Journal of Sciences, Islamic Republic of Iran 20(4): 305-310 (2009) http://jsciences.ut.ac.ir
University of Tehran, ISSN 1016-1104

Stability of Recombinant Proteins in *Escherichia coli*: The Effect of Co-Expression of Five Different Chaperone Sets

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Received: 9 June 2009 / Revised: 27 September 2009 / Accepted: 10 October 2009

Abstract

Chaperones are produced by prokaryotic, yeast and higher eukaryotic cells for various purposes. Over-expression of each chaperone or sets of them affect the production level of a recombinant protein in the cell. On the basis of this hypothesis, five different plasmids with 5 different combinations of 6 chaperones molecule, transformed into *Escherichia coli* along with human basic Fibroblast Growth Factor expression plasmid. Each transformant that contain both plasmids for expression of hbFGF and chaperone combinations was induced with proper concentration of related inducers. Subsequently, total amount of produced hbFGF was analyzed based on SDS-PAGE and ELISA. Our results indicated that "TF" and "DnaK/DnaJ/GrpE" destabilized hbFGF, while "DnaK/DnaJ/GrpE/GroEL /GroES" and "GroEL/GroES" combinations were able to stabilize it. It has also revealed that "GroEL/GroES/TF" combination negatively affected the hbFGF production.

Keywords: Chaperones combination; Recombinant protein stability; hbFGF

Introduction

Escherichia coli has not optimized to produce protein for human applications. Production of recombinant proteins in *E. coli* interferes with the physiology of the cell, forcing reorientation of metabolic activities and adaptation of the gene regulation system. This effect often resembles the cellular response to the environmental stress such as heat shock, amino acid depletion and/or starvation. The ability to carry out appropriate stress response is important for cell survival [1]. However, cells are not able to perform a suitable stress response during recombinant protein over production and that is due to limitation in adaptation capacity of each cell [2]. The most recent strategy for helping host cells to compensate inadequacy of the heat shock proteins during overproduction of heterologous proteins is co-expression of molecular chaperones along with target protein [3]. Chaperones cooperate in the folding of newly synthesized proteins to the native state so they can prevent aggregation and degradation of protein [4, 5].

In the *E. coli* cytosol, the folding of newly synthesized proteins is assisted by the ribosome-associated Trigger Factor, the DnaK system (DnaK with its DnaJ and GrpE cochaperones; KJE), and the GroEL system (GroEL with its GroES cochaperone; ELS) [6-9]. Protein folding and proteolitic degradation are

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Table 1. The list of chaperones plasmid sets which were used in this study

No.	Plasmid	Chaperone	Promoter	Inducer	Resistant Marker
1	pG-KJE8	dnaK-dnaJ-grpE groES-groEL	*araB Pzt1*	L-Arabinose Tetracyclin	Cm
2	pGro7	groES-groEL	*araB*	L-araBinose	Cm
3	pKJE7	dnaK-dnaJ-grpE	*araB*	L-Arabinose	Cm
4	pG-Tf2	groES-groEL-tig	*Pzt1*	Tetracyclin	Cm
5	pTf16	tig	*araB*	L-Arabinose	Cm

efficient way to conserve cellular resources by recycling improperly folded or irremediably damaged proteins into amino acids [10]. Sometimes molecular chaperones can bind and stabilized non-native conformations of other proteins [11, 12].

In some cases over production of chaperones is not only useful for the solubility, but also for total amount of the target protein, as has been shown for the human procollagenase protein [4]. It has also proved that co-expression of GroELS/DnaK along with Cryj2 (an allergen of Japanese Cedar Pollen) results in marked stabilization and accumulation of Cryj2 [13].

This approach has been very successful for a large number of unrelated substrates. In this regard lots of successful applications have been reported by Georgiou and Valax and by Baneyx and Palumbo [14, 15]. On the other hand, there are restrictions that limit common application of this strategy. Sometimes chaperone overproduction enhances tendency of affected proteins to proteolysis and accordingly reduces the total amount of the target proteins [16, 17].

In current study, the effects of 5 different chaperone sets comprising "TF", "DnaK/DnaJ/GrpE", "DnaK /DnaJ/GrpE/GroEL/GroES", "GroEL/GroES" and "GroEL/GroES/TF" on human basic Fibroblast Growth Factor stability was investigated.

Materials and Methods

Bacteria and Plasmids

E. coli OrigamiB (DE3) strain (Invitrogen, California, USA), was used as a host throughout this study. In our previous 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, London, UK) just downstream of its ATG initiation codon [Submitted].

All of the chaperone plasmids were derivatives of pAR3 with a chloramphenicol-resistant marker (Takara, Tokyo, Japan). More details about Takara's chaperone plasmid sets are given in Table 1.

Media, Culture Conditions and Expression

At the first stage chaperone plasmids were separately transformed into the chemically competent cells, subsequently hbFGF expression plasmid (pET-1008) was also transformed into the cells bearing chaperone expression plasmids [18]. To perform co-expression, the transformants were inoculated into 100 ml of LB medium (Merck, New Jersey, USA) containing related antibiotics for plasmid selection (50 µg/ml ampicillin and 20 µg/ml chloramphenicol) and 4 mg/ml L-arabinose and/ or 5 ng/ml tetracycline for induction of chaperone expression and then were shaken in a rotatory shaker at 37°C and 200 rpm. When the OD_{600} reached 0.5, the cultures were induced by addition of 1 mM IPTG. After induction, cells were further shaken at 37°C, 30°C and 25°C until OD_{600} reached 0.2. Two Batch samples were taken at the end of the exponential growth phase and the end of the stationary phase.

Protein Extraction, Detection and Analysis

5 ml of taken culture samples were centrifuged at 6000 rpm for 10 minute. The cell palettes were re-suspended in 200 µl of 50 Mm Tris-HCl buffer (pH 7.2) containing 0.1 mM phenylmethylsulfonyl fluorides (PMSF), 0.5 mM $MgCl_2$, 1 mM EDTA and 250 µg/ml lysozyme. The suspension was kept on ice for 30 minute and then cells were sonicated (3 times, 30 seconds each time) using an ultrasonic processor (Misonix, Framingdale, NY).

Polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on 15% gels according to Laemmli [19]. Protein bands of cell extracts were detected after staining with Coomasie brilliant blue R-250 (Sigma, St. Louis, USA) and the gels were scanned using a densitometer scanner (Bio-Rad, California, USA). Quantification of hbFGF bands was determined by utilization of Quantity-One 1D-Analysis software (Bio-

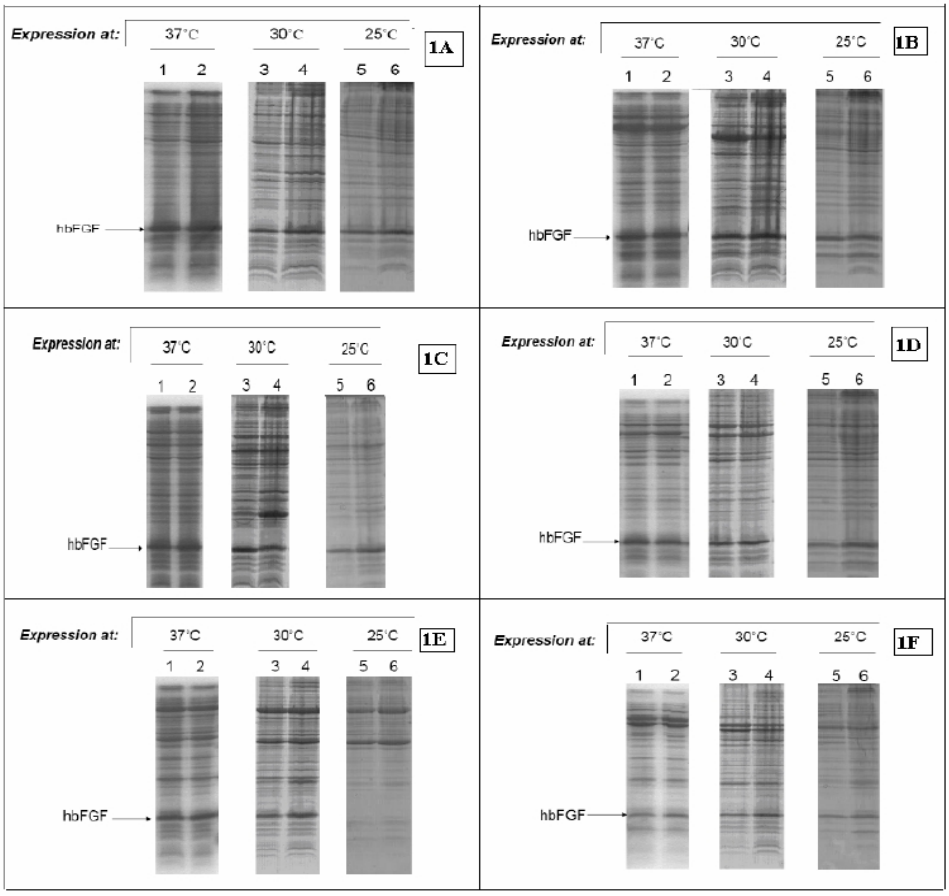

Figure 1. 15% SDS-PAGE of total protein of the cell fractions. Lanes 1, 3, 5: Samples were taken at the end of the exponential growth phase. Lanes 2, 4, 6: Samples were taken at the end of the stationary phase. 1A: Induced OrigamiB (DE3) cell containing pET-1008; 1B: Induced OrigamiB (DE3) cell containing pET-1008 and pGro7; 1C: Induced OrigamiB (DE3) cell containing pET-1008 and pG-KJE8; 1D: Induced OrigamiB (DE3) cell containing pET-1008 and pTf16; 1E: Induced OrigamiB (DE3) cell containing pET-1008 and pKJE7; 1F: Induced OrigamiB (DE3) cell containing pET-1008 and pG-Tf2.

Rad, California, USA). For precise measurement of hbFGF, an ELISA kit (R&D, Minneapolis, USA) was employed.

Results

Over Expression of Trigger Factor and GroEL-GroES

E. coli strain; OrigamiB (DE3) harboring both compatible plasmids for expression of human basic Fibroblast Growth Factor and Trigger Factor were used to study the effects of TF over expression on production of hbFGF. Figure 1A shows the hbFGF production in the absence of chaperone plasmids. The data presented in Figure 1D indicates that when TF was over expressed prior to hbFGF production, it was reduced the total amount of hbFGF. Over expression of groEL/ES along with hbFGF increased total yield (Fig. 1B), whereas over expression of TF together with groEL/ES negatively affected cell growth rate and hbFGF production (Figs. 1F, 2).

Over Expression of DnaJ-DnaK-GrpE and GroEL-GroES

When the DnaK-DnaJ-GrpE chaperones were over expressed along with hbFGF, the yield of target protein reduced (Fig. 1E). In contrast, amount of hbFGF was increased by co-expression of DnaK-DnaJ-GrpE plus GroEL-ES chaperones (Fig. 1C).

The Effect of Growth Temperature and Induction Time

During expression at 37°C, 30°C and 25°C, hbFGF

production was analyzed by densitometry scan of SDS-polyacrylamide gel. The analysis of the 1D-gel bands shows that the appropriate temperature for hbFGF production by all of the expressions systems is 37ºC (Fig. 3).

Results presented in Figure 3 confirm that co-expression of GroELS (pGro7 plasmid) and DnaK-DnaJ-GrpE plus GroELS (pG-KJE8 plasmid) can stabilize hbFGF at 37°C. The hbFGF production was examined at the end of the exponential growth phase and end of the stationary phase. As can be seen in figure 1, the quantity of hbFGF in SDS-PAGE bands proves that the most amount of hbFGF were produced at the end of the stationary phase by all of the expression systems.

hbFGF Quantification in Cells Protein Fractions

Table 2 presents the exact amount of synthesized rhbFGF in 100 milliliter of cell culture through 5 different transformants containing pET-1008 and chaperone plasmids. ELISA results clearly indicate the positive chaperone effect of "GroEL-ES" and "DnaK-DnaJ-GrpE plus GroEL-ES" on hbFGF stability.

Discussion

The assemblies of molecular chaperones and proteases constitute the cellular system for *de novo* folding and quality control of proteins [20]. The role of chaperones and proteases in the quality control of proteins depends on their ability to refold or degrade misfolded proteins. It is already operative under regular growth conditions but becomes particularly important under stress conditions, such as over production of heterologous proteins. Stabilization and folding of recombinant proteins produced in *E. coli* is generally attributed to a limitation in the cell concentration of molecular chaperones elements. This assumption is physiologically supported by the over expression of chaperone genes, in particular of chaperone genes from the heat shock family, in response to recombinant protein over production [21-23].

In this paper, we report the effect and efficiency of 5 different combinations of 6 chaperones on stability of recombinant human basic fibroblast growth factor. We found that over expression of trigger factor can destabilize recombinant hbFGF, hence slight decrease in the total yield was observed. In contrast, co-expression of TF together with GroEL-GroES hardly decreases the total amount, such collaboration is in agreement with previous findings that suggest TF binds to GroEL and increases its affinity for certain proteins in order to promote their folding or degradation [24, 25].

Obtained results indicate that effect of over expression of TF is similar to the effect of DnaK-DnaJ-GrpE chaperone team in destabilization of hbFGF and decrease in total yield. It seems TF and DnaK-DnaJ-GrpE have similar roles during protein folding process, which is coincided with previous data that revealed, the function of DnaK and trigger factor in folding of nascent polypeptide overlap partially [26]. Inhibitory roles of the above-mentioned chaperone teams in

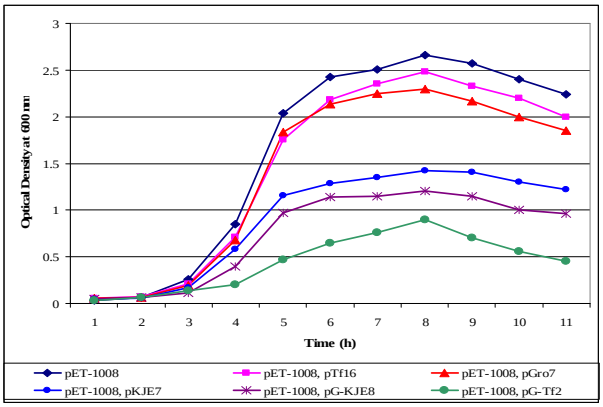

Figure 2. Growth rates of 6 expression systems. During batch cultivation, cell growth of each expression system was monitored by measuring the OD_{600} per hour.

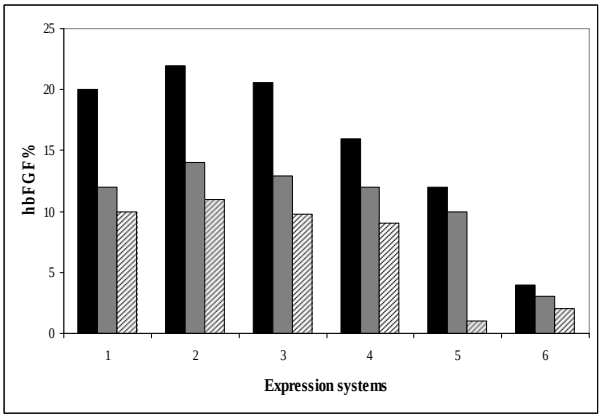

Figure 3. Impact of combined chaperone co-production on hbFGF% in the cellular protein fractions. Densitometry results for six expression systems at 37˚C (black bars), 30˚C (Gray bars) and 25˚C (black on white hachure bars). Column 1: OrigamiB (DE3) cell containing pET-1008; column 2: OrigamiB (DE3) cell containing pET-1008, pGro7; Column 3: OrigamiB(DE3) cell containing pET-1008, pG-KJE8; Column 4: OrigamiB(DE3) cell containing pET-1008, pTf16; Column 5: OrigamiB(DE3) cell containing pET-1008, pKJE7; Column 6: OrigamiB(DE3) cell containing pET-1008, pG-Tf2.

Table 2. ELISA assay. The exact amount of synthesized rhbFGF in 100 milliliter of cell culture through 6 different transformants

hbFGF (mg/100 ml culture)	Cell protein of expression systems
26.5	Induced OrigamiB (DE3) containing pET-1008
29.4	Induced OrigamiB (DE3) containing pET-1008,pGro7
27.3	Induced OrigamiB (DE3) containing pET-1008,pG-KJE8
20	Induced OrigamiB (DE3) containing pET-1008,pTf16
18.7	Induced OrigamiB (DE3) containing pET-1008, pKJE7
5.1	Induced OrigamiB (DE3) containing pET-1008,pGtf2
~ 0.00	Uninduced OrigamiB (DE3) containing pET-1008
~ 0.00	OrigamiB (DE3) without plasmid

recovery of rhbFGF followed by decreases in production, was possibly due to proteolysis enhancement. On the other hand, successfully stabilization of hbFGF by excess co-expression of chaperone teams from pG-KJE8 and pGro7 proves that high levels of GroEL-ES alone and together with DnaK-DnaJ-GrpE can protect nascent hbFGF polypeptides from proteolytic attack.

It has been noted that the DnaK/DnaJ/GrpE chaperones keep nascent polypeptides in unfolded conditions, while the GroEL-ES chaperones are able to interact with incomplete folded polypeptides to help folding process [20]. It is also proved that two chaperone teams (DnaK-DnaJ-GrpE plus GroEL-GroES) in the OrigamiB (DE3) strain stabilize recombinant hbFGF. Thus, these two chaperone teams have supportive roles in assisting protein folding, whereas, over expression of DnaK-DnaJ-GrpE chaperone team alone cause a decrease in stability of the target protein. In contrast, the excess of GroEL-ES stabilizes rhbFGF, suggesting that this chaperone team have partially supportive function in helping protein folding, and over production of GroEL-ES decreases the requirement of the DnaK-DnaJ-GrpE chaperone team and it can act efficiently alone.

In future, progress in understanding the mechanisms and substrate specificities of various chaperone systems will encourage their application in the stabilization of recombinant proteins. When data on proteins and their related chaperones collected, the appropriate chaperone system for co-expression will be chosen and the main decision will not be based upon a trial and error procedure.

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