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Effect of Glycine and Triton X-100 on secretion and expression of ZZ–EGFP fusion protein

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Abstract

Two-factor and three-level fractional factorial design was employed for evaluation of the effect of Glycine and Triton X-100 on the secretion and expression of ZZ–EGFP fusion proteins. Varying contents of glycine (0%, 1%, 2%) and Triton X-100 (0%, 1%, 2%) were added into shaking flasks, respectively, and supplied with appropriate volume of ampicillin (total 9 combinations; group at concentration zero serving as control) to promote more ZZ–EGFP diffuse into liquid culture medium. Fluorescent intensity in the culture supernatant was detected. A standard curve could be generated on the basis of fluorescent intensity and protein concentration. The expression level of ZZ–EGFP fusion proteins was estimated by checking the protein standard curve concentration fluorescene intensity. Results show that when the culture medium contains 2% Glycine and 1% Triton X-100, the expression level of ZZ–EGFP was able to be greatly increased. Further experiments revealed that absorbance value (A_{600}) in the experiment group, whose culture medium contains 2% Glycine and 2% Triton X-100, is significantly lower than other groups in the present experiment. These results indicate that the culture medium containing appropriate quantity of Glycine and Triton X-100 is favourable to the secretion and expression level of ZZ–EGFP in gene-engineering bacteria *Escherichia coli* HB101.

Keywords: Gene-engineering bacteria; *E. coli* HB101; ZZ–EGFP; Triton X-100; Glycine; Secretion

1. Introduction

Green fluorescent protein (GFP) is naturally fluorescent protein in which the chromophore (fluorophore) is derived from posttranslational cyclization of a serine–tyrosine–glycine tripeptide of GFP, followed by dehydrogenation of the tyrosine (Brejc et al., 1997; Kristensen, Sperling-Petersen, Mortensen, & Sfirensen, 2005; Neubauer & Osterrieder, 2004). Since its original discovery in the jellyfish *Aequorea victoria*, it has proven valuable in a plethora of biochemical, cellular, and developmental investigations. Its amazing ability to generate a highly visible, efficiently emitting internal fluorophore is both intrinsically fascinating and tremendously valuable. Thus, GFP requires no exogenous moiety for fluorescence, making it a tremendously useful marker in in vivo studies. High-resolution crystal structures of GFP offer unprecedented opportunities to understand and manipulate the relation between protein structure and spectroscopic function (Brede, Solheim, Stang, & Prydz, 2003; Tirat, Freuler, Stettler, Mayr, & Leder, 2006; Tsien, 1998). GFP has become well established as a marker of gene expression and protein targeting in intact cells and organisms. Mutagenesis and engineering of GFP into chimeric proteins are opening new vistas in physiological indicators, biosensors, and photochemical memories (Heessen, Dantuma, Tessarz, Jellne, & Masucci, 2003).

The usefulness of the fluorescence assay for detecting nisin in food samples was tested with cheese, milk, and salad dressings. At present, the most widely used quantification assay for nisin, the agar diffusion method, which was

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developed by Tramer and Fowler in 1964, is more sensitive than most of the immunological methods; the detection limit for nisin in a sandwich spread in this test was 100 ng/g (Tramer & Fowler, 1964). The major drawback of this assay, however, is its inability to differentiate nisin from other inhibitory substances (Fowler, Jarvis, & Tramer, 1975). The ELISA test described by Falahee and Adams (1992) suffers from the same problem and gives false-positive results when samples contain subtilin, the lantibiotic structurally similar to nisin. In the GFP test the amount of fluorescence is based on the amount of GFP molecules; therefore, a lag phase in growth is an adequate time for measurement of fluorescence. Furthermore, since GFP fluorescence is induced solely by photoactivation, no substrate addition is required. The simplicity of the GFP test allows the analysis of hundreds of samples at the same time to detect 45 ng of nisin per ml in milk, 0.9 μg of nisin per g in cheese, and 1 μg of nisin per g in salad dressings, compared to the levels of more than 1 μg/ml or 1 μg/g used in food manufacturing (de Vuyst & Vandamme, 1994; Thomas, Clarkson, & Delves-Broughton, 2000), and makes it possible to analyse nisin in different kinds of food matrices. Therefore, the GFP assay could be widely used in the food industry, as well as in basic research.

Among the most remarkable attributes of the original green fluorescent protein (GFP) derived from the *A. victoria* jellyfish, as well as the more recently developed palette of colour-shifted genetic variants, is that the entire 27 kDa polypeptide structure is essential for the development and maintenance of fluorescence in this very remarkable family of proteins. The principle fluorophore (often termed a chromophore) is a tripeptide consisting of the residues serine, tyrosine, and glycine at positions 65–67 in the sequence. Although this simple amino acid motif is commonly found throughout nature, it does not generally result in fluorescence. This interactive tutorial explores the molecular rearrangement that occurs during the formation of the enhanced green fluorescent protein (EGFP) fluorophore, which substitutes threonine for serine at position 65 in the amino acid sequence. Prokaryotic secreting expression vectors (pEZZ–EGFP) were constructed in *Escherichia coli* with the helper plasmid pEZZ 18, by recombinant domain ZZ of enhanced green fluorescent protein (EGFP) and staphylococcus protein (SPA) essentially as described previously (Domínguez, Lorenzo, & Blasco, 1998; Ojala, Mottershead, Suokko, & Oker-Blom, 2001; Xi, Lambrecht, Vanderleyden, & Michiels, 1999). In this system, non-induced expression of exogenous gene was determined by both Lac Promoter and SPA Promoter. This is favourable to the secretion of exogenous proteins. SPA signal peptide sequences can help the secretion of expression products of exogenous gene into culture medium. Because ZZ–EGFP in *E. coli* has the common biological characteristics of SPA and EGFP, it may be employed as a common affinity reagent in immunofluorescence assay.

The ZZ–EGFP fusion protein was expressed in *E. coli* with a His tag and purified in high yield by one-step Ni(2+) chelating affinity chromatography. It was then used in the immunoblot analysis of GST and TNFα as well as in immunofluorescent assays of 293T cells transfected with IRF3, an interferon regulatory factor which is localized in cytoplasm without virus infection. The fusion protein also performed effectively in FACS analysis of surface integrin β3 subunit on 293T cells (Huang et al., 2006). The chimeric protein binds various antibodies from different animal sources, directed against a variety of proteins. Thus, ZZ–EGFP showed a broad promise in potential immunological applications. However, ZZ–EGFP was mostly located in cells' periplasmic space in conventional culture conditions. Osmotic pressure of bacteria cells needs to be changed so as to release more objective proteins from cells' periplasmic space. To overcome the problem, except for optimization of expression vectors and signal peptide sequences, it is also necessary to change osmotic pressure in bacteria cells' outer membrane so as to make ZZ–EGFP concentrated in cells' periplasmic space to be released. This is extremely important to later separation and purification.

Both Glycine and Triton X-100 can change osmotic pressure in bacteria cells' outer membrane (Ma, Gao, Mao, Zhou, & Shen, 2004; Monsen, Holm, & Burman, 1983). To better understand their physiological and biological part, we investigated the effect of Glycine and Triton X-100 on the secretion and expression of ZZ–EGFP. We also investigated the expression level of ZZ–EGFP in culture liquid by utilizing fluorescence characteristic of EGFP.

2. Materials and methods

2.1. Main materials and chemicals

The prokaryotic secretion expression vectors pEZZ–EGFP and gene-engineering bacteria *E. coli* HB101/pEZZ–EGFP were constructed by consulting the literature (Fire, Harrison, & Dixon, 1990) and preserved in this laboratory. ZZ–EGFP fusion proteins (electrophoresis purity $\geqslant$ 95%) were prepared in this laboratory by consulting the literature (Zhang, Yoshimatsu, Hildebrand, Frisch, & Goodman, 2003). Peptone and yeast powder were obtained from Oxiod Ltd (England). The Glycine and Triton X-100 were purchased from ShangHai Chemical Reagent Ltd. attached to China Medicine Group (ShangHai, China). BCA proteins test kit was purchased from Pierce Ltd (Rockford, IL, America). Other chemicals were all of homemade analytical grade.

2.2. The preparation of standard curve

The standard curve method is usually based on the values of fluorescent intensity in the culture supernatant. The concentrations of ZZ–EGFP fusion proteins in the culture supernatant were then calculated from the slope of the standard curve. After the initial concentrations of ZZ–EGFP fusion proteins were determined using a BCA protein Test Kit, ZZ–EGFP fusion proteins were diluted with

deionized water. The mean fluorescent intensity of each sample was determined by using F-250 Fluorescence Spectrophotometer (SHIMADZU, Japan). A standard curve is prepared by plotting the fluorescent intensities of the standards against their lot specific protein concentrations (the maximum excitation wavelength/the maximum emission wavelength = 489 nm/511 nm). The standard curve was used to read off the expression level of ZZ–EGFP fusion proteins samples.

2.3. Determination of gene-engineering bacteria culture time

Active gene-engineering bacteria were diluted 10 times with the same medium and inoculated into fresh LB culture medium containing appropriate amount of ampicillin (70 μg/ml) and were cultivated in shaking flasks at 30 °C. The absorbance value and fluorescent intensity in liquid bacterial cultures were then detected (600 nm) in 6 h intervals. Further, the culture of the gene-engineering bacteria was incubated at 30 °C with shaking (150 rpm) until the maximum optical density of the medium at OD600 nm was reached.

2.4. Effect of Glycine and Triton X-100 on the secretion and expression of ZZ–EGFP fusion proteins

Two-factor and three-level fractional factorial design was employed for evaluation of the effect of Glycine and Triton X-100 on the secretion and expression of ZZ–EGFP fusion proteins. Active gene-engineering bacteria were diluted 10 times with the same medium and inoculated into fresh LB culture medium containing appropriate amount of ampicillin (70 μg/ml) and were cultivated at 30 °C with shaking (150 rpm) until the optical density of the medium at OD600 nm reached 0.6. Varying contents of glycine (0%, 1%, 2%) and Triton X-100 (0%, 1%, 2%) were added into shaking flasks, respectively, and supplied with appropriate volume of ampicillin. Further, the culture of the gene-engineering bacteria was cultivated at 30 °C with shaking (150 rpm) for 36 h. Expression of the gene-engineering bacteria was induced by the addition of 2 μl isopropyl-L-D-thiogalactopyranoside at a final concentration of 1 mM. After further incubation for 2 h, cells were harvested by centrifugation. The cell pellets were resuspended in 1:15 volume of a buffer containing 20 mM phosphate, 0.5 M NaCl, and 10 mM imidazole, then cell disruption was performed by ultrasonication. To detect growth condition of the gene-engineering bacteria, appropriate liquid bacteria culture was taken. Fluorescent intensity in the culture supernatant was detected. The expression level of ZZ–EGFP fusion proteins was estimated by checking the protein concentration-fluorescent intensity standard curve.

2.5. Statistics

The fluorescent intensity was considered to be the experimental unit in all statistical analyses performed. Results were expressed as mean ± SE. Data from fluorescent intensity were evaluated by multivariate profile analysis (Bourguignon & Morrisson, 1990). All analyses were performed using the SPSS package version 15.0 for Windows (SPSS Inc., Chicago, IL). The level of statistical significance was set at 5%.

3. Results

3.1. Standard curve of concentration and fluorescence-intensity of ZZ–EGFP fusion protein

The standard curve of concentration and fluorescence-intensity of ZZ–EGFP fusion protein showed that there was a good linearity relationship between concentration and fluorescent intensity in the observed concentration range ($y = 159.06x - 74.707$; $r = 0.997$). The statistical analyses also showed that there was no significant difference between linearity relationship of standard curve of concentration and fluorescence-intensity of ZZ–EGFP fusion protein used as standard sample ($r = 0.997$) and linearity relationship of standard curve of concentration and fluorescene-intensity of monomer EGFP ($r = 0.998$) (Hack et al., 2000), indicating that the standard curve may be used to determine the concentration of ZZ–EGFP fusion protein in the culture supernatant.

3.2. Determination of gene-engineering bacteria culture time

Fig. 1 shows the growth curve of the gene-engineering bacteria *E. coli* and fluorescent intensity curve in the culture supernatant. The gene-engineering bacteria were incubated at 30 °C with shaking (150 rpm) for 36 h until the maximum optical density of the medium at OD600 nm in the culture supernatant was reached. The increased fluorescent intensity was no longer observed with increase in the length of culture time. By contrast, a decreased fluorescent intensity at OD600 nm was detected. This shows that 36 h

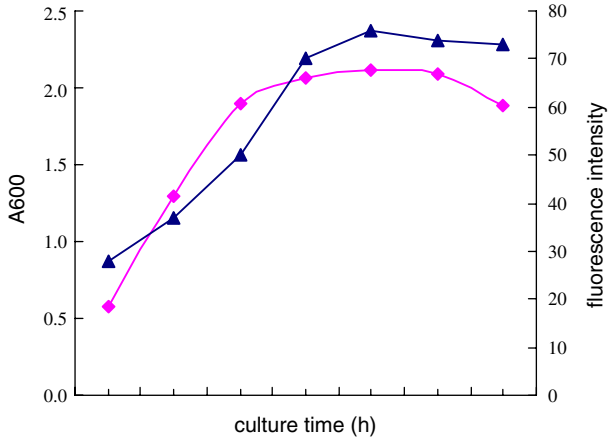

Fig. 1. Growth curve of the recombinant *E. coli* and fluorescent intensity curve in the culture supernatant. ▲: fluorescent intensity, ◆: A_{600}.

of culture time is optimal for the gene-engineering bacteria *E. coli* growth.

3.3. Effect of Glycine and Triton X-100 on the secretion and expression of ZZ–EGFP fusion proteins

Factorial ANOVA has two independent variables which are crossed with each other. This means each value of one variable is paired with every value of the other variable. Two or more individuals are assigned to each combination of values of the independent variables. For this kind of analysis, there is only one dependent variable which is collected from every individual in the study. Table 1 shows the fluorescent intensity value in the culture supernatant in shaking flasks and absorbance value (A_{600}) in liquid bacteria culture medium under different culture conditions. The fluorescent intensity value in the culture supernatant was analysed by employing factorial analysis of variance. The factorial analysis design of variance was randomly assigned to a glycine group and a Triton X-100 group. Data analysis was done on both the glycine group and the Triton X-100 group (Table 2). In glycine level, $F = 10.881$, $P = 0.024$ between Triton X-100 groups; In Triton X-100 level, $F = 12.848$, $P = 0.018$ between glycine groups. Moreover, statistics analysis showed that there was a significant interaction-effect between the glycine groups and the Triton X-100 groups ($F = 5.441$, $P = 0.005$). Maximum fluorescent intensity in the culture supernatant can be detected in culture medium containing 2% Glycine and 1% Triton X-100, as shown in Table 1. At the same time, the expression level of ZZ–EGFP fusion protein could reach 10.4 mg/L, which increased 11 times more than control group (0.94 mg/L).

Table 1
Fluorescent intensity in culture supernatant and A_{600} value of recombinant *E. coli* in different culture conditions

Concentration of glycine (%)	Concentration of Triton X-100 (%)	A_{600}	Fluorescent intensity in culture supernatant
0	0	2.39	74 ± 5
0	1	2.01	622 ± 25
0	2	1.95	733 ± 25
1	0	2.12	283 ± 11
1	1	1.96	1066 ± 43
1	2	1.78	956 ± 35
2	0	1.90	711 ± 19
2	1	1.89	1854 ± 45
2	2	1.06	1199 ± 54

Table 2
Statistics analysis

Factor	DF	*F*	*P*
Glycine	2	10.881	0.024
Triton X-100	2	12.848	0.018
Glycine and Triton X-100	4	5.441	0.005

4. Discussion

SDS-PAGE is usually used for determining the expression level of objective proteins. SDS-PAGE is a denaturing electrophoresis, a new development in this technique is the complete removal and exchange of SDS bound to proteins (including hydrophobic proteins) and restoration of the latter's activity (Tsubone, Yoshikawa, Okada, & Abe, 2007). This involves loading the SDS-protein complexes onto a ceramic hydroxyapatite column, extensive washing of bound proteins with a mild detergent in a phosphate buffer, followed by elution of the retained protein with a phosphate gradient (Hsieh, Calcutt, Chapman, Mitra, & Smith, 2003; Lin, Gangloff, Huang, & Xie, 1999). Complete exchange of SDS with non-ionic detergent such as dodecyl maltoside was achieved with 90–100% protein recovery. It was proposed that the efficiency of SDS removal from protein was due to the combined effect of phosphate ions and the hydrophobic tail of non-ionic detergent (Geueke & Hummel, 2002; Sattarahmady, Khodagholi, Moosavi-Movahedi, Heli, & Hakimelahi, 2007).

In fact, determination of the expression level of objective proteins with coomassie brilliant blue staining has lower sensitivity (0.1–1.0 μg). When protein concentration in sample is below detection limit, it is often necessary to treat sample with a more sensitive staining method to obtain accurate results. When objective proteins possess immunological activity, their expression levels may also be determined by employing an ELISA method. ZZ–EGFP can combine with IgG antibody, so its secretion and expression levels may be determined by employing either SDS-PAGE or ELISA method (Hayhurst et al., 2003). The two methods have been much more difficult to achieve accurate results. The major difficulties come from their complex operation process. Many attempts have been made to improve this technique, but no standard procedures have been accepted for their purification that can be used for sample preparation in electrophoresis. EGFP has an extremely high fluorescence intensity. It is also stable against oxidation and is acid-resistant (Kurien & Scofield, 2003). This was also the case for the quantitative analysis of the expression level of objective gene with EGFP. In the present study, the ZZ–EGFP fusion product was expressed in *E. coli* with excellent solubility and high yield. It can be efficiently purified through single-step Ni^{2+} affinity chromatography, making it a potentially inexpensive reagent for a wide variety of immunoassays. Our present work shows that there exists a good linear relationship between fluorescence intensity and concentration of ZZ–EGFP. Expression level of objective proteins can be quickly calculated by measuring fluorescence intensity in the culture supernatant and checking fluorescence intensity-concentration standard curve.

The cell is a membrane-bound chamber filled with an aqueous milieu that is immensely crowded with two thirds of the proteome. The remaining third of cytoplasmically synthesized proteins escapes to extracytoplasmic loca-

tions. Such hydrophilic proteins can be fully secreted to the cell surface or to the surrounding environment and be hydrolytic enzymes, cytolytic toxins, adhesins, growth factors, hormones or antibodies (Hajitou et al., 2006). This study shows that the extraction of bacterially associated ZZ–EGFP requires cell wall hydrolysis, even though there is no evidence that the proteins are anchored to the cell wall. Accumulation of ZZ–EGFP indicates that the rate of synthesis of ZZ–EGFP exceeds its rate of translocation across the bacterial cell wall. This slow rate of translocation across the cell wall is not a feature of all secreted gene-engineering bacteria *E. coli* HB101 protein. If the expression of exogenous gene in *E. coli* HB101 produces soluble, correctly folded, bioactive proteins, which are easily secreted into the culture medium, this brings great advantage to later protein purification. Prokaryotic Expression Vector (pEZZ–EGFP) is an expression and secretion Vector (Alexeyev & Winkler, 1999). However, efficient translocation of pEZZ–EGFP across the bacterial cell wall occurs upon a decrease in host intracellular pH, which also coincides with some other intracellular and extracellular factors (Raver et al., 1998). In gram-positive bacteria, secretion can be divided in two major steps: translocation across the cytoplasmic membrane and translocation across the cell wall. There are subsets of proteins that translocate across the cytoplasmic membrane, yet remain bacterially associated. Among these are transmembrane proteins, lipoproteins, and cell wall-anchored proteins that can either be covalently or noncovalently anchored to the cell wall (Cabanes, Dehoux, Dussurget, Frangeul, & Cossart, 2002; Navarre & Schneewind, 1999; Sutcliffe & Russell, 1995). Each subset of proteins can be identified by a signature motif within their amino acid sequence. Protein secretion is a multi-stage reaction occurring in the user-unfriendly environment of the membrane. Nevertheless, a combination of biochemical and genetic approaches has already yielded the complete inventory of mechanical parts of the secretion machine, its accessories and the general operational conditions. Glycine and TritonX-100 can destroy integrality of cells' outer membrane so as to ZZ–EGFP leak into culture medium (Harrison, 2004). Our work showed that the expression level of ZZ–EGFP in the culture medium containing glycine (1% or 2%) or Triton X-100 (1% or 2%) is lower. However, the expression level of ZZ–EGFP in the culture medium containing 2% Glycine and 1% Triton X-100 was 11 times more than the former. However, absorbance value (A_{600}) in the experiment group, whose culture medium contains 2% Glycine and 2% Triton X-100, was significantly lower than other groups in the present experiment, indicating that less expression level of ZZ–EGFP is released. Because a higher concentration of Glycine and Triton X-100 seriously destroys cell walls so as to affect bacterial growth. The result suggests that appropriate quantity of Glycine and Triton X-100 in the culture medium is favourable to the secretion and expression level of ZZ–EGFP.

Enhanced green fluorescent protein (EGFP) from *A. victoria* has begun appearing and tools for biophysical biosensors, and photochemical memories have been developed. The combination of the Glycine and Triton X-100 essentially allows ZZ–EGFP to diffuse along the length of the membrane-bound chamber and wall in gene-engineering bacteria *E. coli* HB101. To the best of our knowledge, this is the first time that secretion and expression level of ZZ–EGFP in gene-engineering bacteria *E. coli* HB101 has been induced and raised by using appropriate quantity of Glycine and Triton X-100 in the culture medium. In summary, the appropriate quantity of Glycine and Triton X-100 in the culture medium is demonstrated to meet the high secretion and expression yield of ZZ–EGFP in gene-engineering bacteria *E. coli* HB101. These results provide strong justification for the ongoing studies directed at extraction and purification of ZZ–EGFP in the target species.

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