Purification of Low-Expressed Proteins

Introduction

Recombinant proteins are widely used in the life sciences, biotechnology, and medicine, and there is a growing need for innovative technologies that enable their rapid and cost-effective purification. Some of the most successful protein purification schemes make use of affinity tags that are genetically fused to the protein of interest (POI).[1] A number of such tags have been developed, and include the broadly-used hexahistidine (6xHis) tag that binds to Ni^{2+}-nitrilotriacetic acid (NTA) resin.[2] However, Ni^{2+}-NTA and other commonly used affinity purification resins contain toxic metal ions or reagents that inactivate certain proteins and preclude their use for *in vitro* studies.[3,4,5] In addition to that, the most significant disadvantages to the use of His tags are their high cost and lack of scalability when advancing to manufacturing.[6]

Another common problem during protein purification is the low expression of the POI. Several strategies have been proposed to increase the expression of low-expressed proteins, however most of these require extensive tests and/or several rounds of purification that requires additional time and resources to complete the purification.[7]

A new affinity tag has been developed which meets or exceeds the specifications of His tags in terms of performance – including a significant decrease in cost and the ability to scale from research to preparative to manufacturing. Further development of this technology allowed very low- to moderate-expressed recombinant proteins to be purified in less than 15 minutes with a final purity between 85% to 95% and a final yield of up to 2mg without the use metal ions or toxic reagents.

Three recombinant proteins were expressed and purified using this new affinity tag to demonstrate the applicability of this method for the purification of low-expressed proteins (LEP).

KRAS: KRAS is a human oncoprotein which is mutated in 90% of pancreatic cancer patients and 40% of lung and colorectal cancer patients. Recombinant human KRAS can be expressed at medium-low levels in *E. coli*.

TEV: Tobacco Etch Virus nuclear-inclusion-a endopeptidase (TEV protease) is a valuable reagent that is extensively used to excise affinity tags fused to the N-termini of POIs.[8] Its purification from *E. coli* is

challenging due to its very low solubility that leads either to low yield and low purity or to a lengthy purification process.

PFK: *T. thermophilus* 6-phosphofructokinase is expressed at very low levels in *E. coli* and poses purification challenges both in terms of purity and yield.

We demonstrate the purification of these three proteins using the Proteios Car9 affinity tag and the Proteios LEP Purification Kit. Proteios Car9 is a dodecapeptide with high affinity for silica substrates and whose binding is competitively inhibited by L-Lysine.[9,10] This forms the basis of Proteios' recombinant protein purification technology.

Materials and Methods

Cell Lines

BL21(DE3) (One Shot™ BL21(DE3) Chemically Competent *E. coli*) and Top10 cells (GeneArt™ Gibson Assembly HiFi Cloning Kit, chemically competent cells) were purchased from Thermo Scientific. BL21 Lys Y (T7 Express lysY Competent *E. coli* (High Efficiency)) cells were purchased from New England Biolabs. Plasmid extraction kits were purchased from Thermo Scientific.

Cloning, Cell Growth, Protein Expression and Cell Collection

A dodecapeptide coding for the Proteios Car9 sequence (DSARGFKKPGKR) was introduced either at the N-terminal or at the C-terminal of the POI by PCR. A six amino acid spacer (KLGGGS) was placed between the Proteios Car9 tag and the POI to minimize the effect of the tag. The modified CoDing Sequence (CDS) was introduced into pET24+ plasmid by enzymatic digestion with NdeI and XhoI. Plasmids were amplified in the Top10 bacteria host. BL21 or BL21 lys Y bacteria strains were used to express the desired protein at 37˚C for 3–4 hours under shaking at 200 rpm. Protein expression was induced with 1mM IPTG.

Bacteria cells were collected by centrifugation at 3500g for 10 minutes at 4˚C.

Cell Lysis

Cell pellets from 50mL cultures were destroyed either by three cycles of freeze–thaw or by six cycles of sonication (5sec on, 30sec off, 30% power, in ice) in 4mL of Proteios LEP Lysis Buffer supplemented with 1mM PMSF and 2mM EDTA. Cellular debris was removed at 10000g for 15min at 4˚C. Clarified cell lysate was immediately used or stored at -20ºC for future use.

Protein Purification

A Proteios LEP Purification Kit was used to purify proteins that express from very low to moderate levels according to the manufacturing instructions.

Briefly, the provided spin columns were first pre-washed with 4ml of Proteios LEP Lysis Buffer followed by centrifugation at 500g for two minutes at room temperature. Clarified lysates were loaded on the spin column and spun at 500g for two minutes or until all the lysate flowed through the column. Up to 15mL of clarified

lysate can be loaded at once. The flow-through was discarded and the spin column was washed three times with 4mL of Proteios LEP Wash Buffer. Two minutes of centrifugation at 500g were used for each wash. The POI was eluted in a 50mL collection tube using 4mL Proteios LEP Elution Buffer followed by two minutes centrifugation at 500g. A second elution was performed to elute the remaining protein.

Protein Concentration and Desalting

After elution, the POI was concentrated using centrifugal filter units (Amicon® Ultra-4 Centrifugal Filter Unit Ultracel-10 regenerated cellulose membrane, 4mL sample volume), with a cutoff 10kDa. Buffer exchange was performed using dialysis tubing (SnakeSkin™ Dialysis Tubing, 3.5K MWCO, 16 mm) with a cutoff of 3.5kDa against 20mM Tris pH 8 overnight at 4ºC or using centrifugal filter units.

Purity and Yield Determination

Purity was determined by SDS-PAGE followed by PageBlue protein staining solution (PageBlue™ Protein Staining Solution) and image analysis using ImageJ analysis software. Yield was determined using the Thermo Scientific™ NanoDrop™ OneC Microvolume UV-Vis Spectrophotometer through absorption at 280nm.

Results

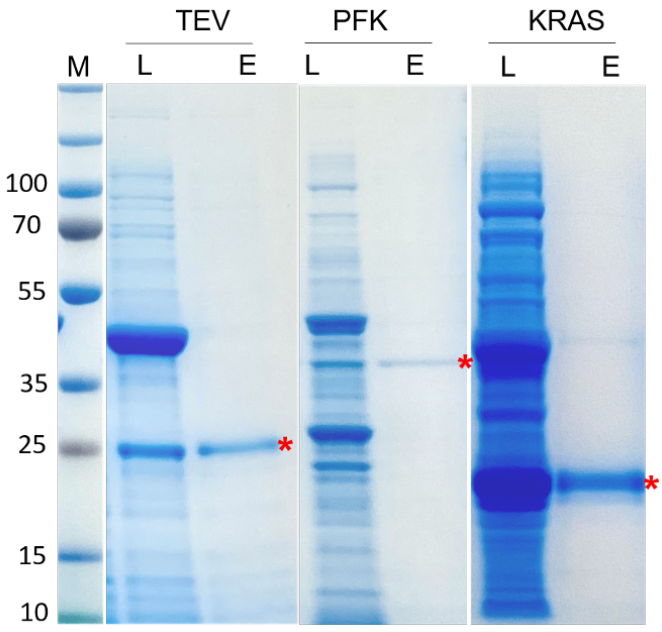

Figure 1: Purification of protein of interest using Proteios LEP Purification Kit for low-expressed protein. MBP-TEV cut site-Car9-TEV (left), PFK-Car9 (middle) and Car9-KRAS (right) are indicated with '*'. M: Protein Ladder (PageRuler™ Plus Prestained Protein Ladder, 10 to 250 kDa), L: Lysate, E: Elution.

The Proteios LEP Purification Kit was able to purify up to 2mg of protein using a single column (**Figure 1**). The purity was affected by the level of the expression of the target protein in the lysate. A purity from 85% to

95% for all the three proteins of interest was achieved without the use of metal ions or toxic reagents (**Table 1**). The protein purification step took less than 15 minutes in total.

Protein	Level of Expression in Lysate	Purity	Yield
KRAS	Moderate	95%	1.1mg/50mL culture
TEV	Low	90%	1.2mg/50mL culture
PFK	Very low	86%	0.9mg/100mL culture

Table 1: Purity and yield obtained using Proteios LEP Purification Kit.

Conclusion

Proteios LEP Purification Kit provides a quick, low-cost alternative to His tag technology to purify up to 2mg of protein that expresses from very low to moderate levels in less than 15 minutes with an average purity between 85% and 95% without the use of metal ions or toxic reagents.

References

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