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Modulation of the Tendency Towards Inclusion Body Formation of Recombinant Protein by the Addition of Glucose in the *araBAD* Promoter System of *Escherichia coli*

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Abstract We attempted to modulate the overall protein expression rate through the addition of a repressor against the *araBAD* promoter system of *Escherichia coli*, in which glucose was used as a repressor. Therefore, 0.5% L-arabinose was initially contained as an inducer in culture medium, and either 2% glucose or 2% glycerol was used as a carbon source, and it was found that the expression of recombinant interferon-α could be observed at the beginning of the batch culture when glycerol was used as a carbon source. However, when glucose was used, the initiation of recombinant interferon-α expression was delayed compared with that when glycerol was used. Furthermore, when the addition of 0.5% glucose was carried out once or twice after 0.5% L-arabinose induction during DO-stat fed-batch culture, the distributions of soluble and insoluble recombinant interferon-α were modulated. When glucose was not added after the induction of L-arabinose, all of the expressed recombinant interferon-α formed an inclusion body during the later half of culturing. However, when glucose was added after induction, the expressed recombinant interferon-α did not all form an inclusion body, and about half of the total recombinant interferon-α was expressed in a soluble form. It was deduced that the addition of glucose after the induction of L-arabinose might lower the cAMP level, and thus, CAP (catabolite activator protein) might not be activated. The transcription rate of recombinant interferon-α in the *araBAD* promoter system might be delayed by the partial repression. This inhibition of the transcription rate probably resulted in more soluble interferon-α expression caused by the reduction of the protein synthesis rate.

Keywords: Modulation of transcription, glucose, *araBAD* promoter, soluble protein expression

It has been reported that the *araBAD* promoter system in *Escherichia coli* (*E. coli*) acts to control the amount of protein expressed by L-arabinose in culture medium [5, 16, 17, 22]. Moreover, the *araBAD* promoter is known to be tightly regulated by the activation and deactivation of two positive regulators, as AraC and catabolite activator protein (CAP) [11, 13, 24, 27, 31]. Although catabolite repression involving CAP has been found in many microorganisms [30], including *E. coli* [3, 26], it has been found that dual positive regulators are involved in the sophisticated control of the *araBAD* promoter system. It was recently demonstrated that the expression level of soluble recombinant protein in *E. coli* could be improved by the modulation of the transcription rate [2, 16, 23]. In these works, the authors adjusted the amount of inducer and/or varied the growth rate in order to modulate transcription. Another example of the modulation of transcription was the expression of recombinant interferon-α at suboptimal temperatures, in which the improvement of soluble protein expression was observed through the inhibition of the overall protein synthesis rate [7]. In this work, we attempted to modulate the overall protein expression rate through the addition of a catabolite repressor, such as glucose, against the *araBAD* promoter system. Because the human-originated heterologous proteins were frequently expressed in the inclusion body [12, 15, 19, 20, 25], it was necessary to modulate the tendency towards inclusion body formation in order to improve the soluble expression level. During this work, we expected that cAMP would be downregulated, and thus, the work of CAP as a positive regulator would be partially inhibited.

In this study, *Escherichia coli* (*E. coli*) MC1061 (F^- *araD139 Δ(ara-leu) 7696 galE15 galK16 Δ(lac) X74 rpsL* (Str^r) *hsdR2* ($r_k^-m_k^-$) *mcrA mcrB1*) that cannot metabolize L-arabinose was used as a host for expression. We used a recombinant *E. coli* in which the expression of human interferon-α was controlled by the *araBAD* promoter. The detailed vector construction procedures have been described previously [14]. Seed culture was performed in a 250-ml Erlenmeyer flask with a culture volume of 100 ml of

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fortified Luria Broth (FLB) medium (tryptone, 10 g/l; yeast extract, 20 g/l; NaCl, 5 g/l; ampicillin, 100 μg/ml) in a shaking incubator for 12 h. Temperature and shaking speed were controlled at 37°C and 150 rpm, respectively. Batch culture and DO-stat fed-batch culture were carried out in a 5.0-l jar fermentor (KoBiotech, Republic of Korea) with an initial volume of 1.5 l. The medium composition of the batch culture was glycerol, 20 g/l; yeast extract, 20 g/l; KH_2PO_4, 2.31 g/l; and Na_2HPO_4, 10.22 g/l. Feed medium consisted of glycerol, 400 g/l; yeast extract, 300 g/l; and $MgSO_4 \cdot 7H_2O$, 5.76 g/l. Ampicillin was added to both media at a concentration of 100 μg/ml. The initial agitation speed and aeration rate were 700 rpm and 1.5 vvm, respectively. Through manual control of the agitation speed and aeration rate, the dissolved oxygen level was maintained so that it did not fall below 20%. The pH was maintained at 7.0 using phosphoric acid solution (10%, v/v) as the acid and ammonia water as the base. After all carbon sources in the initial culture medium were depleted, the feed medium was repeatedly introduced into a fermentor using a DO-stat fed-batch mode in which the DO set-point was 40%. For further analysis, the cell pellets from each sampling were collected by microcentrifugation, in which an optical density of sample broth at 600 nm (OD_{600}) was adjusted to a constant OD_{600} of 2.0 ml with PBS (phosphate-buffered saline), and then 1.5 ml of this cell suspension was microcentrifugation for the preparation of cell pellets. These cell pellets contained a constant amount of cells and were stored at −20°C until Western blot analysis. The culture supernatants were also stored at −20°C for the residual glycerol and glucose analyses.

The cell pellet was disrupted by a nonmechanical method using B-PER II bacterial protein extraction reagent (Pierce, U.S.A.). A mixed solution of both 5 ml of B-PER II reagent and 50 μl of DNase I (Sigma) solution (1 mg/ml) was used in this study. Unless otherwise stated, B-PER II reagent indicates the mixed solution of both B-PER II reagent and DNase I solution. Each cell pellet was mixed with 150 μl of B-PER II reagent, and the mixture was vortexed vigorously and/or pipetted up and down. The soluble fraction was then obtained from the supernatant by microcentrifugation at 13,000 rpm for 5 min, and the insoluble fraction was prepared by resuspending the disrupted cell pellet into 150 μl of B-PER II reagent. Other details for the preparation of soluble and insoluble fractions are described in the instruction manual of the B-PER II bacterial protein extraction reagent (Product number 78260; Pierce). Prior to Western blot analysis, 15% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a SE250 mini-vertical gel electrophoresis unit (Amersham Biosciences). The loading volumes of standard interferon-α and soluble or insoluble fractions were 5 μl and 15 μl, respectively. The protein bands on the gel were transferred to nitrocellulose membranes (Hybond-C Extra; Amersham Biosciences) using a TE22 mini-tank transfer unit (Amersham Biosciences). Mouse monoclonal anti-human interferon-αA (AHC4114; Biosource) as the first antibody and anti-mouse IgG-alkaline phosphatase conjugate (A-4312; Sigma) as the second antibody were used at a dilution of 1:2,000. The nitrocellulose membranes were then stained with BCIP/NBT phosphatase substrate (1-component) (KPL, U.K.). The details of SDS-PAGE and the Western blot procedure have been described previously [14]. The standard human recombinant interferon-α was kindly supplied by Dong-A Pharmaceutical Co. (Republic of Korea) at a concentration of 0.168 mg/ml, and images of the Western blot were analyzed for the measurement of the amount of expressed recombinant interferon-α, for which AlphaEaseFC software (Alpha Innotech, U.S.A.) was used. Cell growth was monitored *via* measurement of OD_{600}, which was performed with a spectrophotometer (Optizen 2120UV; Mecasys, Republic of Korea). Residual glycerol in culture medium was analyzed using a free glycerol determination kit (F6428; Sigma). Residual glucose was determined by *o*-toluidine reagent (T1199; Sigma), in which the assay procedure was performed according to a protocol described for 96-well microplates, which originated from Bioassay Systems (CA, U.S.A.).

Two batch cultures were performed in order to demonstrate the effect of glucose on interferon-α expression. These cultures were performed in a condition in which 0.5% L-arabinose was initially contained as an inducer in the culture medium, and either 2% glucose or 2% glycerol was used as a carbon source. The profiles of cell growth and carbon source consumption are shown in Fig. 1, and indicate that glucose was consumed more rapidly than glycerol, whereas the cell growths were very similar. When glycerol was used as a carbon source, the expression of recombinant

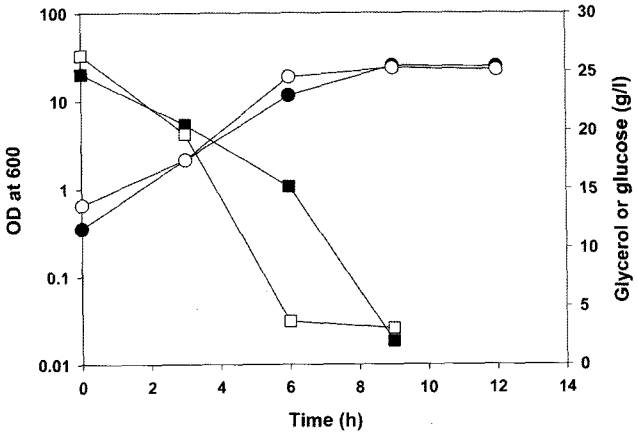

Fig. 1. Profiles of cell growth and carbon source consumption of the batch culture for recombinant interferon-α expression.
Glycerol (2%) or glucose (2%) was used as a carbon source, and L-arabinose (0.5%) was also added initially as an inducer. Cell growths are symbolized by ● (glycerol) and ○ (glucose), and carbon source consumptions are symbolized by ■ (glycerol) and □ (glucose).

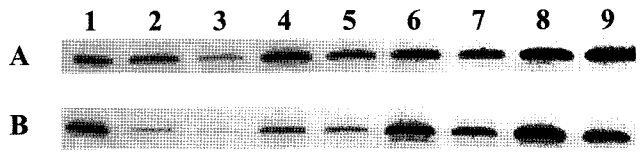

Fig. 2. Western blot analyses of the soluble and insoluble fractions of total cell lysate from Fig. 1.
The nine samples are as follows: lane 1, standard recombinant interferon-α; lanes 2 and 3, soluble and insoluble fractions at an elapsed time of 3 h; lanes 4 and 5, those at 6 h; lanes 6 and 7, those at 9 h; lanes 8 and 9, those at 12 h. Data in **A** and **B** are from the cultures containing glycerol and glucose as a carbon source, respectively.

interferon-α was observed at the beginning of the batch culture (Fig. 2A). However, when glucose was used, the initiation of recombinant interferon-α expression was delayed compared with that when glycerol was used (Fig. 2B). Moreover, its expression level was considerably lower during the early phase of culture, at an elapsed time of 3 and 6 h. The distributions of soluble and insoluble recombinant interferon-α expression were found to be almost the same in those two batch cultures. In the *araBAD* promoter system, both AraC and CAP work as positive regulators, and their activations are necessary for maximum transcription of the *araBAD* promoter to occur. The modulations of transcription of the *araBAD* promoter system were previously observed to be influenced by glucose [5, 30] and D-fucose (L-arabinose analog) [17]. Glucose is able to tightly modulate and control the *araBAD* promoter as a catabolite repressor against CAP, and D-fucose acts against AraC in a similar manner in competition with L-arabinose. In addition, some reports have described the modulation of transcription by transient repression [17, 29], in which the transient addition of glucose in the middle of the culture period led to a transient decrease in the protein expression rate. Although glucose added at the beginning of batch culture worked transiently as a catabolite repressor against the *araBAD* promoter system, it was demonstrated that glucose could also modulate the expression of recombinant interferon-α (Fig. 2).

To examine the usefulness of glucose in the *araBAD* promoter system, glucose was added as a catabolite repressor after the induction of L-arabinose during DO-stat fed-batch culture. In other words, we verified whether the addition of glucose could modulate the tendency toward inclusion body formation. As shown in Fig. 3, the addition of 0.5% glucose was carried out once or twice after 0.5% L-arabinose induction, and the effects were compared with those observed when glucose was not added after L-arabinose induction. In these cultures, the induction was performed at an elapsed time of 3 h, and the medium feeding started at 6 to 8 h after L-arabinose induction. The profiles of cell growth and glycerol consumption were

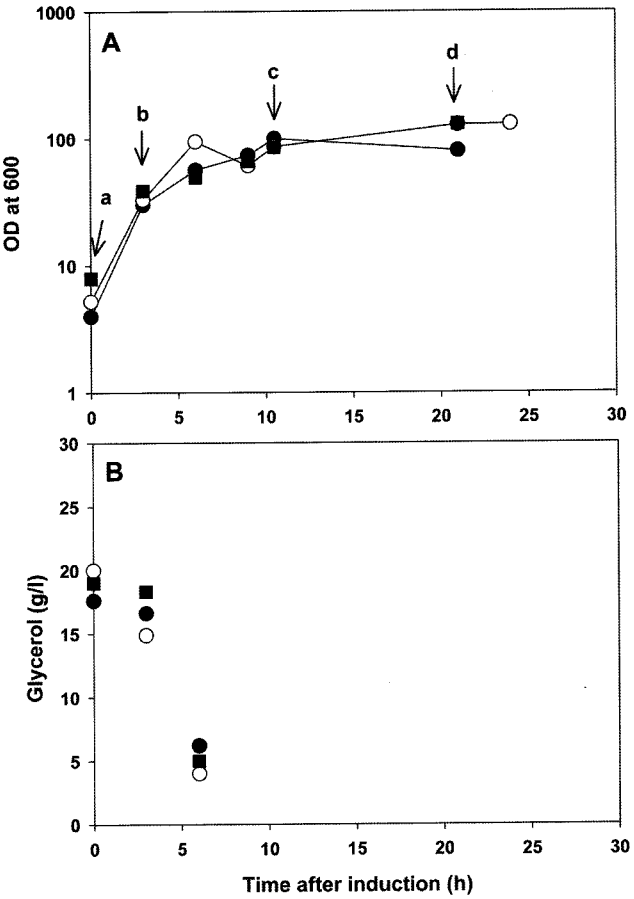

Fig. 3. Profiles of cell growth (**A**) and glycerol consumption (**B**) of DO-stat fed-batch culture with the addition of 0.5% glucose for the modulation of recombinant interferon-α expression.
L-Arabinose induction is indicated by arrow **a**. Medium feeding started at 6 to 8 h after induction. Symbol ● indicates the culture in which L-arabinose induction (arrow **a**) was only performed without the addition of glucose. Symbol ○ describes the culture in which L-arabinose induction (arrow **a**) was performed and two additions of 0.5% glucose (arrows **b** and **c**) were carried out at 3 h and 10.5 h after induction. Symbol ■ indicates the culture in which L-arabinose induction (arrow **a**) was performed and one addition of 0.5% glucose (arrow **c**) was carried out at 10.5 h after induction. Samples (arrow **d**), collected at 21 h after induction, were used for Western blot analyses.

similar. Glucose, however, was rapidly consumed after it was added (Fig. 3), and was only detected in the culture supernatants immediately after its addition. Glucose was completely consumed within 3 h after addition because glucose is a better carbon source than glycerol [1, 8]. When glucose was not added after L-arabinose induction, all of the expressed recombinant interferon-α ultimately formed an inclusion body at 21 h after induction (Fig. 4A). However, when glucose was added after induction, the expressed recombinant interferon-α did not all form an inclusion body, and about half of the total recombinant interferon-α was expressed as a soluble form (Figs. 4B and 4C). Therefore, the distribution patterns of soluble and

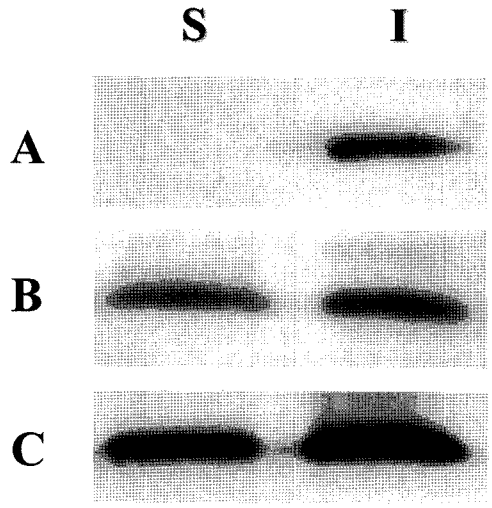

Fig. 4. Western blot analyses of soluble and insoluble fractions of total cell lysate. Samples (arrow **d** in Fig. 3A) were used.
A. Soluble (**S**) and insoluble (**I**) fraction from the culture (●) of Fig. 3A; **B**. Those from the culture (○); **C**. Those from the culture (■).

insoluble fractions of recombinant interferon-α during DO-stat fed-batch culture were investigated in terms of their relative amounts (Fig. 5). The distribution patterns, when induction was performed at a concentration of 0.5% L-arabinose, showed a tendency towards the formation of inclusion bodies (Fig. 5A). As the DO-stat fed-batch culture proceeded into the later portion of culturing, the distributions of recombinant interferon-α in the soluble and insoluble fractions of total cell lysate gradually changed, showing increased expression of interferon-α in an inclusion body. Most of the expressed interferon-α was subsequently found in the insoluble fraction of total cell lysate. However, the distribution patterns differed when 0.5% glucose was added after 0.5% L-arabinose induction. It was shown that the formation of inclusion bodies was impeded by the addition of 0.5% glucose after L-arabinose induction, regardless of whether 0.5% glucose was added once or twice following L-arabinose induction (Figs. 5B and 5C). The addition of glucose inhibited the tendency towards inclusion body formation; only a portion of the recombinant interferon-α was expressed as an inclusion body, although residual glucose was not found at consistent levels in the culture medium. However, the effect of glucose as a repressor lasted throughout the entire period of culturing.

It has been reported that glucose causes catabolite repression by simultaneously downregulating the intracellular levels of both cAMP and CAP in *E. coli* [6, 9, 10], not by a cascade pathway in which the cAMP level is downregulated and thereby CAP cannot be activated. Additionally, it was demonstrated that, when recombinant *E. coli* producing β-galactosidase was added to the medium containing glucose, the enzyme was produced at approximately one-half the rate of that containing glycerol, representing the so-called

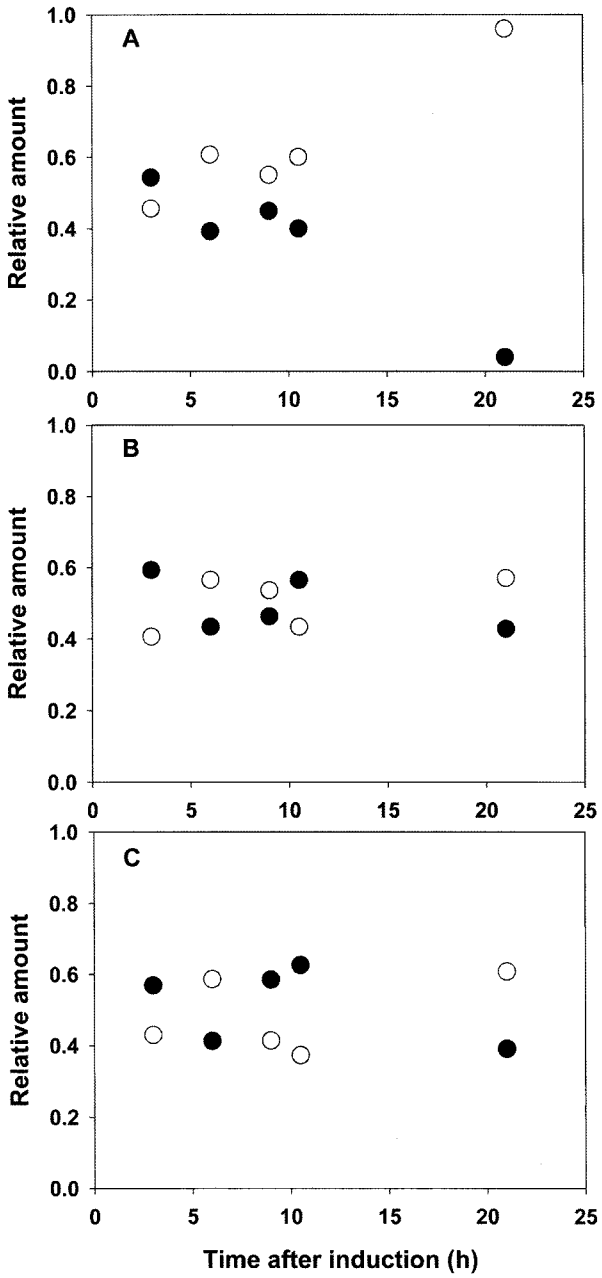

Fig. 5. Distributions of soluble and insoluble fractions of total cell lysate from Fig. 3.
Relative amounts of soluble (●) or insoluble (○) fractions of total cell lysate were calculated from their relative image areas, which were established by Western blot analyses. The total image area was obtained from the sum areas of the soluble and insoluble fractions determined by Western blot analyses, and their relative amounts were calculated by dividing the image area of the soluble or insoluble fraction by the sum area.

phenomenon referred to as "transient repression" [4, 18, 28, 29]. Additionally, in the *araBAD* promoter system, glucose diminished the promoter inducibility because CAP was one regulatory part of the machinery of the promoter system [13, 24, 31]. In those cases, it was deduced that the addition of glucose after L-arabinose induction might

lower cAMP, and thus, the activation of CAP might not occur. The transcription rate of recombinant interferon-α in the *araBAD* promoter system might be delayed by this partial repression. However, complete repression was impossible because another positive regulator, AraC, still functioned in this system. This inhibition of the transcription rate probably resulted in more soluble interferon-α expression owing to the reduction of the protein synthesis rate.

In the previous work [14], the portion of the inclusion body showing recombinant interferon-α expression increased gradually after L-arabinose induction until the end of the DO-stat fed-batch culture, and all of the expressed interferon-α was ultimately found in the form of an inclusion body. A similar result was also shown in this work (Figs. 4A and 5A). However, the tendency toward inclusion body formation changed when 0.5% glucose was added at 10.5 h after induction (Figs. 4B, 4C, 5B, and 5C). As shown in this work (Figs. 4A and 5A) and the previous work [14], the formation of the inclusion body occurred mostly during the later half of culturing. The probable reason for this was that protein expression was maintained at a much higher rate than the cell growth rate during the later half of the culture time. In other words, although protein expression continued during the later half of the DO-stat fed-batch culture, the cell growth rate slowed gradually. On the other hand, at an early phase of culturing, the cell growth rate was not comparatively slow and the probability of the inclusion body formation in the cytoplasm was reduced. Therefore, it was reasonable that the addition of glucose as a repressor was performed during the later half of the culture time (at 10.5 h after induction) because the formation of inclusion bodies was preferable at this time. However, the *araBAD* promoter system was not controlled exclusively by CAP, since AraC also worked as a positive regulator. It was then deduced that the *araBAD* promoter was in the condition of "partial repression" when the glucose was added to the culture medium.

In conclusion, it was found that we could modulate the tendency toward the formation of interferon-α inclusion bodies caused by the addition of glucose during the later half of the DO-stat fed-batch culture in the *araBAD* promoter system. In particular, this principle could be applied to the improvement of the soluble expression level of recombinant therapeutic proteins and/or industrial enzymes in *E. coli*.

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