Original Article

Isolation, cloning, and expression of *E. coli* BirA gene for biotinylation applications

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Abstract

Background: The key enzyme in biotin-(strept) avidin systems, *Escherichia coli* BirA biotin ligase, is currently obtained by overexpression of the long protein-tagged versions of the gene to prevent its toxic effect in *E. coli*. Herein we describe a rather simple and efficient system for expression of *E. coli* BirA without the application of long-tag proteins.

Materials and Methods: The coding sequence of BirA gene was isolated by polymerase chain reaction using DNA extract of *E. coli*-DH5α as template. BirA amplicon harboring a GS-linker at its C-terminal was cloned into *Nde*I-*Xho*I sites of pET24a(+) vector under control of *T7* promoter and upstream of the vector-derived 6xHis-tag. pET24-BirA transformed BL21-cells were induced for protein expression by IPTG and analyzed by SDS-PAGE and Western blotting. Protein expression yields were assessed by image analysis of the SDS-PAGE scans using ImageJ software.

Result: Agarose gel electrophoresis indicated proper size of the BirA gene amplicon (963 bp) and accuracy of the recombinant pET24-BirA construct. Sequence alignment analysis indicated identical sequence (100%) of our isolate with that of the standard *E. coli*-K12 BirA gene sequence (accession number: NC_000913.3). SDS-PAGE and Western blot results indicated specific expression of the 36.6 kDa protein corresponding to the BirA protein. Image analysis estimated a yield of 12% of total protein for the BirA expression.

Conclusions: By application of pET24a(+) we achieved relatively high expression of BirA in *E. coli* without application of any long protein-tags. Introduction of the present expression system may provide more readily available source of BirA enzyme for (strept) avidin–biotin applications and studies.

Key Words: Biotin–(strept) avidin, biotin ligase, BirA, *E. coli*, pET24a(+)

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Received: 26.01.2014, **Accepted:** 12.03.2014

Access this article online	
Quick Response Code:	Website: www.advbiores.net
	DOI: 10.4103/2277-9175.161576

INTRODUCTION

Tagging of proteins with linker-peptides together with application of Immunoaffinity Technologies is a commonly employed approach for detection, purification, cellular imaging, and studying the interaction of proteins among macromolecular complexes. In this context, a number of fusion

How to cite this article: Etemadzadeh MH, Arashkia A, Roohvand F, Norouzian D, Azadmanesh K. Isolation, cloning, and expression of *E. coli* BirA gene for biotinylation applications. Adv Biomed Res 2015;4:149.

protein-tags, such as glutathione-S-transferase (GST), maltose-binding protein (MBA), and has been already employed.[1-3] Another alternative "protein-tagging" method is "biotinylation" of proteins. The strong and extremely tight binding between biotin and (strept) avidin (with Kd [dissociation constant] of 10^{-14}-10^{-16}) is naturally the strongest noncovalent interaction known to date.[4] Due to availability of reagents and simple assays for detection,[5] application of this method for labeling, purification, drug targeting, and nanostructure assembling of (biotinylated) macromolecules,[6-8] has gained wide popularity. Most of these applications exploit tetrameric avidin or streptavidin as strong and noncovalent bridges between two biotinylated molecules.[9] To address the above-mentioned applications of (strept) avidin-biotin–based systems, a proper method to biotinylate biomolecules is the first requirement. Chemical reagents for biotinylation are readily available,[10] but usually they cause random and nonspecific biotinylation that may result in inactivation of proteins. These drawbacks are the reason that exploiting the living cells machinery for biotinylation of proteins (metabolic biotinylation) is becoming more favorable than chemical methods.[11,12] To this end, by adaptation to the naturally occurring cellular biotinylation process, site-selective biotinylation method of proteins using "Biotin Protein Ligase (BPL)" is developed. Indeed, in living organisms, the active form of biotin (vitamin H) is covalently attached to a class of essential enzymes (the biotin carboxylase and decarboxylases, involved in metabolic regulations such as gluconeogenesis and lipogenesis) by enzymatic action of BPL on a specific lysine in certain 60–80 residues known as biotin acceptor (binding) domain (BBD) of these enzymes.[13-16] The application of exogenous BPL of *E. coli*, BirA (that site-specifically biotinylates a lysine residue within a short (15–23 amino acids) acceptor peptide, also known as Avi-tag), and replacement of relatively large BBDs with the 15–23 amino acids biotin acceptor peptide (screened from a library of peptide tags biotinylated by *E. coli* BirA) was described.[17] Because such a biotin acceptor peptide (BAP) are unrecognizable for endogenous mammalian BPLs, it is possible to co-express *E. coli* BirA to biotinylate BAP-fused proteins inside the cell (*in vivo* biotinylation),[6] whereas they can be equally biotinylated *in vitro* by co-addition of biotin and BirA in the presence of Mg2 + and ATP. However, both of the above-mentioned applications (ie, exogenous or endogenous application of *E. coli* BirA for protein biotinylation) require overexpression of this enzyme in heterologous expression systems.[18] To address this concern, different attempts such as codon optimization[19] or truncation[20] of the BirA protein ligase for enhanced expression in mammalian or *E. coli* cells were, respectively, considered. Of note, in earlier reports, overexpression of BirA protein via heterologous systems in *E. coli* resulted in cellular toxicity issues,[21,22] whereas fusion of long linkers such as GST to the *E. coli* BPL (BirA) was later shown to overcome the cytotoxic effect of the protein BirA overexpression.[23] Overexpression of BirA protein ligase by fusion to thioredoxin and MBP linkers via pET-32a and pET-28a expression systems in *E. coli* was reported elsewhere.[24] Although employment of these long linkers resulted in overexpression of BirA protein in *E. coli* and overcame the toxic effect, but removal of the long linker from the final protein product, however, required further downstream processing steps of protease treatment to release the corresponding fusion for purification of the BPL.

In the present study, with the aim of providing a simple system for expression of *E. coli* BirA, the pET-24a(+) vector was employed to express the *BirA* gene isolated from DH5α strain of *E. coli* without utilization of any long linker protein.

MATERIALS AND METHODS

Bacterial strains and plasmids

E. coli DH5α, a derivative of *E. coli* K-12,[25] was used both as target strain for isolation of the BirA DNA fragment and also as the primary host for plasmid manipulations and cloning steps. pTG19-T cloning vector (Vivantis, Selangor DE, Malaysia) was used for TA cloning steps. *E. coli* BL21 (DE3) and pET-24a(+) plasmid (Merck KGaA, Darmstadt, Germany) were used as expression host and vector, respectively. Bacterial strains were cultured in Luria-Bertani medium (supplemented with 50 μg/mL kanamycin when required).

Isolation of BirA gene from *E. coli*

Total DNA was extracted from *E. coli* DH5α according to the standard protocols.[26] In brief, 5 mL of overnight DH5α culture was pelleted by centrifugation and the bacterial pellet was resuspended in 150 μL of distilled water. This suspension was incubated at 95°C for 5 min and 1 μL of 1/250 dilution was used as template to amplify the BirA coding sequence (963 basepairs) in a hot-start polymerase chain reaction (PCR) reaction using *Pfu* DNA polymerase, forward (BirA Fwd; 5′-AAGCTTA**CATATG**AAGGATAACACCGTGCCA-3′) and reverse (BirA Rev; 5′-**CTCGAG**AGAGCCTTTT TCTGCACTACGCAGGGA -3′) primers encoding nucleotides "1–21" and "943–963" of the target sequence (BirA), respectively. Forward and reverse primers harbored *Nde*I and *Xho*I restriction sites

(bold sequences), respectively, for directional cloning of the PCR product, whereas the reverse primer also contained a GS-linker comprising glycine and serine residues (underlined sequence).[27] Primers were designed in this study using the Gene Runner software (version 3.05) and based on the available sequence of *E. coli*-K12 BirA gene (accession number: NC_000913.3).

The PCR program was performed by one cycle of 95°C for 5 min as initial denaturation step followed by 35 cycles of 30 s at 95°C (denaturing), 30 s at 60°C (annealing), and 1 min at 72°C (extension). A final extension step of 72°C for 5 min was also included.

Construction of BirA expressing plasmid (pET24-BirA)

To follow an efficient cloning procedure, the PCR-produced amplicon of the BirA gene was first treated with *Taq* DNA polymerase in the presence of dATP (200 μM) for 20 min at 72°C to add dATPs to the 3′ ends of the blunt-ended double-stranded DNA (PCR product).[28] The *Taq*-treated PCR product was subsequently TA-cloned into the pTG19-T cloning vector (Vivantis, Malaysia) according to the manufacturer's protocol[29] to produce the pTG19-BirA recombinant vector. Finally, the BirA gene fragment was cut from pTG19-BirA vector by *Nde*I and *Xho*I double digestion and ligated into the same sites of the pET-24a(+) plasmid under control of the *T7* promoter to produce the pET24-BirA recombinant expression vector [Figure 1]. All cloning steps were carried out according to the standard protocols[26] or otherwise mentioned. The cloning strategies were designed so that the pre-existing N-terminal T7-tag sequence of pET-24a(+) was eliminated [Figure 1a], and the C-terminal His-tag of the vector was fused to the recombinant protein through a GS-linker [Figure 1b]. The expression cassette authenticity was confirmed by restriction enzyme digestion analysis followed by sequencing (Pasteur Institute of Iran sequencing facility).

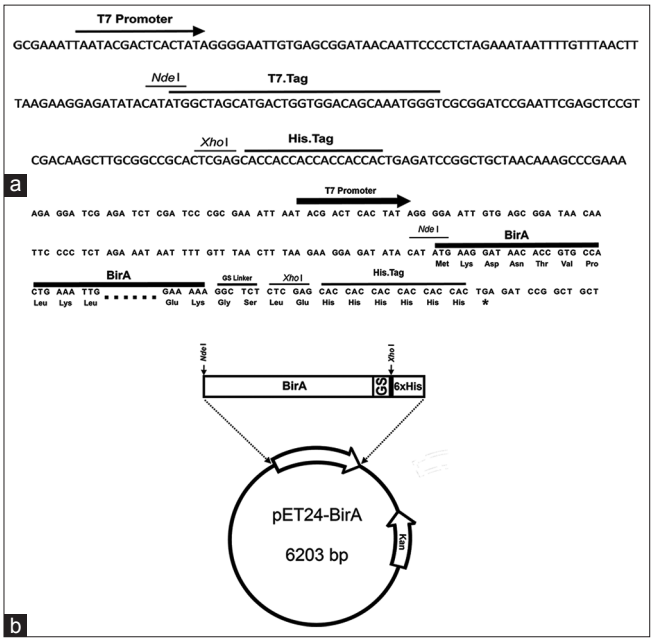

Figure 1: Schematic presentation for construction of pET24-Bir recombinant vector. Nucleotides related to the expression cassette of (a) original pET-24a(+) and (b) its modified version, in which the sequence coding for BirA is cloned into the *NdeI*/*Xho*I sites of the vector

Expression of BirA in *E. coli* BL21 (DE3)

Following transformation of *E. coli* BL21 (DE3) competent cells with pET24-BirA plasmid by standard $CaCl_2$ protocol,[26] expression of recombinant BirA protein was induced by the addition of isopropyl-β-d-thio-galactoside (IPTG) to the final concentration of 1 mM at bacterial concentration of OD_{600} = 0.6. Bacterial culture was incubated for 4 h at 37°C in the presence of IPTG on a rotary shaker incubator at 150 rpm. Subsequently, the optical density of bacterial suspensions were measured at 600 nm for each sample right before they were collected by centrifugation. After washing steps the pellets were saved at – 70°C for further analyses.

SDS-PAGE and Western blot analyses

Bacterial pellet was resuspended in appropriate volume of Laemmli buffer[30] based on their measured OD600 at the time of their collection to normalize the amount of loaded sample (i.e. equalizing their OD measures for the same value) and boiled at 100°C for 10 min and analyzed by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). To visualize the protein bands, gel was stained by Coomassie brilliant blue. After image acquisition by a flatbed scanner (Scanjet™ 3800, HP), the yield of expressed recombinant protein was determined by image analysis using ImageJ software (v. 1.47, Rasband, W.S., ImageJ, U.S. National Institutes of Health, Bethesda, MD, USA, http://imagej.nih.gov/ij/, 1997–2012).[31] To obtain the protein expression yield, the area under the picks was divided by the total area under the curve.

For Western blot analysis, protein bands on the PAGE were transferred in wet condition into the polyvinylidenedifluoride (PVDF) membrane (Roche) according to standard protocols[32,33] and BirA protein was detected by 1:5000 dilution of anti-His (C-term)-HRP antibody (Invitrogen) according to the manufacturer's instructions.

RESULTS

Isolation and cloning of BirA protein ligase from *E. coli* DH5α

The BirA coding sequence was isolated from *E. coli* DH5α by PCR and cloned in pET-24a(+) expression plasmid [Figure 1]. As shown in Figure 2a, agarose

gel electrophoresis results of PCR product (amplicon) indicated a single band of 975 base pair (bp), which was in accordance with the estimated size of the coding sequence of the *E. coli* BirA protein ligase gene [963 bp] with addition of the primer-derived sequences. Agarose gel electrophoresis results of the restriction analysis of pET24-BirA recombinant construct with *Xho*I and *Nde*I double digestion also indicated the expected fragments (975 bp and 5234 bp fragments corresponding to PCR product and vector portions, respectively) and confirmed the proper insertion of the target sequence into the aimed restriction sites of the vector [Figure 2b]. Finally, comparison (alignment) of the sequencing results for the cloned PCR-amplicon (BirA gene isolated from *E. coli* DH5α in our study) with that of the available *E. coli* BirA protein ligase sequences in NCBI Nucleotide database indicated the 100% identity of our cloned gene to *E. coli* K-12 reference sequence and its derivative DH1-BirA protein ligase gene [accession number: NC_000913.3; Figure 3].

Assessment of BirA expression by SDS-PAGE and Western blotting

To evaluate the ability of bacterial colonies to express the recombinant BirA protein, several randomly selected kanamycin-resistant colonies of BL21-DE3 (after transformation by pET24-BirA recombinant vector) were induced for protein expression by addition of IPTG, and the expression level of these colonies were assessed by SDS-PAGE. As shown in Figure 4a, bands corresponding to 36.6 kDa, which was in accordance with the estimated size of the BirA protein ligase of *E. coli* (NCBI Reference Sequence: NP_418404.1) were present in all induced samples compared with un-induced bacterial sample, and there were no significant differences in the expression level of BirA between the randomly selected colonies. Stained gel image analysis by ImageJ software revealed that recombinant BirA comprised 12% of total cell protein. Finally, Western blot analysis of the crude protein extracts from selected IPTG-induced bacterial colonies by tracking the protein of interest through its C-terminal His-tag showed specific bands corresponding to the expected size of 36.6 kDa [Figure 4b].

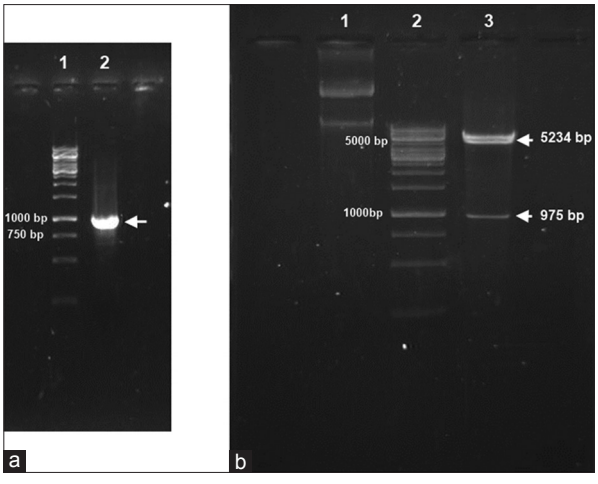

Figure 2: Agarose gel electrophoresis of (A) PCR product corresponding to BirA gene amplicon; Lane 1: DNA size marker (GeneRuler 1 Kb DNA Ladder, Fermentas, Lithuania). Lane 2: BirA gene PCR product (975bp) (B) Restriction enzyme digestion of recombinant plasmid, pET24-BirA, Lane 1: undigested plasmid. Lane 2: DNA size marker. Lane 3: pET24-BirA digested with *Xho*I and *Nde*I (975 bp and 5234 bp fragments corresponding to PCR product fragment and vector respectively). Arrows indicate the target fragments

DISCUSSION

By implementing (strept) avidin–biotin system, researchers are now able to exploit new tools for biomedical researches and applications. Targeted drug delivery and viral vectors modifications are other new areas of interest that can particularly benefit from this technology.[11] The key element in this technology is BirA protein ligase, an enzyme of *E. coli* origin[21] that addition of its recombinant form to the biotinylation substrate is one of the routine methods in utilization of (strept) avidin–biotin system.[34] Therefore, several attempts have been previously undertaken to isolate the corresponding BirA gene from *E. coli* XL-1 Blue,[35] JM109[20] or other *E. coli* K12 strains[36] for heterologous expression studies. In the present study we isolated the BirA gene from *E. coli* DH5α. As shown in Figure 2, the size of the isolated BirA protein ligase in this study (963 bp) and its sequence [Figure 3] was in (100%) accordance with *E. coli* K-12 and its derivative DH1-BirA protein ligase gene sequence (NCBI Nucleotide database accession numbers: NC_000913.3 and CP001637.1, respectively)

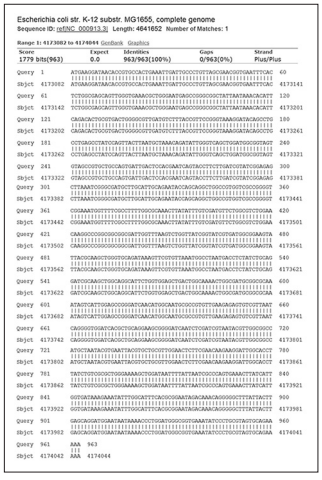

Figure 3: Sequence alignment of the BirA gene isolated from DH5α in the present study (quary) with that of *E. coli* K-12 reference sequence (sequence alignment indicated 100% identity)

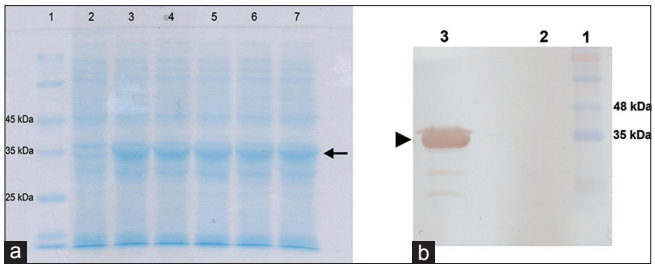

Figure 4: SDS-PAGE (a) and western blot (b) analyses of the recombinant BirA protein expressed via pET24-BirA vector in BL21 *E. coli* cells by addition of IPTG to the final concentration of 1 mM. (a) Lane 1: Protein molecular weight marker (Unstained protein molecular weight marker, Fermentas, Lithuania). Different induced colonies (lane 3–7) showed the same size of BirA (36.6 kDa) expression compared with un-induced sample (lane 2) (b) Western blot analysis of recombinant BirA protein by anti-His antibody specific for C-terminal polyhistidine tag indicated the specific 36.6 kDa protein band. Lane 1: Protein marker (PrestainedProtein Ladder, CinnaGen, Iran) Lane 2: Un-induced bacterial lysate. Lane 3: Recombinant BirA protein (36.6 kDa). Arrows indicate the target protein bands

This identity was expected, because DH5α strain is indeed a derivative strain of *E. coli* K-12[25] whereas biotinylation process itself is also highly conserved throughout the nature.[37]

In earlier studies, the cytotoxic effect of BirA protein ligase overexpression in *E. coli*[21,22] was overcome by fusion of long linkers such as GST,[23] thioredoxin, or MBP employing other expression vectors, such as pET-32a and pET-28a).[24] Removal of these long linkers from the final fusion product, however, required further protease treatment steps to release the corresponding fusion for purification of the BPL. Therefore, in this study, we aimed to provide a novel system for high expression of BirA protein ligase in *E. coli* based on pET24a(+) expression system without use of long protein linkers. To this end, the BirA coding sequence from *E. coli* DH5α was isolated and cloned into pET-24a(+) expression plasmid [Figure 1]. Design of the final construct led to the expression of BirA with the least modification, which was fusion of a C-terminal His-tag through a commonly used GS flexible linker [Figure 2b]. GS flexible linkers have sequences basically consisting of glycine and serine residues that not only provide flexibility and mobility of the connecting domains but also reduce unfavorable interaction between the linker and the protein moieties.[27]

SDS-PAGE [Figure 4a] and Western blot [Figure 4b] results indicated the expression of the predicted 36.6 kDa BirA protein by IPTG-induced BL21 (DE3) cells harboring the pET-24a-BirA recombinant vector, which is in accordance with protein size reported for *E. coli* BirA in prior studies.[21,23,24] By applying an anti-His antibody specific for C-terminal polyhistidine tag with a free carboxyl group (-His-His-His-His-His -His-COOH), the proper reading frame and therefore expression of the desired and complete protein was confirmed [Figure 4b].

Image analysis of the SDS-PAGE protein bands [Figure 4a] by ImageJ software (http://imagej.nih.gov/ij/, 1997–2012)[31] indicated a yield of 12% of total protein expression rate for BirA gene in the present study, which might correspond to 30–40 mg of enzyme from 1 L of a shaking bacterial culture.[23] In prior studies, different levels of BirA expression yields in *E. coli* have been reported, which ranges from lower levels of 3 mg/L of culture[5] or 0.5%–1% of the total cellular protein[21] to higher levels of 15%–20% of total cellular protein[23] or 24.7 and 27.6 mg per liter of culture from thioredoxin and MBP fusion constructs,[24] respectively. Although these higher expression yields were achieved by using long fusion tags in these prior studies, obtaining a yield of 12% of total protein expression rate for BirA gene in the present study without application of any long protein-tag and observation of any toxic effect might clearly attract the attention.

Finally it should be noted that obtaining the 12% expression rate for BirA protein ligase in our study might be further enhanced by employing other strategies such as optimization of nutrient condition[38] or induction parameters in the course of protein expression,[39] which has been already described for enhanced expression of other proteins.

Taken together, in the present study for the first time (to our best of knowledge) we isolated BirA gene from *E. coli* DH5α strain and employed the rather simple pET24a expression system for relatively high expression of BirA protein ligase in *E. coli* without application of any long protein-tags to relieve the potential toxic effects. Introduction of the present expression system for BirA may provide more readily available source of this enzyme for (strept) avidin–biotin applications and studies.

ACKNOWLEDGEMENTS

The present study was funded by Pasteur Institute of Iran (Ph.D. program in Pharmaceutical Biotechnology) as the fulfillment of the Ph.D. thesis of Dr. H. Etemadzadeh.

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Source of Support: Nil, **Conflict of Interest:** None declared.