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SHORT COMMUNICATION

Correlation analysis for non-invasive quantitative monitoring of biological activity of recombinant enzyme using green fluorescence protein in *Escherichia coli* under various culture environments

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Abstract–Monitoring of biological activity for target enzyme is important for its production in recombinant expression systems. Previously, we demonstrated that green fluorescent protein (GFP) as a fusion partner is successfully tooled for facile, *in vivo*, and non-invasive quantification of target enzyme levels based on a linear relationship between GFP fluorescence and enzyme (chloramphenicol acetyltransferase; CAT) activity. Here, we investigated the effects of culture environmental variations (initial glucose amount, surface aeration, and inducer concentration) on correlation between GFP fluorescence and CAT activity, and established a general linear correlation as a unique criterion for quantitative monitoring of CAT biological activity. This general correlation for GFP fusion strategy can be applied for non-invasive and on-line monitoring of recombinant enzyme production under various culture conditions without further experimental calibrations.

Key words: Monitoring of Biological Activity, General Linear Correlation, Green Fluorescent Protein, Recombinant Enzyme, Fusion Protein, *Escherichia coli*

INTRODUCTION

Unique attributes of green fluorescent protein (GFP) as a protein marker are 1) it requires no co-factors or staining for fluorescence, and 2) the fluorescence is readily visible from outside the cells [Chalfie et al., 1994]. Therefore, simple non-invasive monitoring of GFP is possible *in vivo*. In our previous researches, we have constructed and expressed the unique fusion structure $(His)_6$-GFP-EK-X in several expression systems where X represents target foreign protein, $(His)_6$ represents a histidine affinity ligand for simple purification, and EK represents an enterokinase cleavage site for recovering foreign protein from the fusion [Cha et al., 1999a, b, 2000, 2005]. Since this fusion structure has a GFP (specifically, a UV variant, GFPuv) [Crameri et al., 1996], we could *in vivo* monitor expression of foreign protein using a GFP fusion partner. Also, we have successfully demonstrated that facile quantification of foreign protein levels could be achieved based on linear relationship between GFP fluorescence intensity and foreign protein amount [Cha et al., 1999a, b, 2000, 2005]. The slope of the correlation was typically fusion partner dependent [Cha et al., 2000]. We have found that this linear correlation was also established between GFP fluorescence and biological activity of foreign protein, especially for enzyme [Cha et al., 2000]. Therefore, we can apply this correlation into enzyme production in recombinant expression system for *in vivo* and on-line monitoring of its production status.

However, in the previous works, we did not investigate and establish a general linear correlation under variations of culture conditions. The generalization of the linear relationship under various culture environments would be important for practical application of this GFP fusion strategy in recombinant enzyme production. Therefore, in the present work, we established a general linear correlation between fluorescence of GFP fusion reporter and biological activity of target enzyme under various culture environments including different initial carbon source (glucose) amounts, culture volumes (surface aeration), and inducer concentrations. Here, we employed chloramphenicol acetyltransferase (CAT), which is a bacterial enzyme that confers antibiotic resistance to chloramphenicol as a model enzyme. It is widely used as a reporter because its enzymatic activity can be reliably assayed in several ways [Rodriquez and Tait, 1983; Kain and Gauguly, 1995].

MATERIALS AND METHODS

E. coli BL21 (*F' ompT hsdSB* (r_B^- mB^-) *gal dcm*) (Novagen, Madison, WI, USA), which is a derivative of *E. coli* B, was used as the host strain. The recombinant plasmid pTH-GFPuv/CAT [Cha et al., 2000] that contains trc promoter was used for expressing the GFP-CAT fusion protein (Fig. 1). Recombinant *E. coli* was grown to the mid-exponential phase (at ~OD_{600}=0.6) at 37 °C with shaking at 250 rpm in 50 mL or 100 mL M9 minimal medium (12.8 g/L $Na_2HPO_4 \cdot 7H_2O$, 3 g/L KH_2PO_4, 0.5 g/L NaCl, 1 g/L NH_4Cl, 3 mg/L $CaCl_2$, 1 mM $MgSO_4$, and glucose with different concentration of 0.5% or 2% (w/v)) containing 50 μg/mL ampicillin (Sigma) using 250-mL Erlenmeyer flask. These cultures were inoculated (1% (v/v))

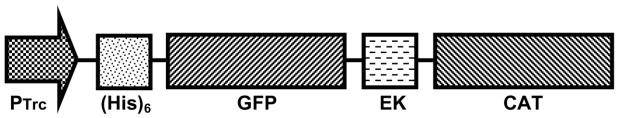

Fig. 1. Structure of the GFP-CAT fusion gene containing histidine affinity ligand $(His)_6$ and enterokinase cleavage site (EK) that is intracellular expressed by *trc* promoter (P_{Trc}) in *E. coli* BL21 [pTH-GFPuv/CAT].

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from 37 °C overnight cultures in the same medium. Recombinant *E. coli* BL21 was induced by the addition of 0.1 or 1 mM isopropyl β-D-thiogalactopyranoside (IPTG; Sigma) to express the fusion protein.

Cell growth was monitored by optical density (at 600 nm, OD_{600}) on a UV/VIS spectrophotometer (UV-1601PC; Shimadzu, Kyoto Japan). GFP fluorescence intensity of whole cells was measured with a fluorescence spectrometer (RF-5301PC; Shimadzu) at an excitation wavelength of 395 nm and emission at 509 nm. CAT activity in cell lysis supernatant was assayed using a UV/VIS spectrophotometer at 37 °C as described by Rodriguez and Tait [1983]; samples with high activity were diluted with deionized water to obtain readings in the linear range. The units of CAT activity were defined as nmoles/min.

RESULTS AND DISCUSSION

Several different culture environments [lower (0.5%) & higher (2%) initial glucose concentration, lower (100 mL working volume in 250-mL flask) & higher (50 mL working volume in 250-mL flask) surface aeration, and lower (0.1 mM) & higher (1 mM) IPTG concentration] were designed to investigate correlations between GFP fluorescence intensities and CAT biological activities in *E. coli* cultures. Through investigation of cell density profiles, we found that lower culture volume (higher surface aeration) and higher glucose amount caused higher cell densities (Fig. 2A). However, higher IPTG concentration caused lower cell density because high inducer levels can provoke metabolic burden on host cells by subsequent higher protein production [Glick, 1995]. These cell density profiles resulted in similar patterns on time profiles of CAT activity (Fig. 2B) and GFP fluorescence intensity (Fig. 2C). All CAT activity measurements were performed from cell lysis supernatants while GFP fluorescence was measured by using whole cells. The cell disruption step for CAT activity assay might cause some variations in the time profiles of CAT activity (Fig. 2B). Higher glucose amount, surface aeration, and inducer concentration showed higher production of GFP-CAT fusion protein, and these results demonstrated that recombinant *E. coli* cells need more carbon source and oxygen molecules, and enough inducer to produce more foreign protein. Therefore, the cultures under all environments with higher glucose, surface aeration, and inducer showed the highest production of fusion protein. Note that we used time (90 min)-shifted GFP fluorescence intensity data in Fig. 2C because it is widely known that synthesized GFP needs a time lag (~90 min) for chromophore formation, which is responsible for its fluorescence after induction and has been shown to be constant or irrespective of the fermenta-

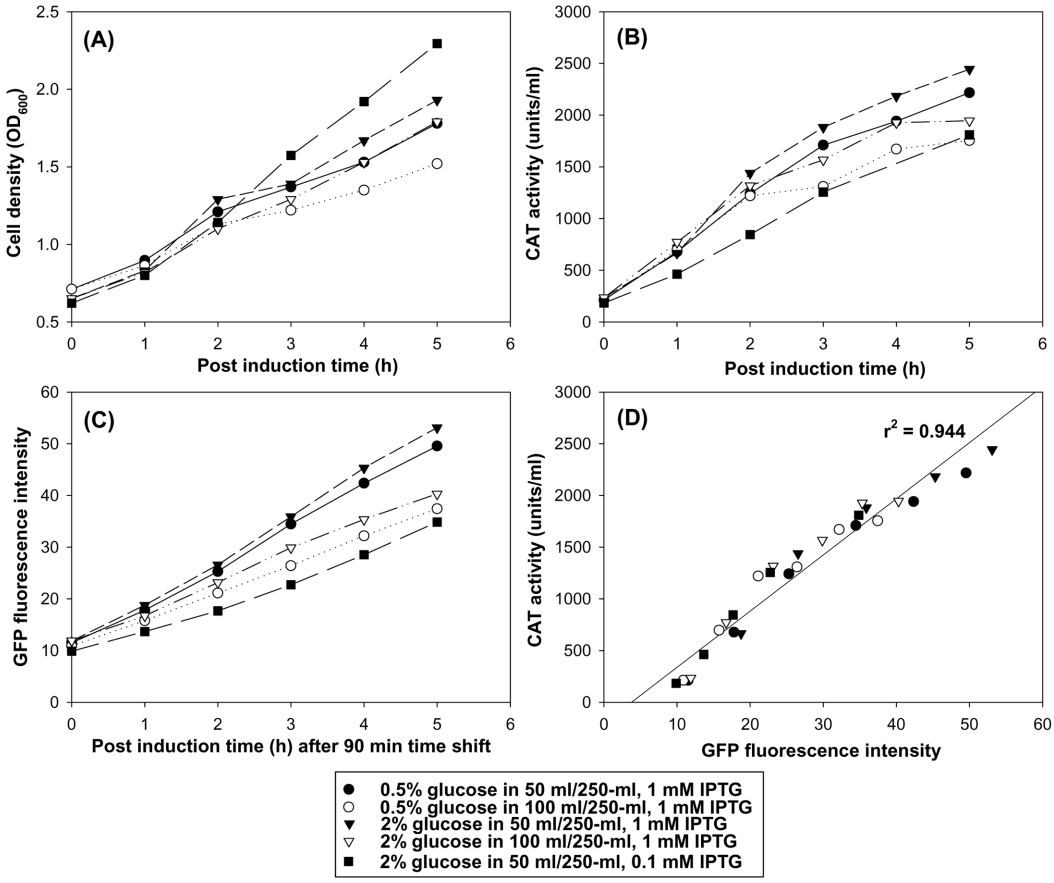

Fig. 2. Time profiles of (A) cell density, (B) CAT activity, and (C) GFP fluorescence intensity under different initial glucose concentrations and culture volumes. (D) Establishment of general correlation between GFP fluorescence intensity and CAT activity from all different cultures. GFP fluorescence intensity was measured by using whole cells by fluorescence spectrophotometer and CAT activity was measured by using cell lysis supernatants by UV/VIS spectrophotometer. Each value represents the mean of two independent experiments.

tion condition [Crameri et al., 1995; Albano et al., 1998], while other synthesized proteins such as CAT can have their biological function without delay after induction. As we reported previously, there was a linear relationship between GFP fluorescence and CAT biological activity when CAT activity was plotted against GFP fluorescence intensity (Fig. 2D). Based on this general linear relationship, we propose that biological activity levels of intracellular CAT can be quantified by simple detection of whole cell GFP fluorescence instead of intracellular GFP.

However, one would need to run calibration experiments under different culture conditions to obtain each correlation between fluorescence signal and product function, as opposed to rote monitoring GFP fluorescence. Importantly, all data from various culture environments showed the same linear correlation (Fig. 2D). This result demonstrates that one can obtain a general linear relationship between GFP fluorescence and biological activity levels of target enzyme. This general correlation can be applied for non-invasive and on-line monitoring of recombinant enzyme production under various culture conditions without further experimental calibrations when production strain and target enzyme are fixed.

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