

Soluble Form Production of Recombinant Human Insulin-Like Growth Factor-1 by NusA Fusion Partner in *E. coli*

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HIGHLIGHTS

- Fusion proteins is a suitable strategy for recombinant production of proteins in the soluble form.
- NusA fusion tag improves the solubility of recombinant proteins expressed in bacterial hosts.
- NusA fusion protein convert IGF-1 insoluble form to soluble form in *E. coli*.

ABSTRACT

Keywords:

E. coli

IGF-1

Fusion protein

NusA

Recombinant production

Insulin-Like Growth Factor-1 (IGF-1) is a small peptide with 70 amino acids and 7.6 kDa molecular weight that acts as the major mediator of growth hormone. According to the previous studies, recombinant production of human IGF-1 (rhIGF-1) in *E. coli* has resulted in an inactive form of protein (inclusion body). There are several strategies to transform inclusion body to a soluble form. Production in the form of fusion proteins as a suitable strategy, helps researcher in recombinant production of proteins in the soluble and active form. In current study, NusA fusion protein was used to produce IGF-1 soluble form, instead of insoluble protein. In previous study, rhIGF-1 was optimally expressed in inclusion body with 1.2 g/L concentration. rhIGF1 -NusA construct was cloned and expressed in *E. coli*, then, cell lysate was analyze by SDS-PAGE and densitometry techniques, to assay soluble and insoluble form of rhIGF-1. Results showed that rhIGF-1 concentration in soluble phase was 0.14 g/L, indicating that about 12% of total expression of rhIGF-1 was in the soluble form through NusA-fusion protein strategy. These results confirmed that some fusion proteins like NusA could improve the solubility of recombinant proteins expressed in heterogeneous bacterial hosts.

Introduction

Insulin-Like Growth Factor-1 (IGF-1) is secreted by different tissues and plays a critical role in metabolism

and growth. Recombinant IGF-1 consists of 70 amino acids and two disulfide bond. *E. coli* is a routine organism for the commercially production of rhIGF-1 that has some advantages and disadvantages compared to eukaryotic host (Ranjbari, 2019; Ranjbari et al., 2015). One of the most important disadvantages of recombinant protein production in *E. coli* is the

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production of proteins in the form of insoluble and inactive inclusion bodies (Babaeipour et al., 2018).

When a foreign recombinant protein is expressed in *E. coli*, the new microenvironment is different from that of the original source in terms of pH, osmolarity, redox potential, cofactors, and folding mechanisms. Also, high level expression under a powerful promoter results in high concentrations of hydrophobic assemblies of polypeptides. All of these factors lead to protein aggregation and formation of inclusion body (Carrio and Villaverde, 2002; Rosano and Ceccarelli, 2014). Another reason for the formation of insoluble form of recombinant protein in *E. coli* is the formation of incorrect disulfide bonds. Correct formation of disulfide bonds are important in the proper three-dimensional conformation and biological activity of recombinant protein (Messens and Collet, 2006; Lobstein et al., 2012). In *E. coli*, oxido-reductase system is in the periplasmic area, where the cysteine oxidation takes place and disulfide bonds are formed. Hence, disulfide bonds formation in the cytoplasmic area is very weak and in incorrect form, therefore, results in the protein misfolding (Derman et al., 1993; Messens and Collet, 2006).

Directing the protein to the periplasm, using the engineered strains with oxidative cytoplasmic environment, cultivation in lower temperature than optimal, tuning inducer concentration and fusing the recombinant protein to a solubility enhancer like fusion partners are useful strategies to increase recombinant protein solubility (Rosano and Ceccarelli, 2014). Thioredoxin, maltose-binding protein, glutathione S-

transferase, ubiquitin, Small ubiquitin-like modifier protein (SUMO) and N-utilization substance protein A (NusA) are the most usual fusion partners in biology. The exact effect of fusion partners as solubility enhancers has not been understood, however, several hypothesis have been proposed (Raran-Kurussi and Waugh, 2012).

In the case of NusA, it was shown that it increases the solubility of foreign proteins. Usually, *E. coli* NusA protein promotes hairpin folding and tagging the N-terminal of insoluble proteins with NusA makes them soluble through expression in *E. coli*. Also, NusA can combined with the His-tag that is useful for purification process (Harrison, 2000; Terpe, 2003).

Materials and Methods

Strain and vector

Origami (B/DE3) was approved as an appropriate strain of *E. coli* for rhIGF-1 production. The protein of rhIGF-1 has two disulfide bonds in its structure. Origami (B/DE3) strain with mutation in thioredoxin reductase (*trxB*) and glutathione reductase (*gor*) genes, provides a suitable microenvironment for the proper formation of disulfide bond. pET-44a and pET-15b plasmids (Novagen), ampicillin resistant plasmids, were used as vector in this study (Fig. 1). pET-44a consists of a NusA tag with 495 amino acids. Synthetic rhIGF-1 gene was cloned in pET-44a plasmid (NusA fused) and pET15b (without fusion tag) under control of strong bacteriophage T7 promoter (Fig. 1).

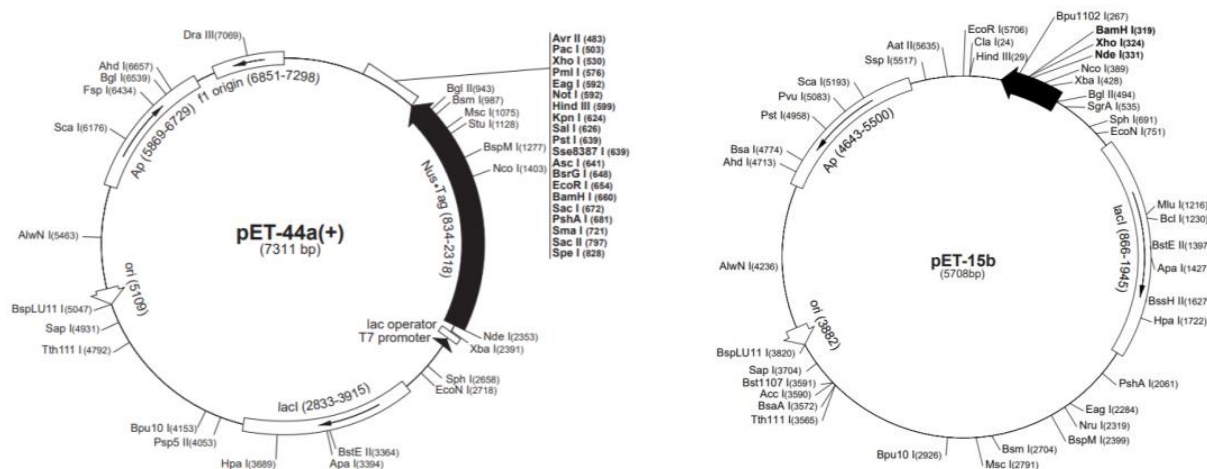


Figure 1. Schematic map of pET44a and pET15b vectors.

Culture condition

According to our previous study, an optimized culture condition included 32y culture medium, 32°C culture temperature and 0.05 mM inducer (IPTG). 32y culture medium composition was: peptone 0.8 % (w/v), yeast extract 3.2% (w/v), NaCl 0.58 (w/v) in Tris-HCl 10 mM (pH=7.6) (Ranjbari et al., 2015).

Transformation and expression of rhIGF-1

Both rhIGF1- pET44a and rhIGF1-pET15b constructs were transformed into Origami (B/DE3) strain by Calcium chloride transformation technique. Transformed colonies were grown in 32y culture medium containing 100 µg/ml ampicillin. Also, protein expression was induced by 0.05 mM IPTG. Samples were collected up to 8h after induction.

Western blot analysis of rhIGF-1

Western blot technique was used for rhIGF1-NusA fusion complex identification. Collected samples after induction were lysed and cell lysate was analyzed by the SDS-PAGE 12.5%, 2 h at 100 V, along with molecular weight markers (Sigma). For Western blotting, proteins resolved by SDS-PAGE were electrophoretically transferred to a nitrocellulose membrane (Wathman, UK). Phosphate buffered saline with 5% nonfat milk was used for blocking the membrane. The membrane immersed in 1:2000 dilution of peroxidase-conjugated anti IGF-1 monoclonal antibody (Abcam, UK) for 2 h at room temperature. Subsequently, it was visualized after color development by chromogenic substrate 3, 3'-diaminobenzidine (DAB) (Roche, Germany).

Protein measurement

In order to quantification of SDS-PAGE data, we used Bradford and densitometry (image J software) techniques. Optical density is the result of darkness of a developed picture and can be expressed absolutely as the number of dark spots in a given area, but usually it is a relative value, expressed in a scale. Densitometry is particularly useful due to its sensitivity, accuracy and versatility, and it can be applied to proteins in gels or on membranes. In addition to being accurate, sensitive and reproducible, the technique is cost-effective, simple, and does not require a high degree of specialized training, yet provides technical advantages over other available tool. The rhIGF-1 expression level was determined by SDS-PAGE 12.5% (w/v). Gels were stained with

Coomassie brilliant blue G250 and then, quantified by gel densitometer. Bradford protein assay was used for the quantitative analysis of total protein with standard protocol (Taylor et al., 2013).

Results and Discussion

Nowadays, proteins are widely produced in *Escherichia coli*, as promising cell factories. The aggregation of exogenous proteins into insoluble inclusion bodies is one of the main limiting factors of the *E. coli* expression system. Protein fusion technology has been widely used for the enhanced production of soluble protein in *E. coli* (Costa et al., 2014). Fusion protein tag of NusA has identified as a highly soluble protein when expressed in *E. coli*. NusA may be a very good tag protein for solubilizing of large recombinant proteins. Tyrosinase, hIFN-γ, Growth Hormone (GH), hIL-3 are good example of NusA fused protein with an efficient production soluble form (Harrison, 2000).

Transformation and expression of IGF1 without fusion tag

DNA sequence encoding human IGF-1 was cloned in pET15b. pET15b-rhIGF1 construct containing the rhIGF-1 gene sequence confirmed by automated DNA sequencing. The expression plasmid was then transformed into *E. coli* strain Origami (B/DE3). In the presence of 0.05 mM IPTG, the expression of the protein was induced. Six hours after induction, transformed cells were analyzed for rhIGF1-NusA expression by SDS-PAGE (Fig. 2). Results showed that rhIGF-1 was produced completely (1.6 mg/ml) as insoluble inclusion body without coproduction of NusA.

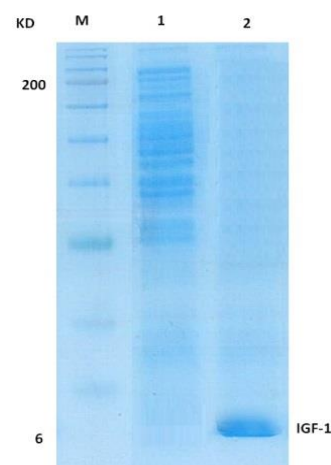


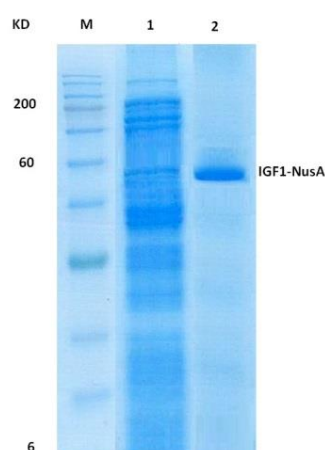
Figure 2. SDS-PAGE 17.5% gel analysis of rhIGF-1 expression in *E. coli*. M: Molecular weight marker, lane 1: soluble phase protein expression pattern, lane 2: insoluble phase protein expression pattern.

Table 1. IGF-1 concentration in soluble and insoluble form.

Protein concentration	Insoluble form				Soluble form			
	Total protein	Total IGF-1	IGF-1 In sediment	IGF1 In supernatant	Total protein	Total IGF1-NusA	IGF1-NusA In sediment	IGF1- NusA In supernatant
	(mg/ml)	(mg/ml)	(mg/ml)	(mg/ml)	(mg/ml)	(mg/ml)	(mg/ml)	(mg/ml)
	5.33	1.6	1.6	0	4	1.2	1.06	0.14

Effect of NusA fusion protein on rhIGF-1 solubility

DNA sequence encoding human IGF-1 was cloned in pET44a. pET44a-rhIGF1 construct containing the rhIGF-1 – NusA gene sequence confirmed by automated DNA sequencing. The expression plasmid was then transformed into *E. coli* strain Origami (B/DE3). In the presence of 0.05 mM IPTG, the expression of the protein was induced. To assess the effect of NusA fusion protein on rhIGF-1 solubility, cultures were harvested 6h post induction, the cell pellets were lysed by sonication, and the lysates were centrifuged and separated into soluble and insoluble fractions. The proteins in both fractions were analyzed by SDS-PAGE 12% followed by Coomassie G-250 staining. Fusion of NusA to the rhIGF-1 protein resulted in the enhanced solubility of rhIGF-1, and approximately 12% (0.14 mg/ml) of rhIGF-1 was produced in soluble form (Fig. 3) (Table1).

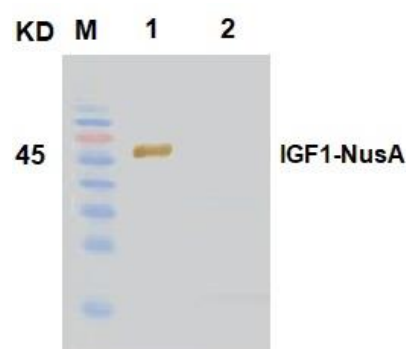
**Figure 3.** SDS-PAGE 12% gel analysis of rhIGF1-NusA expression in *E. coli*. M: Molecular weight marker, lane 1: soluble phase protein expression pattern, lane 2: insoluble phase protein expression pattern.

Western blot analysis of rhIGF-1

rhIGF1-NusA expression was also analyzed by Western blotting using anti-human IGF-1 antibody (Abcam-USA) for further identification (Fig. 4). According to Fig. 3, Western blotting analysis approved production of rhIGF1- NusA complex.

Conclusion

Ease of manipulation, short doubling time, high cell density and cost benefit of production make *E. coli* one of the most widely used host systems for recombinant protein production. RhIGF-1 as a heterogeneous protein had expressed in *E. coli* in the form of inclusion body. Aggregated proteins in inclusion bodies have not proper folding and complete biological activity. Nowadays, various strategies are applied to increase the production of recombinant heterogeneous proteins in soluble form. Optimized inducer concentration, periplasmic expression, use of strains with oxidative cytoplasmic environment, cultivation at lower temperature and the fusion of recombinant protein to a solubility enhancer partners are usual methods to increase recombinant protein solubility. In this study, we used NusA fusion protein to increase the soluble form of rhIGF-1 through recombinant production in *E. coli*. According to the results, NusA could change rhIGF-1 aggregate form towards the soluble form. Therefore, this fusion partner is a promising tag for increasing the soluble form of rhIGF-1 protein in lab or industrial scale.

**Figure 4.** Western blot analysis of NusA fused rhIGF1 expression in *E. coli*. M: Molecular weight marker, lane 1: Western blot analysis of IGF1-NusA expression after induction, lane 2: Western blot analysis of IGF1-NusA expression before induction.

Competing Interests

The authors confirmed that there is no conflict of interest.

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