Romanian Biotechnological Letters *Vol. 21, No. 3, 2016*

ORIGINAL PAPER

Optimization of Recombinant Lipoic Acid Ligase Expression from Bacterial Cells

Received for publication, October 20, 2015
Accepted, November 15, 2015

PAULA ECATERINA FLORIAN[1], GEORGIANA PETRAREANU[2], SIMONA RUTA[3,4], ANCA ROSEANU[1*]

[1]Ligand-Receptor Interactions Department, Institute of Biochemistry of the Romanian Academy, Bucharest 060031, Romania; [2]Enzymology Department, Institute of Biochemistry of the Romanian Academy, Bucharest 060031, Romania; [3]Department of Virology, Carol Davila University of Medicine and Pharmacy, Bucharest 020022, Romania; [4]Emergent Diseases Department, Stefan S. Nicolau Institute of Virology, Bucharest 030304, Romania.
*[*]**Corresponding author:** Anca Roseanu, tel: 004021 223 90 69; fax: 004021 223 90 68 roseanua@gmail.com*

Abstract

Lipoic acid ligase (LplA) is an enzyme used for PRobe Incorporation Mediated by Enzymes (PRIME) labeling of proteins. Based on a previous described protocol for expression and subsequent purification of recombinant LplA ligase, we developed a new procedure in order to obtain recombinant gluthatione-S-transferase (GST)-LplA ligase fusion protein from bacterial cells in larger quantities and with high purity. The recombinant enzyme can be used for further applications such as PRIME labeling of proteins.

Keywords: *lipoic acid ligase, PRIME labeling, protein labeling*

1. Introduction

PRIME (PRobe Incorporation Mediated by Enzymes) labeling is a new, rapid and specific fluorescent labeling method of the protein of interest (UTTAMAPINANT & al. [1]; UTTAMAPINANT & al. [2]; WHITE and ZEGELBONE [3]). The method is based on the enzymatic activity of a ligase derived from *E.coli* lipoic acid ligase (LplA), an enzyme of 338 aminoacids and 38 kDa known to be involved in bacterial oxidative metabolism (CRONAN & al. [4]). It was previously shown that LplA was able to ligate with high specificity an alkyl azide to a target protein previously fused with a recognition sequence for LplA (FERNANDEZ-SUAREZ & al. [5]). This specific sequence is called ligase-acceptor peptide (LAP) and is only 13 aminoacid long. Using site-directed mutagenesis, Uttamapinant et. colab. (UTTAMAPINANT & al. [1]) generated a new ligase able to catalyze the covalent linking of the fluorescent 7-hydroxycoumarin to LAP-fusion proteins. Recently, Liu et al. used computational design to develop a fluorophore ligase derived from *E.coli* LplA to fluorescently label proteins with the red dye resorufin (resorufin ethyl ether) for super-resolution imaging studies (LIU & al. [6]). Based on the previous protocol (UTTAMAPINANT & al. [1]), in this work we reported an optimized procedure which consists in different purification steps by FPLC method in order to obtain high amount of gluthatione-S-transferase (GST)-LplA ligase fusion protein of high purity to be used for downstream applications such as PRIME labeling of proteins.

2. Materials and Methods

Cloning of LpLA ligase

The lplA ligase gene was inserted between the *Nco*I and *Eco*RI sites of the pETM-30 bacterial expression vector (kind gift from Dr. Georgiana Petrareanu), which introduces an N-terminal poly-histidine (His_6)-GST tag, obtaining pETM-30-LplA. Briefly, the situs for *Nco*I restriction enzyme was introduced by PCR using pcDNA3-LplA(W37V) Coumarin PRIME ligase (a gift from Alice Ting, Addgene plasmid # 25845) as a template and forward primers with situs for *Nco*I and reverse primers with situs for *Eco*RI: Forward, ATCGACCATGGCTATGAGCACCCTGAGACTGCTGATC and Reverse, TCGGAGAATTCTTATCTCACGGCGCCGGCC. The PCR product was ligated 'in-frame' to *Eco*RI /*Nco*I digested pETM-30 vector to obtain pETM-30-LplA.

Bacterial expression and purification of recombinant LplA ligase

LplA ligase was expressed as a fusion protein with a His_6-GST tag in N-terminal (GST-LplA) from pETM-30-LplA expression vector and using *E.coli* BL21 competent bacterial cells for quantitative expression of recombinant protein. The bacterial expression vector pETM-30 introduces a His_6-GST tag in N-terminal to the protein of interest which makes possible the purification of the recombinant LplA ligase by both FPLC on Ni^{2+}-Sepharose column and by batch method on a gluthation-Sepharose resin. The pETM-30-LplA vector was transformed into *E.coli* BL21 competent bacterial cells. The bacterial cells were amplified in LB medium supplemented with 50 μg/ml kanamicin at 37 °C until OD_{600}= 0.9. The LplA ligase enzyme expression was induced with 0.4 mM izopropil beta D-1- tiogalactopiranozid (IPTG) for 3h at 37 °C. The bacterial cells were harvested by centrifugation (6000 rpm/10 min/4 °C) and resuspended in lysis buffer (50 mM Tris-base and 300 Mm NaCl, pH 7.8 supplemented with 1 mM PMSF- Phenylmethylsulfonyl fluoride, protease inhibitors, and 1mM DTT- Dithiothreitol). Then the cells were lysed using a Douncer and 3 rounds of French press. The extract was cleared by centrifugation (30000xg/30 min/4 ºC) and the supernatant containing the His_6-GST-LplA fusion enzyme was subjected to purification by fast protein liquid chromatoghraphy (FPLC) on a 1 ml Ni^{2+}-Sepharose HiTrap HP column (AKTA, GE Healthcare). The column was washed with 10 volumes of washing buffer (50 mM Tris-base and 300 mM NaCl, pH 7.8 supplemented with 1 mM DTT and 10 mM imidazole) and eluted 30 min with 0-50% linear gradient of elution buffer (50 mM Tris-base and 300 mM NaCl, pH 7.8 supplemented with 1 mM DTT and 500 mM). The fractions eluted were analyzed by 10 % SDS-PAGE followed by Coomassie Brilliant Blue staining. One of the fractions was run on 10% SDS-PAGE, transferred onto nitrocellulose membrane, blocked with 0.25% skimmed milk in TBS-0.05% Tween-20 (blocking buffer) and immunoblotted using SNAP ID system (Millipore) with rabbit anti-GST Antibody (Sigma Aldrich, dilution 1/500 in blocking buffer) followed by anti-rabbit-HRP conjugated antibody (Santa Cruz, dilution 1/5000 in blocking buffer). The proteins were detected using an enhanced chemiluminescence (ECL) system (Supersignal West Femto ECL, Pierce).

3. Results and Discussions

Immobilized metal-affinity chromatography (IMAC) is a widely used method for rapid purification of proteins containing short affinity tag such as poly-histidine residues. In order to obtain a GST-LplA fusion protein, containing also a His_6-tag in N-terminal and which can be purified by both gluthatione- and Ni^{2+}-Sepharose columns, we first cloned the ligase into

pETM-30 vector. Purification of the ligase was carried out successfully using *E.coli* expression system followed by FPLC on Ni^{2+}-Sepharose column. To increase the purity of GST-LplA fusion protein in the fractions obtained after FPLC method, the standard protocol was optimized by two conditions: first, by adding 1 mM DTT in the lysis buffer and second, by adding imidazole in the washing buffer at a concentration of 10 mM. DTT is a reducing agent which prevents disulfide bonds formation between cysteine residues. DTT also maintains proteins in a stable form and protects them against oxidative process during purification steps. Imidazole at a very low concentration during washing steps prevents non-specific binding of endogenous bacterial proteins enriched in consecutive His residues at the Ni^{2+}-Sepharose resin.—After purification of GST-LplA fusion protein by FPLC on Ni^{2+}Sepharose column a chromatographic profile was obtained (Figure 1A). According to the absorbance curve at 280 nm, after elution with 0-250 mM imidazole gradient several fractions were enriched in proteins (12-18).

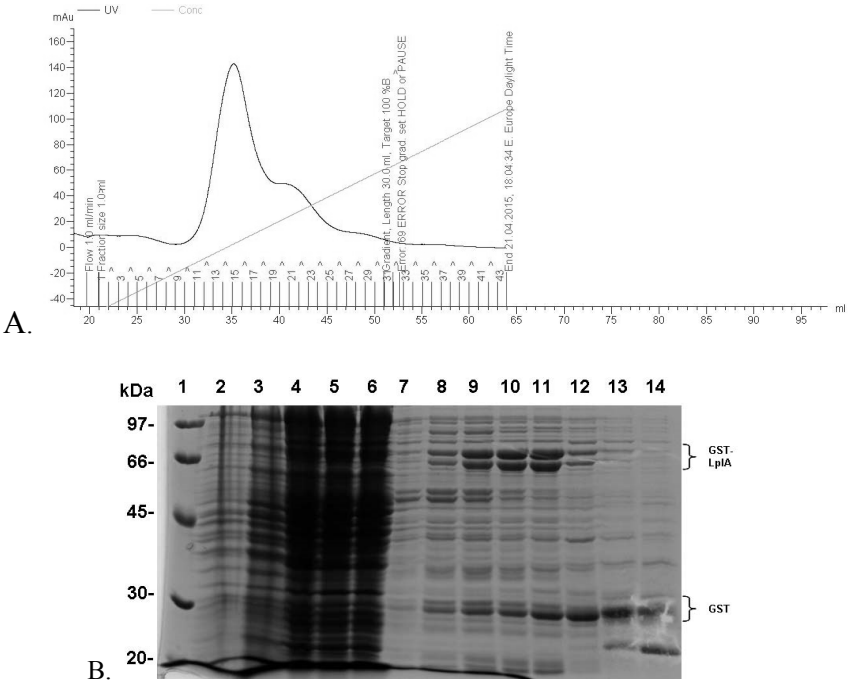

Figure 1. A. Chromatographic profile representing GST-LplA fusion protein purified from bacterial cells by FPLC on Ni^{2+}-Sepharose column. B. SDS-PAGE of eluted fractions after FPLC purification of GST-LplA fusion protein. 1-Marker;2-non-induced by izopropil beta D-1- tiogalactopiranozid (IPTG); 3-induced with IPTG;4-total lysate;5- soluble lysate;6-unbound lysate;7-14 eluted fractions: 12-16, 18, 20, 22.

In order to identify the protein eluted in the fraction pool after FPLC, SDS-PAGE was performed (Figure 1B). The electrophoretic analysis revealed contamination with other endogenous bacterial proteins enriched in consecutive His residues (between 30-66 kDa).
To provide further evidence that we have obtained GST-LplA fusion protein and to reveal the specificity of the purification methods employed, we performed immunoblotting with anti-GST antibody on one of the fractions obtained after FPLC purification. As shown in Figure 2, the fraction contains mostly the recombinant protein GST-LplA.

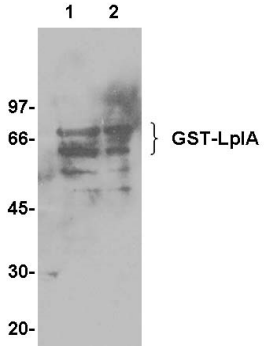

Figure 2. Expression of GST-LplA recombinant fusion protein. Fraction 14 was analyzed by blotting with anti-GST antibody. 1-20 µl from fraction 14; 2-10 µl from fraction 14.

We are currently investigating the functionality of the recombinant GST-LplA enzyme obtained using a protein fused to LAP peptide and 7-hydroxycoumarin.

4. Conclusion

We have developed a new procedure for obtaining recombinant GST-LplA ligase as a fusion protein from bacterial cells, based on a previous protocol for purification of the enzyme. The proposed protocol involves Ni^{2+}-Sepharose FPLC purification. The recombinant enzyme obtained could be a useful tool for PRIME labeling of proteins.

5. Acknowledgments

This paper is supported by the Sectoral Operational Programme Human Resources Development (SOP HRD), financed from the European Social Fund and by the Romanian Government under the contract number POSDRU/159/1.5/S/137390 and by Romanian Academy Project 3 of the Institute of Biochemistry. The authors thank Dr. Costin Ioan Popescu for advice in molecular cloning of LplA ligase, Madalina Icriverzi for technical assistance and Dr. Norica Nichita for careful reading of the manuscript.

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