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INVESTIGATION OF REASONS THAT IMPLY THE DIMINISHED INCLUSION BODIES IN *E. COLI*

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Abstract

We previously reported the cloning and expression of cDNA of Human Basic Fibroblast Growth Factor (hbFGF) in *Escherichia coli* under the control of T7 promoter [6]. In this study we looked for the factor which affects the diminution of Inclusion Bodies (IBs) in *E. coli* cells which produce heterologous proteins (hbFGF), 6 h after induction. It was shown that the amount of the insoluble hbFGF (as inclusion bodies), reached to the highest level 3 h after induction at 37°C and 42°C, however it decreased gradually afterwards to the lowest level at 6 hrs after induction. To address whether this diminution is the result of destruction of IBs by *E. coli* proteases, cell death, cell growth inhibition or changing in other physiological characteristics of *E. coli*, we carried out three experiments. These experiments were continual removal of the *E. coli* cells which loose their plasmids in culture by adding ampicillin; comparing the number of colonies which loose their plasmids to those which keep the plasmids by growing cells on LB agar plates containing ampicillin or without ampicillin; and adding chloramphenicol to culture 3 h after induction in order to cease protein synthesis in the *E. coli* cells. The results showed that neither IBs destruction by proteases nor cell death caused the IBs diminution. We showed that the IBs reduction is the immediate result of overgrowth of plasmid-free cells in culture and inhibition of proliferation of the cells containing plasmids and producing the heterologous proteins. Consequently, the ratio of heterologous protein producing cells to the ones lack this character is decreased.

Keywords: Inclusion bodies; hbFGF; Growth inhibition

Introduction

Inclusion bodies (IBs), which consist of misfolded peptide chains, are frequently produced during the synthesis of heterologous proteins in recombinant *E. coli*. It is believed that misfolded protein aggregation and accumulation in the cell is a result of a kinetic imbalance between digestion and precipitation pathways [1]. In turn, protein aggregation might reduce the availability of the insoluble protein to proteases and thus enhancing its stability [2]. However, in bacteria, it has been proven that loosely aggregated proteins retain

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protease susceptibility [3]. Very little is known about the digestion mechanism of insoluble proteins upon precipitation as IBs, where tighter hydrophobic interactions occur between unfolded chains. In addition, a high protease sensitivity of some IB proteins has been suggested by experimental data [4]. On the other hand, cease of cell growth and re-bFGF formation are observed during prolonged cultivation at 42°C [5], indicating that the synthesis of human bFGF strongly interferes with normal host cell metabolism.

Human basic fibroblast growth factor (hFGF-2) was successfully produced by recombinant *E. coli* using the widely employed pET-3a expression system [6]. In this study, the apparent decrease of IBs was observed 6 hours after induction at 42°C. We revealed that it is happened because of the cease of growth of hbFGF producing cells, as long as continuous proliferation of plasmid-lacking ones. Consequently, the possibility of IBs degradation by protease, as well as cell death due to overexpression of recombinant proteins, assumed to be impertinent.

Materials and Methods

Bacterial Strain and Plasmid

The strain *E. coli* BL21 (DE3) was used as host for the plasmid pET-1005 which was described previously [6]. This plasmid is a derivative of the expression vector pET 3a (Novagen) and, contains the hb*fgf* gene under the control of the T7 promoter.

Cultivation and Purification

A 50 ml cell culture was grown in baffled shake flask at 30°C on Luria Broth (LB) supplemented with 50 mgL^{-1} ampicillin with vigorous shaking. Growth rate of the culture was monitored spectrophotometrically. Heterologous gene expression was induced at an optical density (OD_{600}) of 0.4-0.5 by the addition of 0.5 $mmol\ L^{-1}$ isopropyl-β-D-thiogalactopyranoside = IPTG (the sample without induction was used as negative control). The culture was divided into two parts, one part was incubated at 37°C and the other at 42°C. The samples were withdrawn from each culture after 3, 6 and 18 h of induction and the cells were harvested by centrifugation (5 min, 10^4 rpm). Cell pellets were resuspended in 50 $mmolL^{-1}$ sodium phosphate buffer (pH 7) and diluted to an OD_{600}=5 and then disrupted by ultrasonication at 50 W for 3 min in an ice bath. The soluble and insoluble fractions were collected by centrifugation for 30 min at 22000 rpm. The insoluble fraction was washed with sodium phosphate buffer, centrifuged (30 min at 22000 rpm), and resuspended in the same buffer in one-fourth of the original volume. Supernatant resulted after sonication, which is known as total protein fraction, is a mixture of cytoplasmic soluble protein and insoluble protein particles such as inclusion bodies.

Protein Gel Electrophoresis

Soluble proteins (S.P), insoluble proteins (I.P) and Total Proteins (T.P) fractions were analyzed by SDS-PAGE [7]. Samples were boiled for 10 minutes in sample buffer consisting of 7% (w/v) SDS, 40% (v/v) glycerol, 150 mM DTT (dithiothreitol), 250 mM Tris/HCl (pH 6.8), 0.0025% (w/v) bromophenol blue and electrophoresed immediately using 8-16% precast gels (Pharmacia, Germany). Gels were stained with Coomassie Brilliant Blue R250 and quantified by densitometry (Hirschmann elscript 400, Germany).

Continuing Addition of Ampicillin to the Medium

After cultivation and induction of the cells, they were divided into two shaker flasks, one containing ampicillin (Amp^+) and the other without ampicillin (Amp^-) and they were transferred to 42°C shacking incubator. Every hour a 1.5 ml sample was withdrawn and its OD_{600} was determined. The absorbance of the samples of each culture was plotted as a graph. Ampicillin (50 mg L^{-1}) was then added to the Amp^+ flask each half an hour in order to prevent the growth of plasmid free cells. The samples, taken out from Amp^+ flask at 3, 4.5 and 6 h post-induction, were harvested and the pellets were sonicated. SP and IP isolated from TP were subjected to the SDS-PAGE analysis. But the insoluble protein fraction was finally resuspended in the original volume of PBS buffer.

Culture onto Plates and Colony Count

Every hour after induction, samples were taken from cultures grown in the absence of ampicillin. Each sample was diluted $1/10^5$ and plated on 10 LB-Agar plates, half of them containing ampicillin (100 mg L^{-1}). The plates were incubated at 30°C for 18 h. The colonies of each plate were counted and the colony numbers were plotted.

Protein Synthesis Inhibition by Adding Chloramphenicol

Under the same conditions as described above, the culture was prepared, induced and incubated at 42°C.

Three hours after induction, chloramphenicol (200 mg L^{-1}) was added to arrest the protein synthesis system of cells. The samples were withdrawn after 3, 4.5, 6, 7.5, 9 and 10.5 h. They were treated identically as above to have them prepared for SDS-PAGE, but in this case, insoluble proteins (IBs) were loaded only.

Results

Apparent Decrease of IBs

Isolated IBs, built up by Bl21-pET-1005 (containing hb*fgf* gene) were analyzed by SDS-PAGE (Fig. 1). The results showed that during the first 3h or so of the induction, at 37 and 42°C, the amount of insoluble hbFGF (IBs) fraction was increased substantially. However, 6 h after induction only trace of insoluble hbFGF fraction could be observed. This dramatic decrease in IBs fraction could be the result of cell death, IB destruction by proteases or cell growth inhibition by over expression of heterologous protein (hbFGF).

Continuing Adding of Ampicillin

Because of inhibition of plasmid free cells division by continual adding of ampicillin to the medium, the amount of soluble and insoluble hbFGF (IBs) fractions were remained unchanged even after 6 h of induction (Fig. 2). This means that the quantity of total protein (TP) and insoluble protein fractions in different periods of time (3, 4.5, and 6 h) do not change. Figure 3 shows the data of OD_{600} for both ampicillin containing culture (Amp^+) and culture with no ampicillin (Amp^-). Alternatively adding of ampicillin inhibits the growth of plasmid-free cell showing no increase in OD_{600}, while the OD_{600} from Amp^- flask shows a drastic increase.

Colony Count

The number of colonies grown on both ampicillin containing and ampicillin lacking plates were plotted and the mean values thereof (Fig. 4) show that the proliferation of hbFGF producing cells was ceased. However the plasmid-free cells kept growing and reached to 5 fold greater than their initial count at the end of the 5th h. Thus, the sample that was taken at hour 5 is 10 fold more in plasmid-free cells number than the plasmid-containing ones. Whereas the sample taken at hour 3 has approximately equivalent count of the both kind of cells. Hence, in the 3rd h, half of cells contain heterologous protein but this ratio decreases to 1/10 at hour 5.

Chloramphenicol Addition

Addition of chloramphenicol 3 h after induction actually caused the both plasmid-containing and plasmid-lacking cell growth halt. Despite of that, quantity of IBs not only did not show any significant drop after 9 h, but also the smooth increase of IBs was observed (Fig. 5). This is in contrast to what is known as IB degradation in which a dramatic diminution of IBs after 3 h should be observed. This result is consistent with other findings in this study.

Conclusion

Production of heterologous proteins by recombinant *E. coli* often interferes with normal host cell metabolism. These foreign proteins are frequently recognized as abnormal and are subjected to proteolytic degradation and/or intracellular aggregation [8]. Formation of inclusion bodies and soluble bFGF occurs simultaneously and starts immediately after temperature shift from 30 to 42°C [5]. In addition, Studier *et al.* [9] observed inclusion bodies formation when production occurred at higher growth temperatures.

Culture of Bl21-pET-1005 cells (hbFGF hormone producing) at 37 and 42°C showed that the IBs formation was considerably raised, and correspondingly the loss of soluble hbFGF fraction (3 h after induction) was decreased. A dramatic loss of IBs particles was observed 6 h post-induction.

It has been proven that misfolded proteins undergo a preferent degradation ruled by the housekeeping bacterial proteolytic system, however upon precipitation as IBs their stability dramatically increases [1]. On the other hand, it has been observed that overexpression of heterologous genes in recombinant *E. coli* is frequently associated with growth inhibition [10,11], ribosome destruction and cell death [12,13]. Temperature shift from 30 to 42°C caused the repression of synthesis of ribosomal proteins [8]. In addition, despite bacterial IBs have been often considered as homogeneous, several independent observations reporting enzymatic activity associated to IB particles [14,15], refolding potential of IB proteins [16,17] and both *in vivo* and *in vitro* reversibility of IB formation [16] indicate an unexpected molecular plasticity within these aggregations. Actually, taking into account the other researchers' results, several reasonable possibilities interpreting the notable decreased IBs should be investigated.

Results of the second experiment, counting the grown colonies on LB-Agar plates, clearly showed that the cells carrying plasmid overexpressing the

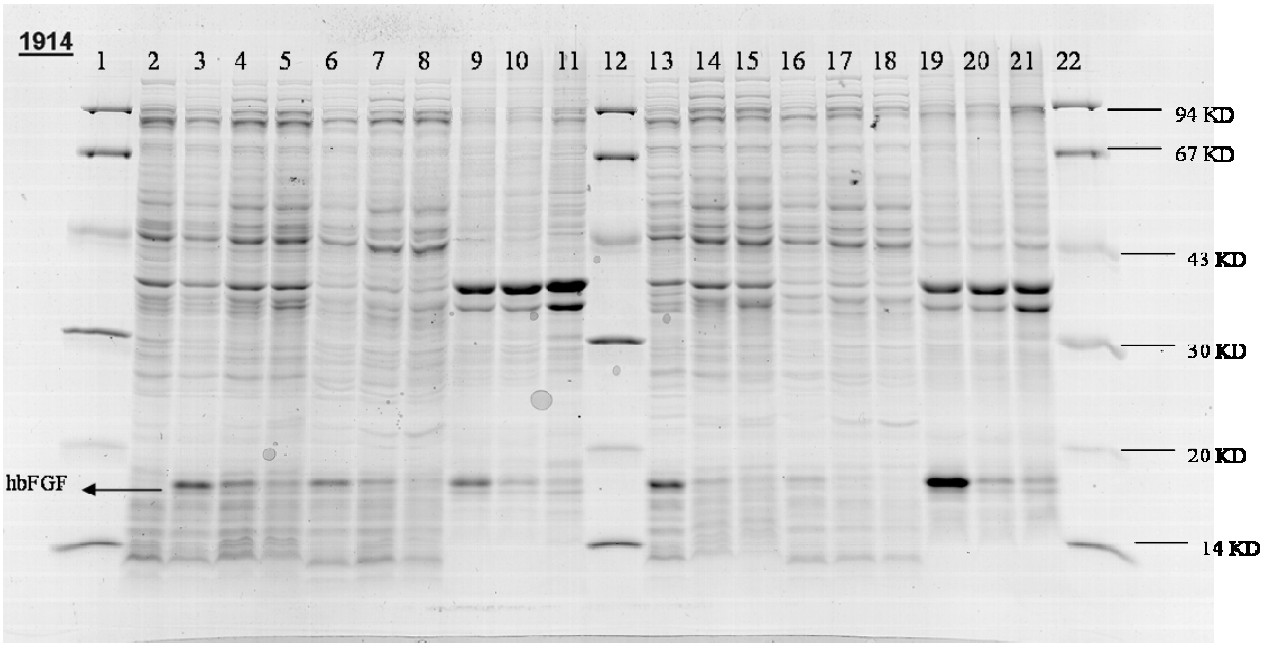

Figure 1. SDS-PAGE (8-16% gradient). The Effect of Different Temperatures (37 & 42°C) on hbFGF Production Level. Lanes 1, 12 & 22: Protein Size Marker. Lane 2: Negative Control. Lanes 3-11: 37°C and Lanes 13- 21: 42°C. Lane 3-5 and 13-15: T.P obtained 3h, 6h and O.N after induction respectively. Lane 6-8 & 16-18: S.P obtained 3h, 6h and O.N after induction respectively . Lane 9-11 & 19-21: I.P obtained 3h, 6h and ON after induction respectively.

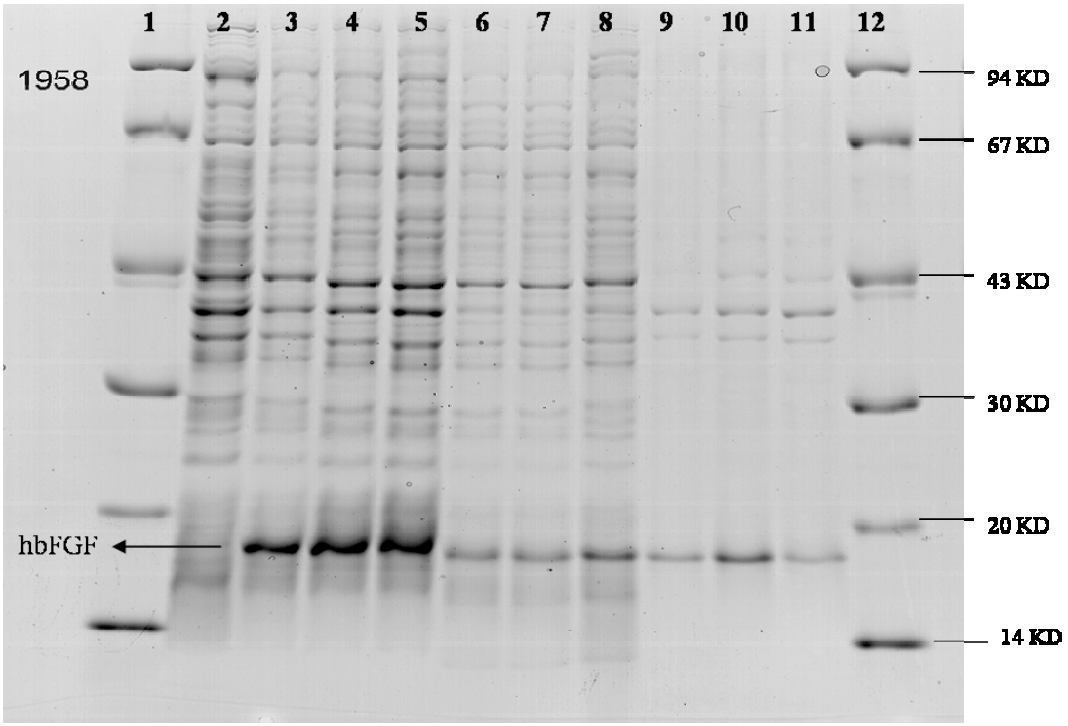

Figure 2. Gradient (8-16%) SDS-PAGE. Adding ampicillin to medium every 30 min at 42°C**.** Lanes 1 and 12: Protein Size Marker. Lane 2: Negative Control. Lanes 3-5, 6-8 & 9-11: T.P, S.P & I.P obtained 3, 4.5 and 6 h after induction respectively.

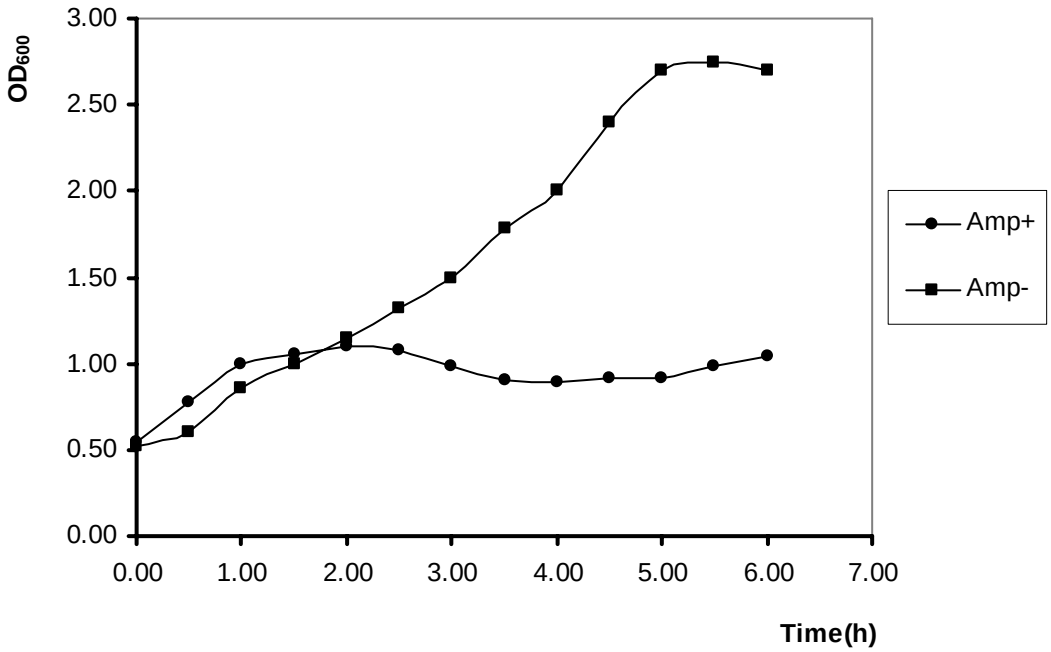

Figure 3. Optical density (OD) measurement of two cultures Amp^+ and Amp^- every half an hour at 42°C after induction by IPTG. Amp^+: continuous addition of ampicillin every half an hour. Amp^-: without ampicillin.

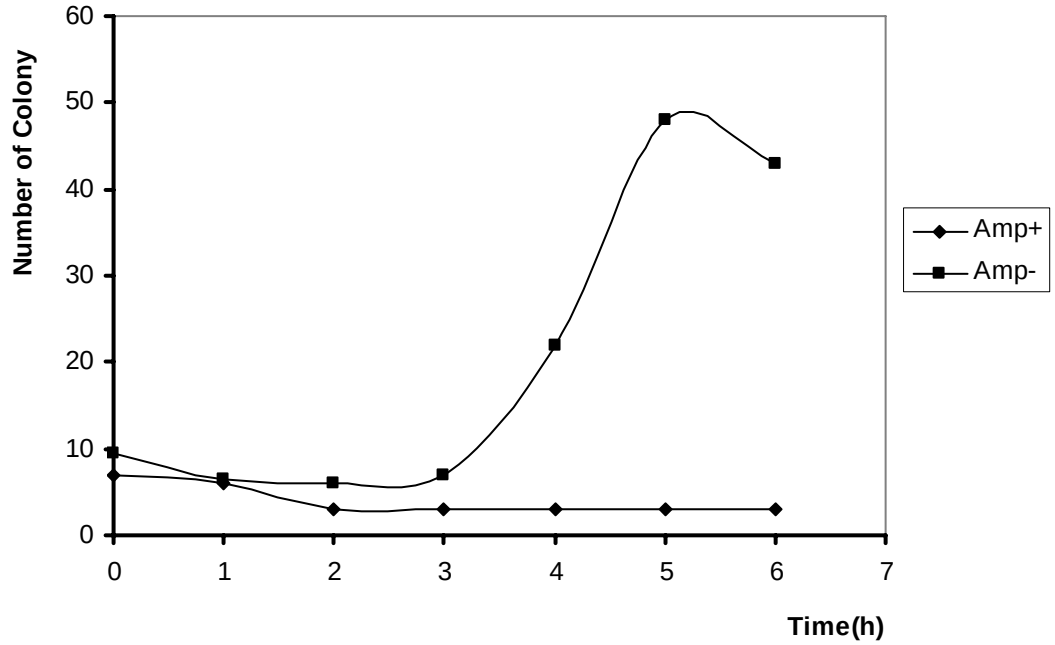

Figure 4. Number of colonies were obtained from hourly growth of culture on both ampicillin containing plates (Amp^+) and ampicillin lacking ones (Amp^-).

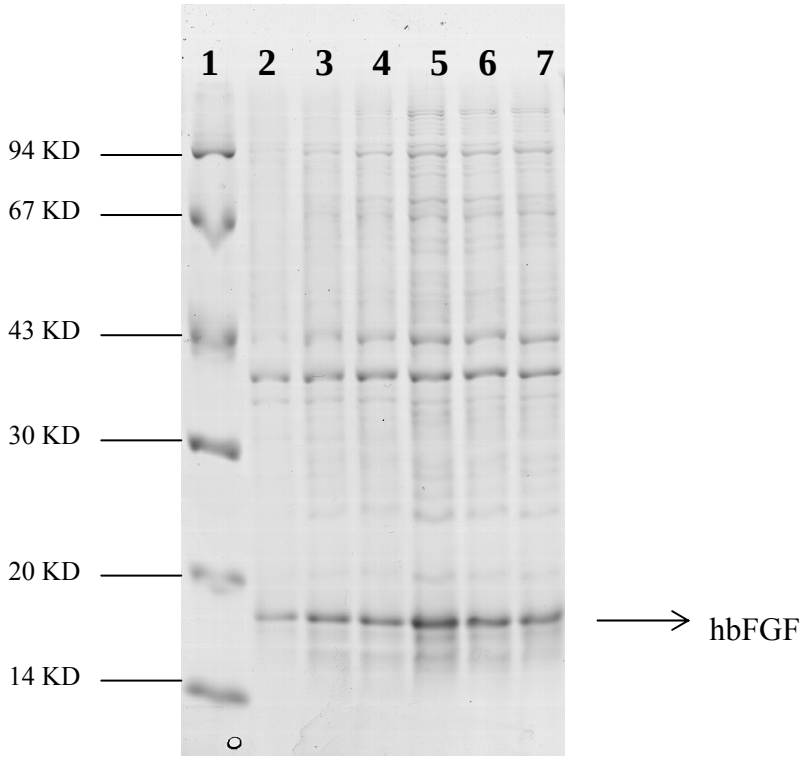

Figure 5. Gradient (8-16%) SDS-PAGE. Adding chloramphenicol to medium 3 h after induction**.** Lane 1: Protein size marker. Lanes 2-7: I.P obtained 3, 4.5, 6, 7.5, 9 and 10.5 h after induction respectively.

heterologous gene have not been subjected to death. Whereas the growth of hbFGF producing cells halted. Similar results were reported by the others [10,11,18]. Inhibition of cell growth may be so strong that immediate cell death occurs resulting from a direct toxicity of the heterologous gene product. Cell growth halt might be caused by diminished or stopped normal cell protein production, as observed by Vind *et al.* [19]. In addition, a reduction of the synthesis rate of ribosomal proteins has been observed in response to bFGF production [8].

Results from continuous addition of ampicillin and chloramphenicol also suggest that IBs were not degraded by proteases.

While it is widely recognized that IB proteins are stable against cell proteases [17], there are numerous papers with contradictive results. Recent studies on protein refolding reveal a deep implication of chaperones activity in the separation of aggregated polypeptide chains [20,21], proving a significant susceptibility of insoluble polypeptides to solubilization. In recombinant bacteria, the arrest of protein synthesis allows the almost complete disintegration of bacterial IBs [16].

Altogether, in our study, there are strong indications that the decrease of insoluble heterologous hbFGF (IBs) in response to temperature upshift from 30°C to 42°C results from the inhibition of hbFGF producing cell growth (after 3 h of induction), simultaneous dramatic continuing proliferation of plasmid-free cells and, consequently, severe reduction of plasmid containing to plasmid-free cells ratio. This cell growth inhibition certainly results from reduction of normal cell protein production, due to overexpression of heterologous protein and temperature augmentation. However, it's not reasonable to completely exclude the other interfering factors within a cell such as proteases that are involved in heat shock response.

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