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Deciphering EGFP production via surface display and self-cleavage intein system in different hosts

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ABSTRACT

In this study, six *E. coli* strains, which harboring enhanced green fluorescence protein (EGFP), an intein (INT) and ice nucleation protein (INP), were used to express the EGFP protein. The effect of *E. coli* strains on the surfaced displayed EGFP via INP-INT system was first investigated. Among these strains, *E. coli* JM109(DE3) was found the best strain for EGFP production (67.9 mg/L), two-fold as compared to that produced by *E. coli* DH1(DE3). It was concluded that lon mutation could stabilize recombinant proteins. Thus, the deletion of the gene coding for proteolysis might be a critical factor when using host for surface-expression production. The optimal conditions for cell cultivation, gene induction and intein cleavage for EGFP production were systematically studied and discussed. The objective function (O) value of 26.5 can be reached at a 12-h induction and 20-h cleavage process, where the EGFP yield is 58.6 mg/L with a purity of 45.3%. Furthermore, results indicated that the higher intein activity was obtained in the shorter cleavage duration; however, the shorter cleavage time sacrificed the production yield as expected. To compromise between the production yield and purity, the optimal conditions for the EGFP production were: 12-h induction and 20-h cleavage process is for the production of higher purity EGFP.

1. Introduction

Various systems have been mentioned with respect to displaying foreign proteins on the *E. coli* membrane surface based on anchoring motifs derived from outer-membrane proteins such as lipoproteins or autotransporters [1–7]. One anchoring motif derived from the ice-nucleation protein (INP) was found in several plant pathogenic bacteria [8]. The INP can employ a glycosylphosphatidylinositol (GPI) anchor in the N-terminal domain to couple on the cell surface and let the C-terminal domain be exposed to the outer membrane [8]. The INP-based display system had been employed to express proteins such as levansucrase [6], viral antigen [6,9], organophosphorus hydrolase [10–12], chitinase [13] and NADPH-cytochrome P450 oxidoreductase [14]. As compared to other outer-membrane anchoring proteins, such as OmpA, OmpT and PgsA, the INP is more preferentially fit for the expression of larger proteins with unique structural and functional features.

Intein (INT), an intervening segment, catalyzes its own excision from a protein precursor [15]. Owing to its characteristics, the target protein can be generated from the fusion protein under controllable cleavage conditions such as pH or temperature shifts [16–21]. In general, the process applying INT to produce target protein still requires the cell disruption process to harvest the soluble proteins and the purification process to obtain the purified target protein [19]. A simple process using the truncated INP portion of *Xanthomonas campestris* [22] with the Ssp DnaB intein from the *Synechocystis* species has been developed to express enhanced green fluorescent protein (EGFP) on *E. coli* DH1(DE3) [23,24]. The process for protein production could be carried out only via cultivation and centrifugation, without the addition of any proteases or chemical reagents.

To efficiently express the recombinant proteins in *E. coli*, the host strains play an important role in the production system. Chou et al. investigated the effects of the host on penicillin acylase (PAC) expression. Results showed that *E. coli* JM109 harboring the PAC gene construct gave significantly higher expression than that of HB101 when glycerol was used as the carbon source [25]. Miroux et al. applied three *E. coli* host strains, BL21(DE3), C41(DE3) and C43(DE3), to the toxic proteins' production. Results indicated that C41(DE3) and C43(DE3) were superior to BL21(DE3) for the production [26]. Zhang et al. studied the effect of different *E. coli* hosts on the surface-displayed organophosphorus hydrolase (OPH) activity. Results showed that *E. coli* JM109 exhibited the highest whole-cell OPH activity, indicating

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Table 1
E. coli strains used in this study.

Strain	Relevant characteristics	Source
DH5α	F^- endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169, hsdR17(r_K^- m_K^+) λ–	Novagen, USA
BL21 (DE3)	F^- ompT gal dcm lon hsdS_B(r_B^- m_B^-) λ^-(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5])	Novagen, USA
BL21(DE3)LPS truncated	F^- ompT gal dcm lon hsdS_B(r_B^- m_B^-) λ^-(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5]) Δ*waaC*	Dr. Chao Yun-Peng's gift, Taiwan
DH1 (DE3)	endA1 recA1 gyrA96 thi-1 glnV44 relA1 hsdR17(r_K^- m_K^+) λ^-(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5])	New England Biolabs, USA
ER 2566	F-λ- fhuA2 [lon] ompT lacZ::T7 gene 1 gal sulA11 Δ(mcrC-mrr)114::IS10R(mcr-73::miniTn10-TetS)2 R(zgb-210::Tn10)(TetS) endA1 [dcm]	New England Biolabs, USA
JM109 (DE3)	endA1 glnV44 thi-1 relA1 lon::IS186 mcrB+Δ(lac-proAB) e14-[F' traD36 proAB+ $lacI^q$ lacZΔM15] hsdR17(r_K^- m_K^+)λ^- (DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5])	Promega, USA
Rosetta(DE3) pLysSRARE	F^- ompT hsdS_B ($R_B^-m_B^-$) gal dcm λ (DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5]) pLysSRARE (Cam^R)	Novagen, USA

the screening of the *E. coli* host was crucial for better expression of fused protein [27].

In this study, six *E. coli* hosts were applied to test the effect on the surface-displayed EGFP production via the INP-INT system. The best production stain was selected, and the optimal conditions for cell cultivation, induction and intein cleavage durations for EGFP production were systematically studied. The host effect on the surface expression and production of EGFP via the INP-INT system was revealed for the first time.

2. Materials and methods

2.1. Bacterial strains and plasmids

E. coli strains DH5α, BL21(DE3), BL21(DE3) LPS truncated, DH1 (DE3), ER2566, JM109(DE3) and Rosetta(DE3)pLysSRARE were respectively used as hosts for recombinant DNA manipulation and recombinant protein expression. Their genotypes are listed in Table 1. *E. coli* BL21(DE3) LPS truncated was obtained from Dr. Chao Yun-Peng at Feng Chia University, Taiwan. The *E. coli* BL21(DE3) LPS truncated strain is a *waaC* null mutant. The *waaC* gene is coded for a heptosyltransferase, which plays a key role in the synthesis of the inner core region of LPS. The *waaC*-deficient strains express truncated LPS molecules at the cell surface. Plasmid pINP-INT-EGFP was constructed by Wu et al. [23]. In brief, the DNA fragments of the genes INT and EGFP were cloned from plasmids pTwin1 and pEGFP using gene-specific primers. The amplified products, INT-EGFP1 and INT-EGFP2, were used as templates to conduct an overlap extension PCR to obtain INT-EGFP fragments, which was further ligated into the EcoRI and XhoI sites of pInaXNC1 (a plasmid with INP gene) to generate pINP-INT-EGFP [23]. All recombinant DNA manipulations were performed following the standard procedures [28].

2.2. Fusion protein expression and induction conditions

E. coli BL21(DE3), BL21(DE3) LPS truncated, DH1 (DE3), ER2566, JM109(DE3) and Rosetta(DE3)pLysSRARE carrying plasmid pINP-INT-EGFP were constructed, respectively. An overnight culture of each strain was inoculated into 100 ml of Luria-Bertani (LB) medium containing 50 μg/ml kanamycin and grown at 37 °C until OD_{600} reached 0.7. The cells were induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). To study the induction conditions, various temperatures (15–37 °C) and incubation durations after induction (1–24 h) were tested. The collected samples were used for protein and EGFP assays.

2.3. Cleavage condition

The induced cells were collected via centrifugation at 3000 g for 10 min. According to the instruction of the IMPACT^{TM} system, the optimal pH value for INT cleavage is at 6.0–7.5 [19]. To perform the cleavage reaction, the pellets were washed twice with sterilized water and resuspended in a cleavage reaction buffer (20 mM Tris, pH7.0 containing 1 mM EDTA) and incubated at 37 °C for various durations. The solution was centrifuged at 3000 g for 10 min to remove the cells. Supernatants were collected for protein analysis.

2.4. Analytical methods

Protein analysis was carried out by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and commassie blue staining according to the method of Laemmli [29]. To comparatively measure the EGFP fluorescence intensity from different samples, the cell density of culture broth for each sample was concentrated to OD_{600}=10. The induced cells were collected via centrifugation at 3000 g for 10 min. To perform the cleavage reaction, the pellets were resuspended in a cleavage reaction buffer at 37 °C for various durations. The solution was centrifuged at 3000 g for 10 min. The released EGFP in the supernatant was measured by a fluorescence spectrometer. The concentration of EGFP was calculated according to a calibration curve obtained via the prepared EFGP standard solution. The unit of EGFP yield is defined as one mg EGFP produced per liter (mg/L) in the culture broth. The purity of EGFP was estimated according to the method of Wu et al. [23,24] via computer software (TotalLab, v2.0) to analyze the image of the EGFP band compared to that of other proteins in SDS-PAGE. All the experimental data were performed in triplicate, and the results were statistically expressed as the mean±SD.

3. Results and discussion

3.1. Host effects

To investigate the host effects, plasmid pINP-INT-EGFP was transformed into six *E. coli* hosts, BL21(DE3), BL21(DE3) LPS truncated, DH1(DE3), ER2566, JM109(DE3) and Rosetta(DE3)pLysSRARE, respectively, followed by the cultivation and IPTG-induction. All the transformed *E. coli* cells carrying the pINP-INT-EGFP displayed a green fluorescence color, suggesting the fusion EGFP protein was expressed correctly. The SDS-PAGE analysis on IPTG-induced *E. coli* cells revealed one predominant band of about 74 kDa, and was confirmed by western blot experiment (data not shown), which is exactly the molecular weight (MW) of the recombinant INP-INT-EGFP protein (Fig. 1). These results show the gene construct is able to successfully express the INP-INT-EGFP protein in all the hosts.

The six strains carrying pINP-INT-EGFP were induced with 1 mM IPTG, respectively. The induction conditions were set at 15 °C for 24 h, and the cleavage reactions were mediated at 37 °C for 24 h. The EGFP production was measured by a fluorescence spectrometer. Table 2 lists the values of EGFP yield, biomass and specific EGFP yield. For the six *E. coli* strains, the EGFP yield and specific EGFP yield have the same