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# High-level Production of Recombinant Human IFN-α2a with Co-expression of $tRNA^{Arg(AGG/AGA)}$ in High-cell-density Cultures of *Escherichia coli*

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**Abstract** The co-expression of the *arg*U gene in a double-vector expression system of recombinant *Escherichia coli* BL21(DE3)[pET-IFN2a+pAC-argU] significantly enhanced the production level of recombinant human interferon-α2a (rhIFN-α2a) in high cell density cultures, compared to a recombinant *E. coli* culture containing only the single expression vector, pET-IFN2a. The dry cell mass concentration increased to almost 100 g/L, and more than 4 g/L of rhIFN-α2a was accumulated in the culture broth. Evidently, the synthesis of rhIFN-α2a was strongly dependent on the pre-induction growth rate and more efficient at a higher specific growth rate. The additional supply of $tRNA^{Arg(AGG/AGA)}$ enhanced the expression level of the rhIFN-α2a gene in the early stage of the post-induction phase, yet thereafter the specific production rate of rhIFN-α2a rapidly decreased due to severe segregational instability of plasmid vector pET-IFN2a. It would appear that the plasmid instability, which only occurred to pET-IFN2a in the double vector system, was related to the effect of translational stress due to the overexpression of rhIFN-α2a.

*Keywords*: codon usage, co-expression of *arg*U gene, *Escherichia coli*, high-cell-density cultures, human interferon-α2a, plasmid instability

## INTRODUCTION

Foreign gene expression in recombinant *E. coli* results from the interplay of many factors that can be largely classified into genetic micro-factors (*e.g.* promoter strength, gene sequence structure in the vicinity of the ribosome binding site, codon usage, plasmid stability and copy number, mRNA stability and transcriptional efficiency, etc.) and environmental macro-factors (*e.g.* specific growth rate, pH, temperature, medium composition, feed strategy, induction method, etc.) [1-3]. Sometimes a high content of *E. coli* rare codons in human genes has been reported to cause a low expression level of recombinant human proteins (*e.g.* tissue-type plasminogen activator, pro-urokinase, granulocyte-macrophage colony stimulating factor and gp41 or HIV) in *E. coli* [4,5]. Human IFN-α2a consisting of 165 amino acids also contains 9 arginine residues encoded by the *E. coli* low-usage codons AGG or AGA, which are the least used codons in *E. coli* [6]. The minor arginine $tRNA^{Arg(AGG/AGA)}$ encoded by the *arg*U gene has been shown to be a limiting factor in the translation of rhIFN-α2a in *E. coli* [7].

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Human IFN-α2a is one of 15 different IFN-α subtypes that are closely related proteins exhibiting antiviral, antiproliferative, and immunomodulatory activities [8]. As such, based on its biological properties, hIFN-α has recently been developed as a therapeutic for the treatment of human hepatitis B. This article reports that a high-level production of human IFN-α2a was facilitated in the recombinant *E. coli* cultures with a high cell concentration via the simultaneous expression of the *arg*U gene to supplement the minor arginine $tRNA^{Arg(AGG/AGA)}$.

## MATERIALS AND METHODS

### Recombinant *E. coli* Strains

The cloning of human IFN-α2a and *E. coli arg*U genes and the co-transformation of *E. coli* BL21(DE3) with the two plasmids, pET-IFN2a and pAC-argU have already been described in detail in a previous report [9].

### Media and Bioreactor Operation for High Cell Density Culture and Recombinant Gene Expression

The preparation of stock cultures and an inoculum

for each bioreactor experiment was the same as described in a previous report [10,11]. The synthetic medium used for the initial batch cultures contained per liter, (a) 13 g $KH_2PO_4$, 4 g $(NH_4)_2HPO_4$, 1.7 g citric acid, 20 mL trace metal solution [17], 0.1 g thiamine-HCl; (b) 1.2 g $MgSO_4.7H_2O$; and (c) 20 g glucose. Components a, b, and c were autoclaved separately and 200 mg of each of ampicillin and chloramphicol was added per liter through a syringe filter (0.2 μm). For the growth-phase fed-batch operation, a glucose-salt synthetic medium was used as the feed with the addition of ammonium hydroxide as both the nitrogen source and the pH-regulator. The feed media used for the recombinant protein synthesis contained per liter, (a) 1.5 g $(NH_4)_2SO_4$, 211 g yeast extract; (b) 1 g $MgSO_4.7H_2O$; and (c) 274 g glucose. The each feed media contained 1 g of each of ampicillin and chloramphicol per liter and components a, b, and c were also autoclaved separately.

All batch and fed-batch bioreactor experiments were conducted in 5-L Bioflo III laboratory fermenters (New Brunswick Scientific, USA). In the growth (or pre-induction) phase of the fed-batch operation, all recombinant strains were grown at a specific growth rate, 0.05 or 0.15 $h^{-1}$ via the exponential feeding of glucose-salt defined media. Ammonium hydroxide was added to the culture as both the nitrogen source and the pH regulator only in the growth phase. When the cell concentration reached around 40 g/L, the recombinant gene expression was initiated with the addition of IPTG (3.0 mmoles per g cell). The post-induction medium feed was determined depending on the bacterial cell mass in the bioreactor using the following correlation: $F = (0.45) \cdot XV$ where $F$, $X$, and $V$ represent the volumetric medium feed rate (mL/h), recombinant cell mass concentration (g/L), and culture volume (L), respectively. Unless otherwise mentioned, the bioreactors were operated at pH 6.75 (only in the growth phase) and 37°C. The dissolved oxygen was controlled above 40% air saturation to avoid oxygen limitation. The data acquisition and control of various operating parameters (e.g. temperature, dissolved oxygen concentration, pH, rpm, and medium flow rate) were effectively achieved by using the Bioflo III's electronic control module and software, AFS (Advanced Fermentation System).

### Analytical Methods

The glucose concentration was measured using a YSI glucose analyzer (model 2300 STAT PLUS, Yellow Springs Instruments, Yellow Springs, OH, USA). The acetic acid and ammonia concentrations were measured by employing an acetate (Boehringer Mannheim) and ammonia (Sigma) assay reagent as per the procedure suggested by the supplier. The optical density of each culture sample was measured at 600 nm using a Pharmacia Ultrospec III spectrophotometer. The *E. coli* cell mass concentration was then obtained using a previously developed correlation between the optical density and the dry cell mass concentration (*i.e.* dry cell mass concentration (g/L) = 0.417 $OD_{600}$).

The expression level of recombinant hIFN-α2a (rhIFN-α2a) was measured by subjecting samples taken from the fermenter to denaturing gradient sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (10-20% tricine gel, NOVEX™, San Diego, USA). 3 μg of bovine serum albumin (BSA) was separately loaded on each gel with culture samples as the standard for protein quantification. The resulting protein bands were scanned with a laser densitometer Ultroscan XL (Pharmacia LKB Biotechnology, Sweden). From this analysis, the percentage of recombinant protein from the total cellular protein was determined, and the amount of rhIFN-α2a produced was also calculated from a previously developed correlation between the BSA concentration and the measured band area [13]. A non-reducing SDS-PAGE with inclusion bodies separated from the cell lysates was also conducted to estimate the molecular interaction of the recombinant protein aggregates.

The plasmid stability was determined in the post-induction cultures growing at a high growth rate (*i.e.* 0.15 $h^{-1}$). After being properly diluted, culture samples taken from the bioreactor were transferred onto LB-agar plates ($amp^-$) and incubated at 37°C for 12-15 h. Fifty colonies were randomly picked, aseptically transferred onto LB-agar plates (containing 100 mg ampicillin/L), and incubated at 37°C for 12-15 h. The plasmid stability was estimated on the basis of the number of colonies formed on the $amp^+$ LB-agar plates.

## RESULTS AND DISCUSSION

### High-Level Production of rhIFN-α2a in High-Cell-Density Cultures with Co-expression of *arg*U Gene

Fig. 1(a) shows that the expression level of recombinant hIFN-α2a was extremely low in the fed-batch cultures of *E. coli* BL21(DE3)[pET-IFN2a] at high cell density. The production of recombinant proteins in *E. coli* can be affected by many cellular factors, such as the gene sequence structure, mRNA stability and translational efficiency, folding efficiency, degradation of recombinant protein by host proteases, codon usage, toxicity of heterologous protein to the host, etc. [1-3,12]. Among the factors described above, codon usage was considered to be responsible for the low-level production of rhIFN-α2a (Fig. 1(a)) [7,9], because there is a significant difference between the rhIFN-α2a and native *E. coli* codons. As presented in Table 1, human IFN-α2a contains 9 arginine residues encoded by AGG or AGA codons which are the least used in *E. coli*. To enhance the translational efficiency of rhIFN-α2a in *E. coli*, Jeong and Shin [9] introduced the *arg*U gene into a multicopy plasmid pACYC184, which is compatible with a pET-3a vector, as a result, the $tRNA^{Arg(AGG/AGA)}$ was expressed together with rhIFN-α2a in the same recombinant cell. In the high-cell-density culture of recombinant *E. coli* BL21(DE3) [pET-IFN2a+pAC-argU], the much higher expression level of rhIFN-α2a was proba-

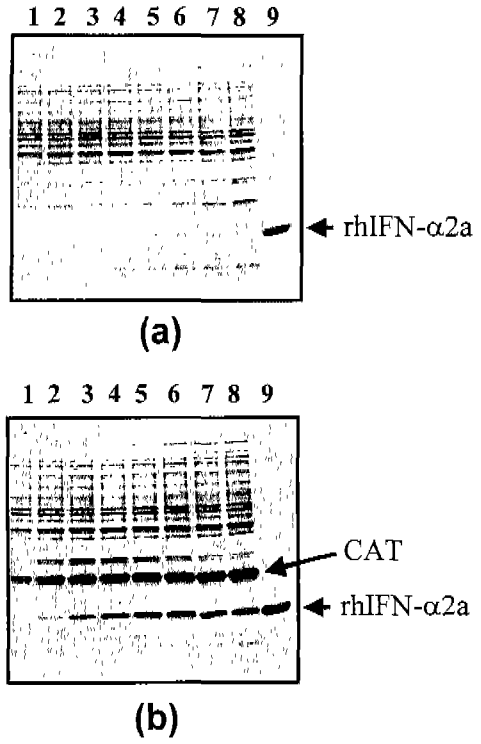


**Fig. 1.** (a) Time-course analysis of recombinant gene expression by reducing SDS-PAGE, using whole cell lysate samples from post-induction fed-batch cultures of recombinant *E. coli* BL21(DE3)[pET-IFN2a]. (lanes 1-8, culture samples taken at 0.0, 2.3, 5.5, 8.5, 11.8, 14.5, 17.5, and 20.5 h post-induction time, respectively; lane 9, standard hIFN-α2a), (b) Time-course analysis of recombinant gene expression by reducing SDS-PAGE, using whole cell lysate samples from post-induction fed-batch cultures of recombinant *E. coli* BL21(DE3)[pET-IFN2a+pAC-argU] (lanes 1-8, culture samples (10 μL) taken at 0.0, 3.3, 6.3, 9.5, 12.3, 15.3, 18.3, and 21.3 h post-induction time, respectively; lane 9, standard hIFN-α2a; CAT: chloramphicol transferase).

bly facilitated due to the enhanced translational activity as a result of the help of the supplemented minor $tRNA^{Arg(AGG/AGA)}$ (Fig. 1(b)), as compared to the recombinant *E. coli* culture containing only the single expression vector, pET-IFN2a (Fig. 1(a)). The same feeding strategies were applied to both fed-batch experiments, where the pre-induction growth rate was controlled at 0.15 $h^{-1}$, and the post-induction feed rate was determined according to the correlation defined in Materials and Methods. The dry cell mass concen-tration increased to around 100 g/L, and more than 4 g/L of recombinant hIFN-α2a was accumulated in the culture broth with the co-expression of the *arg*U gene.

From Fig. 2 (lanes 1-4), it is evident that the rhIFN-α2a was expressed as insoluble inclusion bodies. In the SDS-PAGE gel under reducing condition, rhIFN-α2a appeared as a single band (lanes 1 and 3 in Fig. 2). How ever, when the same culture samples were analyzed by

**Table 1.** Codons used by *E. coli* at frequency of < 1%

| Rare Codons | Encoded amino acid | Frequency per 1000 codons | Number found in hIFN-α2a |
|---|---|---|---|
| AGG/AGA | Arg | 1.5/2.6 | 4/5 |
| CGA | Arg | 3.6 | 0 |
| CUA | Leu | 3.9 | 2 |
| AUA | Ile | 5.0 | 1 |
| UGU | Cys | 5.2 | 3 |
| CCC | Pro | 5.2 | 3 |
| CGG | Arg | 5.4 | 0 |
| UGC | Cys | 6.4 | 4 |
| CCU | Pro | 7.1 | 3 |
| UCA | Ser | 7.6 | 6 |
| ACA | Thr | 7.7 | 5 |
| GGA | Gly | 8.3 | 2 |
| CCA | Pro | 8.4 | 0 |
| UCG | Ser | 8.6 | 0 |
| UCC | Ser | 8.9 | 2 |
| AGU | Ser | 8.9 | 3 |
| UCU | Ser | 9.3 | 9 |
| ACU | Thr | 9.5 | 4 |
| CAC | His | 9.9 | 1 |

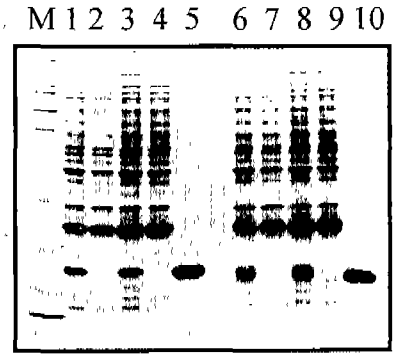


**Fig. 2.** Reducing (lanes 1-5) and non-reducing (lanes 6-10) SDS-PAGE analyses of post-induction culture samples from the high-cell-density fed-batch culture ($FB_B$ in Table 2) of recombinant *E. coli* BL21(DE3)[pET-IFN2a+pAC-argU]. The culture samples were taken at 12.3 h (lanes 1,2,6,7) and 18.3 h (lanes 3,4,8,9) post-induction time. After the cell disruption of each culture sample, both the total cell lysate (lanes 1,3,6,8) and the supernatent (lanes 2,4,7,9) were analyzed by a reducing and non-reducing SDS-PAGE (M, protein size marker; lanes 5 and 10, standard rhIFN-α2a).

non-reducing SDS-PAGE, the rhIFN-α2a bands were significantly smeared, *i.e.* became much broader yet less intensive (lanes 6 and 8 in Fig. 2). Since the electrophoretic migration of the other (host) proteins was exactly the same in both the reducing and non-reducing gels, the appearance of the smeared rhIFN-α2a bands in the non-reducing gel did not appear to be due to covalent multimers formed between rhIFN-α2a and the *E. coli* native proteins. Jeong and Shin [9] reported that several kinds of variants, such as partially oxidized, a native

IFN-α2a, and covalent oligomers, were formed during the refolding process of denatured rhIFN-α2a. The formation of inclusion bodies (*i.e.* off-pathway aggregation) is known to result from the mis-folding of the folding intermediates. Therefore, it would seem more reasonable to presume that the dispersed rhIFN-α2a band in the non-reducing gel resulted from heterogeneity in the reduction/oxidation status of mis-folded rhIFN-α2a. Since human IFN-α2a has two disulfide bonds (Cys1-Cys98 and Cys29-Cys138), four different reduced/oxidized forms can be produced in the course of the intramolecular S-S bond formation of rhIFN-α2a, *i.e.* fully reduced, fully oxidized, and two partially reduced (or oxidized) forms. Considering the possibility of mis-matched disulfide bonds in the fully and partially oxidized forms of rhIFN-α2a, aggregated rhIFN-α2a may include a substantial level of complex heterogeneity in terms of its reduction/oxidation status.

### Effect of Pre-induction Growth Rate on Production of rhIFN-α2a and Plasmid Stabilities in Fed-batch Cultures

In the pre-induction phase of the fed-batch cultures, the specific growth rate of recombinant *E. coli* was well controlled using the exponential feed method (Materials and Methods). The post-induction medium feed was determined based on *E. coli* cell mass in the bioreactor using the following correlation: $F = (0.45) \cdot XV$ where $F$, $X$, and $V$ represent the volumetric medium feed rate (mL/h), recombinant cell mass concentration (g/L), and culture volume (L), respectively. Table 2 shows the results of the rhIFN-α2a production in the two fed-batch cultures ($FB_A$ and $FB_B$), which differed in their pre-induction growth rate, i.e. $\mu_A$ and $\mu_B$ being 0.05 and 0.15 $h^{-1}$, respectively. After the gene expression was induced, the specific growth rate was maintained within almost the same range between 0.04 and 0.08 $h^{-1}$ in both experiments. During the early period of the post-induction phase, the specific production rate of rhIFN-α2a (g rhIFN-α2a synthesized per g cell per hour) in the fed-batch culture $FB_B$ was about three times higher compared to the fed-batch culture $FB_A$. As previously discussed by Shin *et al.* [13] concerning the production of a recombinant human growth hormone and glucagon in *E. coli*, the strong dependence of rhIFN-α2a production on the specific growth rate is presumed to be related to the effect of the ribosomal machinery on the rate of protein synthesis. The ribosome concentration within cells is known to increase with an increasing specific growth rate [14], which allows the cells to synthesize proteins faster, thereby maintaining constant internal concentrations of growth and maintenance proteins. In the present study, the specific growth rate was increased in the pre-induction phase, and it is reasonable to assume that the ribosomal machinery facilitating a faster protein production was already activated even before the recombinant gene expression, which would explain the rapid production of rhIFN-α2a in the early stage of the post-induction phase.

In the fed-batch culture $FB_B$, the specific rhIFN-α2a production rate rapidly decreased to a far lower value soon after reaching the maximum rate (Table 2). From the results presented in Table 3, the plasmid vector harboring the rhIFN-α2a gene (*i.e.* pET-IFN2a) suffered from severe segregational instability in the post-induction fed-batch cultures of recombinant *E. coli* BL21(DE3)[pET-IFN2a($amp^+$)+pAC-argU($cm^+$)] even when ampicillin was supplied through the feed medium. For the first 6 h after induction, the average supply rate of ampicillin was 46 mg/h, meanwhile the total cell mass increased from 92 to 135 g during the same period. Probably the degradation rate of ampicillin was higher than the slow addition rate of ampicillin in the high cell density cultures, and hence the growth of pET-IFN2a-negative *E. coli* cells was not effectively repressed. Table

**Table 2.** Results of rhIFN-α2a production in two fed-batch cultures of recombinant *E. coli* BL21(DE3)[pET-IFN2a+pAC-argU], $FB_A$ and $FB_B$ differing in their pre-induction specific growth rate (μ), *i.e.* $\mu_A$ and $\mu_B$ at 0.05 and 0.15 $h^{-1}$, respectively

| Fed-batch culture | Post-induction time (h) | Dry cell mass concentration (g/L) / specific cell growth rate ($h^{-1}$) | rhIFN-α2a concentration in culture broth (g rhIFN-α2a / L) | Specific production rate of rhIFN-α2a (g rhIFN-α2a / g dry cell mass / h) |
|---|---|---|---|---|
| $FB_A$ | 0.0 | 37 / 0.045 | 0.2 | |
| | 3.0 | 42 / 0.055 | 0.4 | 0.011 |
| | 7.0 | 50 / 0.067 | 1.3 | 0.020 |
| | 11.0 | 61 / 0.075 | 2.5 | 0.021 |
| | 13.5 | 68 / 0.065 | 2.7 | 0.014 |
| | 16.0 | 75 / 0.057 | 3.0 | 0.023 |
| | 18.0 | 78 / 0.044 | 2.7 | 0.016 |
| $FB_B$ | 0.0 | 42 / 0.134 | 0.5 | |
| | 3.3 | 50 / 0.068 | 0.8 | 0.010 |
| | 6.3 | 55 / 0.053 | 2.4 | 0.069 |
| | 9.5 | 63 / 0.068 | 4.1 | 0.051 |
| | 12.3 | 69 / 0.065 | 4.1 | 0.010 |
| | 15.3 | 79 / 0.079 | 4.1 | 0.008 |
| | 18.3 | 90 / 0.080 | 4.2 | 0.009 |
| | 21.3 | 99 / 0.070 | 3.6 | 0.003 |

**Table 3.** Estimation of segregational plasmid stabilities in two post-induction fed-batch cultures of recombinant *E. coli*, BL21(DE3)[pET-IFN2a+pAC-argU] and BL21(DE3)[pET-IFN2a], respectively

| Recombinant *E. coli* culture | Post-induction time (h) | Number of colonies transferred from antibiotic-free to antibiotic (ampicillin or chloramphenicol)-containing LB agar plate | Number of colonies formed on | |
|---|---|---|---|---|
| | | | Ampicillin + LB agar plate | Chloramphenicol + LB agar plate |
| BL21(DE3) [pET-IFN2a] | −4.5 | 50 | 50 | − |
| | 0.0 | 50 | 50 | − |
| | 8.5 | 50 | 49 | − |
| BL21(DE3) [pET-IFN2a + pAC-argU] | −6.0 | 50 | 48 | 50 |
| | 0.0 | 50 | 46 | 50 |
| | 8.3 | 50 | 3 | 42 |

3 shows that in the post-induction culture of recombinant *E. coli* BL21(DE3)[pET-IFN2a], the segregational instability of plasmid pET-IFN2a never occurred. The same growth conditions, including the medium composition and feed and DO control strategies were applied to both recombinant *E. coli* strains (BL21(DE3)[pET-IFN2a+pAC-argU] and BL21(DE3)[pET-IFN2a]). Therefore it would appear that the growth conditions, known to induce plasmid loss such as nutrient and oxygen starvation [15,16] did not result in the segregational instability of the pET-IFN2a vector. Recently, it has been reported that high specific production rate of recombinant hIL-2 induced significant translational stress in a *rec*A$^+$ *E. coli* host and hence severe plasmid instability [11]. As evidenced in this study, the additional supply of $tRNA^{Arg(AGG/AGA)}$ enhanced the translational activity of the rhIFN-α2a gene in the early stage of the post-induction phase, yet thereafter the specific production rate of rhIFN-α2a rapidly decreased due to the severe plasmid instability of pET-IFN2a. Seemingly, the segregational instability, which only occurred to plasmid pET-IFN2a in the double-vector system, was related to the effect of translational stress resulting from the overexpression of rhIFN-α2a. Accordingly, on the basis of the above presumption, to further increase the production level of rhIFN-α2a, a process for modulating *argU* gene expression needs to be developed to prevent the plasmid instability and hence the decrease in the specific production rate of rhIFN-α2a.

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