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ORIGINAL PAPER

Methyl α-D-glucopyranoside enhances the enzymatic activity of recombinant β-galactosidase inclusion bodies in the *araBAD* promoter system of *Escherichia coli*

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Abstract In this study, we utilized a catabolite repressor to improve the enzymatic activity of recombinant β-galactosidase inclusion bodies (IBs) produced in *Escherichia coli* under the *araBAD* promoter system. Specifically, we employed methyl α-D-glucopyranoside (α-MG) to lower the transcription rate of the β-galactosidase structural gene. In deepwell microtiter plate and lab-scale fermentor culture systems, we demonstrated that the addition of α-MG after induction improved the specific β-galactosidase production, even though β-galactosidase was still produced as an IB. Particularly, the addition of 0.0025% α-MG led to the most significant increase in the specific activity of the β-galactosidase. Interestingly, the β-galactosidase IBs obtained in the presence of 0.0025% α-MG were more loosely packed, as determined by IB solubilization in guanidine hydrochloride solution. We propose that the reduced gene transcription rate was responsible for the increased specific β-galactosidase activity and the loose packing that characterized the IBs produced in the presence of α-MG. This principle could be applied throughout the enzyme bioprocessing industry in order to enhance the activity of aggregate-prone enzymes within IBs.

Keywords Methyl α-D-glucopyranoside · β-Galactosidase inclusion body · *araBAD* promoter system · Protein expression rate · *Escherichia coli*

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Introduction

Insoluble protein aggregates, also known as inclusion bodies (IBs), frequently result from unfavorable protein-folding environments, such as when very high translation rates overburden the protein-folding machinery [21, 23]. Typical IBs are insoluble with heterogeneous conformational structures, and they are mainly comprised of misfolded, functionally inactive protein. One interesting exception is β-galactosidase [26] and its fusion proteins [8, 9], which have demonstrated enzymatic activity from within IBs. Previous studies have observed biological activity from green or blue fluorescent protein (GFP and BFP)-fused β-galactosidase IBs [9], and *Clostridium thermocellum* endoglucanase D [22] in *Escherichia coli* (*E. coli*) expression systems. Surprisingly, these IBs demonstrated relatively high enzymatic activity compared to soluble fractions [8, 9]. In support of these observations, other studies have suggested that IBs contained not only nonnative intermolecular β-sheet-rich structures, but also properly-folded polypeptides, resulting in the observed conformational variability [3, 11, 24]. It is conceivable, therefore, that some or all of these properly folded polypeptides could exhibit enzymatic activity.

In one analysis of IBs containing an *E. coli*-expressed GFP fusion protein, the final amount of biologically active protein in the IBs depended on how fast the aggregation event occurred [6]. The faster the protein aggregated, the lower the resulting fluorescence emission. At low temperatures (16–30 °C), the conformational quality of the aggregated GFP improved in a temperature-

dependent manner; lower temperatures enhanced the GFP fluorescence emission and enriched the *a*-helix structure within the GFP aggregates [25]. It is possible that lower temperatures improved the functional quality of the proteins within the IBs because the rates of transcription, translation, protein folding, and aggregation decrease as a function of temperature. In addition, others have reported that presence of the DnaK chaperone modulated the specific fluorescent activity of insoluble GFP [16]. Moreover, host organisms with cytosolic chaperone deficiencies produced GFP that was much less soluble, but much more fluorescent [10]. Interestingly, a new biosynthetic approach to produce human granulocyte-colony stimulating factor at low temperature yielded nonclassical IBs from which correctly folded protein could be extracted [12].

In agreement with these previous reports [8, 9, 22, 26], we observed herein that recombinant β-galactosidase expressed as IBs in *E. coli* had biological activity. Based on previous observations [6, 25], we sought to enhance the production of functional β-galactosidase within these IBs by changing the protein expression rate, specifically by reducing the transcription rate with methyl α-D-glucopyranoside (α-MG,), a non-metabolizable glucose analog known to be a catabolite repressor [1] and a modulator of glucose uptake [4]. We used the *araBAD* promoter system to express β-galactosidase in *E. coli*, a system in which induction of expression is mediated by the addition of L-arabinose. This promoter system is known to be sophisticatedly regulated by the activation and deactivation of dual positive regulators, such as AraC and catabolite activator protein (CAP) [15, 19, 21, 27]. We hypothesized that the addition of α-MG after L-arabinose induction would downregulate cAMP, due to partial inhibition of CAP, and as a consequence the transcription rate (and thus the protein expression rate) of β-galactosidase would be lowered. Based on the previous observations cited above, we predicted that this reduced protein expression rate would alter the structure and enhance the enzymatic activity of the resulting β-galactosidase IBs.

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 into the pBAD/*Myc*-His/*lacZ* vector (7.2 kb), a control vector from the pBAD/*Myc*-His expression kit (V440-01, Invitrogen). *E. coli* MC1061 was used as an expression host, and recombinant β-galactosidase (120 kDa) expression was induced by the addition of L-arabinose. More details, including the plasmid map, multi-cloning site, and *araBAD* promoter system, are described in the pBAD/*Myc*-His expression kit manual and in a previous report [13].

Deepwell microtiter plate cultures

The 96-deepwell microtiter plate culture system [7] was purchased from Kühner AG (Swiss) and utilized according to the manufacturer's instructions. Fortified Luria Broth (FLB) medium (tryptone 10 g/L; yeast extract 20 g/L; NaCl 5 g/L; ampicillin 100 μg/mL) was used as the culture medium. Cultures were maintained in a 37 °C shaking incubator (250 rpm). For each microplate culture, we dispensed 1 mL of sterile FLB into each of 60 deepwells per plate (we excluded the 36 deepwells along the outer edges), and 50 μL of seed culture was inoculated into each deepwell. To induce expression of recombinant β-galactosidase, 0.05% L-arabinose (Sigma) was added at the elapsed time of 3 h. Following induction, α-MG (Sigam) was added where indicated.

Lab-scale fermentor cultures

Fermentor cultures were conducted in a 5.0-L jar fermentor (KoBiotech, Republic of Korea) with a volume of 1.5 L, under conditions described previously [13, 14]. 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 used for positive selection (100 μg/mL). L-arabinose induction was performed at 0.05%, at the elapsed time of 3 h. If necessary, α-MG was added at the elapsed time of 3.5 h. For further analysis, cell pellets from each condition were collected by centrifugation, and the optical density at 600 nm (OD_{600}) was adjusted to 2.0 with phosphate-buffered saline(PBS), in order to standardize the amount of cell per unit of volume. For each sample, 1.5 mL of this standardized cell suspension was centrifuged, and the resulting cell pellets 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 the deepwell microtiter plate was monitored by OD_{595} (optical density at 595 nm) in a microplate reader (Model 680, RioRad, USA) as follows: 20 μL of culture broth from each well was collected and diluted 40-fold in PBS. For analysis, 60 μL of the diluted culture was transferred to a flat-bottomed 96-well microplate (SPL Life Sciences, Republic of Korea) and OD_{595} was measured for each sample. Cell growth in the fermentor was monitored

via OD_{600} measurements taken with a spectrophotometer (Optizen® 2120UV, Mecasys, Republic of Korea).

Assay of β-galactosidase activity

To measure β-galactosidase activity in the deepwell microtiter plate cultures, we conducted colorimetric assays in 96-well microplates, based on protocols from the yeast β-galactosidase assay kit manual (Product number 75768, Pierce) and from Miller's method [17, 18]. Color development was measured at OD_{415} using a microplate reader. For the lab-scale fermentor cultures, we measured β-galactosidase activity using the colorimetric assay described by Miller [18]. Color development for this assay was measured at OD_{420} using a spectrophotometer, and β-galactosidase activity was calculated as follows:

$$\beta - \text{Galactosidase (units/mL)} = \frac{(\text{OD}_{420})}{(0.0045) \times (1) \times (15)}$$

where OD_{420}, 0.0045, 1, and 15 are the OD measurement at 420 nm, molecular extinction coefficient ($nmole^{-1}$ mL cm^{-1}) of *o*-nitrophenol, light path length (cm), and reaction time (min), respectively. One unit of β-galactosidase was defined as the amount of enzyme required to produce 1 nmole of *o*-nitrophenol in 1 min at 28 °C and pH 7.

Separation of soluble and insoluble fractions

Cell pellets were disrupted non-mechanically using a cocktail containing 5 mL of B-PER® II bacterial protein extraction reagent (Product number 78260, Pierce) and 50 μL of 1 mg/mL DNase I (Sigma). The pellets were resuspended by vigorous vortexing and pipetting, and the resulting homogenous suspensions were centrifuged at 13,000 rpm for 5 min. After removal of the supernatants (soluble fractions), the pellets of cell debris (insoluble fractions) were resuspended in 150 μl of the B-PER® II reagent, as described in the manufacturer's instructions.

His-tag staining and western blotting

Proteins of interest were separated by 12.5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using a SE250 mini-vertical gel electrophoresis unit (Amersham Biosciences). Recombinant His-tagged β-galactosidase was visualized by a GelCode® 6 × His tag staining kit (Pierce), according to the manufacturer's instructions. For western blotting, proteins were transferred to nitrocellulose membranes (Hybond-C™ Extra, Amersham Biosciences) using a TE22 mini tank transfer unit (Amersham Biosciences), followed by sequential immunoprobing with 1:5,000 rabbit anti-β-galactosidase (Molecular Probes, USA) and 1:10,000 goat anti-rabbit IgG-HRP (horseradish peroxidase) conjugate (Abcam, UK). Immunoreactive bands were visualized using SuperSignal® West Pico chemiluminescent substrate (Pierce) followed by exposure on Hyperfilm® ECL (Pierce).

IB purification

Insoluble fractions were prepared and resuspended in 150 μL of B-PER® II reagent, as described above, and combined with 6 μL of 10 mg/mL lysozyme. The suspensions were vortexed for 1 min, combined with 1.0 mL of 1:20 diluted B-PER® II reagent, and vortexed for an additional minute. The resulting purified IBs were harvested by centrifugation at 15,000 rpm for 10 min. These purified IBs were used for the IB solubilization in guanidine hydrochloride. For the β-galactosidase assay of IB solution, the purified IB was prepared by twice washings, using 1:20 diluted B-PER® II reagent, and then resuspended in 1.0 mL of B-PER® reagent. To determine protein concentrations of the IB solutions, samples were subjected to 12.5% SDS-PAGE alongside a 2 mg/mL bovine serum albumin (Pierce) protein standard. Concentrations were quantified by gel image analysis using AlphaEase®FC software (Alpha Innotech, USA).

IB solubilization in guanidine hydrochloride solution

Purified IBs were resuspended in 1.0 mL of 10 mM Tris·HCl buffer (pH 7.5) containing 2–8 M guanidine hydrochloride and incubated in a room-temperature reciprocal shaker. After a 24-h incubation, 350 μL of the suspensions was transferred to 96-well microplates. Solubility was expressed as the turbidity of the suspension at 595 nm, which was measured using a microplate reader [2, 5, 20].

Results

Effect of α-MG on recombinant β-galactosidase production

To investigate the effect of the addition of α-MG on β-galactosidase production, we performed deepwell microtiter plate cultures of β-galactosidase-expressing *E. coli*, where α-MG, from 0.0 to 0.02%, was added after L-arabinose induction. As shown in Fig. 1a and b, the cell growth and β-galactosidase production were not severely inhibited by the addition of α-MG at 4 and 8 h after induction, compared to those at 24 h. However, the relative specific β-galactosidase productions were somewhat higher at 4 and 8 h after induction than when α-MG was added (Fig. 1c); although the effects were not directly proportional

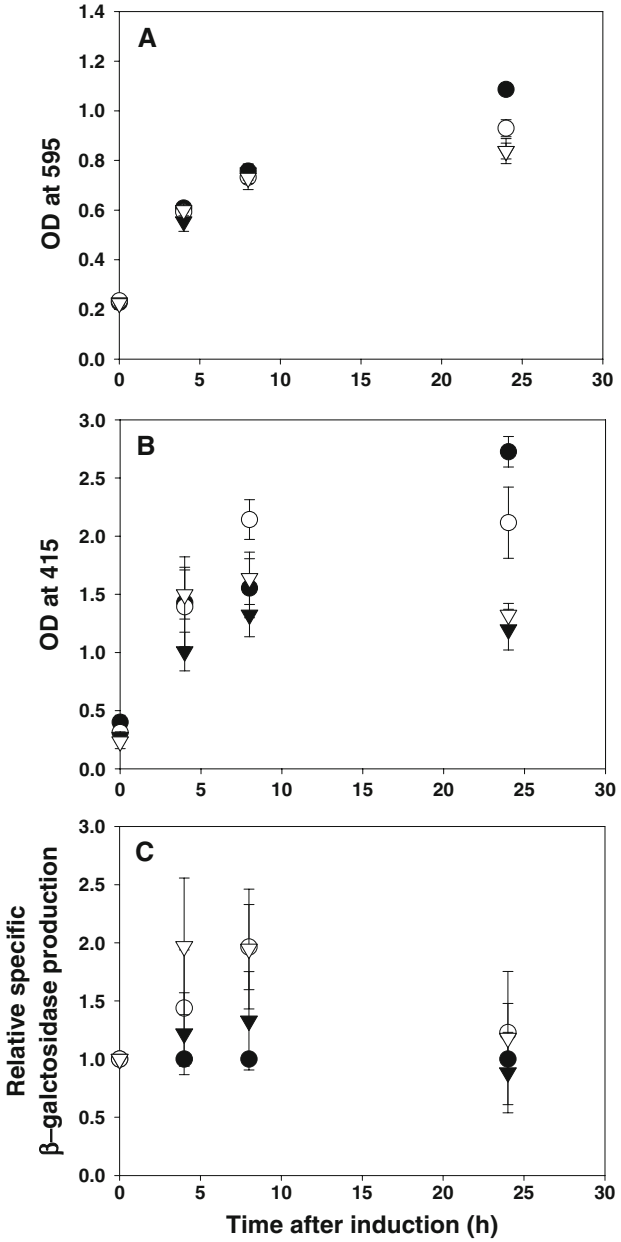

Fig. 1 Profiles of cell growth (**a**), β-galactosidase production (**b**), and relative specific β-galactosidase production (**c**) in deepwell microtiter plate culture. For the induction, 0.05% L-arabinose was added at the elapsed time of 3 h. At 3 h after induction, 0.005 to 0.02% α-MG was added. Symbol *filled circle* indicates no α-MG addition, and the other symbols indicate the following: the addition of 0.005% (*open circle*), 0.01% (*inverted filled triangle*), and 0.02% (*open square*) α-MG. OD at 415 nm (**b**) means readings from the β-galactosidase assay, indicating β-galactosidase production. Relative specific β-galactosidase production (**c**) is defined as specific β-galactosidase production normalized to untreated controls; i.e. measurements without α-MG were set at "1". In addition, the other values (*open circle*, *inverted filled triangle*, *open triangle*) in (**c**) were estimated based on the normalization of specific β-galactosidase production at zero time as "1". Shown are means and standard deviations calculated from six parallel cultures per condition

to the concentration of α-MG. Moreover, β-galactosidase was found in the insoluble fraction of the total cell lysate, that is, as an IB. These β-galactosidase productions as IBs were demonstrated in the results from His-tag staining and western blotting (Fig. 2a, b). Then the soluble fraction did not have enzymatic activity (data not shown). In the deepwell microtiter plate culture, it was demonstrated that the α-MG addition enhanced specific β-galactosidase production, although β-galactosidase was produced as an IB.

To verify the data from the deepwell microtiter plate culture, based on the same rationale, the effects of adding α-MG after induction on cell growth and β-galactosidase production were investigated in a lab-scale fermentor. Cell growth was fairly inhibited by the addition of 0.01% α-MG, and the 0.00125–0.005% α-MG additions led to moderate inhibitions of cell growth (Fig. 3a). However, β-galactosidase production and specific β-galactosidase production were relatively higher when 0.0025% α-MG was added as compared to when no α-MG was added (Fig. 3b, c). These values that occurred when 0.00125, 0.005, and 0.01% α-MG were added were not higher than those when no α-MG was added. We also observed that the β-galactosidase in the lab-scale fermentor culture was produced as an IB, regardless of the concentration of α-MG that was added (data not shown).

Specific activity of the IB solution

In the deepwell microtiter plate culture and lab-scale fermentor culture, it was shown that β-galactosidase production and its specific production were enhanced by the addition of 0.0025% α-MG after induction, and β-galactosidase was expressed as an IB in the insoluble fraction of the total cell lysate. In order to verify whether a change occurred in the specific β-galactosidase activity as a result of adding α-MG, and whether the β-galactosidase IB had enzymatic activity, the specific activity of the β-galactosidase IB solution was measured. Figure 4 shows the specific β-galactosidase activities of the IB solution when α-MG was added after induction. The specific β-galactosidase activity of the IB solution when 0.0025% α-MG was added was approximately four times higher than when α-MG was not added. It was also shown that the specific β-galactosidase activities of the IB solution increased by the addition of α-MG, up until the α-MG was added at the concentration of 0.0025%. Activity then started to decrease at 0.005% α-MG, and at 0.01% α-MG it was much less than when no α-MG was added. Overall, it was clearly demonstrated that the addition of 0.0025% α-MG after induction in the lab-scale fermentor culture led to an increase in the specific activity of β-galactosidase IB.

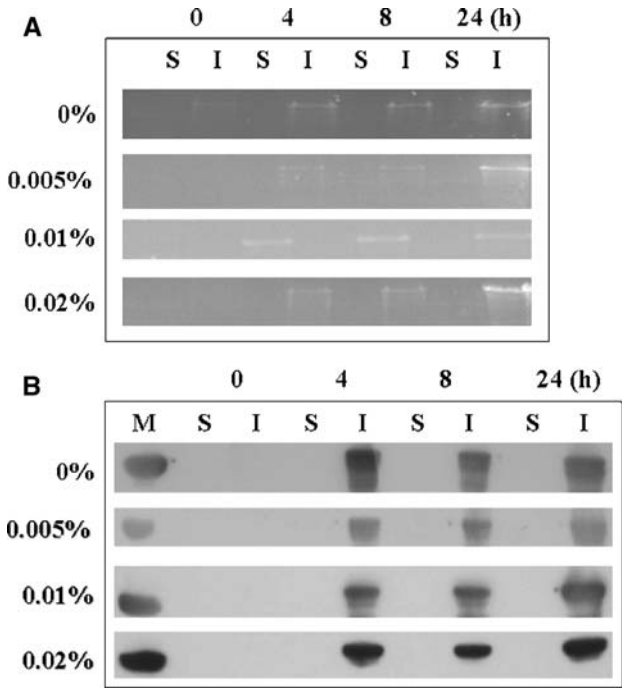

Fig. 2 Hig-tag staining (**a**) and western blotting (**b**) of the soluble and insoluble fractions of the total cell lysate from deepwell microtiter plate cultures. Times (0, 4, 8, 24 h) indicate the elapsed times after 0.05% L-arabinose induction. Total cell lysates obtained at the timepoints indicated were fractionated into soluble (*S*) and insoluble (*I*) fractions. The concentrations of α-MG (0–0.02% α-MG) added after L-arabinose induction are indicated to the left side of the blots. Sample loading volumes were 20 μL for Hig-tag staining and 3 μL for western blotting, respectively. Commercially-available *β*-galactosidase protein (1 mg/mL, Sigma), designated as "M," was used as a positive control for Western blotting

IB solubilization in guanidine hydrochloride solution

To better understand why the specific activity of the *β*-galactosidase IB solution was increased when 0.0025% α-MG was added following induction, we explored the structural property of the IBs, such as their physical compactness or hardness, by determining the stability of the IB in a chaotropic agent. As shown in Fig. 5, the stabilities of the IBs as their resistance to solubilization in guanidine hydrochloride, represented by the normalized turbidity of the IB solution, were measured. These profiles of the IBs showed an overall decrease by increasing concentrations of guanidine hydrochloride. The IB obtained from the cell grown when 0.0025% α-MG was added after induction was more rapidly solubilized in the guanidine hydrochloride solution as compared to that when α-MG was not added, although, in the 2-M guanidine hydrochloride solution, the turbidity increased by approximately 60% as compared to that when guanidine hydrochloride was not added.

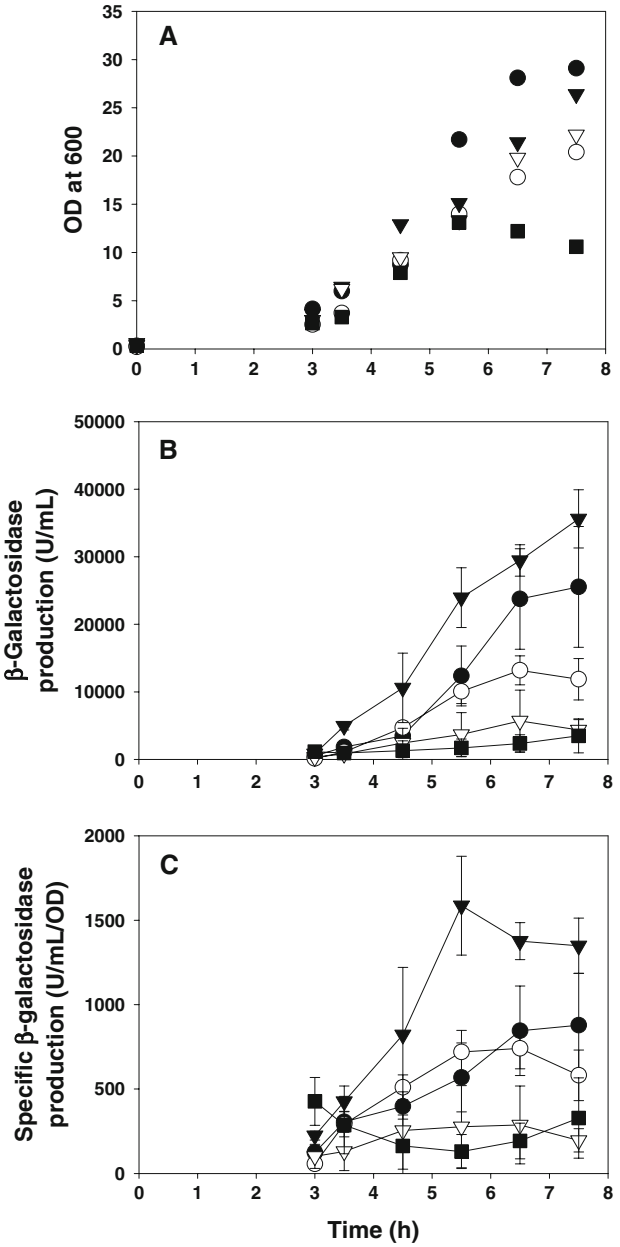

Fig. 3 Cell growth (**a**), *β*-galactosidase production (**b**), and specific *β*-galactosidase production (**c**) in the lab-scale fermentor. At the elapsed time 3 and 3.5 h, 0.05% L-arabinose induction and α-MG addition (0.00125to 0.01%) were performed. Symbol *filled circle* indicates no α-MG addition, and the other symbols indicate the following: the addition of 0.00125% (*open circle*), 0.0025% (*inverted filled triangle*), 0.005% (*open square*), and 0.01% (*filled square*) α-MG. The *β*-galactosidase productions were measured six times using the same sample, and means and standard deviations were calculated

Discussion

It has been observed that increasing the growth temperature above 37 °C reduces the specific activity of IBs of GFP fusion protein, while lowering the growth temperature

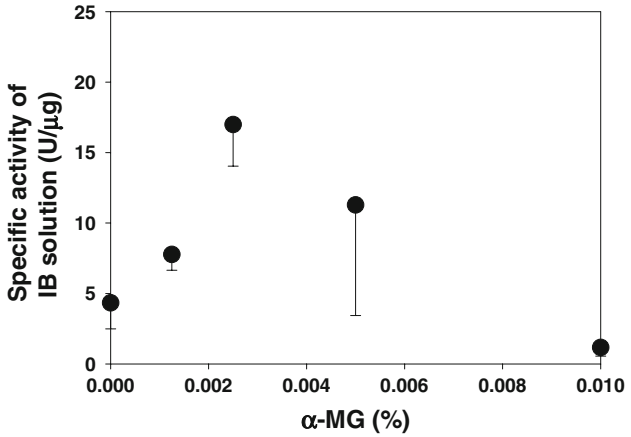

Fig. 4 Specific activity of the β-galactosidase IB solutions. IBs were obtained from samples of the lab-scale fermentor culture at the elapsed time of 5.5 h. β-Galactosidase assays of the IB solution were performed three times using the same sample, and their specific activities were estimated from dividing the β-galactosidase assays of the IB solution by their protein concentrations

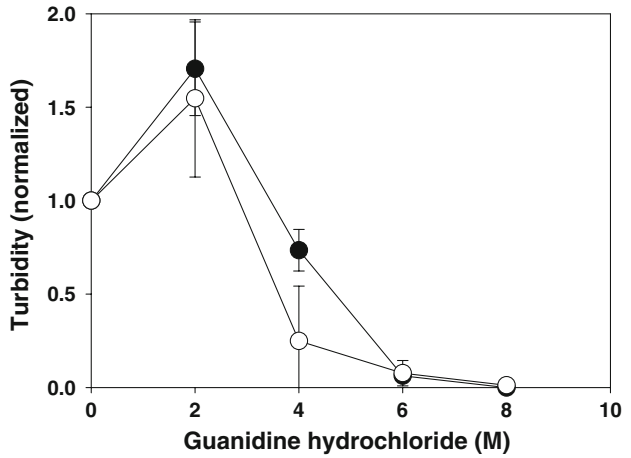

Fig. 5 Solubilization of β-galactosidase IB in guanidine hydrochloride solution. IBs were obtained from samples of the lab-scale fermentor culture at the elapsed time of 5.5 h. Symbols *filled circle* and *open circle* indicate no α-MG addition and the addition of 0.0025% α-MG, respectively. Turbidity values were normalized to the values measured in negative control samples (0 M guanidine hydrochloride). Turbidity at 8 M guanidine hydrochloride was used as a background. Means and SD of three independent experiments are shown

significantly increases the fluorescence emission of this IB protein [4]. Furthermore, the IBs formed at 37 and 42 °C exhibit higher resistance to proteolytic digestion and greater stability against denaturant. Additionally, it was found that a lower fluorescent emission of GFP fusion protein was attributed to faster protein aggregation [5]. Consequently, these results suggest that the structure of IBs, as well as their physical properties, could be controlled by parameters such as the growth temperature or growth rate, and thereby, these controls could cause a change in the biological activity of IBs. In this work, we attempted to change the β-galactosidase activity produced by IBs via the addition of a catabolite repressor, α-MG, after induction, rather than by temperature reduction. The β-galactosidase production rate was controlled by the addition of α-MG, where α-MG led to the repression of the *araBAD* promoter system, expecting that the overall β-galactosidase production rate had decreased. This decrease caused changes in the biological and physical properties of the β-galactosidase IBs.

The reason for the increase in the specific production of β-galactosidase by the addition of 0.0025% α-MG in the lab-scale fermentor was because the specific activity of the β-galactosidase IB solution was maximized although the cell growth was moderately inhibited (Figs. 3, 4). However, at the concentrations of 0.005 and 0.01% α-MG, it may be that cell growth inhibition began, and the specific β-galactosidase production decreased, because α-MG adversely works as a catabolite repressor against other protein expression systems. As a result, it was deduced that the addition of α-MG after induction repressed the function of CAP as a positive regulator in the *araBAD* promoter system, and then, the overall expression rate of β-galactosidase was lowered; more loosely packed β-galactosidase IBs were formed thereafter. However, this repression did not lead to the complete repression of the *araBAD* promoter system, because another positive regulator, AraC, still existed. Therefore, when 0.0025% α-MG was added after induction, the IBs had the enhanced β-galactosidase activity (Fig. 4).

In Fig. 5, it was elucidated that these IBs were more easily solubilized in guanidine hydrochloride solution as compared to those when α-MG was not added after induction. This result demonstrated that the IBs obtained when 0.0025% α-MG was added were more loosely packed as compared to those when α-MG was not added. As shown in previous reports [5, 6], it was deduced that the increase of activity for the β-galactosidase IB solution when 0.0025% α-MG was added, was attributed to their structural property changes. In this work, we controlled this change by the addition of a catabolite repressor, α-MG, and not by temperature reduction. However, when the β-galactosidase IBs were solubilized in a 2-M guanidine hydrochloride solution, the turbidity was increased in both cases of IBs. In these cases, it was deduced that the IBs were transiently inflated by the guanidine hydrochloride.

The addition effects of repressor or inducer analog against *araBAD* promoter system were recently reported. We observed a modulation toward an increase of soluble expression within 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 [14]. This may have been attributed to the slowing of the overall protein production rate, particularly

to the partial repression. In addition, we observed that D-fucose, an L-arabinose analog, also worked as α-MG did in this study. In that case, it was demonstrated that the addition of D-fucose after L-arabinose induction changed a portion of the α-helix and β-sheet structure within β-galactosidase IBs. It is expected that the structural changes of the β-galactosidase IBs after adding α-MG can also be observed by structure analysis.

Many recombinant industrial enzymes are produced as IBs in bacterial expression systems, and refolding processes are cost-intensive and time-consuming. These points make the existence of enzymatically active IBs a very attractive concept. Herein, we increased the enzymatic activity of β-galactosidase IBs by altering the protein production rate, using a catabolite repressor against the *araBAD* promoter system. Importantly, all β-galactosidase activities reported herein were measured from IBs, not from soluble protein. Our model dictates that by slowing the protein production rate, we increased the overall enzymatic activity of our protein of interest without having to create soluble protein. We expect that this principle will apply to other aggregate-prone enzymes when expressed in bacteria; therefore, our findings have unearthed a process that could save valuable time and production cost throughout the bioprocessing industry.

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