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A simple method for production and purification of soluble and biologically active recombinant human leukemia inhibitory factor (hLIF) fusion protein in *Escherichia coli*

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ABSTRACT

Mouse embryonic stem cells (mESCs) rely on a cytokine named leukemia inhibitory factor (LIF) to maintain their undifferentiated state and pluripotency. However, the progress of mESC research is restricted and limited to highly funded laboratories due to the cost of commercial LIF. Here we presented the homemade hLIF which is biologically active. The hLIF cDNA was cloned into two different vectors in order to produce N-terminal His_6-tag and Trx-His_6-tag hLIF fusion proteins in Origami(DE3) *Escherichia coli*. The His_6-hLIF fusion protein was not as soluble as the Trx-His_6-hLIF fusion protein. One-step immobilized metal affinity chromatography (IMAC) was done to recover high purity (>95% pure) His_6-hLIF and Trx-His_6-hLIF fusion proteins with the yields of 100 and 200 mg/l of cell culture, respectively. The hLIF fusion proteins were identified by Western blot and verified by mass spectrometry (LC/MS/MS). The hLIF fusion proteins specifically promote the proliferation of TF-1 cells in a dose-dependent manner. They also demonstrate the potency to retain the morphology of undifferentiated mESCs, in that they were positive for mESC markers (Oct-4, Sox-2, Nanog, SSEA-1 and alkaline phosphatase activity). These results demonstrated that the N-terminal fusion tags of the His_6-hLIF and Trx-His_6-hLIF fusion proteins do not interfere with their biological activity. This expression and purification approach to produce recombinant hLIF is a simple, reliable, cost effective and user-friendly method.

1. Introduction

Since the 1980s, mouse embryonic stem cells (mESCs) have been used to study the characteristics of ESCs, which are expected to be used as a renewable source of therapeutic cells for use in regenerative medicines (Teo and Vallier, 2010). ESCs are usually propagated in complex culture medium that is supplemented with several growth factors and cytokines (Zweigerdt, 2009). LIF is one of the cytokines essential to maintain mESCs in an undifferentiated state. The high price of LIF and several other growth factors/cytokines is one of the major costs in ESC research. Recombinant DNA technology has the potential to produce homemade growth factors and cytokines at a lower cost to support stem cell research.

Many studies have successfully used *Escherichia coli* to produce several human growth factors and cytokines, such as interleukin-1 (Furutani et al., 1985), interleukin-4 (van Kimmenade et al., 1988), epidermal growth factor (Shimizu et al., 1991; Abdull Razis et al., 2008), fibroblast growth factor (Squires et al., 1988; Gasparian et al., 2009) and LIF (Gearing et al., 1989; Samal et al., 1995; Tomala et al., 2010) for therapeutic and clinical purposes. Although, recombinant human LIF (hLIF) is available and has been successfully produced in *E. coli* expression systems, they still require many steps of downstream processing. In addition, these systems have also shown multiple disadvantages: (1) high amounts of affinity resins are needed, (2) expensive proteases are required to cleave the target protein from the affinity tag, and (3) additional chromatography steps are needed to remove the proteases (Gearing et al., 1989; Tomala et al., 2010). From these reasons, these methods are not cost effective for long-term production of recombinant proteins. In the present study, we described an easy and simple strategy to produce biologically active hLIF fusion proteins in *E. coli*. This method allows rapid purification at low cost of human LIF protein that is both soluble and biologically active, suggesting that it will have widespread usage as a tool in the pro-

Abbreviations: hLIF, human leukemia inhibitory factor; Trx-tag, thioredoxin tag; His_6-tag, hexahistidine-tag; IMAC, immobilized metal affinity chromatography; mESCs, mouse embryonic stem cells; MEF, mouse embryonic fibroblast; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

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duction of several other growth factors and cytokines for stem cell research.

2. Materials and methods

2.1. Construction of expression vector

Total mRNA was isolated from human ovarian tissue, obtained from patients undergoing ovariectomy for benign gynecologic reasons with informed consent (Department of Obstetrics and Gynecology, Maharat Nakhon Ratchasima Hospital, Nakhon Ratchasima, Thailand) by Dynabeads mRNA purification kit (Invitrogen Dynal AS, Oslo, Norway). The first strand cDNA synthesis was done using Super Script III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and Oligo dT primer. The primers used to amplify hLIF were designed from GenBank Accession No.: NM_002309 nucleotides 182–721 to exclude the signal peptide coding sequence. The PCR amplification was carried out with GoTaq® Flexi DNA Polymerase (Promega, Madison, WI, USA), in a GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, CA, USA). The thermocycling protocol included pre-incubation at 95 °C for 2 min and 30 cycles at 95 °C for 30 s, 58 °C for 30 s, 72 °C for 45 s and finally at 72 °C for 2 min.

2.1.1. Construction of the pCOLD I/hLIF vector

The coding region of hLIF mature protein was amplified with a forward primer contained a *Sac*I site (underlined) 5′-CCGAGCTCAGCCCCCTCCCCATCACC-3′ and the reverse primer that incorporated the stop codon and an added *Xba*I site (underlined) 5′-GGTCTAGAAGGCCTGGGCCAACACGG-3′. The PCR product was digested with *Sac*I and *Xba*I restriction enzymes (Promega), and then subcloned into *Sac*I/*Xba*I digested pCOLD I (Takara, Shiga, Japan) to produce the pCOLD I/hLIF expression vector that encodes the His_6-hLIF fusion protein (Fig. 1A). The sequence of pCOLD I/hLIF was confirmed by DNA sequencing (Macrogen Inc., Seoul, Korea).

2.1.2. Construction of the pET32a/hLIF vector

The coding region of the hLIF mature protein was amplified by PCR. The forward primer used was 5′-CACCAGCCCCCTCCCCATCACC-3′, which introduced a directional cloning site at the 5′ end (underlined), and the reverse primer was the same to the reverse primer used above. The PCR product was cloned into the pENTR-D/TOPO Gateway entry vector, according to the supplier's directions (Invitrogen). The cDNA insert in the pENTR-D/TOPO vectors was transferred into the pET32a (+)/DEST Gateway expression vector (Opassiri et al., 2006) by LR Clonase recombination. The generated expression vector, named pET32a/hLIF, was created to encode the sequence of a thioredoxin (Trx)-His_6-hLIF fusion protein (Fig. 1B). The resulting vector was sequenced to ensure that the coding sequence of hLIF was correct and in-frame with the Trx gene.

2.2. Recombinant fusion protein expression and purification

The constructs of pCOLD I/hLIF and pET32a/hLIF vectors were transformed by electroporation into Origami(DE3) *E. coli* (Novagen, Madison, WI, USA), and positive clones were selected on a lysogeny broth (LB) agar plate with antibiotics (15 μg/ml kanamycin, 12.5 μg/ml tetracycline and 100 μg/ml ampicillin). For recombinant fusion protein expression, the selected clones were grown overnight in LB with antibiotics at 37 °C. The overnight cultures were then diluted 1:100 in fresh LB containing antibiotics and grown at 37 °C until the OD_{600} reached 0.8. Recombinant fusion protein expression was then induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.2 mM and the cells were incubated for 6 h or longer at 15 or 25 °C, with 200 rpm shaking. The induced cells were harvested by centrifugation at 4000 × *g* for 15 min and then the cell pellets were frozen at −70 °C until use for protein purification.

Prior to purification, the cell pellets were thawed and resuspended in 10 ml per g cells lysis/binding buffer [20 mM Tris–HCl (pH 8.0), 1 mM phenylmethylsulfonylfluoride (PMSF), 150 mM NaCl, 0.1% Triton X-100, 10% glycerol, 2 mM imidazole and 200 μg/ml lysozyme], and incubated at room temperature for 30 min. The lysate was further disrupted by sonication on ice until the sample was no longer viscous. The cell debris was precipitated by centrifugation at 16,000 × *g* for 20 min and the supernatant was used for the purification. The expressed His_6-hLIF and Trx-His_6-hLIF fusion proteins were purified by immobilized metal affinity chromatography (IMAC). The supernatant was mixed with IMAC Sepharose 6 Fast Flow (GE Healthcare, Freiburg, Germany) resin that had been equilibrated with 50 mM $CoCl_2 \cdot 6H_2O$. The mixtures were then mixed by gentle shaking at room temperature for 30 min to ensure that all of the fusion proteins bound to the resin. Then, the mixtures were packed into a PD-10 column (GE Healthcare). The column was washed with 5 column volumes (CV) of washing buffer (20 mM Tris–HCl (pH 8.0), 150 mM NaCl), to remove non-specifically bound proteins. The bound proteins were eluted with 5 CV of each of a series of wash buffers with increasing imidazole concentrations (10, 50, 100, 250 and 500 mM). All elution fractions were collected and analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Fractions containing fusion proteins were desalted and concentrated in 10 kDa-cut-off centrifugal ultra filtration membranes (YM-10, Millipore, Bedford, MA, USA), and 20 mM Tris–HCl (pH 8.0), 150 mM NaCl. The protein concentration was determined by the Bradford method. The purified His_6-hLIF and Trx-His_6-hLIF fusion proteins were filter sterilized (0.2 μm) and stored at −20 °C until further use.

2.3. SDS-PAGE and Western blot analysis

The samples of elution fractions of identical volume were mixed with equal volumes of 2× loading buffer [100 mM Tris–HCl (pH 6.8), 4% SDS, 0.2% bromophenol blue, 20% glycerol and 200 mM β-mercaptoethanol] and heated at 95 °C for 5 min and then analyzed by SDS-PAGE on a 15% (w/v) separating gel followed by staining with 0.1% Coomassie brilliant blue R-250. The percent of recombinant fusion protein compared with total protein was quantified by densitometry of the gel band intensities using Quantity One software (Bio-Rad, Hercules, CA, USA). Coomassie brilliant blue stained protein bands of interest were cut out from the SDS-PAGE gel. An in-gel trypsin digestion was performed. The tryptic peptides were resuspended with 0.1% formic acid and then analyzed by liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS).

After electrophoresis, proteins were transferred onto polyvinylidene difluoride (PVDF) membrane (Bio-Rad) in transfer buffer (25 mM Tris–HCl (pH 8.0), 192 mM glycine, 0.1% SDS, and 15% methanol) for Western immunoblotting. The membranes were exposed to blocking buffer (5% skim milk in phosphate buffered saline (PBS) containing 0.05% (v/v) Tween-20 (PBST)) for 1 h at room temperature, then washed three times with blocking solution and then incubated with 1:1000 HRP-linked INDIA His probe (Pierce Biotechnology, Rockford, IL, USA) overnight at room temperature. After incubation, the membranes were washed three times with PBST and then the membranes were developed with 3,3′,5,5′-tetramethylbenzidine (TMB, Sigma, St. Louis, MO, USA).