Protein Expression and Purification 63 (2009) 58–61

Contents lists available at ScienceDirect

Protein Expression and Purification

journal homepage: www.elsevier.com/locate/yprep

High-throughput T7 LIC vector for introducing C-terminal poly-histidine tags with variable lengths without extra sequences

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ARTICLE INFO

Article history:
Received 28 July 2008
and in revised form 3 September 2008
Available online 14 September 2008

Keywords:
Histidine tag of variable lengths
Ligation independent cloning
Immobilized metal ion affinity chromatography
T7 bacterial expression
High-throughput protein purification

ABSTRACT

Immobilized metal ion affinity chromatography (IMAC) has become one of the most popular protein purification methods for recombinant proteins with a hexa-histidine tag (His-tag) placed at the C- or N-terminus of proteins. Nevertheless, there are always difficult proteins that show weak binding to the metal chelating resin and thus low purity. These difficulties are often overcome by increasing the His-tag to 8 or 10 histidines. Despite their success, there are only few expression vectors available to easily clone and test different His-tag lengths. Therefore, we have modified *Escherichia coli* T7 expression vector pET21a to accommodate ligation-independent cloning (LIC) that will allow easy and efficient parallel cloning of target genes with different His-tag lengths using a single insert. Unlike most LIC vectors available commercially, our vectors will not translate unwanted extra sequences by engineering the N-terminal linker to anneal before the open reading frame, and the C-terminal linker to anneal as a His-tag.

Introduction

Immobilized metal ion affinity chromatography (IMAC) has become one of the most popular recombinant protein purification methods [1–5]. By adding six histidines at the N- or C-terminus of a protein, this allows the metal affinity resin charged with nickel or some other transition metal ion to selectively capture the target protein by metal–ligand covalent bonding. By increasing the concentration of imidazole in the purification buffer or stripping the metals off the resins with strong metal chelators such as EDTA, easy controlled release of captured proteins is achieved. Furthermore, the interaction between the resin and protein can also occur in buffer conditions containing urea thus allowing on-column refolding [6–10].

Despite all the benefits of IMAC, there can be problems like weak or no interaction between the resin and target. This is often caused by concealed His-tags, and could be alleviated by switching the position of the His-tag to the other terminus of the protein. For some cases this does not help either, presumably because the "free" portion of the His-tag is still not long enough for strong binding. We encountered this situation with an *Escherichia coli* chemotaxis receptor. This can be resolved by increasing the binding affinity of the target protein thus leading to increased purity of the target. Many researchers have increased the length of His-tags on their targets with good results [11–15]. Despite these successes, there are not many expression vectors that allow easy and efficient cloning of proteins with different His-tag lengths. Therefore, we modified EMD Biosciences' pET21a, an *E. coli* T7 protein expression vectors to allow introduction of variable length His-tags using ligation-independent cloning (LIC).

Materials and methods

Materials

The vectors were derived from *E. coli* expression vector pET21a and expression was performed using BL21(DE3) Star *E. coli* cells (Novagen, La Jolla, CA). All synthesized oligos and PCR primers were from Integrated DNA Technologies (Coralville, IA) and dNTP, dGTP and dCTP were from Promega (Madison, WI). Deep Vent DNA Polymerase, T4 DNA Polymerase and restriction enzymes StuI came from New England Biolabs (Beverly, MA). Mutations were introduced using QuikChange II Site-Directed Mutagenesis (Stratagene, La Jolla, CA). MachT1 DH5α *E. coli* competent cells were used for cloning (Invitrogen, Carlsbad, CA). Carbenecillin (United States Biological, Swampscott, MA) was used at a concentration of 100 μg/mL. *n*-Dodecyl-β-D-maltopyranoside was purchased from Anatrace (Maumee, OH). HisTrap 5 mL column was purchased from GE Healthcare (Piscataway, NJ).

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doi:10.1016/j.pep.2008.09.005

Construction of variable His-tag length LIC vectors pJL

The vectors pJL-H6, pJL-H8 and pJL-H10 were derived from pL, a pET21a derived LIC vector for C-term His-tag cloning (Not published). Briefly, pL vector was constructed by modifying the multiple cloning site of pET21a to allow LIC cloning of the gene with a C-terminal six histidine tag. All three vectors were made by QuikChange site directed mutagenesis of pL using primers,

- pJL-H6: 5′-GAAGGAGATATAAGGCCTCACCATCACCATCATCACTGAGATCCGG-3′ and 5′-CCGGATCTCAGTGATGATGGTGATGGTGAGGCCTTATATCTCCTTC-3′,
- pJL-H8: 5′-GAAGGAGATATAAGGCCTCACCATCACCATCATC ACCA CCACTGAG-3′ and 5′-CTCAGTGGTGGTGATGATGGTGATGGTGAGGCCTTATATCTCCTTC-3′,
- pJL-H10: 5′-GAAGGAGATATAAGGCCTCACCATCACCATCATCA CC ACCACCACCACTGAG-3′ and 5′-CTCAGTGGTGGTGGTGGTGATGA TGGTGATGGTGAGGCCTTATATCTCCTTC-3′.

All constructs were confirmed by DNA sequencing.

Parallel cloning and expression of Tsr receptor in pJL with different His-tag lengths

LIC ready vector stocks were made by digesting each pJL vector with StuI and then purified by agarose gel electrophoresis. The purified excised vectors were made LIC ready by treating with T4 DNA polymerase with dCTP. The LIC ready vectors were stored at −20 °C until use. *E. coli* serine chemotaxis receptor (Tsr) was amplified from DH5α genomic DNA using Deep Vent DNA polymerase and primers 5′-GAAGGAGATATAAGGATGTTAAAACGTATCAAAATT GTGACC-3′ and 5′-ATGATGGTGATGGTGAAATGTTTCCCAGTTCTCCT CG-3′. In these primers, 5′-GAAGGAGATATAAGG-3′ is the N-terminus annealing sequence and 5′-ATGATGGTGATGGTG-3′ is the C-terminal annealing sequence for the LIC reaction. The C-terminus annealing sequence is compatible with all three pJL-H6, pJL-H8 and pJL-H10 vectors to form hexa-, octa- and deca-histidine tags, respectively. For cloning hepta-, nona- and undeca-histidine tags fused Tsr, Tsr was amplified using 5′-ATGGTGATGGTGGTGAAATGTTTCCCAGTTCTCCTCG-3′ as the C-terminal primer where 5′-ATGGTGATGGTGGTG-3′ is the insert linker sequence. Purified PCR products were treated with T4 DNA polymerase and dGTP, and mixed with LIC ready vector at a 1:2 ratio (vol/vol) of vector to insert (estimated 20–40 ng, respectively) before transformation into MachT1 DH5α cells. A single colony was picked, plasmid purified by miniprep, and sequence confirmed. Expression level was tested using Studier's auto-inducing media [16]. Briefly, pJL clones and the native pJL vector (negative control) were transformed into BL21(DE3) Star cells, plated onto a Medium phosphate, aspartate (D) containing 17 Amino acid supplemented, Glucose containing (MDAG) plate with Carbenicillin. A single colony was inoculated into 2 mL ZYM-5052 autoinduction media. The culture was incubated at 37 °C overnight with shaking at 200 RPM. Ten microliters of the overnight culture was mixed with 3X Laemmli sample buffer, boiled for 5 min, then run on 4–20% Tris–HCl SDS–PAGE gel. The gel was visualized using 0.12% Coomassie Blue R-250 stain. Expression levels per liter were estimated by estimating the intensity of the 60 kDa presumptive Tsr expression band in comparison to the 75 kDa molecular marker band (1 μg). Expression levels per gram of wet weight cells were estimated by converting expression level per liter using the conversion factor of 8 grams per liter of wet cells which is empirically determined by harvesting twelve liters of pET21 Tsr grown in ZYM-5052.

Immobilized nickel ion affinity chromatography of pJL Tsr with different His-tag lengths

Each pJL Tsr with different His-tag lengths were grown in 2 L ZYM-5052, harvested by centrifugation and stored at −80 °C until use. Frozen cell pastes were resuspended in an ice cold resuspension buffer (20 mM Tris–HCl, pH 8, 0.1 M NaCl, 1 mM PMSF), and cells were disrupted using a microfluidizer (Microfluidics, Newton, MA). The detergent *n*-dodecyl-β-D-maltopyranoside (DDM) was directly added to the cell lysate (1% w/v final concentration), and stirred for a half hour to extract membrane proteins which includes Tsr. The detergent extracted cell lysate was cleared by centrifugation at 100,000*g*. The imidazole concentration of cleared lysate was raised to 40 mM before loading onto a Histrap 5 mL column equilibrated with 20 mM Tris–HCl, pH 8, 0.1 M NaCl, 40 mM imidazole and 0.02% w/v DDM (equilibration buffer). After loading the lysate, the column was washed with 70 mL of equilibration buffer. Tsr was eluted using a 40–300 mM imidazole gradient in 50 mL, collecting 3 mL fractions. Protein concentrations of eluates from UV 280 nm absorbance peak fractions were determined by Bradford protein assay [17], and fraction purities were analyzed by a 12% Tris–HCl SDS–PAGE gel.

Results and discussion

pL, the parent of pJL, was intended to facilitate the high-throughput cloning, expression, and purification of transmembrane proteins with a C-terminal His-tag without any unwanted cloning sequence in the open-reading frame (ORF) in the cloned gene. We realized that pL could be further improved by allowing one insert to generate different length variations of His-tag fusions (Fig. 1) for optimizing purification of difficult protein targets that do not or weakly bind to the IMAC resins as demonstrated by many researchers [11–15]. For pJL, the N-terminal annealing sequence for the insert is 5′-GAAGGAGATATAAGG-3′ and the cloner must include the start codon for the gene to be translated. The C-terminal annealing sequence for the insert (5′-ATGATGGTGATGGTG-3′) generates a hexa- (pJL-H6), octa- (pJL-H8) and deca- (pJL-H10) histidine tag fusion protein. LIC ready pJL vectors can be prepared by excising with StuI, and treating with T4 DNA polymerase with dCTP. LIC ready inserts can be prepared by treating purified PCR products with T4 DNA polymerase with dGTP.

Cloning *E. coli* serine chemotaxis receptor (Tsr) yielded all three constructs with the correct length of His-tags confirmed by DNA sequencing (Data not shown). This demonstrates successful cloning into pJL. Furthermore, by inserting a single extra histidine before the linker sequence allows cloning of hepta-, nona- and undeca-histidine tags fused proteins. On the other hand, by inserting the stop codon before the linker sequence prevents translation of the His-tag demonstrating complete His-tag length control. pJL also allows protein translation rate control by changing the length of the spacer between RBS and the start codon [18] by shortening or lengthening the 3′ end of the insert's N-terminal LIC linker. Testing expression in BL21(DE3) Star in ZYM autoinduction media showed clear protein expression at the correct molecular weight for *E. coli* serine chemotaxis receptor (Fig. 2). Expression level reached up to 200 mg/L or 25 mg/g of wet cells.

Immobilized nickel ion affinity chromatography of Tsr with six, eight, and ten histidine tag demonstrates higher binding affinity as the length of the His-tag increases (Fig. 3A). As the His-tag length increases, the elution peak shifts toward a higher imidazole concentration and the peaks also broaden. The purity of the UV 280 nm maximum fractions determined by a SDS–PAGE gel indicates some increase in the purity as the length of the His-tag increases (Fig. 3B). Some impurities may be binding to Tsr tightly