J. Microbiol. Biotechnol. (2008), **18**(3), 434–442

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Enhanced Enzyme Activities of Inclusion Bodies of Recombinant β-Galactosidase *via* the Addition of Inducer Analog after L-Arabinose Induction in the *araBAD* Promoter System of *Escherichia coli*

Jung, Kyung-Hwan*

Division of Food and Biotechnology, Chungju National University, Chungju, Chungbuk 380-702, Korea

Received: July 20, 2007 / Accepted: September 27, 2007

We observed that an inclusion body (IB) of recombinant β-galactosidase that was produced by the *araBAD* promoter system in *Escherichia coli* (*E. coli*) showed enzyme activity. In order to improve its activity, the lowering of the transcription rate of the β-galactosidase structural gene was attempted through competition between an inducer (L-arabinose) and an inducer analog (D-fucose). In the deep-well microtiter plate culture and lab-scale fermentor culture, it was demonstrated that the addition of D-fucose caused an improvement in specific β-galactosidase production, although β-galactosidase was produced as an IB. In particular, the addition of D-fucose after induction led to an increase in the specific activity of β-galactosidase IB. Finally, we confirmed that the addition of D-fucose after induction caused changes in the structure of β-galactosidase IB, with higher enzyme activity. Based on these results, we expect that an improved enzyme IB will be used as a biocatalyst of the enzyme bioprocess, because an enzyme IB can be purified easily and has physical durability.

Keywords: Partial repression, inducer analog, inclusion body, β-galactosidase

Protein insoluble aggregates, referred to as inclusion bodies (IBs), are commonly produced in the cytoplasm of *Escherichia coli* (*E. coli*) during the overexpression of recombinant proteins. These structurally complex aggregates are frequently formed in the unfavorable protein-folding environment, in which the protein expression rate is very high and proteins crowd in the cytoplasm at concentrations of several hundred milligrams per liter, thus overburdening the protein-folding machinery [23, 27]. Several strategies have been attempted to circumvent the formation of IBs during protein expression, and these have recently been reviewed [23]. In the case of the expression of fusion protein containing β-galactosidase in *E. coli*, it was observed that the induction temperature and pH of the cultivation medium had a profound effect on the formation of IBs [25]. In other cases of the expression of this protein in *E. coli*, the complex medium effectively prevented IB formation at 42°C [14]. Additionally, this report demonstrated that the slow production in the high-cell density fed-batch mode enabled lower IB formation of fusion proteins. More specifically, both the control of the transcription level and the lowering of the rate of translation caused the slowing of protein synthesis, and the soluble expression level of human therapeutic protein thereby increased in *E. coli* [19]. As another approach to preventing the formation of IBs, fusion tag technology was developed to increase both the solubility of expressed protein in the cytoplasm and the purification efficiency of expressed protein [6, 18, 24]. In particular, molecular chaperones (*e.g.*, DnaK, DnaJ, GroEL, GroES, GrpE, ClpB, IbpA, IbpB, *etc.*) involving protein refolding and protein secretion were thoroughly discussed and reviewed [4, 21, 23] in terms of the coexpression of molecular chaperones and aggregate-prone proteins, particularly their overexpressions in the cytoplasm as an attractive methodology to prevent protein aggregation.

In general, IBs are characterized by their insolubilities, biological disabilities, and structurally heterogeneous conformational natures. However, interesting observations of the biological activities of IBs of β-galactosidase [30] and its fusion protein [10, 11], green- and blue-fluorescent protein (GFP and BFP) fused-proteins [11], as well as *Clostridium thermocellum* endoglucanase D [26] in *E. coli* have been reported. Moreover, they were found to have relatively high activities compared to those in soluble fractions [10, 11]. It was suggested that this was because the heterogeneous IB protein population contained properly folded polypeptides as well as an intermolecular β-sheet-rich structure, which indicated its conformational variability [5, 28]. In the event that a fusion protein containing GFP was expressed in *E.*

*Corresponding author
Phone: 82-43-841-5246; Fax: 82-43-841-5240;
E-mail: khjung@cjnu.ac.kr

coli, it was observed that the final amount of active protein in an IB of GFP depended on how fast the aggregation event occurred [8]. The faster a protein aggregates, the lower its fluorescence emission, and vice versa.

In this work, we observed that the recombinant β-galactosidase expressed as an IB in *E. coli* had biological activity similar to that shown in previous reports [10, 11, 26, 30]. Then, based on the findings of one report [8], in order to improve β-galactosidase activity, we attempted to induce a change by changing the protein aggregation rate, in which the transcription rate was lowered by competition between an inducer and an inducer analog (D-fucose). In other words, we expected that the inducer analog would partially repress the transcription of the β-galactosidase structural gene after induction, leading to the slowing of its protein expression rate and thereby changing its heterogeneous complex IB structure toward a higher enzyme activity. In particular, we used the *araBAD* promoter system of *E. coli* for the expression of β-galactosidase, and the induction was carried out by the addition of L-arabinose. In this system, AraC is involved as a positive regulator when L-arabinose is present in the medium [22], and it can be expected that the addition of an inducer analog after induction causes a reduction of the function of AraC. Thus, the protein expression rate will be lowered *via* the slowing of the transcription rate.

Fourier transform infrared (FT-IR) analysis of samples obtained from biological resources has been reported in the characterization of molecular structure [3, 16]. It is known that IBs lead to changes in FT-IR absorbance spectra around the Amide I band; these changes mainly result from carbonyl C=O stretching in the peptide bond of a protein [1, 2, 15]. It was observed that the second derivative FT-IR spectra of the IB had the characteristic peaks between 1620 and 1660 cm^{-1} when a protein aggregate was formed during the overexpression of recombinant proteins in *E. coli* [1, 2, 11, 12, 15]. Those were explained as the results of the conformational change of protein in terms of α-helix and β-sheet structure during the formation of protein aggregates. In order to elucidate why changes in enzyme activities occurred through partial repression, we also investigated the structural changes of β-galactosidase IBs using FT-IR spectrometry.

MATERIALS AND METHODS

Recombinant β-Galactosidase-expressing *E. coli*

In this study, we used a recombinant *E. coli* in which the expression of β-galactosidase was controlled by the *araBAD* promoter. The β-galactosidase gene was cloned in an expression vector, pBAD/*Myc*-His/*lacZ* (7.2 kb), which is one of the control vectors provided in the pBAD/*Myc*-His expression kit (Invitrogen). This expression vector contains the structural gene of β-galactosidase (*lacZ*), which is directed by the *araBAD* promoter and inserted into the SfuI site in a multicloning site. In addition, the positive regulator gene (*araC*), pBR322 origin, and ampicillin-resistant marker were contained in this vector. More details are described in the manual of the pBAD/*Myc*-His kit (Invitrogen). As an expression host, *E. coli* MC1061 (F⁻*araD139 Δ(ara-leu) 7696 galE15 galK16 Δ(lac) X74 rpsL (Str^r) hsdR2* ($r_k^-m_k^-$) *mcrA mcrB1*) was used, in which the recombinant β-galactosidase (120 kDa) was expressed by the addition of L-arabinose.

Culture of β-Galactosidase-expressing *E. coli* in Deep-Well Microtiter Plates

For the deep-well microtiter plate culture [9], 96-deep-well microtiter plates, sandwich covers, and cover clamps were purchased from Kühner AG (Swiss). When this culture system was used in a shaking incubator, the cover clamp (CR 1300, Kühner AG) was mechanically attached to the shaking plate of the shaking incubator, and the deep-well microtiter plate (CR 1302), which was covered with its sandwich cover (CR 1200), was then fixed on the shaking plate using the cover clamp. Seed culturing for the deep-well microtiter plate culture was performed in a 250-ml Erlenmeyer flask with a culture volume of 100 ml of Luria Broth (LB) medium (tryptone, 10 g/l; yeast extract, 5 g/l; NaCl, 10 g/l; ampicillin, 100 μg/ml) in a shaking incubator for 12 h. For the deep-well microtiter plate culture, the deep-well microtiter plate with a sandwich cover and the fortified Luria broth (FLB) medium (tryptone, 10 g/l; yeast extract, 20 g/l; NaCl, 5 g/l) were autoclaved separately. Then, 1.0 ml of sterile FLB medium was dispensed into each deep well of a 96-deep-well microtiter plate, and 50 μl of seed culture was inoculated into each deep well using a multichannel pipette. For the culture of the β-galactosidase-expressing *E. coli*, 100 μg/ml of ampicillin was also added prior to inoculation. In this study, 60 deep wells of a 96-deep-well microtiter plate, excluding the wells around the outer edge of the plate (36 deep-wells), were used for culture. Six cultures in the same vertical column were performed under the same culture conditions. For induction of the recombinant β-galactosidase, 0.05% L-arabinose was added at an elapsed time of 3 h. If necessary, D-fucose (inducer analog) was added. Both seed flask cultures and deep-well microtiter plate cultures were carried out in a shaking incubator at 37°C and 250 rpm.

Culture of β-Galactosidase-expressing *E. coli* in a Lab-Scale Fermentor

Seed culturing for β-galactosidase expression in a fermentor was performed in a 250-ml Erlenmeyer flask with a culture volume of 100 ml of 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, in which temperature and shaking speed were controlled at 37°C and 150 rpm, respectively. Culturing in the fermentor was carried out in a 5.0-l jar fermentor (KoBiotech, Republic of Korea) with a volume of 1.5 l. The medium composition was as follows: glycerol, 20 g/l; yeast extract, 20 g/l; KH_2PO_4, 2.31 g/l; and Na_2HPO_4, 10.22 g/l. Ampicillin was also added at a concentration of 100 μg/ml. The agitation speed and aeration rate were 700 rpm and 1.5 vvm, respectively. The dissolved oxygen level was maintained, so that it did not decrease below 20%, through the manual control of the agitation speed and aeration rate. pH was maintained at 7.0 using phosphoric acid solution (10%,v/v) as acid and ammonia water as base. L-Arabinose induction was performed at 0.05% at an elapsed time of 3 h. If necessary, D-fucose (inducer analog) was added at an elapsed time of 3.5 h. For further analysis, the cell pellets from each sampling were collected by microcentrifugation,

where the optical density of the sample broth at 600 nm (OD_{600}) was adjusted to a constant OD_{600} of 2.0 with PBS (phosphate-buffered saline), and then, 1.5 ml of this cell suspension was microcentirifuged for the preparation of the cell pellets. These cell pellets had 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.

Cell Growth

Cell growth in deep-well microtiter plates was monitored by reading the OD_{595} (optical density at 595 nm) in a microplate reader (Model 680; RioRad, U.S.A.). At the sampling time, 20 μl of culture broth in a 96-deep-well microtiter plate was transferred to a flat-bottomed 96-well microplate (SPL Life Sciences, Republic of Korea), and a 40-fold dilution with phosphate-buffered saline (PBS) was then performed. After the dilution was completed, 60 μl of diluted culture broth was transferred to a new 96-well microplate. Measurement of OD_{595} was then performed. Cell growth in the fermentor was monitored *via* measurements of OD_{600}, which were taken with a spectrophotometer (Optizen® 2120UV, Mecasys, Republic of Korea).

Assay of β-Galactosidase Activity

For the deep-well microtiter plate culture, the protocol for the β-galactosidase assay using a 96-well microplate was tryptone created based on two principles from both the yeast β-galactosidase assay kit (Product number 75768, Pierce) and the protocol described by Hardin *et al.* [13]. The principles and protocol are based on Miller's protocol [20]. At first, the (2×) β-galactosidase assay buffer, which was a 1:1 mixture of both (2×) Z-buffer ($Na_2HPO_4 \cdot 7H_2O$, 32.2 g/l; $Na_2PO_4 \cdot H_2O$, 11 g/l; KCl, 1.5 g/l; $MgSO_4 \cdot 7H_2O$, 0.492 g/l; β-mercaptoethanol, 5.4 ml/l; ONPG (*o*-nitrophenyl-β-D-galactoside), 2.7 g/l; pH 7.0) and B-PER II, was prepared. Sixty μl of (2×) assay buffer was then dispensed into a flat-bottomed 96-well microplate, followed by the addition of 60 μl of a 40-fold dilution of culture broth from a deep-well microtiter plate culture. This microplate was incubated in a shaking incubator for 30 min at 50 rpm. This color development step was then stopped by the addition of 50 μl of 1 M Na_2CO_3, and OD_{415} was measured in a microplate reader. For the lab-scale fermentor culture, a β-galactosidase assay was performed on the basis of the protocol described by Hardin *et al.* [13]. In particular, the culture broth was diluted appropriately using PBS, and Z-buffer was also used as an assay buffer. The cell debris was removed by microcentrifugation at 15,000 rpm for 10 min before the measurement of optical density. Other details have been described by Hardin *et al.* [13]. For the calculation of units of β-galactosidase, OD_{420} was measured using a spectrophotometer. The production of β-galactosidase was calculated as follows:

$$\beta\text{-Galactosidase (Units/ml)} = \frac{(OD_{420})}{(0.0045) \times (1) \times (15)}$$

where OD_{420}, 0.0045, 1, and 15 indicate the OD measurement at 420 nm, molecular extinction coefficient ($nmol^{-1}$ ml cm^{-1}) of *o*-nitrophenol, light pathlength (cm), and reaction time (min), respectively. A unit of β-galactosidase was defined as the amount of enzyme that produced 1 nmole of *o*-nitrophenol per 1 min at 28°C, pH 7.

Separation of Soluble and Insoluble Fractions

The cell pellet was disrupted by a nonmechanical method that utilized the B-PER II bacterial protein extraction reagent (Pierce). In this study, a mixed solution of 5 ml of B-PER II reagent and 50 μl of DNase I (Sigma) solution (1 mg/ml) was used. Each cell pellet was mixed with 150 μl of B-PER II (+ DNase I) solution, and the mixture was then vortexed vigorously and pipetted up and down. Next, the soluble fraction was obtained from the supernatant by microcentrifugation at 13,000 rpm for 5 min, and the insoluble fraction was prepared by resuspension of the disrupted cell pellet in 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).

His-Tag Staining and Western Blotting

Prior to His-tag staining and Western blotting, 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using an SE250 mini-vertical gel electrophoresis unit (Amersham Biosciences). The 6xHis-tag of expressed recombinant β-galactosidase was stained with a GelCode 6xHis tag staining kit (Pierce). For Western blotting, 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). Anti-β-galactosidase (rabbit IgG fraction; Molecular Probes, U.S.A.) as the first antibody and goat anti-rabbit IgG-HRP (horseradish peroxidase) conjugate (Abcam, U.K.) as the second antibody were used at dilutions of 1:5,000 and 1:10,000, respectively. For band detection, SuperSignal West Pico Chemiluminescent substrate (Pierce) and Hyperfilm ECL (Pierce) were used as a peroxidase substrate and as a film for exposure, respectively.

IB Purification

After the insoluble fraction of expressed recombinant β-galactosidase was prepared by the above-described protocol, the insoluble fraction was resuspended in 150 μl of B-PER II reagent. Six μl of 10 mg/ml lysozyme was subsequently added to that suspension, which was vortexed for one minute; 1.0 ml of 1:20 diluted B-PER II reagent was subsequently added. After additional vortexing for one minute, the purified IBs were collected by microcentrifugation at 13,000 rpm for 10 min. Finally, the purified IB was prepared by two more washes with 1:20 diluted B-PER® II reagent. For the β-galactosidase assay, the purified IB was resuspended in B-PER® reagent, and for FT-IR measurement, the purified IB was resuspended in distilled water after three more washes with distilled water.

Protein Assay

The protein concentration of the IB solution was quantified by image analysis of the gel following 12.5% SDS-PAGE. Bovine serum albumin (2 mg/ml, Pierce) was used as a protein standard, and AlphaEase FC software (Alpha Innotech, U.S.A.) was utilized for image analysis.

FT-IR Spectrometry

Five μl of the purified IB suspension was deposited onto a KRS-5 window and dried at room temperature. FT-IR absorption spectra from 1,500 to 1,800 cm^{-1} were acquired by a FTS-6000 spectrometer (Bio-Rad) in the absorbance mode at 2 cm^{-1} resolution, 20 kHz speed, 256 scan co-addition, and triangular apodization. The spectra of the IB solutions were obtained as the data were separated from the background spectrum of distilled water. The nonlinear regression curves were obtained using the SigmaPlot 8.0 program (SPSS Inc.,

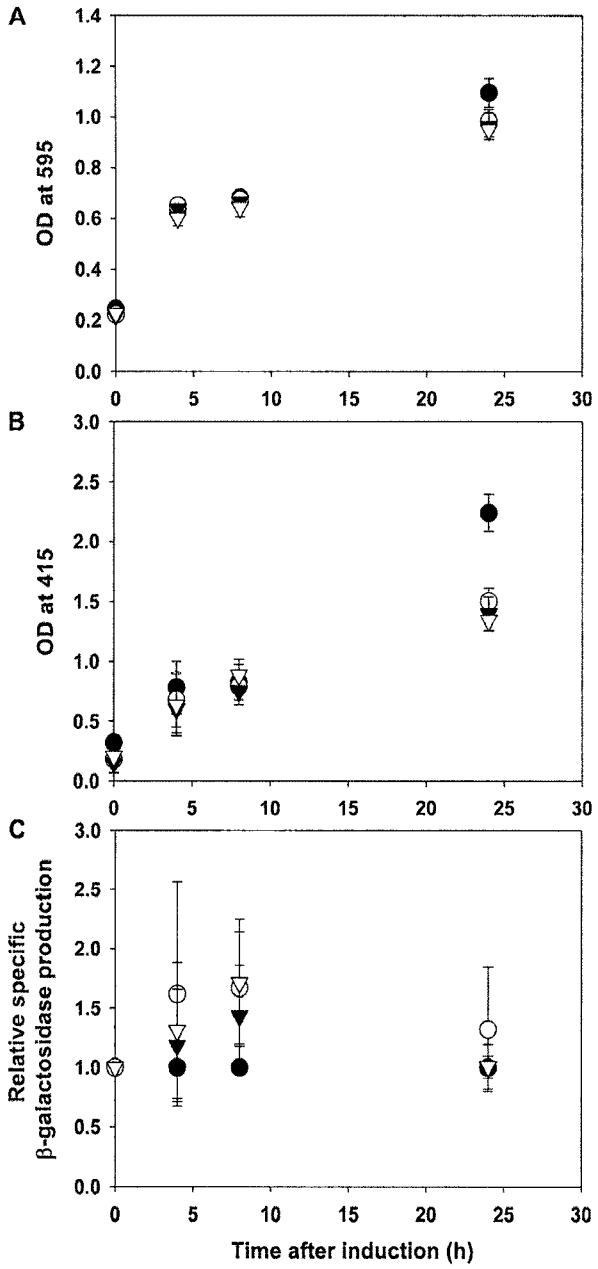

Fig. 1. Profiles of cell growth (**A**), β-galactosidase production (**B**), and relative specific β-galactosidase production (**C**) in deep-well microtiter plate culture.

For the induction, 0.05% L-arabinose was added at an elapsed time of 3 h. Times on the x-axis indicate the elapsed times after L-arabinose induction. At 3 h after induction, 0.0025% to 0.01% D-fucose was added. Symbol ● indicates no D-fucose addition, and the other symbols indicate the addition of D-fucose as follows; the addition of 0.0025% (○), 0.005% (▼), or 0.01% (▽) D-fucose. OD at 415 nm (**B**) refers to the readings of the microplate reader from the β-galactosidase assay, indicating β-galactosidase production. Relative specific β-galactosidase production (**C**) was estimated based on the normalization of specific β-galactosidase production as "1" when D-fucose was not added (●). In addition, the other values (○, ▼, ▽) in (**C**) were estimated based on the normalization of specific β-galactosidase production at zero time as "1". Before the measurements of cell growth (OD at 595) and β-galactosidase production (OD at 415), the culture broth was diluted 40-fold using phosphate-buffered saline (PBS), and FLB medium was used as a blank. Data from six cultures in the same vertical column of deep-well microtiter plate were used to calculate both the average and the standard deviation.

U.S.A.), and the second derivatives of those regression curves were obtained using the differentiation menu of that program.

RESULTS

Effect of the Addition of D-Fucose on Recombinant β-Galactosidase Production in Deep-Well Microtiter Plate Culture

Both to investigate the effect of the addition of D-fucose on β-galactosidase production and to screen the range of the effective concentration of D-fucose, we performed deep-well microtiter plate cultures of β-galactosidase-expressing *E. coli*, in which D-fucose was added at 0.0% to 0.01% after L-arabinose induction. As shown in Figs. 1A and 1B, the cell growth and β-galactosidase production were slightly inhibited by the addition of D-fucose, except for the sample from 24 h after induction. However, the relative specific β-galactosidase productions, that is, the values relative to those when no D-fucose was

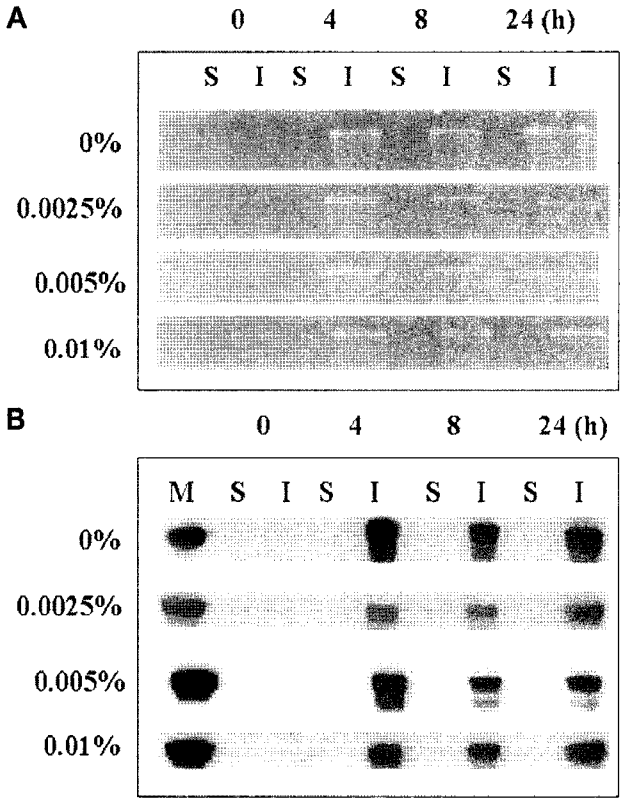

Fig. 2. His-tag staining (**A**) and Western blotting (**B**) of the soluble and insoluble fractions of the total cell lysate from deep-well microtiter plate culture.

Times (0, 4, 8, and 24 h) indicate the elapsed times after 0.05% L-arabinose induction. The samples obtained at those times were fractionated into the soluble (**S**) and insoluble (**I**) fractions of the total cell lysate. The concentrations of added D-fucose (0 to 0.01% D-fucose) after L-arabinose induction are described on the left side. M indicates β-galactosidase standard in Western blotting, in which its concentration was 1 mg/ml. Sample loading volumes were 20 μl for His-tag staining and 3 μl for Western blotting, respectively.

added, were somewhat higher after the addition of D-fucose (Fig. 1C), although the effects were not directly proportional to the concentration of D-fucose. Moreover, β-galactosidase was found in the insoluble fraction of total cell lysate, that is, as an IB. The production of β-galactosidase as IBs was shown in the results of His-tag staining and Western blotting of the soluble and insoluble fractions of the total cell lysate (Figs. 2A and 2B). In the deep-well microtiter plate culture, it was demonstrated that the addition of D-fucose caused an improvement in specific β-galactosidase production, although β-galactosidase was produced as an IB.

Effect of the Addition of D-Fucose on Recombinant β-Galactosidase Production in Lab-Scale Fermentor Culture

The data from the deep-well microtiter plate culture were verified in a lab-scale fermentor. Cell growth showed similar profiles regardless of the amount of D-fucose (Fig. 3A) added, and β-galactosidase production was considerably higher following the addition of 0.01% D-fucose (Fig. 3B). Fortunately, the specific β-galactosidase production as well as its relative value clearly demonstrated higher β-galactosidase production following the addition of 0.01% D-fucose (Fig. 4A). In view of the relative specific β-galactosidase production, the additions of 0.005% and 0.01% D-fucose showed improvements in β-galactosidase production, whereas 0.0025% and 0.02% D-fucose did not. However, from the results of Fig. 3 and Fig. 4, the addition of 0.01% D-fucose could be chosen as the best in concentration in terms of β-galactosidase production, because the overall production of β-galactosidase and its specific production acted as determinants. Additionally, β-

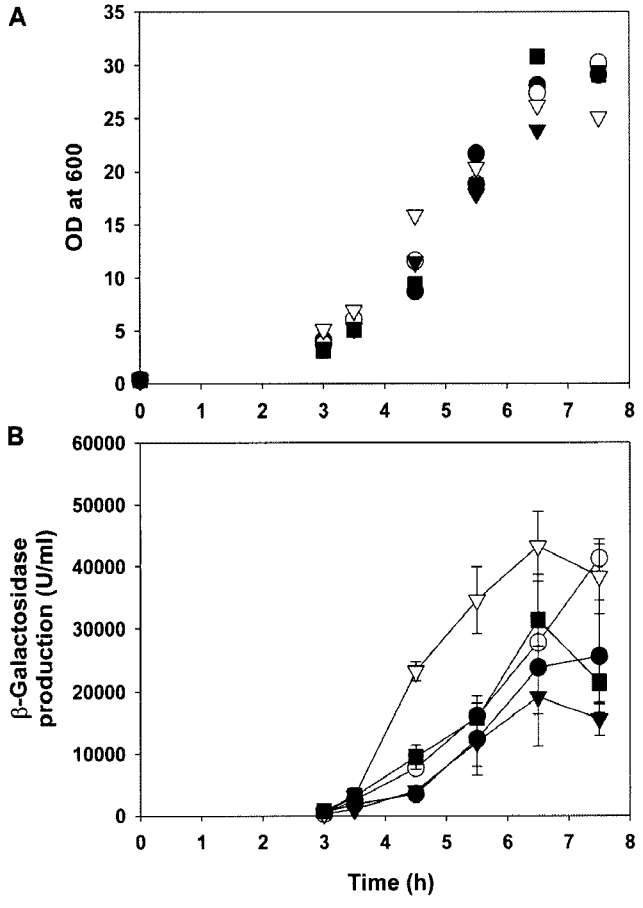

Fig. 3. Cell growth (**A**) and β-galactosidase production (**B**) in the lab-scale fermentor.
For induction, 0.05% L-arabinose was added at an elapsed time of 3 h. At 30 min after induction, 0.0025% to 0.02% D-fucose was added. Symbol ● indicates no D-fucose addition, and the other symbols indicate the addition of D-fucose as follows; the addition of 0.0025% (○), 0.005% (▼), 0.01% (▽), or 0.02% (■) D-fucose. The measurements of β-galactosidase production were performed three times using the same sample, and the average and standard deviation were calculated.

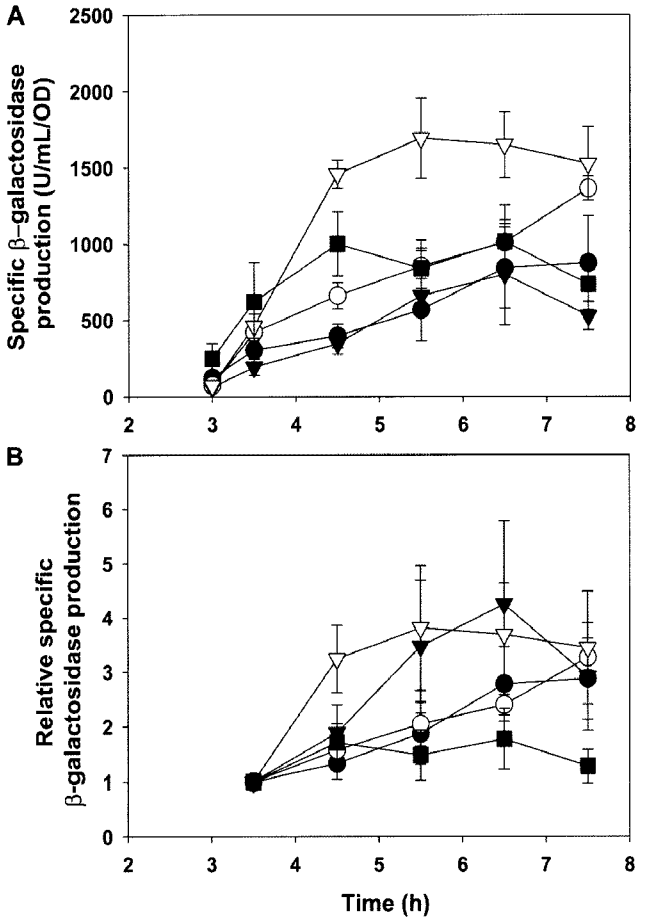

Fig. 4. Specific β-galactosidase production (**A**) and relative specific β-galactosidase production (**B**) in the lab-scale fermentor.
For induction, 0.05% L-arabinose was added at an elapsed time of 3 h. At 30 min after induction, 0.0025% to 0.02% D-fucose was added. Symbol ● indicates no D-fucose addition, and the other symbols indicate the addition of D-fucose as follows; 0.0025% (○), 0.005% (▼), 0.01% (▽), or 0.02% (■) D-fucose. Relative specific β-galactosidase production (**B**) at each condition was estimated based on the normalization of specific β-galactosidase production at "1" at an elapsed time of 3.5 h, that is, at the time when D-fucose was added after induction. Both specific β-galactosidase and relative specific β-galactosidase production were calculated from the data presented in Fig. 3.

Fig. 5. Western blotting of the soluble and insoluble fractions of the total cell lysate from the lab-scale fermentor culture.
Times (3, 3.5, 4.5, 5.5, 6.6, and 7.5 h) indicate the elapsed times of cultures. The samples obtained at those times were fractionated into the soluble (**S**) and insoluble (**I**) fractions of the total cell lysate. The concentrations of added D-fucose (0 to 0.02% D-fucose) at an elapsed time of 3 h are described on the left side. M indicates β-galactosidase standard in Western blotting.

galactosidase in a lab-scale fermentor culture was also produced as an IB regardless of what concentration of D-fucose was added (Fig. 5), as in the deep-well microtiter plate culture.

Specific Activity of IB Solution

In deep-well microtiter plate culture and lab-scale fermentor culture, it was shown that the specific production of β-galactosidase was changed by the addition of D-fucose after induction, and β-galactosidase was expressed in the insoluble fraction of the total cell lysate, that is, as an IB.

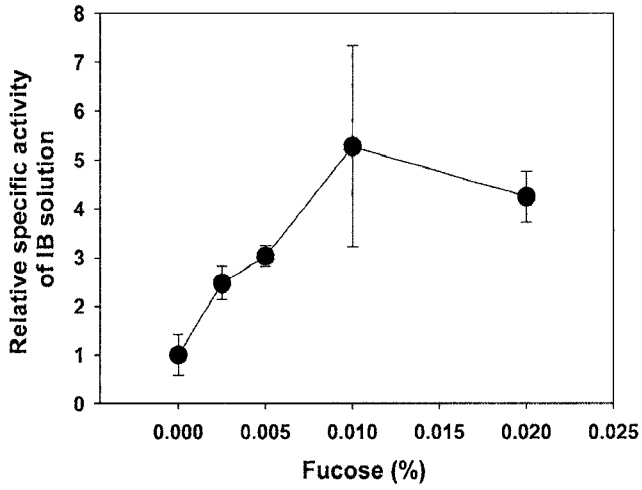

Fig. 6. Relative specific activity of β-galactosidase IB solution.
IBs were obtained from samples of the lab-scale fermentor culture at an elapsed time of 5.5 h. β-Galactosidase assays of IB solution were performed three times using the same sample, and their specific activities were estimated by dividing the β-galactosidase activities of IB solution by their protein concentrations. Their relative values were calculated based on the normalization of the specific activity of the β-galactosidase IB solution at "1" when D-fucose was not added. Data were described as averages and standard deviations.

In order to verify whether the IB had enzyme activity and whether a change occurred in the specific β-galactosidase activity as a result of the addition of D-fucose, the purification of β-galactosidase IB was performed using B-PER II reagent, and the specific activity of β-galactosidase IB solution was measured. Fig. 6 shows the relative specific β-galactosidase activities of IB solution when D-fucose (0.0025%, 0.005%, 0.01%, or 0.02%) was added after induction. β-Galactosidase IBs were purified from the cells of the lab-scale fermentor culture (Figs. 3 and 4) at an elapsed time of 5.5 h. The relative specific β-galactosidase activity of IB solution when D-fucose was added at concentration of 0.01% was approximately five times higher than that when D-fucose was not added. In addition, it was shown that the relative specific β-galactosidase activities of IB solution were increased by the addition of D-fucose, from 0.0025% to 0.01%, and reached a plateau at 0.01 % and 0.02% D-fucose. It was clearly demonstrated that the addition of D-fucose after induction in a lab-scale fermentor culture led to an increase in specific activity of β-galactosidase IB solution, regardless of the concentration of D-fucose that was added.

FT-IR Analysis of IBs

To elucidate the reason that the specific activity of the β-galactosidase IB was increased by the addition of D-fucose after induction, the characteristic peak of IB solution in the second derivative of the FT-IR spectra was investigated. In this experiment, we used the purified IBs obtained from the cells of the lab-scale fermentor culture (Figs. 3 and Fig. 4) at an elapsed time of 5.5 h. Using the five IB solutions shown in Fig. 6, five absorbance spectra were measured, and these were differentiated twice in order to plot the second derivatives of the FT-IR spectra. It was

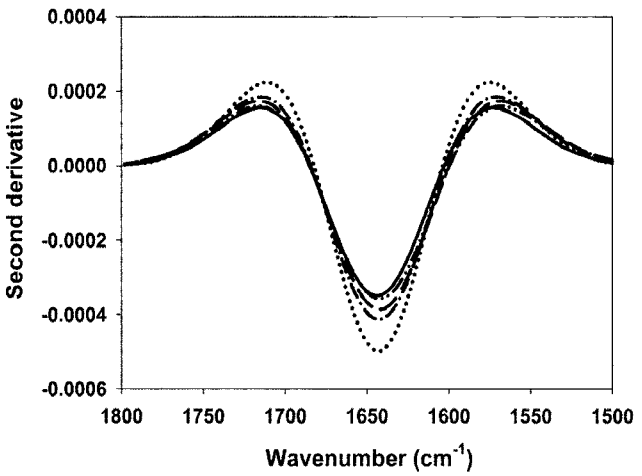

Fig. 7. FT-IR analysis of β-galactosidase IB solution.
Second derivatives were obtained by differentiating the spectra twice when D-fucose was added at a concentration of 0.0% (——), 0.0025% (·····), 0.005% (– – –), 0.01% (-·-·-·), or 0.02% (–··–··).

observed that the five spectra showed the characteristic peaks of the protein aggregate around a wavenumber of 1,650–1,660 cm^{-1} (Fig. 7). In addition, the heights of these characteristic peaks toward the negative value were changed according to the addition of D-fucose after induction. Although the peak heights were not proportionally changed according to the amounts of D-fucose added, the changes toward more negative values were observed when D-fucose was added after induction. Based on the idea that the peak around a wavenumber of 1,650–1,660 cm^{-1} indicates an α-helix structure of protein conformation, which is also changed toward more positive values by the formation of an IB [2, 29], we could confirm that the addition of D-fucose after induction caused changes in the structure of β-galactosidase IB.

Discussion

It was observed that the activity of the protein in IBs is strongly influenced by the temperature of cultivation [7]. Increasing the growth temperature above 37°C results in a reduction in specific activity, whereas lowering the temperature significantly increases the fluorescence emission of the GFP fusion protein. IBs that formed at 42°C and 37°C exhibited higher resistance to both proteolytic digestion and stability against denaturants such as guanidinium hydrochloride, which indicates that a more densely packed structure exists in the aggregated species. Additionally, it was predicted that the amount of active protein in an IB was increased by the reduction of the protein aggregation rate, and it was also observed that the lower fluorescent emission of the GFP fusion protein could be attributed to the faster protein aggregation [8]. Consequently, these results suggest that the structures of IBs, including their physical properties, can be controlled by parameters such as growth temperature and growth rate, and thus, these controls caused changes in the biological activities of IBs. In other words, the control of the protein aggregation rate results in altered biological activities of IBs. Ultimately, it can be concluded that the controls of the transcription or translation rate may be used as a specific strategy for the inhibition of the protein expression rate. In our previous paper [17], we observed an increase in the soluble fraction of total cell lysate of α-interferon-expressing *E. coli*, which was directed by the *araBAD* promoter system following the addition of glucose as a repressor after L-arabinose induction. This might be attributed to the slowing of the overall protein production rate, particularly to the partial repression resulting from the addition of glucose.

Then, in this work, we attempted to change the β-galactosidase activity produced in IBs through the addition of an inducer analog, D-fucose. Our strategy was to change the properties of β-galactosidase IBs by controlling the protein expression rate. The β-galactosidase production rate was controlled by the addition of D-fucose, wherein D-fucose led to partial repression by competing with the inducer, L-arabinose. The addition of D-fucose finally resulted in partial repression of the promoter system, and the overall β-galactosidase production rate subsequently decreased. This reduction caused changes in the properties of β-galactosidase IBs in terms of biological and physical properties. In this work, four kinds of culture conditions were examined in a deep-well microtiter plate. In other words, the effects of the addition of D-fucose were all investigated simultaneously. Otherwise, at least four kinds of flask cultures were performed. Interestingly, it was observed that β-galactosidase was produced in the form of IBs (Fig. 2) in all of the deep-well microtiter plate cultures. Because the samples from the deep-well microtiter plate cultures were prepared on the basis of the constant amount of cell, and β-galactosidase production levels did not differ according to the protein concentration, as shown in Fig. 2, it was deduced that the specific activities of β-galactosidase IBs were improved by the addition of D-fucose after induction. In addition, in lab-scale fermentor cultures, the addition of D-fucose after induction improved β-galactosidase production and their specific productions (Fig. 3 and Fig. 4), and β-galactosidase was also produced in the form of IBs (Fig. 5). These results co-incided with those from the deep-well microtiter plate cultures, except that the effect of the addition of D-fucose after induction was maximized at a concentration of 0.01%. This D-fucose concentration could be deduced from data for the overall and specific production of β-galactosidase (Fig. 3 and Fig. 4). That is, all of these data were optimized at a concentration of 0.01% D-fucose. For the estimation of the specific activities of β-galactosidase IB solution (Fig. 6), it was necessary that the protein quantification assay was performed. However, consistent data were not obtained when the BCA protein assay kit (Pierce) was used (data not shown). The reason for this might be that the IB solution solubilized in B-PER (Pierce) was the suspension of a heterogeneous protein aggregate, although no precipitates were visually observed. Therefore, for the estimation of the specific activity of the IB solution, the data for the protein concentration of the IB solution were obtained from the image analysis of SDS-PAGE with a protein standard. As shown above (Fig. 6), the increase in specific activities of IB solutions by the addition of D-fucose after induction despite the only slight differences in the protein expression levels suggested that an exploration of the changes that occurred in IBs was necessary. It was deduced that the reason for the structural changes in IBs by the addition of D-fucose after induction was probably indicated by the previous reports [7, 8], in which the changes in the aggregation rates of fusion proteins resulted in their specific activities.

The second derivatives of the FT-IR spectra of the IB solution showed changes in the heights of characteristic

peaks around a wavenumber of 1,650–1,660 cm^{-1} (Fig. 7). The definite changes in the peak heights toward more negative values resulted from the addition of D-fucose. Some reports [2, 29] suggested that these changes were attributed to the change in α-helix structure of the protein aggregate by the formation of the intermolecular β-sheet structure, and the peak heights were changed toward more positive values by the formation of IB. However, in this work, the direction of changes in the peak heights after the addition of D-fucose was the opposite, compared with that by the formation of IB. Although these second derivative spectra did not show a quantitative degree of increases in specific activity by the addition of D-fucose, it could be deduced that structural changes in β-galactosidase IBs occurred toward the opposite direction of IB formation. In other words, the addition of D-fucose after induction might cause a more loose structure of β-galactosidase IBs. Other investigators have performed absorbance curve smoothing by the Savitzky-Golay method (Third polynomial, 13 smoothing points) using the GRAMS/32 software (Galactic Industries, U.S.A.) [1, 2]. In addition, to compare data from different cultures, the second derivative FT-IR spectra were normalized around a band of certain amino acids. On the other hand, we did not perform normalization, because a constant amount of cell mass was always used. There might be a difference in protein concentration between the IBs solution and the inaccuracy in the measurement of protein concentration by image analysis. Because of these constraints of FT-IR analysis, our results could not be compared with those of other reports [1, 2, 12, 29]. However, using the FT-IR analysis, we were able to acquire evidence of changes in the structures of β-galactosidase IBs toward the improvement of their specific activities by the addition of D-fucose after L-arabinose induction in the *araBAD* promoter system. As shown in the previous report [8], it was deduced that a more densely packed β-galactosidase IB was lost by the addition of D-fucose, and a subsequent increase in the portion of biologically active β-galactosidase occurred.

Based on the results of this work and a previous report [11], we now believe that enzyme IBs can be used as biocatalysts of the enzyme bioprocess, because enzyme IBs can be purified easily and have physical hardness compared with the purified and solubilized recombinant enzyme. Further studies must elucidate the changes in the β-galactosidase IB structure resulting from the addition of D-fucose after induction, in order to precisely control the enzyme activity of β-galactosidase IBs and to apply this principle to other aggregate-prone enzyme systems.

Acknowledgments

This work was supported by a Korea Research Foundation Grant funded by the Korean Government (MOEHRD, Basic Research Promotion Fund) (KRF-2006-331-D00174). The author thanks Ms. Ji-Hyeon Yeon and Mr. Sung Hun Kim for their technical assistance.

References

1. Ami, D., L. Bonecchi, S. Calì, G. Orsini, G. Tonon, and S. M. Doglia. 2003. FT-IR study of heterologous protein expression in recombinant *Escherichia coli* strains. *Biochim. Biophys. Acta* **1624:** 6–10.
2. Ami, D., A. Natalello, P. Gatti-Lafranconi, M. Lotti, and S. M. Doglia. 2005. Kinetics of inclusion body formation studied in intact cells by FT-IR spectroscopy. *FEBS Lett.* **579:** 3433–3436.
3. Bramhachari, P. V., P. B. Kavikishor, R. Ramadevi, R. Kumar, B. R. Rao, and S. K. Dubey. 2007. Isolation and characterization of mucous exopolysaccharide (EPS) produced by *Vibrio furnissii* strain VB0S3. *J. Microbiol. Biotechnol.* **17:** 44–51.
4. Bukau, B., J. Weissman, and A. Horwich. 2006. Molecular chaperones and protein quality control. *Cell* **125:** 443–451.
5. Carrió, M., N. González-Montalbán, A. Vera, A. Villaverde, and S. Ventura. 2005. Amyloid-like properties of bacterial inclusion bodies. *J. Mol. Biol.* **347:** 1025–1037.
6. Davis, G. D., C. Elisee, D. M. Newham, and R. G. Harrison. 1999. New fusion protein systems designed to give soluble expression in *Escherichia coli*. *Biotechnol. Bioeng.* **65:** 382–388.
7. de Groot, N. S. and S. Ventura. 2006. Effect of temperature on protein quality in bacterial inclusion bodies. *FEBS Lett.* **580:** 6471–6476.
8. de Groot, N. S. and S. Ventura. 2006. Protein activity in bacterial inclusion bodies correlates with predicted aggregation rates. *J. Biotechnol.* **125:** 110–113.
9. Duetz, W. A., L. Rüedi, R. Hermann, K. O'Connor, J. Büchs, and B. Witholt. 2000. Methods for intense aeration, growth, storage, and replication of bacterial strains in microtiter plates. *Appl. Environ. Microbiol.* **66:** 2641–2646.
10. Garcia-Fruitos, E., M. M. Carrio, A. Aris, and A. Villaverde. 2005. Folding of a misfolding-prone β-galactosidase in absence of DnaK. *Biotechnol. Bioeng.* **90:** 869–875.
11. García-Fruitós, E., N. González-Montalbán, M. Morell, A. Vera, R. M. Ferraz, A. Arís, S. Ventura, and A. Villaverde. 2005. Aggregation as bacterial inclusion bodies does not imply inactivation of enzymes and fluorescent proteins. *Microb. Cell Fact.* **4:** 27.
12. González-Montalbán, N., E. García-Fruitós, S. Ventura, A. Arís, and A. Villaverde. 2006. The chaperone DnaK controls the fractioning of functional protein between soluble and insoluble cell fractions in inclusion body-forming cells. *Microb. Cell Fact.* **5:** 26.
13. Hardin, C., J. Edwards, A. Riell, D. Presutti, W. Miller, and D. Robertson. 2001. Time course assay of β-galactosidase, pp. 292–293. *In*: *Cloning, Gene Expression, and Protein Purification; Experimental Procedures and Process Rationale.* Oxford University Press.
14. Hoffmann, F., J. van den Heuvel, N. Zidek, and U. Rinas. 2004. Minimizing inclusion body formation during recombinant protein production in *Escherichia coli* at bench and pilot plant scale. *Enzyme Microb. Technol.* **34:** 235–241

15. Jevševar, S., V. Gaberc-Porekar, I. Fonda, B. Podobnik, J. Grdadolnik, and V. Menart. 2005. Production of nonclassical inclusion bodies from which correctly folded protein can be extracted. *Biotechnol. Prog.* **21:** 632–639.
16. Joo, J.-H. and J.-W. Yun. 2005. Structural and molecular characterization of extracellular polysaccharides produced by a new fungal strain, *Trichoderma erinaceum* DG-312. *J. Microbiol. Biotechnol.* **15:** 1250–1257.
17. Lee, Y.-J. and K.-H. Jung. 2007. Modulation of the tendency towards inclusion body formation of recombinant protein by the addition of glucose in the *araBAD* promoter system of *Escherichia coli*. *J. Microbiol. Biotechnol.* **17:** 1898–1903.
18. Kapust, R. B. and D. S. Waugh. 1999. *Escherichia coli* maltose-binding protein is uncommonly effective at promoting the solubility of polypeptides to which it is fused. *Protein Sci.* **8:** 1668–1674.
19. Koo, T. Y. and T. H. Park. 2007. Expression of recombinant human growth hormone in a soluble form in *Escherichia coli* by slowing down the protein synthesis rate. *J. Microbiol. Biotechnol.* **17:** 579–585.
20. Miller, J. M. 1973. *Experiments in Molecular Genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
21. Mogk, A., M. P. Mayer, and E. Deuerling. 2002. Mechanisms of protein folding: Molecular chaperones and their application in biotechnology. *Chembiochem* **3:** 807–814.
22. Schleif, R. 2000. Regulation of the L-arabinose operon of *Escherichia coli*. *Trends Genet.* **16:** 559–565.
23. Sørensen, H. P. and K. K. Mortensen. 2005. Advanced genetic strategies for recombinant protein expression in *Escherichia coli*. *J. Biotechnol.* **115:** 113–128.
24. Sørensen, H. P., H. U. Sperling-Petersen, and K. K. Mortensen. 2003. A favorable solubility partner for the recombinant expression of streptavidin. *Protein Expr. Purif.* **32:** 252–259.
25. Strandberg, L. and S.-O. Enfors. 1991. Factors influencing inclusion body formation in the production of a fused protein in *Escherichia coli*. *Appl. Environ. Microbiol.* **57:** 1669–1674.
26. Tokatlidis, K., P. Dhurjati, J. Millet, P. Béguin, and J. P. Aubert. 1991. High activity of inclusion bodies formed in *Escherichia coli* overproducing *Clostridium thermocellum* endoglucanase D. *FEBS Lett.* **282:** 205–208.
27. van den Berg, B., R. J. Ellis, and C. M. Dobson. 1999. Effects of macromolecular crowding on protein folding and aggregation. *EMBO J.* **18:** 6927–6933.
28. Ventura, S. and A. Villaverde. 2006. Protein quality in bacterial inclusion bodies. *Trends Biotechnol.* **24:** 179–185.
29. Vera, A., N. González-Montalbán, A. Arís, and A. Villaverde. 2007. The conformational quality of insoluble recombinant proteins is enhanced at low growth temperatures. *Biotechnol. Bioeng.* **96:** 1101–1106.
30. Worrall, D. M. and N. H. Goss. 1989. The formation of biologically active β-galactosidase inclusion bodies in *Escherichia coli*. *Aust. J. Biotechnol.* **3:** 28–32.