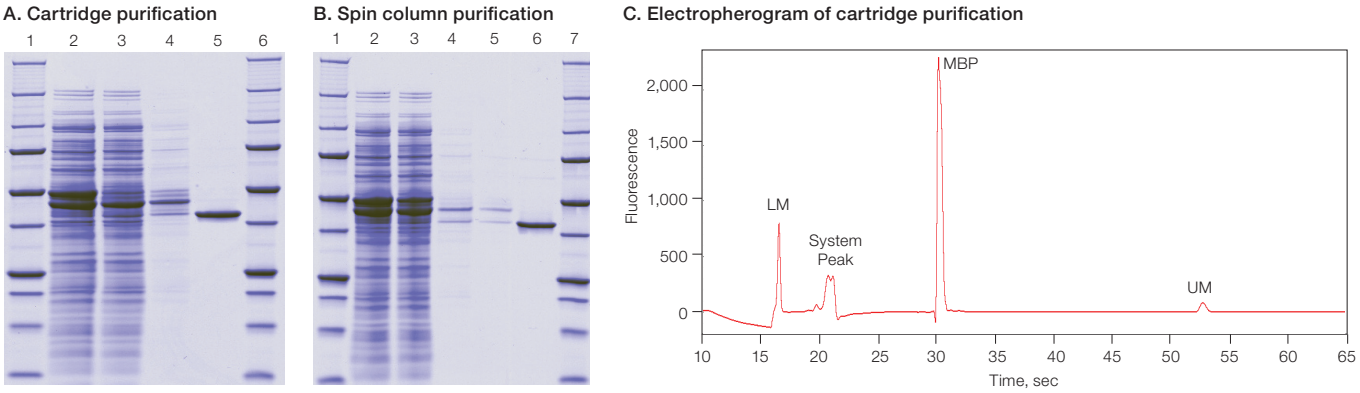

Fig. 3. Purification of native MBP protein. A, SDS-PAGE analysis of fractions taken from the purification of MBP using a 1 ml Bio-Scale Mini Profinity eXact cartridge on a BioLogic DuoFlow system. Lane 1, Precision Plus Protein standards; lane 2, crude *E. coli* extract; lane 3, host protein contaminants in the flowthrough fraction; lane 4, host protein contaminants washed from the cartridge by Profinity eXact bind/wash buffer; lane 5, eluted MBP; lane 6, Precision Plus Protein standards; **B**, SDS-PAGE analysis of fractions taken from the purification of MBP using a Profinity eXact mini spin column. Lane 1, Precision Plus Protein standards; lane 2, crude *E. coli* extract; lane 3, host protein contaminants in the flow-through fraction; lane 4, host protein contaminants removed from the spin column by Profinity eXact bind/wash buffer in wash fraction 1; lane 5, host protein contaminants removed from the spin column by Profinity eXact bind/wash buffer in wash fraction 2; lane 6, eluted MBP; lane 7, Precision Plus Protein standards; **C**, electropherogram showing purity of MBP eluted from the Profinity eXact cartridge and analyzed with an Experion Pro260 analysis kit on the Experion automated electrophoresis system. LM, lower marker; UM, upper marker.

Stability and Reusability of Profinity eXact Purification Resin

The Profinity eXact purification resin can be regenerated and used repeatedly for the purification of tagged proteins. This was demonstrated by five consecutive preparations of MBP with the same 1 ml Bio-Scale Mini Profinity eXact cartridge on a BioLogic DuoFlow system. The Profinity eXact tag retained by the immobilized protease during target protein elution was effectively stripped off the resin by 0.1 M phosphoric acid. This cartridge was then reequilibrated with Profinity eXact bind/wash buffer for successive rounds of purification. The chromatograms of five consecutive purifications of MBP are overlaid in Figure 4A. Detailed quantitative analysis revealed that this cartridge retained 90% of its binding capacity after five cycles of use (Figure 4B), while the purity of MBP from each preparation, determined using relatively high amounts of protein (3 µg) to visualize low-level contaminants, was unchanged at levels over 99% (Figure 4C).

Purification of Recombinant Proteins in the Presence of Urea

While *E. coli* has been the workhorse of heterologous expression, aggregation of overproduced recombinant proteins is a major obstacle for generating functional molecules, requiring initial solubilization of the expressed proteins using strong denaturants, such as urea and guanidine hydrochloride (GuHCl), before chromatographic separation. To test whether the Profinity eXact system could be applied to these situations, we evaluated the performance of Profinity eXact purification resin using the control MBP protein lysate in the presence of increasing concentrations of urea. We found that this resin retained its binding capacity, selectivity, and cleavage activity even in the presence of 4 M urea (Figure 5 and Table 2). However, the inclusion of more than 4 M urea in the binding buffer resulted in an elevated level of contaminants (decreased purity) and a significantly lower yield of target protein in the elution fraction (data not shown). It is worth

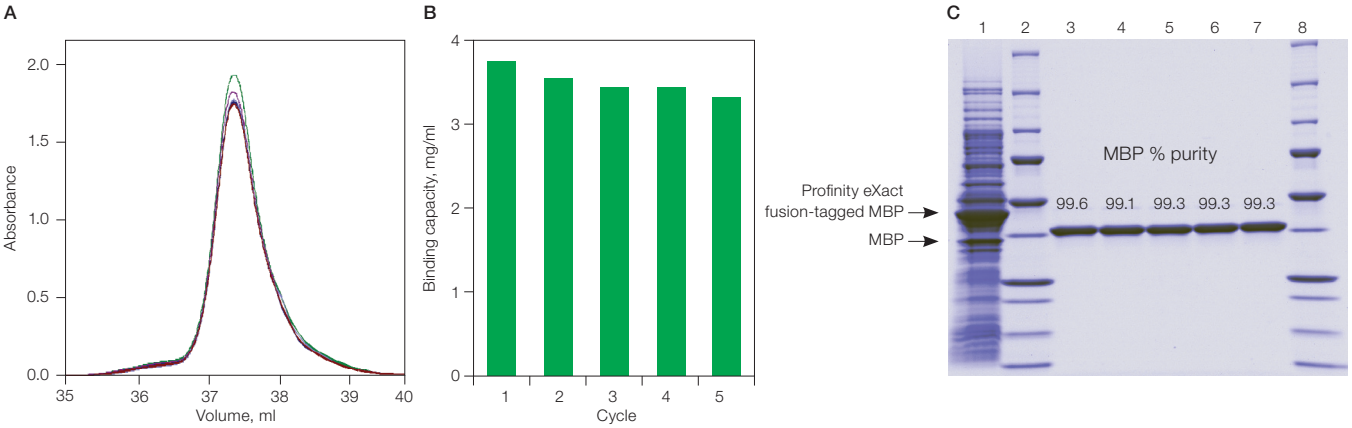

Fig. 4. Stability and reusability of Profinity eXact purification resin. A, overlaid chromatograms from MBP purification using a 1 ml Profinity eXact cartridge on a BioLogic DuoFlow system; **B**, change of binding capacity for MBP during repeated use of a 1 ml Profinity eXact cartridge; **C**, SDS-PAGE purity analysis of MBP eluted from the cartridge. Lane 1, *E. coli* lysate containing Profinity eXact fusion-tagged MBP; lane 2, Precision Plus Protein standards; lanes 3–7, 3 µg of MBP eluted from the cartridge during five consecutive uses; lane 8, Precision Plus Protein standards.

mentioning that GuHCl is not suitable for the purification of Profinity eXact fusion-tagged proteins. At the concentrations of GuHCl required to solubilize recombinant proteins, the Cl^- counterion can trigger the premature cleavage of fusion protein by the immobilized subtilisin mutant during the initial capturing step, leading to the loss of the desired protein.

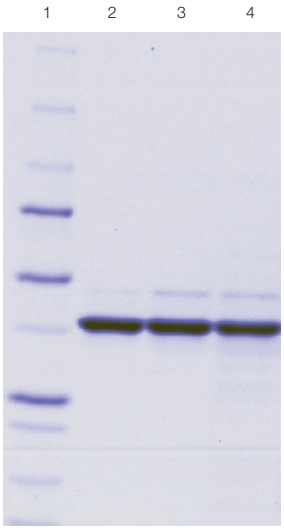

Fig. 5. SDS-PAGE analysis of MBP purified under either native or denaturing conditions with a 1 ml Bio-Scale Mini Profinity eXact cartridge on a BioLogic DuoFlow system. Lane 1, Precision Plus Protein standards; lane 2, 3 µg of MBP eluted with Profinity eXact elution buffer; lane 3, 3 µg of MBP eluted with Profinity eXact elution buffer plus 2 M urea; lane 4, 3 µg of MBP eluted with Profinity eXact elution buffer plus 4 M urea.

Table 2. Purification of MBP using Profinity eXact purification resin in the presence of urea.

Urea Concentration, M	Binding Capacity, mg/ml	Purity of Eluted MBP, %
0	3.7	100
2	3.7	98.1
4	2.5	96.2

Summary

We have demonstrated the purification of a variety of tag-free recombinant proteins using the Profinity eXact fusion-tag system. This is the only system currently available for fusion protein purification and complete tag removal in a single chromatographic step using a truly generic protocol. Preparation of a tag-free protein, without any additional N-terminal residues, can typically be accomplished in about an hour, in contrast to other affinity-tag purification and tag-removal systems that usually require substantial condition optimization and lengthy protocols. The stability of this new affinity resin allows multiple uses of a cartridge for target purification, making the system cost effective. We also showed that the binding and on-column cleavage properties of the Profinity eXact purification resin were compatibile with up to 4 M urea. Therefore, this resin is potentially amenable to the purification of recombinant proteins accumulated in the form of insoluble aggregates. The efficiency and consistency of the Profinity eXact fusion-tag system makes it a universal platform for recombinant tag-free protein purification in a high-throughput setting.

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