Periplasmic Expression and Purification of Recombinant Proteins From *E. coli* Using the Profinity eXact™ Fusion-Tag System

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Introduction

Expression of recombinant proteins in *E. coli* cytoplasm is widely used. However, improper folding of many target proteins may occur during this process. Improper folding often results in the formation of inclusion bodies despite attempts to optimize growth conditions. One possible approach to obtaining a correctly folded recombinant protein is to export the protein into the *E. coli* periplasm. Some successful examples of this approach include production of soluble and functional human growth hormone (Soares et al. 2003), human interferon-γ (Balderas Hernández et al. 2008), and murine CMP-sialic acid transporter (Maggioni et al. 2007). Secretion of recombinant proteins to the periplasm of *E. coli* has several advantages over intracellular production. These advantages include simplified downstream processing, enhanced biological activity, higher product stability and solubility, and N-terminal authenticity of the expressed protein (Mergulhão et al. 2005).

The Profinity eXact fusion-tag system offered by Bio-Rad Laboratories provides an efficient one-step purification-cleavage protocol for bacterial recombinant protein production. The Profinity eXact affinity tag, in an N-terminal fusion with a target protein, binds to the Profinity eXact resin and, upon on-column cleavage, releases the purified target protein in the elution step. The highly specific, controlled cleavage generates a recombinant protein containing the desired native N-terminal amino acid sequence with the tag still attached to the resin, eliminating the need for tag and protease removal. A protein of interest can be cloned into the pPAL7 vector and expressed in *E. coli* as a fusion protein under control of the T7 promoter.

In order to utilize the Profinity eXact fusion-tag system for expression of recombinant proteins in the *E. coli* periplasm, we constructed a new expression vector by adding the OmpA signal peptide upstream of the Profinity eXact tag. We report successful periplasmic expression and purification of human interferon-α 2a (hIFNα2a).

Methods

Plasmid Construction

The OmpA signal sequence was constructed by annealing two phosphorylated primers encoding all 21 amino acids of the signal peptide (underlined): forward 5'-TATG ATG AAA AAG ACA GCT ATC GCG ATT GCA GTG GCA CTG GCT GGT TTC GCT ACC GTA GCG CAG GCC CA-3' and reverse 5'-TATG GGC CTG CGC TAC GGT AGC GAA ACC AGC CAG TGC CAC TGC AAT CGC GAT AGC TGT CTT TTT CAT CA-3'. A mixture of 10 µl (0.5 µg/µl) of each primer was incubated for 5 min at 75°C. The mixture was then slowly cooled at room temperature. Annealed product containing NdeI cohesive ends was ligated into the NdeI site of the pPAL7 vector (Bio-Rad Laboratories, Inc.), incorporating the OmpA signal peptide at the Profinity eXact tag's N-terminus (Figure 1) to generate plasmid pOPAL7.

The cDNA encoding hIFNa2a was amplified using restriction-independent cloning (RIC) as described in the Profinity eXact system instruction manual (bulletin 10011260). Then the RIC hIFNa2a fragment was cloned into the SapI and EcoRI sites of pOPAL7. The resulting pOPAL-IFNα2a plasmid generates an OmpA signal-Profinity eXact tag-IFNα2a fusion with a Thr-Ser linker to minimize premature elution of the target due to the natural N-terminal Cys residue present in the mature interferon (see bulletin 10011260).

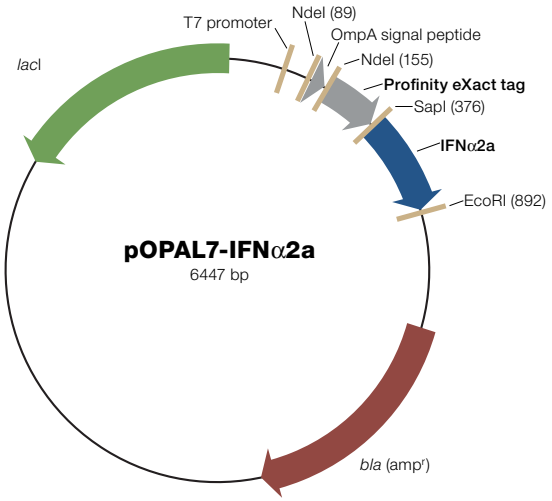

Fig. 1. Map of pOPAL7-IFNα2a plasmid.

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Bacterial Strains and Cultivation

E. coli C-Max™ 5α competent cells (Bio-Rad) were used as a primary host for transformation and propagation of plasmids. *E. coli* BL21(DE3) (Bio-Rad) were used for protein expression. A noninducing medium (Studier 2005) containing glucose, salts ($MgSO_4$, $(NH_4)_2SO_4$, KH_2PO_4, Na_2HPO_4, $CaCl_2$), and trace metals (Mn, Fe, Zn, Co, Cu, Ni, Mo, Se, B) was used for overnight starter culture. Expression was carried out in M9 minimal medium containing 3 g/L Na_2HPO_4, 1.5 g/L KH_2PO_4, 0.5 g/L NH_4Cl, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, and 0.4% glucose. Both media were supplemented with 100 µg ampicillin/ml.

Protein Expression and Purification

Expression plasmids were transformed into *E. coli* BL21(DE3) cells, and the resulting transformants were grown overnight at 37°C in 5 ml noninducing medium. From this overnight culture, 2.5 ml was used to inoculate 250 ml M9 medium; the cell culture was grown at 37°C until OD_{600} reached 0.5. Protein expression was induced by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.1 mM. The induced culture was grown for 16 hr at 20°C and 100 rpm. Periplasmic proteins were released by osmotic shock (Thorstenson et al. 1997). Briefly, the cell pellet was resuspended with 10 ml of 0.1 M Tris-acetate buffer (pH 8.0) containing 20% sucrose, 0.1 mM phenylmethanesulfonyl fluoride (PMSF), and 0.5 mM EDTA and incubated on ice for 15 min. The cell pellet was collected by centrifugation at 6,000 × g for 10 min and the supernatant was stored on ice. Cells were resuspended in 10 ml of ice-cold deionized water and incubated on ice for 15 min. After centrifugation at 6,000 × g for 10 min, the recovered supernatant was combined with supernatant from the sucrose wash. The resulting periplasmic fraction was purified using Bio-Scale™ Mini Profinity eXact cartridges (Bio-Rad). After purification, 500 µl of periplasmic solution was loaded onto prepacked mini spin columns pre-equilibrated with ice-cold 0.1 M Tris-acetate buffer (pH 8.0). The resin was washed three times with 500 µl of the same buffer and incubated at room temperature in 500 µl of cleavage buffer (0.1 M Tris-acetate, pH 8.0, and 10 mM NaN_3). After incubation for 30 min, the eluate containing tag-free target protein was collected.

SDS-PAGE Analysis

Protein samples were analyzed on 4–20% Criterion™ Tris-HCl gels followed by staining with Bio-Safe™ Coomassie stain (Bio-Rad). Image acquisition and analysis were done using a Molecular Imager® GS-800™ calibrated densitometer and Quantity One® 1-D analysis software (Bio-Rad).

Results and Discussion

Periplasmic Expression of the Profinity eXact Tag in *E. coli*

Periplasmic translocation is one of the widely employed approaches to improve soluble recombinant protein expression in *E. coli*. The oxidizing environment of the periplasmic space facilitates the formation of disulfide bonds, promoting correct folding and protein stability. Purification of periplasmically expressed recombinant proteins benefits from the lower content of bacterial proteins in the periplasm and easy osmotic shock extraction protocols.

In order to develop an alternative strategy for expression of Profinity eXact fusion-tagged recombinant proteins in *E. coli*, we investigated the incorporation of an N-terminal OmpA signal sequence as a means of targeting fusion proteins into the periplasmic space. Many soluble and active proteins have been successfully produced using the OmpA signal sequence (Mergulhão et al. 2005).

The vast majority of *E. coli* secreted proteins use the SecB-dependent pathway for translocation across the inner membrane (Mergulhão et al. 2005). These proteins contain an amino-terminal signal peptide that functions as a targeting and recognition signal. The OmpA signal peptide targets the *E. coli* outer membrane protein A to the Sec translocase (the *E. coli* translocation machinery in the inner membrane), permitting the translocation of OmpA into the periplasm, where it is removed by a signal peptidase (Binet et al. 1997).

To confirm that the OmpA signal peptide can direct the Profinity eXact tag into the *E. coli* periplasm, we expressed the tag fused with and without the signal peptide. The periplasmic deposition was optimal at the following conditions: 20°C culturing, minimal medium, gentle shaking of induced cell culture, and induction with 0.1 mM IPTG. We speculate that using less than optimal growth conditions favors transportation of a target protein directed by the signal peptide into the periplasm where it could be correctly folded. After induction with IPTG and expression, the harvested cell pellet was subjected to osmotic shock in order to release periplasmic proteins. All fractions were analyzed by SDS-PAGE as shown in Figure 2.

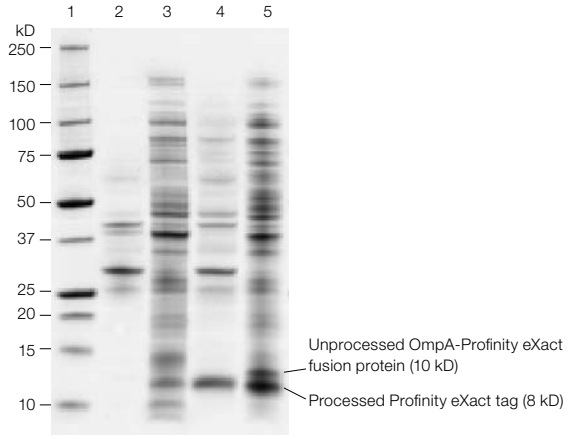

Fig. 2. SDS-PAGE analysis of the Profinity eXact tag distribution between periplasmic and intracellular fractions. Profinity eXact tag (8 kD) was expressed in *E. coli* alone (Profinity eXact tag) or fused with the OmpA signal peptide (OmpA-Profinity eXact fusion protein). Lane 1, Precision Plus Protein™ unstained standards; lanes 2 and 4, periplasmic fraction (supernatant) of Profinity eXact tag and OmpA-Profinity eXact fusion protein, respectively; lanes 3 and 5, intracellular fraction (pellet) of Profinity eXact tag and OmpA-Profinity eXact fusion protein, respectively. The 8 kD mass of the Profinity eXact tag has been confirmed by mass spectrometry (data not shown) and has an apparent electrophoretic mass of approximately 12 kD.

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The Profinity eXact tag, as expressed without the signal peptide using the unmodified pPAL7 vector, accumulates in the cytoplasm and cannot be released by osmotic shock (Figure 2, lanes 2 and 3). The presence of the OmpA signal peptide (pOPAL7 vector) changes the localization of the Profinity eXact tag, targeting it to the periplasm (Figure 2, lane 4). Similar mobility of the Profinity eXact tag from the periplasmic fraction (Figure 2, lane 4) and intracellularly expressed tag without OmpA signal peptide (Figure 2, lane 3) suggests that the OmpA signal peptide was processed by the signal peptidase. The presence of processed Profinity eXact tag in the pellet fraction may be explained by incomplete periplasmic extraction (Figure 2, lane 5). Efficiency of periplasmic extraction can be improved by performing additional osmotic shock (Sletta et al. 2007).

Judging from gel mobility, some of the Profinity eXact tag encoded by pOPAL7 in the pellet fraction is not proteolytically processed. This can be explained by cytoplasmic retention of the OmpA-fused Profinity eXact tag.

Comparing band intensities on the gel, the amount of Profinity eXact tag released by osmotic shock under the described conditions is 15 mg/L. Expression yield of the Profinity eXact tag is significantly higher when it is expressed with the OmpA signal peptide. This phenomenon needs further investigation, but similar observations have been made by other researchers (Sletta et al. 2007).

Expression and Purification of Human Interferon-α 2A

Interferon-α 2A belongs to the IFN family of cytokines, which can induce antiproliferative, immunomodulatory, and potent antiviral activities against a wide range of mammalian viruses (Pestka et al. 1987). It has pharmaceutical value and is used to treat several diseases, including some types of cancer and hepatitis, in particular hepatitis C.

Expression of human IFNα2a either untagged or with the His6 tag usually results in insoluble protein accumulation and inclusion body formation (Beldarraín et al. 2001, Piehler and Schreiber 1999). We used hIFNα2a as a model protein to examine targeting of Profinity eXact-tag fusion protein into the periplasm and subsequent purification.

After expression, periplasmic proteins were extracted by osmotic shock. In this experiment, we also included a wash step prior to periplasmic extraction to check for periplasmic leakage. A portion of the cell pellet was washed twice with cold 0.1 M Tris-acetate buffer (pH 8.0). All fractions were analyzed separately by SDS-PAGE, including sucrose wash and ice-cold water extraction (Figure 3).

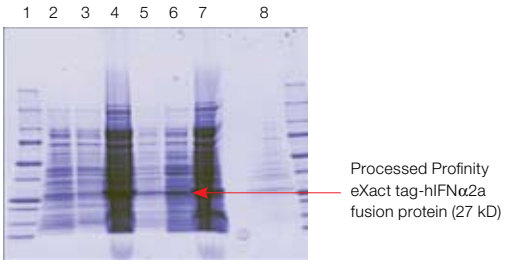

Fig. 3. SDS-PAGE analysis of the periplasmic secretion of the Profinity eXact tag-hIFNα2α fusion protein. Lane 1, Precision Plus Protein unstained standard; lanes 2 and 3, proteins extracted by a 0.1 M Tris-acetate buffer (pH 8.0) wash; lane 4, total cell proteins remaining after Tris-buffer wash; lane 5, proteins extracted with 0.1 M Tris-acetate buffer (pH 8.0), 20% sucrose, 0.1 mM PMSF, and 0.5 mM EDTA; lane 6, proteins extracted with ice-cold water; lane 7, proteins remaining after osmotic shock (water) extraction; lane 8, spent culture medium.

Based on the results shown in Figure 3, it is believed that the OmpA signal peptide was processed off the initial OmpA-Profinity eXact tag-hIFNα2a fusion protein. The gel image shows soluble Profinity eXact tag-hIFNα2a can be released by osmotic shock (lanes 4 and 5). The presence of Profinity eXact tag-hIFNα2a and other bacterial proteins in the Tris-buffer wash fractions (lanes 2 and 3) as well as in the growth medium (lane 8) suggests periplasmic leakage. The periplasmic leakage may have several causes. During cell division, leakage of periplasmic contents can happen prior to the formation of the individual outer membrane (Mergulhão et al. 2004). The accumulation of recombinant protein in the periplasm may cause an osmotic pressure buildup, which can be a driving force for transport across the outer membrane (Hasenwinkle et al. 1997). Periplasmic secretion may also induce cell lysis, resulting in the release of periplasmic content (Lee and Bernstein 2001).

About 40% of the expressed Profinity eXact tag-hIFNα2a was released by the osmotic shock and 20% was secreted into the culture medium. The amount of secreted Profinity eXact fusion-tagged hIFNα2a estimated from the gel is 10 mg/L.

hIFNα2a encoded by pPAL7 vector lacking signal peptide was used as a negative control for periplasmic targeting. No band corresponding to Profinity eXact tag-hIFNα2a can be detected in the periplasmic fraction of the cell expressing interferon without signal peptide (Figure 4).

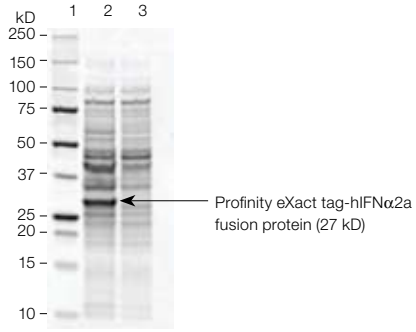

Fig. 4. SDS-PAGE analysis of the periplasmic proteins extracted from the cells expressing Profinity eXact tag-hIFNα2a with and without OmpA signal peptide. Lane 1, Precision Plus Protein unstained standard; lane 2, Profinity eXact tag-hIFNα2a (27 kD) with OmpA signal peptide; lane 3, Profinity eXact tag-hIFNα2a (27 kD) without OmpA signal peptide.

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Periplasmic hIFNα2a was purified using Bio-Scale Mini Profinity eXact cartridges (Figure 5). The detailed analysis of recombinant protein purification using the Profinity eXact system is described elsewhere (see bulletin 5652). On-column cleavage releases tag-free hIFNα2a and presents a great advantage in periplasmic protein purification. Since specific cleavage occurs between the last amino acid of the Profinity eXact tag and the N-terminal amino acid of the target protein, N-terminal heterogeneity due to possible errors in signal peptide processing of the target is eliminated.

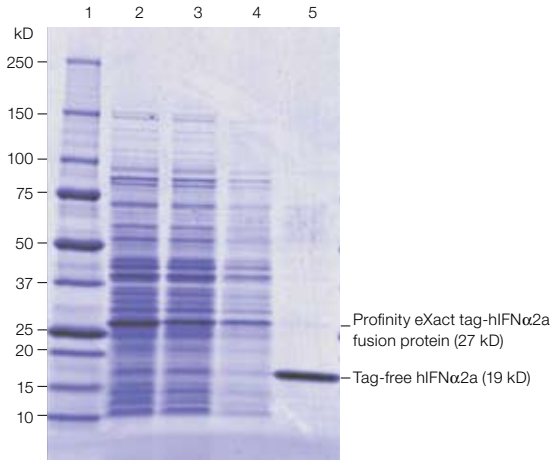

Fig. 5. Bio-Scale Mini Profinity eXact cartridge purification of periplasm targeted Profinity eXact fusion-tagged hIFNα2a. Lane 1, Precision Plus Protein unstained standard; lane 2, periplasmic proteins extracted by osmotic shock, containing Profinity eXact tag-hIFNα2a fusion protein (27 kD); lane 3, flowthrough from spin column; lane 4, wash; lane 5, eluted tag-free hIFNα2a (19 kD).

Conclusions

In this paper we report the applicability of the Profinity eXact fusion-tag system for purification of periplasmic proteins. Results show that hIFNα2a in fusion with the Profinity eXact tag and with an N-terminal OmpA signal peptide can be successfully translocated into the periplasm, suggesting utility with other proteins of interest. Fusion protein can then be natively purified using the Profinity eXact purification resin. Precise on-column cleavage of the Profinity eXact tag facilitates affinity purification of the target and eliminates the need to maintain compatibility of the leader peptide with target proteins for periplasmic targeting and correct signal peptide processing.

The secretory production of recombinant proteins by *E. coli* has been proven to be a successful method to facilitate downstream processing, folding, and in vivo stabilization, enabling the production of soluble and biologically active proteins. Our interest in developing new protein purification techniques and generating native soluble proteins of interest led us to explore the possibility of targeting Profinity eXact fusion-tagged proteins into the *E. coli* periplasmic space.

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