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Green Fluorescent Protein : Evolutionary Reporter for Bioprocess ?

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INTRODUCTION

Now, for the first time, an appropriate marker exists for visualizing gene expression in real time. This marker is the green fluorescent protein (GFP). GFP was originally isolated from the jellyfish, *Aequorea victoria*, and was first described in 1962 (Shimomura et al., 1962). The cDNA of GFP was cloned from *Aequorea victoria* in 1992 (Prasher et al., 1992). GFP has suddenly become one of most exciting and useful proteins in the field of biotechnology. This excitement is originated from the recent discovery that the gene for GFP can be expressed in foreign cells as a fully fluorescent product (Chalfie et al., 1994). The value of this discovery is that the GFP can serve as a non-invasive marker of gene expression in living cells, tissues, and organisms. GFP applications abound in such areas as cell and developmental biology, neurobiology, and cytology.

GFP emits bright green light when simply exposed to UV or blue light, unlike other bioluminescent reporters. The emission of green light is due to the transfer of energy from the photoprotein, aequorin, of the organism to GFP (Johnson et al., 1962). GFP is a 238 amino acid protein with a molecular weight of 27 kDa (Chalfie et al., 1994). GFP has a major absorption peak at 395 nm and a minor peak at 470 nm with a single emission peak at 509 nm (Chalfie et al., 1994). GFP has several advantages such as its fluorescence is species-independent and requires no substrate, cofactor, or additional proteins for illuminating green light. As such, GFP has been studied recently as a novel genetic reporter molecule. GFP has been succesfully expressed in several host organisms and cells such as *E. coli*, yeast, mammalian cells, insect cells (Drosophila, Mosquitoes, Sf9), and plant cells. Unlike other reporter tags such as luciferase, b-galactosidase, or fluorescent-tagged antibodies, GFP does not require fixation techniques that are toxic to the cells under investigation (Chalfie et al., 1994).

In this research, we used a GFP variant, GFPuv, which was optimized for UV excitation (Crameri et al., 1996). GFPuv is 18 times brighter than wild-type GFP and can be easily detected by the naked eye when excited with standard, long-wave UV light (e.g., source for many DNA transilluminator light tables). This variant contains additional amino acid mutations which also increase the translational efficiency of the protein. GFPuv has the same excitation and emission maxima as wild-type GFP. GFPuv expressed in *E. coli* is a soluble, fluorescent protein even under conditions in which the majority of wild-type GFP is expressed

in a nonfluorescent form agglomerated into inclusion bodies.

In the present work, we have investigated the expression of a gene fusion, designed to facilitate product detection using GFP as a fusion partner. We demonstrated the use of GFP (specifically a UV variant, GFPuv (Crameri et al., 1996)) as a quantitative marker of protein level in several recombinant expression system including *Trichoplusia ni* insect larvae (Cha et al., 1997; Cha et al., 1999a; Cha et al., 1999b), Sf-9 insect cells (Cha et al., 1999c), *Escherichia coli* (Cha et al., 2000), and *Pichia pastoris*. In this work, the product-of-interest is human interleukin-2 (hIL-2). IL-2, initially known as T-cell growth factor (TCGF), is a powerful immunoregulatory lymphokine that has been evaluated as a therapeutic agent in the treatment of cancer (Rosenburg et al., 1984). IL-2 is also used extensively as a tissue culture reagent.

MATERIALS AND METHODS

Strains and Medium. The Sf-9 insect cell line (available from ATCC, CRL-1711) was used in cell culture experiments. The culture medium was either Grace's Insect media (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Sigma Chemical Co., St. Louis, MO) or serum-free Sf-900 II SFM (Life Technologies, Gaithersburg, MD). Sf-9 cells were routinely subcultured every three to four days. Total cell counts were performed with a hemacytometer (Fisher Scientific, Pittsburgh, PA), and viability was determined by Trypan blue (Sigma) exclusion using a 0.4% (w/v) solution. The insect larvae used were cabbage looper, *Trichoplusia ni*. The eggs (Entopath, Inc., Easton, PA) were hatched in Styrofoam cups containing solid food (Entopath) at 30°C, and the fourth instar larvae were used for infection experiments (Pham et al., 1999). *Escherichia coli* BL21 (*F' ompT hsdSB* (r_B^- *mB*$^-$) *gal dcm*; Novagen, Madison, WI) and JM105 (*F' Δlac-pro thi strA endA sbcB15 hspR4 tra36 proAB*$^+$ *lacI*^{q}*-ZΔM15*) were used for expressing the fusion proteins. Recombinant *E. coli* strains were grown to mid-exponential phase (at ~ OD_{600} = 0.6) at 37°C in 200 mL of LB (Luria broth) medium (5 g/L yeast extract (Sigma Chemical Co., St. Louis, MO), 10 g/L bacto-tryptone (Difco Lab., Detroit, MI), and 10 g/L NaCl) containing 50 μg/mL ampicillin (Sigma). These cultures were inoculated (5% (v/v)) from 37°C overnight cultures in the same medium. At OD_{600} = 0.6, *E. coli* strains were induced by the addition of 1 mM isopropyl β-D-thiogalactopyranoside (IPTG; Sigma) to express the fusion proteins. *Pichia pastoris* GS115 (*his4*) (Invitrogen) was used for protein expression. Recombinant *P. pastoris* strains were grown at 30°C in 100 ml of BMM (Buffered Minimal Methanol; 100 mM potassium phosphate, pH 6.0, 1.34% (w/v) yeast nitrogen base, $4x10^{-5}$% (w/v) biotin (Sigma Chemical Co., St. Louis, MO), and 0.5% methanol) and BMMC (BMM with 1% casamino acids (Sigma)) after resuspending from harvesting with 50 ml overnight cultures that were grown in MGY (Minimal Glycerol Medium; 1.34% YNB, 1% glycerol, and $4x10^{-5}$% biotin).

Analytical Assays. The total protein assay was performed using a protein assay kit (Bio-Rad Lab., Hercules, CA) with bovine serum albumin as a standard. The GFPuv whole cell assay was performed by measuring fluorescence intensity using a fluorescence spectrometer (LS-3B; Perkin-Elmer Ltd., Beaconsfield, Buckinghamshire, England) at an excitation wavelength of 395 nm and emission at 509 nm. The quantities of hIL- was determined using pure recombinant hIL-2 (Life Technologies) as a calibration standard on Western blots.

RESULTS AND DISCUSSION

GFP Fusion Techniques in *Trichoplusia ni* Insect Larvae. Time course larvae samples were examined under UV illumination. Until 60 hour post infection (hpi), no fluorescence was seen. However, at 74 hpi and 84 hpi, larvae emitted bright green fluorescence upon UV excitation, which implied that the GFPuv/hIL-2 fusion protein was expressed. The average larva mass increased continuously throughout the culture (fourth instar). To quantify the GFPuv fluorescence and hIL-2 mass, we homogenized the larvae in buffer, centrifuged, and saved the supernatants as soluble lysates. The fluorescence of GFPuv in these lysates was insignificant until 60 hpi, but increased rapidly to a maximum at 74 hpi. It then rapidly decreased by 63% due to proteolysis; this was almost identical to our previous report on GFPuv expression as a single protein (Cha et al., 1997). Quantification of hIL-2 mass was performed by densitometric scanning of Western blots. Importantly, the hIL-2 profile was closely tracked the GFPuv fluorescence intensity.

More importantly, because of the sharp peaks in product yield apparent at *each* virus loading, it became obvious that harvest time was critical for obtaining a high fusion protein yield. A linear correlation was obtained between GFPuv fluorescence intensity and hIL-2 mass in several samples from several infection experiments. Interestingly, this correlation was obtained on the actual undiluted samples, spanning the entire observed range of fluorescence. Based on these correlations, the *in vivo* quantification of hIL-2 was made possible by simple measurement of GFPuv fluorescence intensity, obviating the need for laborious Western blot and enzyme linked-immuno-sorbent (ELISA) assays.

GFP Fusion Techniques in Sf-9 Insect Cells. The expression of hIL-2 as a fusion protein with GFPuv and as a single protein in suspended Sf-9 insect cells was investigated in spinner flask cultures. As expected, the final cell density of the uninfected cells (control) was higher than those of infected cells by recombinant viruses *v*PH-GFPuv:hIL2 and *v*PH-hIL2. The viabilities of infected cells rapidly de-creased after 60 hpi. There was no significant difference in either the final cell concentration or the cell death rate due to the addition of the GFPuv portion of the fusion protein. In the case of the fusion with GFPuv, the green fluorescence intensity increased significantly after 48 hpi and reached a maximum at 84 hpi. The decrease in fluorescence

intensity after 84 hpi was likely due to proteolysis (Cha et al., 1997) as well as cell lysis. Notably, after 84 hpi the green fluorescence intensity in the culture medium (extracellular) increased exponen-tially, which is consistent with cell lysis as the viability dropped precipitously at that time. The hIL-2 expression profile, as determined by Western blot, is depicted. Both infected Sf-9 cells had identical profiles with maxima at 72 hpi. The maximum concentration of hIL-2 expressed as a single protein was 0.27 μg/mL, while the maximum concentration of hIL-2 as a GFPuv fusion was 0.31 μg/mL, suggesting a slight increase (~15%) in the soluble fraction was af-forded by fusion with GFPuv. The hIL-2 in both was approximately 0.01% of the total protein in the soluble lysates. The profile of hIL-2 concentration from GFPuv fusion was virtually identical to that of GFPuv fluorescence intensity. Therefore, GFP levels serve as an indicator of hIL-2 expression in the suspended Sf-9 cell culture.

GFP Fusion Techniques in *Escherichia coli.* The cell densities of BL21 cultures were generally higher than those of JM105 strains, which was consistent with previous reports (eg, VandeWalle and Shiloach, 1988). Interestingly, there was no large difference in growth rate for cells producing the GFP fusion when compared to cells only producing the product (CAT ; chloramphenicol acetyltransferase). Hence, the addition of GFP did not pose a large metabolic burden on the cells, which is a positive result for the use of GFP as a fusion partner. The fluorescence intensity of GFPuv increased with culture time after IPTG induction in both strains. Strain BL21 had a higher initial fluorescence intensity, implying that the expression was not tightly repressed even though the parent pTrcHis plasmid contains the *lacI*^{q} gene for tight regulation of the *trc* promoter. We also observed this leaky expression in BL21 from the plots of CAT concentration determined by Western blot. Conversely, the foreign protein expression was relatively tightly regulated in JM105 (*F' lacI*^{q} in combination with plasmid *lacI*^{q}). We observed significantly lower (4 times less) CAT activity in the fusion constructs when expressed on a per μg CAT basis. Thus, we suspected the CAT monomer was not as active when bound to the GFP reporter. There was roughly two-fold less total volumetric CAT in the cells which, because they are similar molecular weight, corresponded roughly to the contribution of GFP in the fusion (at similar expression rate of recombinant protein which was confirmed by Western blot). However, there was a 4-fold decrease in activity of the expressed CAT suggesting that the GFP-bound CAT was 2-fold lower in activity than the native protein.

Linear regression analyses of GFPuv fluorescence intensity with the concentration of the product proteins (CAT, OPH (organophosphorous hydrolase), hIL-2) and enzymatic activity (CAT, OPH) are shown. Because of the time lag (~95 min) for GFPuv chromophore cyclization, which has been shown to be constant or irrespective of the fermentation condition, the GFPuv fluorescence intensity was shifted 95 min. Linear correlations were obtained in both

amount and activity for both GFPuv/CAT and GFPuv/OPH samples. Interestingly, the correlation between fluorescence and CAT was obtained for samples of two different *E. coli* strains BL21 and JM105, demonstrating that the correlation was independent of the host strain. One observation, however, was that the slope of the correlation was typically fusion partner dependent. That is, one would need to run a calibration experiment to obtain the fluorescence signal that correlates with product quantity, as opposed to rote monitoring GFP fluorescence.

GFP Fusion Techniques in *Pichia pastoris*. Several profiles of GFP fluorescence intensity were depicted for the Mut^S intracellular expression system using 0.5% methanol induction. The methanol was present in the media from time zero. The hIL-2 in the GFP fusion protein was analyzed from the soluble lysate fraction. A polyclonal anti-hIL-2 antibody was used for detection. The hIL-2 amount increased and reached maximum (1.17 μg/mL) at around 72 h, while the fluorescence increased steadily throughout. We suspect that this might be due to the partial proteolysis of hIL-2 either as part of the fusion or as a clipped intact molecule. We have also shown that some cleavage between the GFP and hIL-2 occurs in insect larvae (Cha et al., 1999a). On the other hand, GFP is known for its stability (Chalfie et al., 1994). The hIL-2 was quantified by Western blot in several fractions and the corresponding fluorescence intensity was plotted as a function of hIL-2. A linear relationship between the two, particularly in the middle of the fermentation. For the later points, proteolysis was likely, while in the early stages, the data was close to lower sensitivity limits of Western blots. Importantly, the relationship between hIL-2 and fluorescence was quite good for the bulk of the fermentation (12 to 72 h). Therefore, hIL-2 can be quantified by simple detection of GFP fluorescence. This is particularly attractive in yeast since monitoring intracellular protein production requires significant effort as yeast cell walls are quite resistant to lysis.

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