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Full Length Research Paper

Improving recombinant protein solubility in *Escherichia coli*: Identification of best chaperone combination which assists folding of human basic fibroblast growth factor

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Manipulating the cytoplasmic folding environment by increasing the intracellular concentration of folding modulators, such as chaperone molecules, causes the convenient production of heterologous proteins. Wrong selection of chaperones will negatively affect the host cells physiology and the production of heterologous proteins. Due to this reason, type and combination of chaperone molecules are crucial to produce more soluble and active form of target protein. In the current study, the co-overproduction of five different combinations of 6 chaperones, comprising "DnaK/DnaJ/GrpE/GroES/GroEL", "GroES/GroEL", "DnaK/DnaJ/GrpE", "GroES/GroEL/TF" and "TF" along with recombinant human basic fibroblast growth factor (rhbFGF) were studied. As a result, we proved that none of these combinations was able to completely prevent the formation of inclusion bodies, but co-overexpression of the bacterial chaperone system TF along with rhbFGF could significantly enhance the yield of soluble protein. Recombinant soluble hbFGF that co-expressed with TF was then purified from the cells and was found to be identical to the active rhbFGF expressed alone with respect to size and spectral properties.

Key words: Human basic fibroblast growth factor (hbFGF), chaperone co-expression, chaperone combination, inclusion body, protein folding.

INTRODUCTION

Over-expression of heterologous proteins in the cytoplasm of *E. coli* is often accompanied by their accumulation into insoluble aggregates. The aggregated proteins lose their correct conformations and are deposited to inclusion bodies known to be in the inactive form of the protein (Baneyx and Mujacic, 2004).

Although inclusion body formation can greatly simplify protein purification, there is no guarantee that the *in vitro* refolding will produce large amounts of biologically active product. On the other hand, conditions for refolding the aggregated protein to its native form have to be standardized for each protein separately and this is often both time-consuming and expensive (Tsumoto et al., 2003). Thus, while targeted inclusion body formation may be valuable, being able to produce over-expressed soluble proteins *in vivo* remains a critical concern. It is now clear that solubility is the best criterion of recombinant protein quality. Therefore, acquisition of solubility is the main goal in protein production processes.

Under normal circumstances of *in vivo* folding, molecular chaperones modify the kinetic partitioning between productive folding and off-pathway reactions (Gething

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and Sambrook, 1992). During over-expression of proteins in *Escherichia coli*, the bacterial chaperones might get titrated out and thus fail to meet the demand of an overload of nascent polypeptides (Schweder et al., 2002). In this regard, increasing the chaperone supply seems a noticeable solution to the problems arising from formation of inclusion bodies during over-production of recombinant proteins (Schlieker et al., 2002).

Goloubinoff et al. (1989) first demonstrated that chaperone co-overexpression is a powerful tool to improving the folding of heterologous proteins by showing that the yield of active *Aspergillus nidulanse* RUBISCO was increased 10-folds in the cells harboring *groELS* operon. Subsequently, lots of attempts were made to increase the yields of correctly folded and soluble recombinant proteins by the co-overproduction of chaperones combination in producing cells.

It is still a trial and error process to find a compatible chaperone system to increase the solubility of the target protein. For instance, GroELS or DnaKJ were effective for some proteins such as RUBISCO, human procollagenase, *E. coli* glutamate racemase but along with chloramphenicol acetyltransferase, yeast N-myristoyl transferase and β-galactosidase-bovine somatotropin fusion protein both chaperone systems demonstrate the lack of effect (Goloubinoff et al., 1989; Lee and Olins, 1992; Ferreyra et al., 1993). Nishahara et al. (2000) reported the effect of chaperone combinations on the three different aggregation prone proteins. They revealed that while over-production of DnaKJ plus GrpE or trigger factor alone was sufficient to improve the solubility of mouse endostatin, DnaKJ plus GrpE or trigger factor plus GroELS were effective for human oxygen-regulated protein ORP150 and only trigger factor plus GroELS were capable of improving solubility ratio of human lysozyme. All these studies show that while this approach has been very successful for a large number of unrelated substrates, there is no guarantee that chaperones co-overproduction will improve the folding of a recombinant protein.

For the first time in this study, the capability of the molecular chaperone combinations inside the cell for the production of soluble well qualified recombinant human basic fibroblast growth factor (rhbFGF) was examined. For that reason, we used 5 different combinations of 6 chaperone molecules. At the end, we report the conformational quality of soluble rhbFGF produced by the best co-transformants using spectroscopic studies.

MATERIALS AND METHODS

Strain and plasmids

E. coli OrigamiB (DE3) (Invitrogen, USA) was used as host. In ourprevious work, an expression vector for human basic fibroblast growth factor, pET-1008, was constructed as follows: A 450 bp DNA fragment containing the coding region for the mature cytoplasmic isoform of hbFGF was inserted into pET-22b vector (Novagen, UK) just downstream of its ATG initiation codon. Expression of the hb*fgf* gene was under the control of the *T7* promoter, induced by isopropyl-β-D-thio-galactoside (IPTG) (Alibolandi et al., 2010).

The plasmids containing the chaperone gene(s) were purchased from Takara Company (Japan). All the chaperone plasmids were derivatives of pAR3 with a chloramphenicol-resistant marker.

Plasmid pG-KJE8 contains the *dnaK-dnaJ-grpE* and *groES-groEL* operons, respectively, fused with *araB* and *Pzt-l* promoters, while pG-Tf2 plasmid consists of trigger factor gene and *groES-groEL* operon under control of tetracycline inducible promoter (*Pzt-1*). pTF16 and pKJE7 plasmids, with the L-arabinose inducible (*araB*) promoter, were employed to co-express trigger factor and DnaK-DnaJ-GrpE chaperones.

Media and cultivation condition

Co-transformed bacteria with pET-1008 and chaperone plasmids were grown in Lauria Bertani (LB) agar (Merck, USA) plate containing ampicillin (50 µg/ml) and chloramphenicol (20 µg/ml). The plates were incubated at 37°C for approximately 15 h. For preparing the seed cultures, an isolated single colony of each co-trasformant bacteria was picked to be inoculated into 5 ml of LB medium (Merck, USA). The medium was supplemented with ampicillin (50 µg/ml) and chloramphenicol (20 µg/ml) which was then incubated at 37°C overnight.

Expression and analytical methods

In order to achieve high yields of soluble rhbFGF, different incubation time, temperatures and concentration of inducers were tested in the expression procedure. To perform co-expression of rhbFGF along with each combination of chaperone, 2 ml of prepared seed cultures were inoculated into 100 ml of LB medium in the presence of related antibiotics for plasmid selection and 0.5 to 4 mg/ml L-arabinose and/or 2 to 5 ng/ml tetracycline for the induction of chaperone expression, then were shaken in a rotatory shaker at 37°C and 200 rpm. When the A_{600nm} reached 0.5, the cultures were supplemented with 1 mM IPTG to induce the expression of the *rhbfgf* gene regulated by the *T 7* promoters. After induction, the cultures were further shaken at 25, 30, 37°C and 200 rpm. Batch samples were taken every hour until OD_{600} reached 0.2.

2.5 ml of culture samples were centrifuged at 6000 rpm for 10 min. The cell pellets were re-suspended in 100 µl of 50 mM phosphate buffer (pH 7.2) containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 0.5 mM $MgCl_2$, 1 mM EDTA and 250 µg/ml lysozyme. The suspension was kept on ice for 30 min and cells were then sonicated (3 times, 30 seconds each time) using an ultrasonic processor (Misonix, NY). The cell extracts were centrifuged at 4°C and 10000 rpm for 15 min.

For precise measurement of soluble hbFGF, the supernatants containing soluble proteins were assayed by the enzyme-linked immunosorbent assay (ELISA) kit (R&D, USA). The pellets containing insoluble proteins were re-suspended in 100 µl of 50 mM phosphate buffer (pH 7.2). For measurement of insoluble hbFGF, the same ELISA kit was employed. All ELISA tests were done in triplicate. Soluble and insoluble fractions were normalized with respect to cell density and loaded on 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions (Laemmli, 1970) and stained with coomassie brilliant blue R250 (Sigma, USA).

The protein concentrations in the samples were determined with Bradford method (Bradford, 1976) using bovine serum albumin as the concentration standard. For Western blotting assay, soluble and insoluble fractions of 6 expression systems lysate in the gel were transferred to a 0.2 µm pore size nitrocellulose membrane

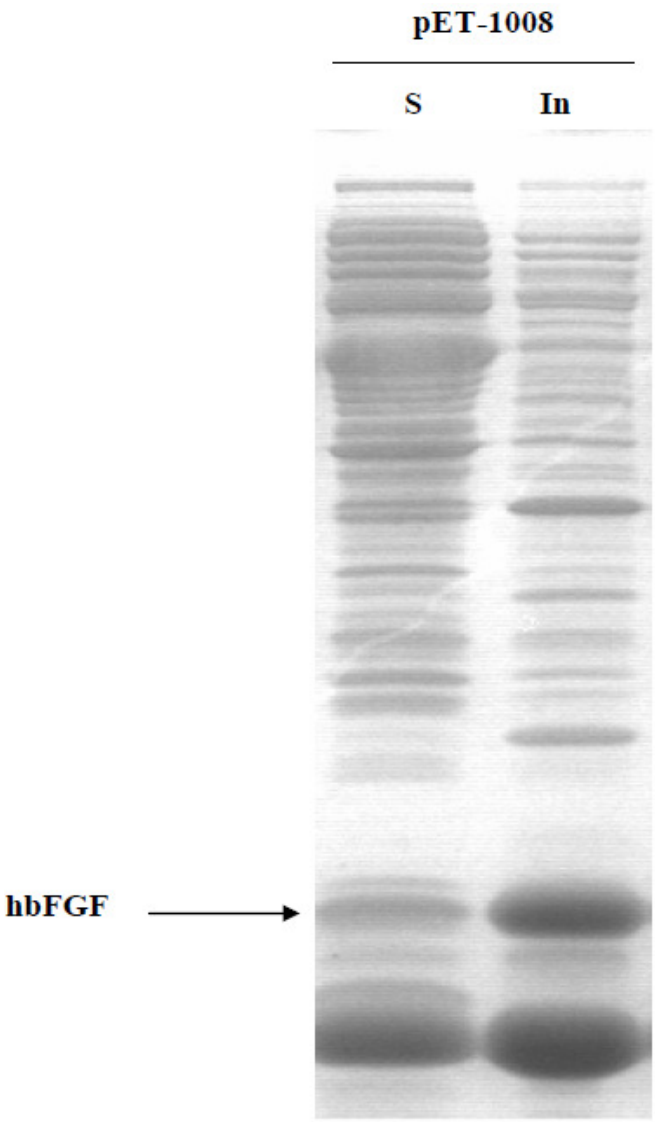

Figure 1. SDS-PAGE analysis. The soluble (S) and insoluble (In) cell proteins of induced *E. coli* OrigamiB (DE3) transformed with pET-1008.

(schlecher and Schuell, USA) using a semi-dry electroblotting apparatus (Bio-Rad, USA) at 12 V for 45 min. Rabbit anti-hbFGF anti serum (Sigma, USA) served as primary antibody and the secondary antibody, horse radish peroxidase conjugated goat-anti-rabbit antibody (Sigma, USA) was added followed by incubation at room temperature for 2 h. The immunoreactions were visualized using 0.5 mg/ml diaminobenzidine HCl and 0.01% H_2O_2.

Purification of recombinant hbFGF

The lysates obtained from 1 liter of induced cell cultures for best transformant and co-transformant, were centrifuged at 4°C and 10000 rpm for 15 min. Supernatants containing the soluble fraction were ultrafiltrated using centricon plus-70 (ultracell cut off 30000 Da, Millipore, USA), at 3000 g for 15 min. The proteins that passed through the ultrafilter were run on a 25 ml CM sepharose column (Pharmacia, Sweden) under native condition according to the protocol of Alibolandi et al. (2010).

The purity of the recombinant protein and yields of purified protein were quantified by densitometry scan of 1-D gels using Quantity-One 1D-gel analysis software (Bio-Rad, USA). Thermal denaturation was used in order to prepare denatured protein for spectroscopic studies. For preparation of 0.5 mg denatured hbFGF, 0.8 mg purified hbFGF in phosphate buffer pH = 7 was incubated at 65°C for about 15 min.

Circular dichroism (CD)

CD spectroscopy was used to determine the recombinant hbFGF secondary and tertiary structures using JASCO J-810 spectro-polarimeter. All spectra were recorded using 1mm pathlength cuvette, scanning rate of 200 nm/min and 1 nm bandwidth. The protein concentration of about 0.5 mg/ml was used for each CD measurement. The CD spectra were corrected for buffer contributions.

Fluorescence spectroscopy

Determination of protein fluorescence spectrum was performed with a Hitachi F-4010 fluorescence spectrophotometer using a quartz cuvette with 1 cm light path-length. Excitation and emission slits were set as 10 nm. Fluorescence spectra were then recorded with an excitation of 295 nm and recording the emission spectra from 200 to 500 nm.

RESULTS

Expression of recombinant human basic fibroblast growth factor

Figure 1 shows the soluble/insoluble fractions of pET-1008 (rhb*fgf*-cDNA inserted into pET-22b) expression under the regulation of the *T 7* promoter in the OrigamiB (DE3) strain. The result implies that rhbFGF can be properly expressed but process to form native form of the protein is deficient. Under optimal conditions for production of soluble hbFGF (Table 1), more than 80% of produced rhbFGF were deposited to insoluble fraction. As the formation of insoluble rhbFGF suggested the existence of a protein-folding issue for this expression system, the effect of several chaperone combinations on hbFGF expression was investigated.

The effect of trigger factor and/or GroEL – GroES on solubility ratio of rhbFGF

In order to determine if trigger factor or GroELS alone could assist in the folding of hbFGF, we co-expressed pET-1008 along with pTf16 and pGro7 plasmids. Co-expression studies were initiated by co-transforming pET-1008 plus pTf16 or pGro7 into the same *E. coli* host. The optimal culturing conditions for co-expressing of hbFGF with mentioned chaperone combinations are given in Table 1.

The hbFGF amount in the soluble fractions was measured

Table 1. Optimal conditions for production of highest amount of soluble rhbFGF by expression systems.

Expression system	Concentration of inducer	Temp. (°C)	Induction time (h)
OrigamiB (DE3) containing pET-1008	1 mM IPTG	37	5
OrigamiB (DE3) containing pET-1008,pGro7	1 mM IPTG, 2 mg/ml L-arabinose	37	5
OrigamiB (DE3) containing pET-1008,pG-KJE8	1 mM IPTG, 4 mg/ml L-arabinose, 2 ng/ml tetracycline	37	5
OrigamiB (DE3) containing pET-1008,pTf16	1 mM IPTG, 4 mg/ml L-arabinose	37	5
OrigamiB (DE3) containing pET-1008, pKJE7	1 mM IPTG, 4 mg/ml L-arabinose	30	7
OrigamiB (DE3) containing pET-1008,pG-Tf2	1 mM IPTG, 2 ng/ml tetracycline	30	7

measured, and it showed that co-expressing rhbFGF with GroEL-GroES decreased solubility ratio, while the other system which co-expresses TF along with hbFGF, significantly increased solubility ratio of target protein (Figure 2B). Furthermore, analysis of the extract was made from insoluble material which indicated that the vast majority of the rhbFGF was in insoluble fraction when hbFGF is co-expressed with GroELS; thus this system enhances accumulation of hbFGF. In contrast, trigger factor co-over-production could decrease hbFGF accumulation (Figure 2A, Figure 2B).

The plasmid that could express TF together with GroEL-GroES (pG-Tf2) was employed to examine the effects of TF plus GroEL-GroES combination on the production of soluble hbFGF. Under the optimal condition (Table 1) remarkable decrease in hbFGF amount in both soluble and insoluble fractions occurred using this combination (Figure 2C).

The effect of DnaK/DnaJ/GrpE alone and together with GroEL – GroES on solubility ratio of rhbFGF

Similar experiments carried out with pKJE7 and pG-KJE8 plasmids, respectively included DnaK-DnaJ-GrpE and DnaK-DnaJ-GrpE-GroELS chaperones (Table 1). As shown in figure 3A, when the DnaK-DnaJ-GrpE chaperones were co-expressed with hbFGF, it was recovered from insoluble to soluble fraction and the amount of hbFGF in soluble fraction was increased. Furthermore, the remarkable decrease of hbFGF in insoluble fraction was observed. Co-expression of both DnaK-DnaJ-GrpE and GroEL-GroES chaperones (pG-KJE8 plasmid) along with rhbFGF increased the amounts of hbFGF in both soluble and insoluble fractions (Figure 3B).

ELISA / western blot tests for rhbFGF

Western blotting analysis demonstrated that produced recombinant hbFGF in soluble and insoluble fractions of 6 expression systems could bind with the rabbit anti-human basic fibroblast growth factor (bFGF) polyclonal antibody (Figure 4). The differences in quantity of produced soluble hbFGF by expression systems are clearly shown in Figure 4. The ELISA test revealed that all the chaperone combinations were not able to prevent the accumulation of hbFGF in insoluble fractions, but DnaK-DnaJ-GrpE, GroEL-GroES plus DnaK-DnaJ-GrpE and especially TF chaperone systems were able to increase the solubility ratio of rhbFGF. On the other hand, co-expression of GroEL-GroES and DnaK-DnaJ-GrpE plus GroEL-GroES along with rhbFGF also could increase accumulation of hbFGF in insoluble fraction (Table 2).

Purification and Structural characterization of soluble rhbFGF

The soluble fraction of two expression systems comprising OrigamiB (DE3) containing pET-1008 and OrigamiB (DE3) containing pET-1008, pTF16 were purified. Pre-purification was made using 30000 Da molecular weight cut off ultrafilter. The proteins that passed through the ultra-filter containing recombinant protein were run on carboxymethyl (CM) sepharose column. Recombinant hbFGF was bound to the column and eluted with 0.4 M NaCl. The purity of rhbFGF was more than 95% in SDS-PAGE lane (Figure 5). Summary of purification steps of rhbFGF is given in Table 3.

Circular dichroism spectroscopy was applied to characterize the secondary and tertiary structures of rhFGF, using denatured hbFGF as control. The CD profiles of the hbFGF and hbFGF that co-expressed with TF overlap and showed significant presence of beta sheet (Figure 6). Fluorescence spectroscopy was employed to investigate the quality of rhbFGF folding. Both produced hbFGF exhibited very low fluorescence emission while denatured hbFGF revealed high fluorescence emission (Figure 7).

DISCUSSION

Efficient folding of newly synthesized proteins relies on support from molecular chaperones, which is employed to prevent protein misfolding and aggregation in the cell. Chaperones that bind nascent chain polypeptide, including trigger factor and heat shock protein (Hsp) 70, stabilize the extension of polypeptide chains on ribosomes. Folding in the cytoplasm is attained by releasing

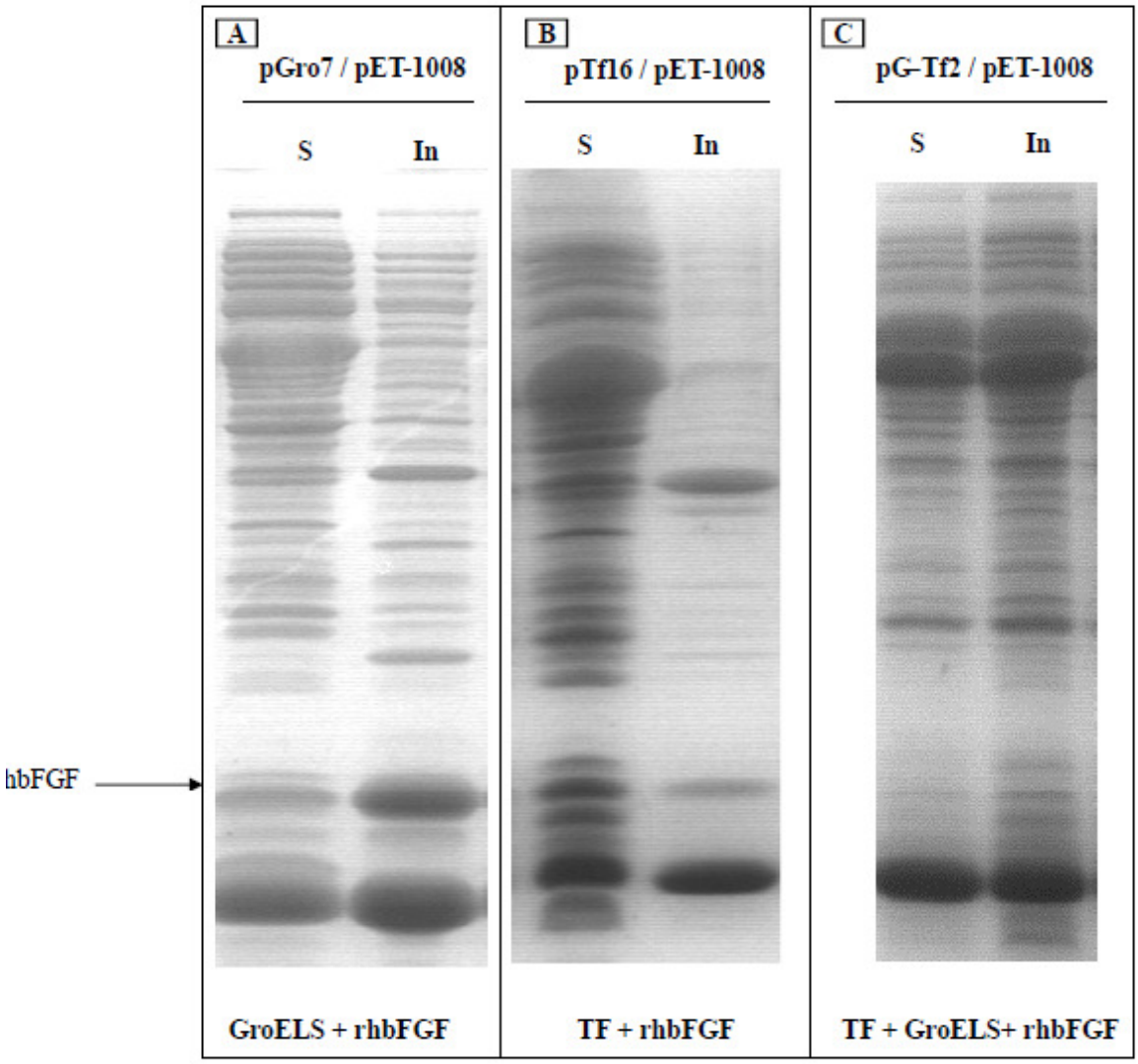

Figure 2. SDS-PAGE analysis. The soluble (S) and insoluble (In) cell proteins. (A) Induced *E. coli* OrigamiB (DE3) co-transformed with pET-1008 and pGro7; (B) induced *E. coli* OrigamiB (DE3) co-transformed with pET-1008 and pTf16; (C) induced *E. coli* OrigamiB (DE3) co-transformed with pET-1008 and pG-Tf2.

polypeptides from these factors or after transport of newly synthesized proteins to chaperonins (Gething and Sambrook, 1992; Hartl, 1996; Frydman, 2001). Although the substrate specificity of these chaperone systems has been characterized and an enormous progress in understanding chaperone-mediated protein folding processes has been achieved, the success of chaperone co-production in increasing soluble protein is not predictable from properties of recombinant protein (Baneyx and Mujacis, 2004; Hoffmann and Rinas, 2004; Ventura and Villaverde, 2006).

Trigger factor is a 48 kDa protein which binds to ribosome and interacts with nascent chains polypeptides (Goldberg, 2003). We found that over expression of trigger factor can remarkably increase production of soluble hbFGF, hence decrease the amount of insoluble hbFGF. In contrast, co-expression of TF along with GroEL-GroES significantly decreases both soluble and insoluble hbFGF, such observation is in agreement with previous findings that TF binds to GroEL and increases its affinity for certain proteins in order to promote their folding or degradation (Kondor et al., 1995).

According to our experiments, it seems that the effect of over expression of TF is similar to the effect of DnaK-DnaJ-GrpE chaperone team in solubilization of rhbFGF and reduction of protein aggregation. Our data also coincident with another studies suggesting TF and DnaK DnaJ-GrpE have similar roles during protein folding process and TF has an overlapping chaperone function with the Hsp70, DnaK and DnaJ, in holding polypeptide

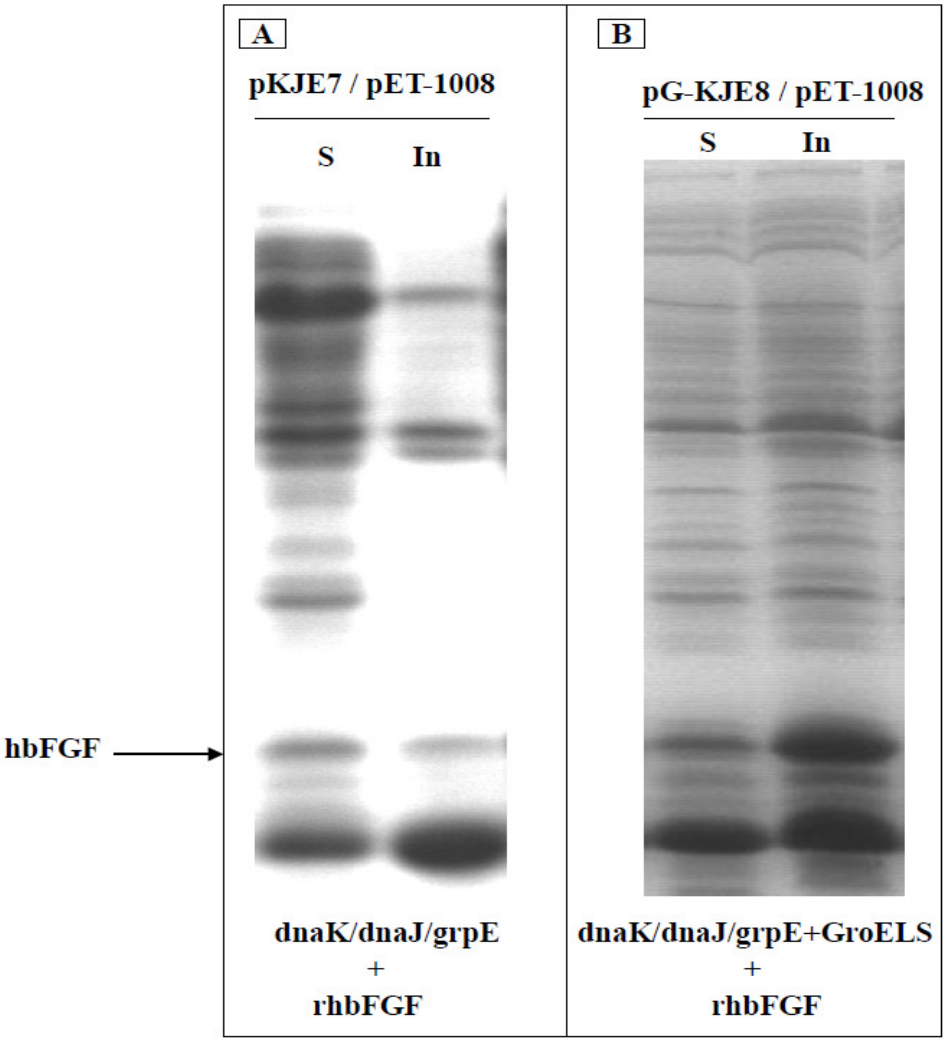

Figure 3. SDS-PAGE analysis. The soluble (S) and insoluble (In) cell proteins. (A) Induced *E. coli* OrigamiB (DE3) co-transformed with pET-1008 and pKJE7; (B) induced *E. coli* OrigamiB (DE3) co-transformed with pET-1008 and pG-KJE8.

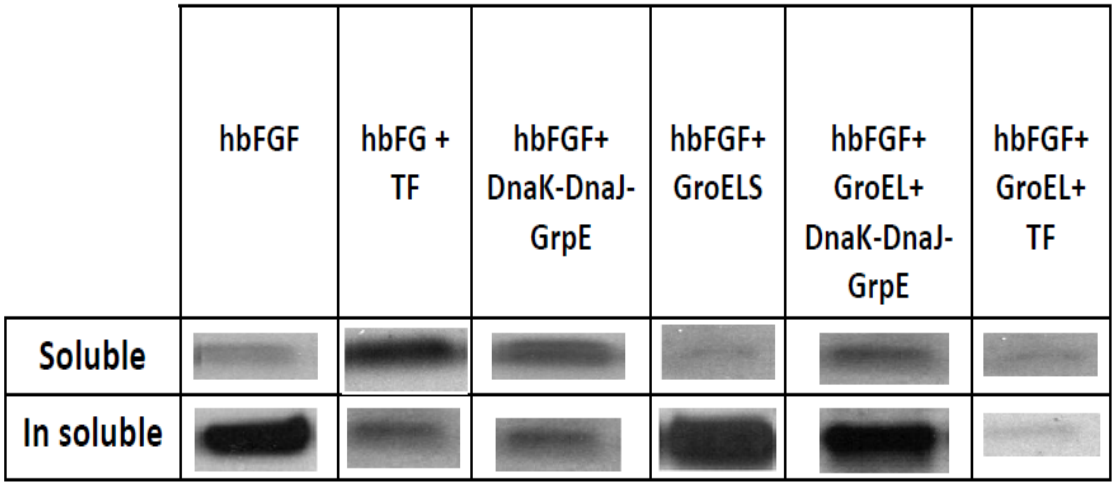

Figure 4. Western blot analysis of soluble and insoluble fractions of six expression systems.

Table 2. The amount of hbFGF in the soluble and insoluble cell fractions of induced *E. coli* OrigamiB (DE3) strain co-transformed with pET-1008 and chaperone plasmids was quantified by ELISA kit.

Cell protein of expression system	Soluble hbFGF (mg/ l culture)	Insoluble hbFGF (mg/l culture)
Induced OrigamiB (DE3) containing pET-1008	34	256
Induced OrigamiB (DE3) containing pET-1008,pGro7	15.3	284.95
Induced OrigamiB (DE3) containing pET-1008,pG-KJE8	54	263.2
Induced OrigamiB (DE3) containing pET-1008,pTf16	187	2.3
Induced OrigamiB (DE3) containing pET-1008, pKJE7	84	6.1
Induced OrigamiB (DE3) containing pET-1008,pGtf2	0	0
Uninduced OrigamiB (DE3) containing pET-1008	0	0
OrigamiB (DE3) without plasmid	0	0

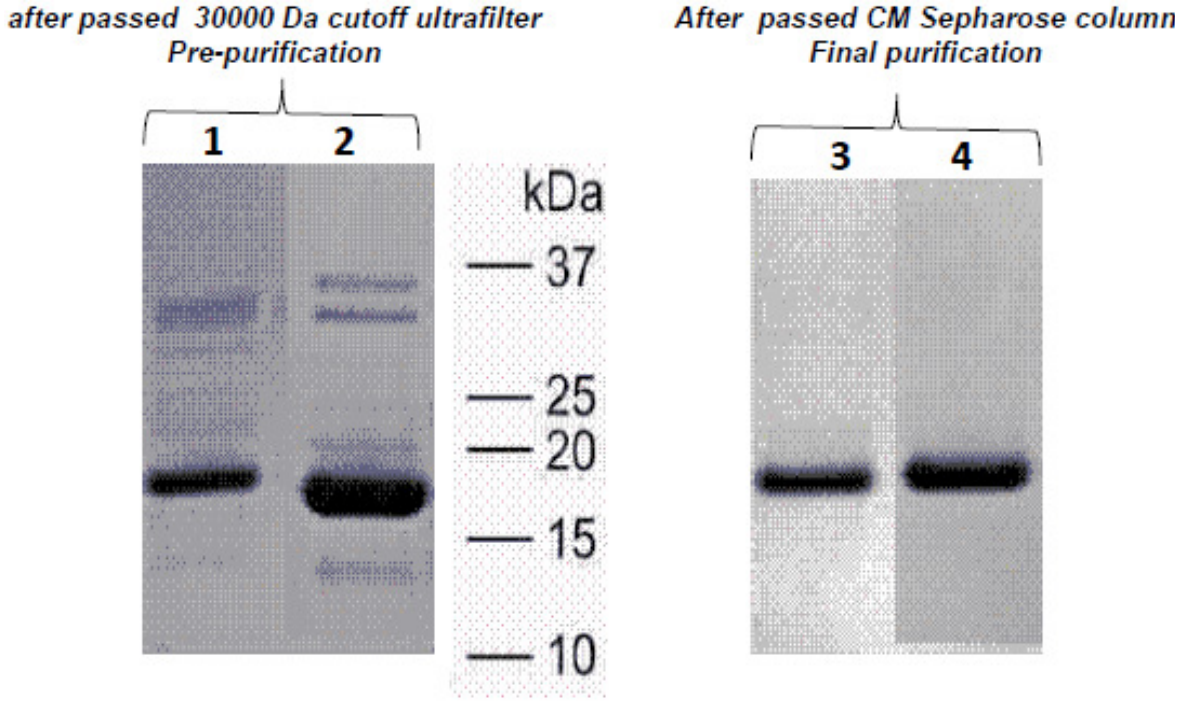

Figure 5. Lane 1, 3: Purification of supernatant of 1 L cell (induced OrigamiB (DE3) containing pET-1008) lysate; Lane 2, 4: purification of supernatant of 1 L cell (induced OrigamiB (DE3) containing pET-1008, pTF16) lysate.

chains in a state competent for folding (Deuerling et al., 1999; Teter et al., 1999).

Solublization of rhbFGF by co-expression of chaperone teams from pG-KJE8 and pGro7 prove that high levels of GroEL-GroES individually and together with DnaJ/DnaK/ GrpE can enhance accumulation of hbFGF in inclusion bodies. Co-production of chaperones comprises DnaK and GroEL family with recombinant proteins been traditionally examined in order to increase the amount of soluble protein (Goloubinoff et al., 1989; Blum et al., 1992; Baneyx and Palumbo, 1999). Moreover, in some cases during over expression of DnaK, even accumulation of inclusion bodies can occur (Petersson et al., 2004).

DnaK and GroEL family also associate with inclusion bodies. DnaK is localized preferably on the surface of inclusion bodies. GroEL is distributed in the cytoplasm, found inside of inclusion bodies (Carri'o and Villaverde, 2005). The precise function of the DnaK and GroEL in inclusion body metabolism is not yet understood. Empirical results are even consistent with an effective role of GroEL in the deposition of misfolded protein into inclusion bodies (Carri'o and Villaverde, 2003).

In the current study, co-production of GroELS alone or GroELS in addition to DnaK-DnaJ-GrpE enhanced accumulation of hbFGF in inclusion bodies but co-expression of DnaK-DnaJ-GrpE along with hbFGF could decrease aggregation.

Table 3. (A) Summary of purification steps of 1 L induced OrigamiB (DE3) containing pET-1008 culture; (B) summary of purification of 1 L induced OrigamiB (DE3) containing pET-1008, pTF16 culture.

Purification step	Volume	Total protein (mg/ml)	Recovery (%)
A			
Supernatant	1 L	174	100
30000 Da Ultrafilter	540 ml	57.8	72.3
CM Sepharose	10 ml	3.46	66.2
B			
Supernatant	1 L	183	100
30000 Da Ultrafilter	540 ml	94	75.3
CM Sepharose	10 ml	12.38	66.7

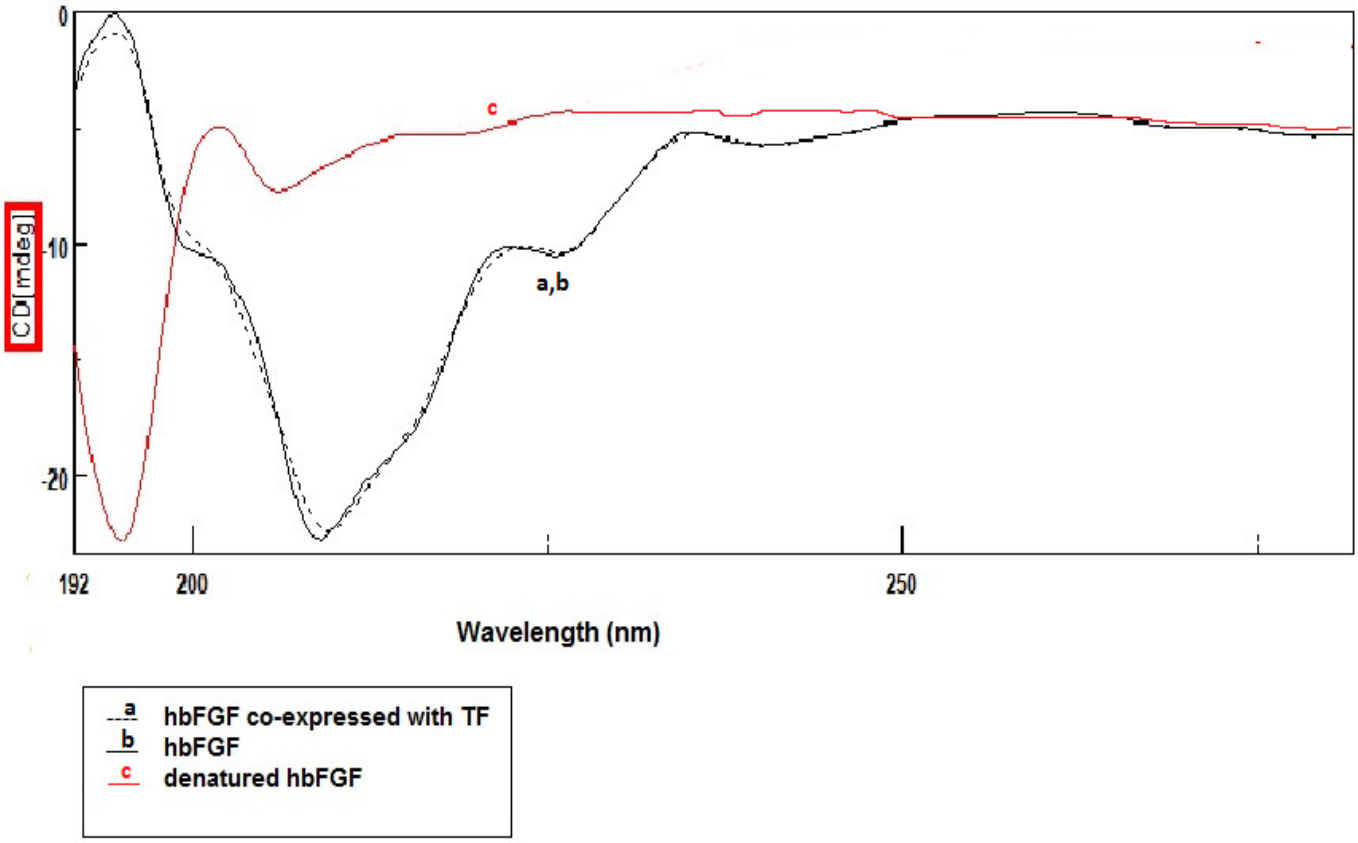

Figure 6. The CD spectra of recombinant hbFGF co-expressed with TF, recombinant hbFGF and thermal denatured hbFGF.

Our results strongly suggest the important role of GroEL in stabilization of rhbFGF in inclusion bodies, while the DnaK-DnaJ-GrpE or TF operating systems reveal a direct role in disaggregating target protein. This clearly shows that GroELS chaperonins are not essential for the formation of soluble hbFGF, but DnaK family and more importantly trigger factor are involved in promoting rhbFGF folding and, in promoting degradation of the abnormal and unstable protein in case folding and refolding is not possible. Using trigger factor we obtained approximately 4 times more soluble protein than in cells producing only hbFGF. In our previous work, we found that rhbFGF produced by induced OrigamiB (DE3) containing pET-1008 culture is biologically active and has good quality comparable with commercial one (Alibolandi et al., 2010).

Identical patterns in CD spectra between rhbfgf expressed alone and rhbfgf co-expressed with TF suggests their secondary and tertiary structure is similar. Quantitative analysis of the CD spectra also indicate that

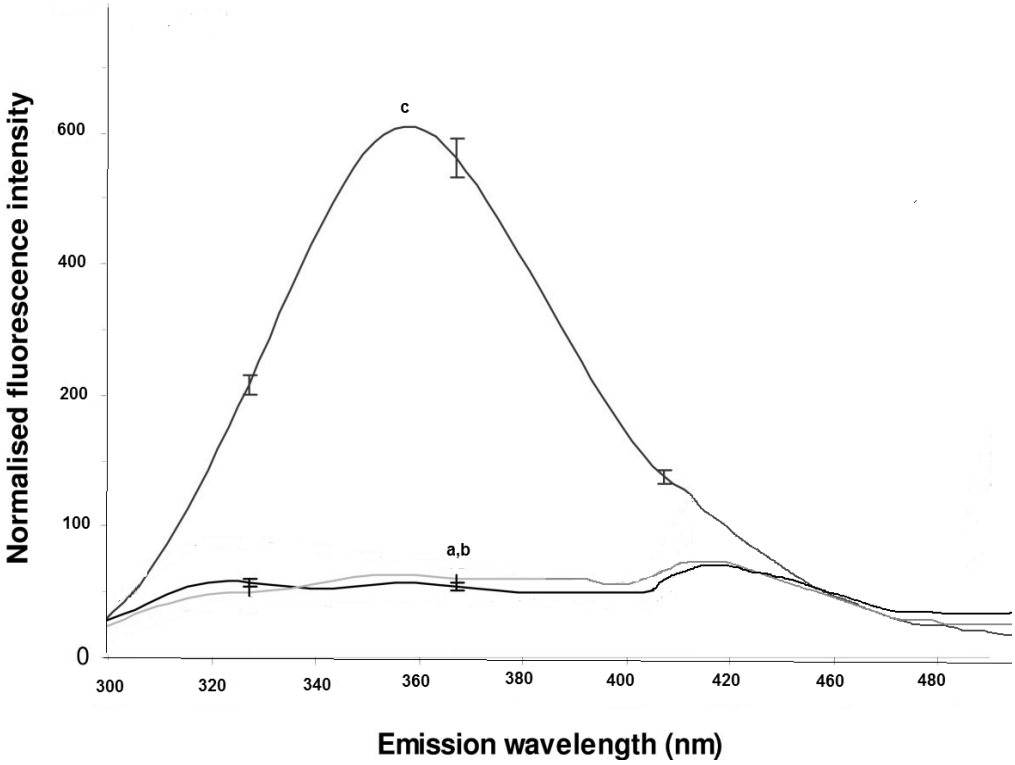

Figure 7. Relative fluorescence intensity of recombinant hbFGF produced in *Escherichia coli* cells with or without TF (a,b) and thermal denatured rhbFGF (C).

rhbFGF (produced by both expression systems) are predominantly in beta-sheet and other conformations with very little alpha-helix fraction. These results are in agreement with earlier observations (Prestrelski et al., 1991).

It is well established that fluorescence intensity obtained at an excitation wavelength of 295 nm correlates with the exposure of hydrophobic tryptophan residues on the surface of the polypeptide (Ladokhin et al. 2000) and hence the extent of protein unfolding. hbFGF carry 8 tryptophan residues (Dubey et al. 2005). Fluorescence emission of purified recombinant hbFGF exhibited an emission characteristic that was comparable to native hbFGF. Here, we showed purified recombinant hbFGFs had very low emission in comparison with denatured hbFGF.

Both CD spectra and fluorescence emission indicate that the significant increase in solubility ratio did not alter the secondary and tertiary structure of rhbFGF. This outcome paves the way for broad use of molecular chaperone in the *in vivo* folding of recombinant therapeutic proteins, where structure conservation is pre-requisite for regulatory approval.

Abbreviations

rhbFGF, Recombinant human basic fibroblast growth factor; **LB,** Lauria Bertani; **PMSF,** phenylmethylsulfonyl fluoride; **ELISA,** enzyme-linked immunosorbent assay; **CD,** circular dichroism; **SDS-PAGE,** sodium dodecyl sulfate polyacrylamide gel electrophoresis; **Hsp,** heat shock protein.

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