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Original Article

Efficient expression of EpEX in the cytoplasm of *Escherichia coli* using thioredoxin fusion protein

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Abstract

Recombinant epithelial cell adhesion molecule extracellular domain (EpEX) has a high potential as a candidate for passive and active immunotherapy as well as cancer vaccination. In the present study, EpEX was expressed as a thioredoxin fusion protein in *Escherichia coli* (*E. coli*). The effect of different hosts and expression conditions on the expression level of the fusion protein was also evaluated. Moreover, the effect of temperature and isopropyl-β-d-thiogalactopyranoside (IPTG) concentration on protein solubility was assessed. The codon optimized-synthetic gene was cloned into pET32a (+) expression vector and transformed into *E. coli* BL21 (DE3), Rosetta™ (DE3), and Origami™ (DE3). The protein expression was confirmed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting. Lowering the expression temperature to 16 °C and IPTG concentration to 0.5 mM also dramatically increased the volumetric productivity of the fusion protein. In optimum culture condition, high-level expression of the target fusion protein was detected in Rosetta™ (DE3) and Origami™ (DE3) (207 and 334 µg/mL, respectively), though they were expressed as inclusion bodies. No improvement was observed in the solubility of the fusion protein by reducing the temperature or IPTG concentration even when expressed in a TrxB/gor mutant strain. Results showed that Trx tag combined with other strategies utilized here could be effective to achieve high level of protein production but not effective in solubility improvement. However, new approaches might be necessary to enhance the solubility of EpEX in the *E. coli* system.

Keywords: EpCAM; EpEX; *Escherichia coli*; Expression; Thioredoxin.

INTRODUCTION

The epithelial cell adhesion molecule (EpCAM; also called CD326, TROP-1, EGP-2, or ESA) is a transmembrane, 40-kDa, 314-amino acid-long glycoprotein. Although its function is still largely unknown, in addition to cell adhesion, it is involved in cell migration, proliferation, and differentiation. The activation of intracellular signaling cascade of EpCAM is associated with the cleavage of this protein followed by EpCAM extracellular domain (EpEX) release (1). Although, EpCAM is abundantly expressed in various epithelial tumors such as colon, rectum, breast, stomach, prostate, biliary tract, hepatocellular, and pancreas, it is also expressed in the corresponding normal epithelia, albeit at lower expression levels than found in tumors. An increased EpCAM expression has been associated with a poorer prognosis in a wide variety of different carcinomas including breast and gallbladder (2). These data emphasize the potential utility of EpCAM as a suitable target for passive immunotherapy with monoclonal antibodies and in active immunotherapy with anti-idiotypic antibodies or with recombinant protein for the most abundant human cancers.

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Recently, cancer vaccination has become an important emphasis of oncology. Cancer vaccination leads to a direct cytotoxic response against cancer cells after stimulation by tumor-associated antigens such as EpCAM. An immune response generated by active immunization is potentially against multiple epitopes in contrast to passive immunotherapy, which targets only a single epitope with a monoclonal antibody. Furthermore, due to a strong cellular response in addition to a humoral response, more efficient tumor cell killing may be resulted by active immunization. Yet, EpEX has been used as a target of cancer vaccination in several clinical trials. When applied as a recombinant protein in colorectal carcinoma patients, both cellular and humoral immune responses were observed (3). The EpEX used in research and early clinical trials has been expressed in the baculovirus expression vector system. In spite of several advantages of this system, such as reproducible and well-controlled expression rates, there are some pitfalls. Scaled-up protein production in baculovirus expression vector system is cost-intensive as well there is a risk of the introduction of pathogens into the product (4). Due to easy handling, large-scale production, and inexpensive cultivation, *Escherichia coli (E. coli)* is the preferred host for rapid and low-cost mass production of recombinant proteins. However, heterologous eukaryotic protein production in *E. coli* system has been always challenging, especially for proteins containing disulfide bonds. Disulfide bonds are crucial for proper protein folding, activity, and stability. In general, the reducing environment of *E. coli* cytoplasm is not favorable for disulfide bond formation. Misfolding and insolubility followed by aggregation of the expressed protein result in the formation of inactive inclusion bodies. However, it is well known that *in vitro* refolding of protein in inclusion bodies is often unpredictable and time consuming as well the overall yield of biologically active protein from inclusion bodies is low in many cases. Therefore, generation of soluble protein is the preferred choice (5-8). Thus, several approaches have been used to overcome these obstacles to improve soluble expression of different disulfide-bonded proteins in the *E. coli* cytosol. One strategy is to change the cytoplasmic thiol-redox equilibrium environment *via* alteration in reducing pathways such as thioredoxin reductase. Various types of mutant strains including SHuffle (New England Biolabs) and Origami™ (DE3) (Novagen, Germany) which lack glutathione reductase (Δgor), thioredoxin reductase, and/or glutathione biosynthesis pathways are commercially available. For example, functional single-chain antibody fragment (scFv) and antibody Fab were produced efficiently in the cytoplasm of the mutant *E. coli* cells (9). Another widely adapted strategy is to use a fusion protein. Fusion partners such as thioredoxin (Trx), glutathione S-transferase small ubiquitin related modifier, and maltose-binding protein (MBP) generally result in enhanced solubility and high productivity in cytoplasmic expression. TrxA, one of the *E. coli* thioredoxins, demonstrates inherent thermal stability and high solubility in the *E. coli* cytoplasm. It has been used as a C- or N- terminal fusion protein to increase recombinant proteins solubility. Moreover, TrxA has been shown to prevent protein degradation (10). Optimization of the conditions for expressing recombinant proteins in *E. coli* can also improve the productivity and solubility of the products. The solubility of heterologous proteins has been shown to be increased by prolonged induction with decreased amounts of isopropyl-β-d-thiogalactopyranoside (IPTG) at low temperatures (11). As mentioned above, a Trx fusion method has been shown to be useful for soluble production of recombinant proteins. However, folding and disulfide bond formation of Trx fusion proteins might be further enhanced by using a thioredoxin reductase B mutant strain. Here, a Trx fusion tag was attached to the N-terminus of the EpEX protein. The fusion protein was expressed in three *E. coli* mutant strains, and protein solubility was assessed after optimization of the culture condition. The effect of temperature and IPTG

concentration on protein expression level and solubility was also evaluated.

MATERIALS AND METHODS

Cloning of EpEX in pET32a (+) as a Trx fusion tag

The DNA sequence encoding EpEX was codon-optimized for *E. coli* and synthesized (Generay Biotech Co, China). The synthesized DNA containing the EpEX gene (813 bp) was digested with *Nco*I and *Xho*I restriction endonucleases, gel extracted using high pure PCR product purification kit (Roche, Germany), and then cloned into similarly digested ends of pET32a (+) vector, achieving pET32a-Trx-EpEX. *E. coli* DH5-α was transformed with the ligation mixture. The construct was confirmed by DNA sequencing (Macrogen, Korea). A hexa-histidine tag was fused to the C terminus of the expressed EpEX for protein detection and purification purposes. The recombinant DNA techniques and methods described by Sambrook *et al*. (12) were used in the present study.

Expression of the EpEX

E. coli strains of BL21 (DE3), Rosetta™ (DE3), and Origami™ (DE3), purchased from Pasteur institute of Iran, Tehran, I.R. Iran, were used as expression hosts. Hosts were transformed with the expression plasmid pET32a-Trx-EpEX. Briefly, proper amount of desired plasmid (usually 100-500 ng) was added to aliquot of competent cells and placed on ice for 20-30 min. The cells were then heat shocked at 42 °C for 60 sec, in heating block, and placed on ice for another minute. One mL Luria-Bertani (LB) medium was added to each tube and incubated at 37 °C for 1 h. Then 100 µL of cell suspension was spread on LB plate. Plates were incubated overnight at 37 °C. For protein expression, a single colony harboring pET32a-Trx-EpEX was inoculated into 3 mL of LB broth containing 100 µg/mL ampicillin. The culture was shaken at 37 °C overnight, and then transferred to LB medium including 100 µg/mL ampicillin at a ratio of 1:10. Once the optical density of cells at 600 nm reached to 0.7-0.9, the expression of Trx-EpEX was induced by adding 1 mM IPTG (Sinaclon, I.R. Iran) and the induced culture was shaken for another 3, 5, 7, and 18 h under the same conditions. For optimization of Trx-EpEX expression, cultivations were performed under different IPTG concentrations (0.25, 0.5, 1 or 2 mM) and temperatures (16, 25, 30 or 37 °C). Optical density of each sample was measured at the final expression time. The cell pellets were harvested by centrifugation at 10,000 rpm for 5 min. After centrifugation, the total bacterial pellet was lysed (lysis buffer = 100 mM NaH2PO4, 10 mM urea, pH= 8). The protein samples were prepared in a gel loading buffer (0.25 M tris-HCl, pH 6.8, 5 % glycerol, 5 % 2-mercaptoethanol, 3 % sodium dodecyl sulfate (SDS), and 0.2 mg/mL bromophenol blue) and were heated to 95 °C for 5 min to denature proteins. Proteins were then separated by SDS-polyacrylamide gel electrophoresis (80 V for 5 % gel and 150 V for 12 % gel).

SDS-PAGE and western blot analysis

The expression of fusion protein was assessed by standard SDS-PAGE method. After centrifugation, the total bacterial pellet was lysed and prepared as described above. Proteins were then separated by SDS-PAGE (80 V for 5 % gel and 150 V for 12 % gel). The expression level of the recombinant protein was determined by densitometry analysis of polyacrylamide gels using TL120 software (Nonlinear Inc, Durham NC, USA). For western blotting, bacterial lysates were separated by 12% SDS-PAGE and electrophoretically transferred to polyvinylidene fluoride membrane. The transferred membrane was blocked overnight with 2% bovine serum albumin (BSA) in phosphate-buffered saline with 0.1% Tween® 20 and incubated for 1 h with 1/10,000 dilution of anti-His Tag polyclonal antibody as the primary antibody (Sigma, UK; Catalog No. H1029). After washing, membrane was incubated in a 1.5/10,000 dilution of anti-mouse HRP conjugated immunoglobulin (Sigma, UK; Catalog No. A0168) as the secondary one. The blot was developed

using 3,3'-diaminobenzidine (DAB) substrate. The prestained protein ladder (SinaClon, I.R. Iran; Catalog No. SI7002) was used for assessment of protein size.

Solubility assessment of Trx-EpEX

For solubility assessment of recombinant Trx-EpEX, pellets were harvested by centrifugation at 10,000 *g* for 15 min and resuspended in buffer (50 mM NaH2PO4, 300 mM NaCl, and 10 mM imidazole). Cells were incubated in the presence of 1 mg/mL lysozyme (Cinagene, I.R. Iran) on ice for 30 min. This lysate was further lysed by sonication (6 x 10 s with 10 s pauses at 200-300 W). The sonicated samples were centrifuged at 10,000 *g* for 25 min at 4 °C. The soluble fraction was collected after the insoluble debris was pelleted. The insoluble pellet was resuspended in 5 mL lysis buffer. This is a suspension of the insoluble matter (inclusion bodies). Five µL of 2 × SDS-PAGE sample buffer was added to 5 µL of soluble and insoluble extracts. These samples were heated along with the frozen noninduced and induced cell samples at 95 °C for 5 min. The samples were centrifuged at 15,000 *g* for 1 min. Twenty µL of the noninduced and induced cell samples, and all of the extract samples were loaded on a 12% SDS-PAGE gel and protein bands were visualized by coomassie brilliant blue staining. To identify the effect of temperature and IPTG concentration on solubility, cultivations were performed under different IPTG concentrations (0.25, 0.5, 1 or 2 mM) and temperatures (16, 25, 30 or 37 °C) and the soluble and insoluble fractions were prepared as described before.

Determination of protein concentration

The total protein concentrations were determined via the bicinchoninic acid (BCA) assay and BSA was used as standard (Takara, Japan). For sample preparation, the total bacterial pellets were lysed in lysis buffer (100 mM NaH2PO4, 10 mM urea, pH= 8). Before measurement, the working solution was prepared by mixing BCA reagent A and B at a 100:1 ratio. Dilutions of BSA standard solution were prepared according to protocol. Twenty-five µL of each dilution of the BSA standard solution as well as samples were dispensed into microtiter plate. For each concentration duplicate measurements were performed. Two hundred µL of the working solution was added to microtiter plate and mixed immediately. The plate was incubated for 30 min in a 37 °C water bath. The absorbance was measured at 562 nm using a Biotek microplate spectrophotometer (Biotek Instruments, Power Wave XS, Bad Friedrichshall, Germany). The standard curve was generated based on concentrations of BSA standard samples. The concentration of the total protein was determined using the standard curve as a reference. Total protein samples were also electrophoresed on a 12% SDS-PAGE gel. The band intensity of recombinant protein was analyzed using TL120 software (Nonlinear Inc, Durham NC, USA). Based on estimated intensity, TL120 calculates the quantity of recombinant protein as a percentage of total protein. According to total protein concentrations obtained from BCA assay and percentage of recombinant protein obtained from TL120 analysis, the concentration of recombinant protein can be calculated.

RESULTS

Construction of pET32a-Trx-EpEX

As shown in Fig. 1, the expression vector, pET32a-Trx-EpEX, was constructed successfully. The fusion protein must have 432 aa with a theoretical molecular weight of 47.77 kDa. Using restriction enzyme analysis, the recombinant plasmid expressing Trx-EpEX protein was confirmed. The electrophoresis results showed clear bands of 5846 bp and 827 bp when recombinant plasmid was digested with *Nco*I and *Xho*I (Fig. 1B). Nucleotide sequencing verified correct assembly of the fusion gene.

Expression and detection of Trx-EpEX fusion protein

The pET32a-Trx-EpEX plasmid was transformed into various *E. coli* strains including BL21 (DE3), Rosetta™ (DE3), and Origami™ (DE3) competent cells.

Expression of Trx-EpEX was driven by the T7 promoter, which is induced by the addition of IPTG. After IPTG induction, the expected band was not observed in case of BL21 (DE3) strain even after optimization of culture condition (data not shown). In this study, we also used Rosetta™ (DE3) and Origami™ (DE3) *E. coli* strains. Analysis of bacterial lysates *via* SDS-PAGE after cultivation for Trx-EpEX expression at the 1 mM IPTG at 37 °C resulted in detection of protein bands at the theoretically expected molecular weight (47.77 kDa) for Rosetta™ (DE3) and Origami™ (DE3) *E. coli* strains compared to the negative control (Fig. 2A). By screening various incubation times (3, 5, 7, and 18 h), the highest protein expression level was obtained at 3 and 5 h after induction for Rosetta™ (DE3) and Origami™ (DE3), respectively (data not shown). Based on densitometry and BCA analysis, a good expression was detected in Origami™ (DE3) and Rosetta™ (DE3) up to 22.8 and 22.3% of the total protein (547 and 333 µg/mL), respectively. However, protein expressed in Origami™ (DE3) had a higher level (125 µg/mL) than Rosetta™ (DE3) (74.3 µg/mL) (Fig. 2A). Western blotting analysis indicated that expressed Trx-EpEX was a his-tagged fusion protein using the anti-his (C-terminal) antibody (Fig. 2B and C).

Cultivation temperature and IPTG concentration effects on Trx-EpEX expression level

IPTG concentration needs to be optimized on a case-by-case basis due to its toxicity to *E. coli* and high cost. In this study, various IPTG concentrations (0.25, 0.5, 1 or 2 mM) were examined. Densitometry analysis showed that the highest expression level of fusion protein was achieved by 0.5 mM IPTG induction in both Rosetta™ (DE3) (Fig. 3) and Origami™ (DE3) (Fig. 4) strains. The productivity of Trx-EpEX fusion protein was decreased using higher IPTG concentrations. The highest expression level of Trx-EpEX was attained 3 and 5 h postinduction time at 16 °C for Rosetta™ (DE3) (Fig. 3) and Origami™ (DE3) (Fig. 4), respectively. Lowering the expression temperature to 16 °C increased the expression level of tag-fused EpEX, especially in Origami™ (DE3) in which the recombinant protein concentration was increased to more than 50% of the total protein in all tested IPTG concentrations (Fig. 4A).

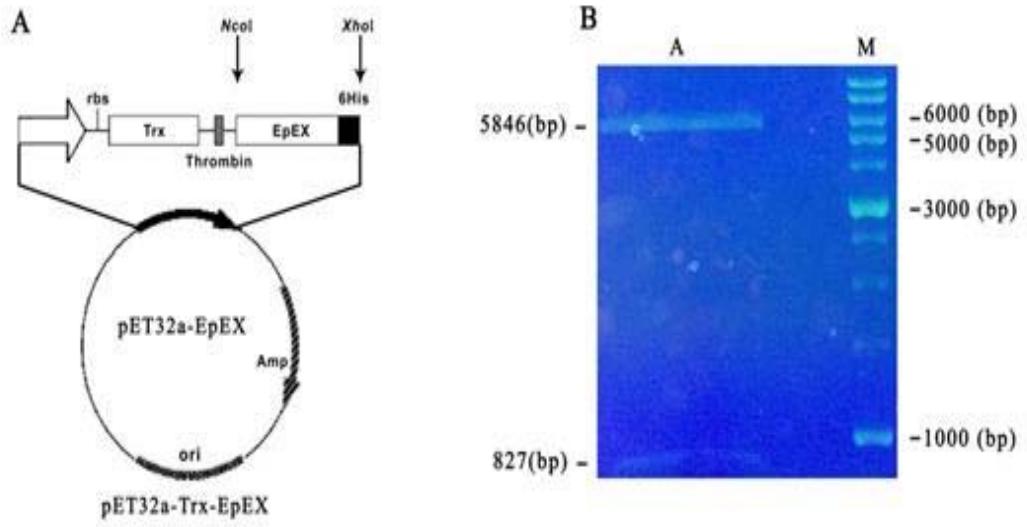

Fig. 1. Cloning of EpEX in pET32a (+) as a Trx fusion tag. (A) Schematic representation of the pET32a (+) expression vector harboring gene encoding EpEX protein. The cleavage sites of restriction enzymes are indicated by the arrows; (B) restriction enzyme analysis of recombinant pET32a-Trx-EpEX expression vector. Lane A, the electrophoresis results showed clear bands of 5846 bp and 827 bp when recombinant plasmid was digested with *Nco*I and *Xho*I; lane M, DNA marker 1 kb. EpEX, Epithelial extracellular domain; Trx, thioredoxin.

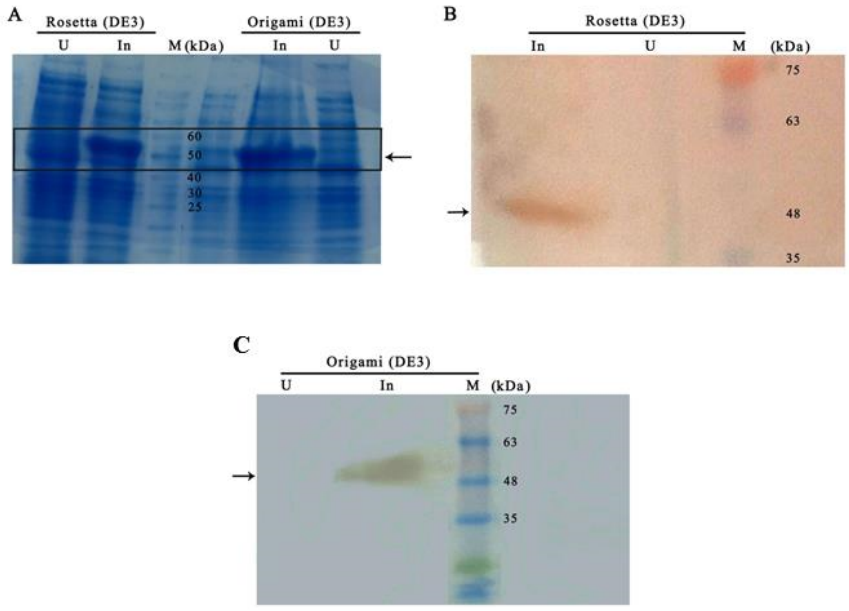

Fig. 2. Expression analysis of the Trx-EpEX protein. (A) Proteins were separated on a 12 % SDS-PAGE gel. Total protein was extracted from *Escherichia coli* Rosetta™ (DE3) and Origami™ (DE3) containing pET32a-Trx-EpEX plasmid before induction and after induction with 1 mM IPTG for 3 and 5 h, respectively at 37 °C. (B and C) Western blotting analysis of the recombinant Trx-EpEX protein. BL21 (DE3) bacterial lysates of *Escherichia* coli Rosetta™ (DE3) and Origami™ (DE3) before and after induction were treated with the anti His antibody. The Trx-EpEX protein (47.77 kDa) is denoted by an arrow. EpEX, Epithelial extracellular domain; Trx, thioredoxin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; IPTG, isopropyl-β-d-thiogalactopyranoside; U, before induction; In, after induction; M, protein marker.

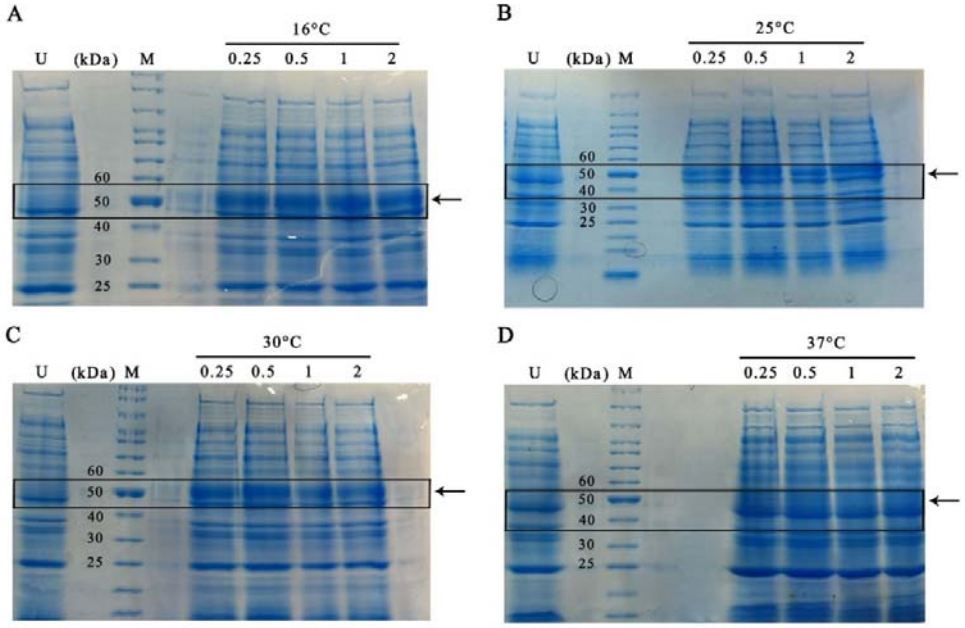

Fig. 3. The effects of IPTG concentration (mM) and incubation temperature on Trx-EpEX fusion protein expression in *Escherichia coli* Rosetta™ (DE3). After separation on a 12 % SDS-PAGE gel, protein bands were visualized by coomassie brilliant blue R250 staining. Protein expression was induced with different concentration of IPTG (0.25, 0.5, 1 or 2 mM) at (A) 16 °C, (B) 25 °C, (C) 30 °C, and (D) 37 °C for 3 h. Lane M, protein marker and lane U, bacterial culture before induction. The arrows indicate the position of Trx-EpEX. IPTG, isopropyl-β-d-thiogalactopyranoside; EpEX, epithelial extracellular domain; Trx, thioredoxin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

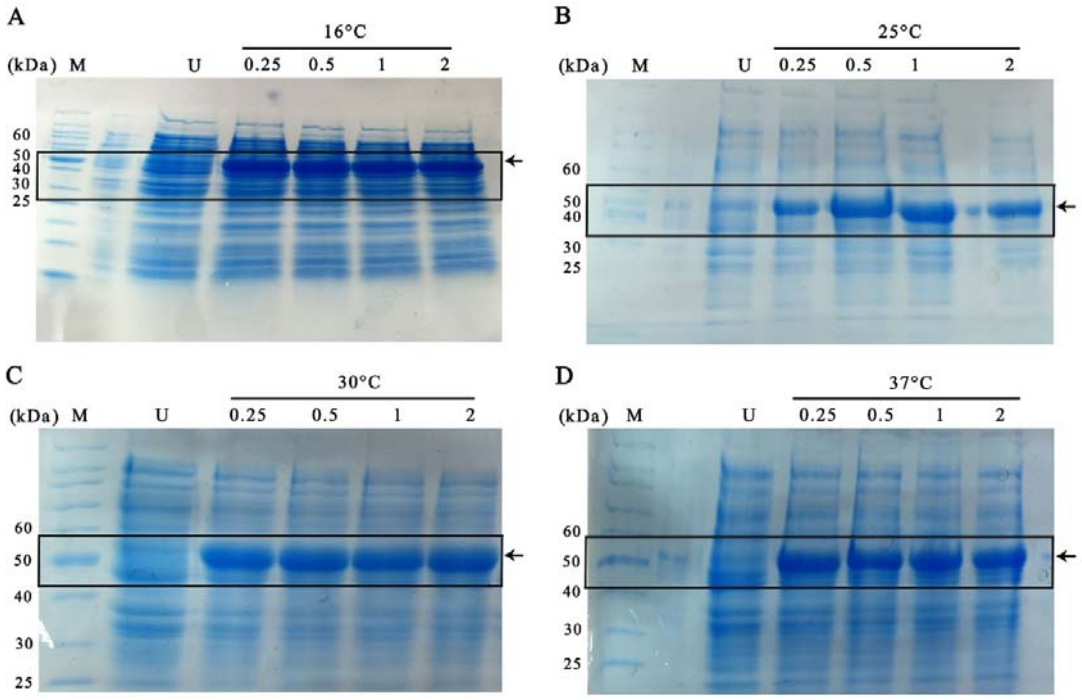

Fig. 4. The effects of IPTG concentrations (mM) and incubation temperature on Trx-EpEX fusion protein expression in *Escherichia* coli Origami™ (DE3). After separation on a 12 % SDS-PAGE gel, protein bands were visualized by coomassie brilliant blue R250 staining. Protein expression was induced with different concentration of IPTG (0.25, 0.5, 1 or 2 mM) at (A) 16 °C, (B) 25 °C, (C) 30 °C, and (D) 37 °C for 5 h. Lane M, protein marker; lane U, bacterial culture before induction. The arrows indicate the position of Trx-EpEX. EpEX, Epithelial extracellular domain; Trx, thioredoxin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; IPTG, isopropyl-β-d-thiogalactopyranoside.

Under the above optimal conditions (cultivation at 16 °C and 0.5 mM IPTG), a high percentage of the target fusion protein was expressed in Rosetta™ (DE3) and Origami™ (DE3) (49 and 56% of the total protein (414 and 564 µg/mL), respectively). The volumetric productivity of fusion protein reached 207 and 334 µg/mL in Rosetta™ (DE3) and Origami™ (DE3), respectively. Therefore, Origami™ (DE3) showed increased overall production of recombinant Trx-EpEX compared to Rosetta™ (DE3) (Fig. 5).

Solubility assessment of the expressed protein

In this study, the gene coding for Trx was N-terminally fused to EpEX coding sequence to improve the expression and solubility of recombinant protein. We assessed the solubility of Trx-EpEX in optimum culture condition of the two strains. Using SDS-PAGE, soluble and insoluble fractions were analyzed after cell disruption. Densitometry analysis using TL120 software was performed for protein quantification. As shown in Fig. 5, over-expression of Trx-EpEX in the cytoplasm of both strains in optimized condition contributes to a high-level accumulation of the recombinant protein in the insoluble cell extract, and no protein band was detected in the soluble fraction. Therefore, it seems that the high molecular weight and hydrophobicity of Trx-EpEX caused the inclusion bodies to be formed quickly in *E. coli* cytoplasm after protein translation.

Effect of temperature and IPTG concentration on Trx-EpEX solubility improvement

To test whether temperature and IPTG concentration can affect the solubility of the expressed protein, we examined the solubility level of Trx-EpEX at several temperatures (16, 25, 30, and 37 °C) (Fig. 6A and B) and IPTG concentrations (0.25, 0.5, 1, and 2 mM) (Fig. 6C and D), respectively. Almost all the fusion protein was sequestered in the insoluble pellets, and no protein band could be detected in the supernatant portion (Fig. 6 A-D).

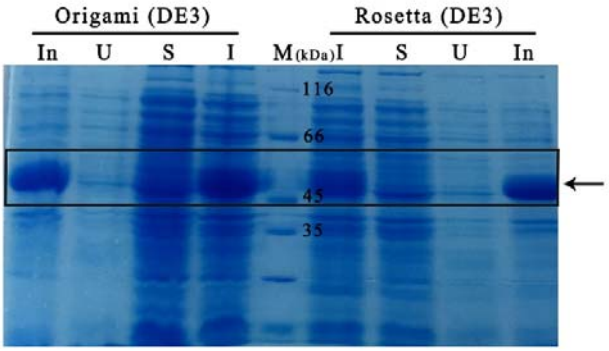

Fig. 5. Expression and solubility assessment of the Trx-EpEX fusion protein in optimum culture condition in two strains. Protein expression was induced with 0.5 mM IPTG at 16 °C and the cells were harvested from cultures after 3 h (in Rosetta™ (DE3) and 5 h in Origami™ (DE3)). The solubility of Trx-EpEX was assessed in optimum culture condition of two strains. Lane M, protein marker; lanes U and In, bacterial culture before and after induction in Rosetta™ (DE3) and Origami™ (DE3), respectively; lanes I and S, insoluble and soluble fractions. The arrow indicates the position of Trx-EpEX. EpEX, Epithelial extracellular domain; Trx, thioredoxin.

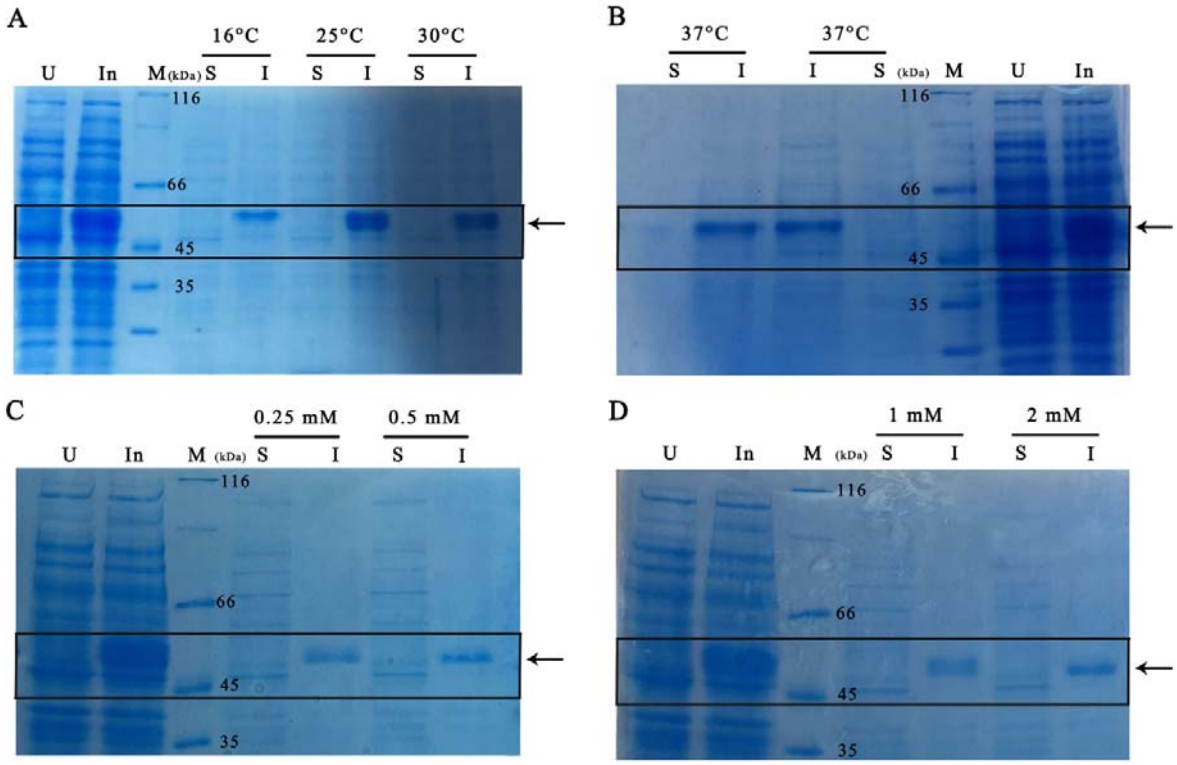

Fig. 6. Solubility profiles of Trx-EpEX fusion protein at different temperatures (16-37 °C) and IPTG concentrations (0.25-2 mM) in *Escherichia coli* Origami™ (DE3). (A and B) Cells were harvested from cultures induced with 0.5 mM IPTG after 5 h at each temperature; (C and D) after 5 h, cells were harvested from cultures induced at 16 °C with different IPTG concentrations. The arrows indicate the position of Trx-EpEX. I, Insoluble; S, solubale; U, uninduced lysate, In, induced lysate; EpEX, Epithelial extracellular domain; Trx, thioredoxin; IPTG, isopropyl-β-d-thiogalactopyranoside.

DISCUSSION

Cancer vaccination has become an important focus of oncology in recent years. Active immunization with tumor-associated antigens such as immunogenic epitopes of EpCAM is thought to potentially overcome the reoccurrence of metastasis. EpCAM EpEX epitopes are of immunogenic epitopes expressed by the EpCAM antigen (3,13). Due to fast growth rate, cheap fermentation media, and clear genetics we tested *E. coli* as a preferred host for the expression of the EpEX as a potential colorectal cancer vaccine. In the current research, first, the expression of recombinant Trx-EpEX fusion protein was confirmed at 37 °C and 1 mM IPTG in Origami™ (DE3) and Rosetta™ (DE3) *E. coli* strains. However, protein expressed in Origami™ (DE3) had a higher level than Rosetta™ (DE3) and the expected band was not detected in case of BL21 (DE3) strain even after optimization of culture condition (data not shown). Consistently, Baghbani-arani *et al* reported that the ARFP/F protein has not been achieved in the BL21 (DE3) pLysS or BL21 CodonPlus (DE3)RIL strains, but has been effectively expressed in the Rosetta™ (DE3) *E. coli* strain (14). Although there is no clear explanation for these results, optimization of the vector/host combination might be one of the most important issues that should be considered in recombinant protein expression.

A large amount of purified recombinant proteins is required for their biochemical, functional, and structural characterization. Up to now several fusion tags have been described to be able to enhance the yield of recombinant proteins via facilitation of translation initiation. As previously reported, Trx is the fusion tag of choice in expression of a protein with disulfide bonds (9). Jurado *et al.* indicated that Trx was able to enhance the expression level of Trx-scFv fusion protein (60-100 μg/mL of induced culture in a shake flask). They also reported that MBP-scFv fusions were expressed at yields (~30-40 μg/mL) lower than that of Trx-scFv fusion proteins (15). In good agreement with these previous studies, here, the EpEX gene fused with Trx gave a good expression in Origami™ (DE3) and Rosetta™ (DE3) up to 22.8 and 22.3% of the total protein, respectively. However, protein expressed in Origami™ (DE3) had a higher expression level (125 μg/mL) than Rosetta™ (DE3) (74.3 μg/mL) which may be due to higher oxidizing environment of the bacterial cytosol which is provided by mutations in two proteins (thioredoxin reductase and glutathione reductase) in Origami™ (DE3). Based on published results, this strain is able to increase the expression of proteins with multiple disulfide bonds (16).

Optimization of culture conditions can also play an important role in obtaining a maximum level in protein expression. Here, the effect of different temperatures (16, 25, 30 or 37 °C) as well as IPTG concentrations (0.25, 0.5, 1 or 2 mM) was assessed on recombinant protein expression in all three strains (Figs. 3 and 4). Based on the results presented here, the highest expression level of Trx-EpEX was obtained with 0.5 mM IPTG at 16 °C for 3 and 5 h for Rosetta™ (DE3) (207 μg/mL) and Origami™ (DE3) (334 μg/mL), respectively. Interestingly, a low IPTG concentration for induction improved expression of Trx-EpEX in all temperatures (Figs. 3 and 4). The expression level of Trx-EpEX was gradually decreased as the IPTG concentration increased over 0.5 mM, suggesting the toxicity of IPTG to *E. coli*. In agreement with our results, a significant decrease for synthase was observed by increasing the IPTG concentration over the 0.6 mM (17). Accordingly, the lower level of IPTG (0.1 mM) led to a higher level of recombinant Man i 1 (rMan i 1) (one major mango allergen has been identified) expression in *E. coli* (18). The IPTG toxic effect may be related to higher metabolic load as well as induction of bacterial proteases, which may degrade recombinant proteins (19). Here an optimal expression of recombinant protein occurred at a low growth temperature of 16 °C in both strains (Rosetta™ (DE3) and Origami™ (DE3)). This may be due to less activity of the proteases at lower temperatures reported in previous studies (18). Our results are in agreement with one report indicating

that the highest amount of rMan i 1 was obtained after induction at 16 °C (18). Conclusively, although at lower temperature there was a general trend for less expression, the temperature effect on protein expression level was not as straightforward as was expected. Increasing the solubility of the expressed proteins is often desirable. Different approaches including fusion-expression strategy decrease in IPTG concentration for induction and growth at lower temperatures have been developed to reduce the production of inclusion bodies (9). A number of tags such as yeast small ubiquitin related modifier, MBP, and thioredoxin have been shown to enhance the solubility of the fusion proteins (20). We assessed the solubility of Trx-EpEX in optimum culture condition of two strains (Rosetta™ (DE3) and Origami™ (DE3)) (Fig. 5). Our results showed no improvement in solubility of EpEX fused with Trx even when expressed in a trxB/gor mutant strain. In accordance with our results, Sonoda *et al*. found that the majority of the expressed Trx-scFv protein in Origami™ (DE3) cells was accumulated in the insoluble form (16). Consistently, Sun *et al.* reported that MBP or NusA was able to enhance the solubility of the fused anti-interleukin-17A scFvs while the Trx could not improve the solubility of the recombinant fusion protein (21). Besides fusion-expression strategy, other approaches such as growth at lower temperatures as well as decrease in IPTG concentration for induction were shown to be effective in solubility improvement. At a lower temperature, cell processes at a milder manner leading to reduced rates of transcription, translation, and cell division (22). Here, unlike in past studies, our results demonstrated that lower post induction temperatures as well as lower IPTG concentration could not improve the solubility of EpEX-Trx fusion protein (Fig. 6). These data are in good agreement with results published by Liu *et al.* They showed that DREBIII-1, a plant specific transcriptional factor, was mainly sequestered in the insoluble pellets when expressed in *E. coli* and the solubility level was not affected by either a decrease in IPTG concentration or a reduction of growth temperature (23). Here, the reason for insolubility of expressed protein maybe due to its large size or its sequence. The theoretical pI of the fusion protein was 5.94. Based on published data, in *E. coli,* there is a certain molecular weight dependency regarding solubility especially for proteins larger than 60 kD. Increasing molecular weight was shown to decrease the probability of soluble expression. For example, Dyson *et al*. (20) evaluated properties of 95 proteins expressed in *E. coli*. They revealed that in *E.coli,* small proteins (~ 22.8 kD) were often soluble while larger ones (~ 40.4 kD) were expressed mainly in insoluble form. The solubility of expressed protein was also shown to be affected by the starting and ending residues of the recombinant protein. For example, in a study by Klock *et al*., a highly soluble protein was turned into being insoluble by deleting four residues at either the N- or C terminus of the protein (24).

CONCLUSION

In conclusion, for the first time, the EpEX gene cloned and the recombinant EpEX fused to thioredoxin, was successfully expressed in Rosetta™ (DE3) and Origami™ (DE3) strains of *E. coli.* Different induction parameters, such as inducer concentration, growth temperature, and incubation time were modified to improve the expression levels and solubility of EpEX. The results revealed that a maximum yield of total protein expression was achieved by induction with 0.5 mM IPTG and 3 and 5 h after induction at 16 °C in Rosetta™ (DE3, 207 µg/mL) and Origami™ (DE3, 334 µg/mL), respectively. Furthermore, our data showed that the Trx tag combined with other strategies utilized here could be effective to improve protein expression level but not effective in solubility improvement. Before performing biological and biophysical analysis as well as structural characterization, the Trx-EpEX fusion protein should be applied to affinity chromatography and the Trx tag should be cleaved off by proteases. However, based on previous reports published by Niiranen *et al*.,

larger amounts and more soluble protein can be achieved by using MBP and NusA compared to Trx (25). Therefore, large tags including MBP and NusA may be more suitable candidate for soluble expression of EpEX in *Ecoli*.

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