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Comparative production of human interleukin-2 fused with green fluorescent protein in several recombinant expression systems

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Abstract

The selection of an optimal recombinant expression system is important for successful protein production. Here, we compared production of human interleukin-2 (hIL-2)-green fluorescent protein (GFP) fusion proteins in several expression systems such as bacteria *Escherichia coli*, yeast *Pichia pastoris*, insect *Spodoptera frugiperda* Sf-9 cells, insect *Tricoplusia ni* larvae, and insect *Drosophila melanogaster* S2 cells. Due to the highly hydrophobic nature of hIL-2, the GFP/hIL-2 fusion protein was expressed as an inclusion body in the *E. coli* system, resulting in minimal green fluorescence; however, Western blot analysis revealed the proper fusion band. In all other cases, the fusion proteins were expressed intracellularly or secreted as a functional form; green fluorescence was observed in each of these expression systems. We determined the linear relationships between GFP fluorescence and hIL-2 concentration in each case and used these correlations for comparison of the various expression systems in terms of production yield, productivity, product solubility (for intracellular expression systems), secretion efficiency (for secretion systems), and even functionality by simple measurement of GFP fluorescence. Even though the culture conditions were not optimized for each expression system, this comparison can be used as preliminary criteria for the selection of a proper expression system for recombinant protein production.

Keywords: *Escherichia coli*; *Pichia pastoris*; *Spodoptera frugiperda* Sf-9 cells; *Tricoplusia ni* larvae; *Drosophila melanogaster* S2 cells; Green fluorescent protein fusion; Human interleukin-2

1. Introduction

Recombinant protein expression systems have been developed in bacterial, yeast, insect, plant, and mammalian cells for use in the production of desired foreign proteins. The selection of an optimal expression system is very important for the success of overall protein production, leading to better functionality, higher production yield, higher purification yield, and/or lower production costs. However, this selection is often based on complex factors such as the purpose (usage) of the desired protein product (e.g., pharmaceutical, industrial enzyme, human food, animal food, etc.), the functionality of the expressed protein, the physicochemical properties of the protein (e.g., solubility), the required expression levels, the desired post-translational modification (e.g., glycosylation, disulfide bond formation, or proteolytic cleavage of a precursor form), secretion, folding, growth rate, growth density, production cost, and so on. Thus, it is not always easy to choose a proper expression system; each has its advantages and disadvantages (Table 1).

For many years, the bacteria *Escherichia coli* has been used as a host microorganism for production of recombinant proteins, owing to its superior properties for protein production compared to those of many other organisms [13,14]. However, the formation of inclusion bodies in the *E. coli* expression system can be a serious obstacle for foreign gene

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Table 1
Comparison of recombinant expression systems

	E. coli	Yeast	Insect	Mammalian
Growth rate	Very fast	Fast	Slow	Slow
Expression yield (based on dry weight)	High (1–5%)	High (>1%)	Very high (30%)	Very low (<1%)
Productivity	Very high	High	High	Low
Media cost	Very low	Low	High	Very high
Culture techniques	Very easy	Easy	Difficult	Very difficult
Production cost	Very low	Low	High	Very high
Protein folding	Fair	Good	Very good	Very good
Simple glycosylation	No	Yes	Yes	Yes
Complex glycosylation	No	No	Yes[a]	Yes
Secretion	Poor	Very good	Very good	Very good
Functionality of expressed eukaryotic protein	Poor	Good	Very good	Very good
Availability of genetic systems	Very good	Good	Fair	Fair
Pyrogen problem	Possible	No	No	No
References	[1–3]	[4–6]	[7–9]	[10–12]

[a] Glycosylation patterns differ from mammalian cells.

expression, as the recombinant proteins must then be refolded into their native functional conformations [15,16].

Methylotrophic yeast *Pichia pastoris* has the ability to use methanol as a sole carbon source [17]. Adaptation to growth on methanol is associated with induction of alcohol oxidase, which can account for over 30% of the cell protein in methanol-grown cells [17]. This level of induction, as well as the fact that *P. pastoris* is well suited for fermentative growth to high cell density levels, provides the basis for abundant recombinant protein expression [6,18]. Specifically, the *P. pastoris* expression system is convenient for the expression of eukaryotic foreign proteins [19], because it is as easy to manipulate as *E. coli*, yet can also carry out post-translational modifications and protein folding.

Insect cells have been used as a higher eukaryotic expression system capable of overcoming some deficiencies in the prokaryotic system (e.g., lack of post-translational modification) and mammalian cell system (e.g., very low expression level). The baculovirus infection system has been widely used for foreign protein production in insect lepidopteran cells such as *Spodoptera frugiperda* and *Trichoplusia ni* [9,20–23]. These suspended insect cell systems are convenient and relatively simple to use on the bench scale, but are difficult to use for larger-scale protein production. For example, oxygen transfer becomes limiting at the larger scales (e.g., damaging air sparging becomes necessary) and contamination problems can be more frequent and costly. The use of insect larvae for recombinant protein production is a feasible and cost effective alternative to insect cells [24]; scale-up is simple, contamination problems are minimal, and larvae are inexpensive. However, despite the popularity of both cell- and larvae-based insect/baculovirus systems, downstream protein purification can be problematic because the producing cells are destroyed by the viral infection [9].

Schneider S2 cells derived from insect *Drosophila melanogaster* have been developed as a plasmid-based insect cell system [25]. In this plasmid-based non-lytic expression system, high copy numbers of recombinant plasmid vectors are inserted into the host cell genome, with the advantage that foreign proteins are stably expressed without destruction of the protein-producing cells [26,27].

Human interleukin-2 (hIL-2; 18 kDa), our target protein, has been evaluated as a therapeutic agent in the treatment of cancer because of its role in promoting proliferation of anti-tumoral lymphocytes [28,29]. In addition, hIL-2 is used extensively as a tissue culture reagent, as it is required for survival of cultured T-lymphocytes. Glycosylation of hIL-2 at its *O*-glycosylation site is an important post-translational modification of this protein in vivo. However, this glycosylation is not critical for the protein's in vivo biological activity [30], meaning that hIL-2 can be successfully produced even in the *E. coli* recombinant protein expression system.

In the present work, we compared recombinant hIL-2 expression in five expression systems: *E. coli* (intracellular expression), *P. pastoris* (intracellular expression or secretion), Sf-9 cells (intracellular expression), *T. ni* larvae (intracellular expression), and S2 cells (secretion). For facile comparison of expression systems, we employed a unique fusion strategy in which green fluorescent protein (GFP) acts as a quantitative monitoring fusion partner.

We have previously reported the use of this unique fusion structure in a number of recombinant expression systems, including *E. coli* [31], *P. pastoris* (this work), *S. frugiperda* Sf-9 cells/baculovirus [22], *T. ni* larvae/baculovirus [32], and *D. melanogaster* S2 cells [33]. The structure consists of $(His)_6$-GFP-EK-X, where X represents a desired foreign protein, $(His)_6$ represents a hexahistidine affinity ligand used for simple purification, and EK represents an enterokinase cleavage site for recovering the target protein. GFP (27 kDa) was chosen as the monitoring reporter because it requires no cofactors or staining for fluorescence, the fluorescence is readily visible from outside the cells, and it does not present a large metabolic burden to the host [34]. We have previously demonstrated the use of GFP (specifically a UV variant, GFPuv) as a quantitative fusion marker of protein levels [22,31–33]. Here, we used this unique fusion construct to show

comparison of recombinant hIL-2 expression in five expression systems.

2. Materials and methods

2.1. Strains, plasmids, culture conditions, and sample preparation

E. coli BL21 (*F′mpT hsdSB* (r_B^- mB^-) *gal dcm*) (Novagen, USA) and recombinant plasmid pTH-GFPuv/hIL2 [31] (Fig. 1A) that contains isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible *trc* (Trc) promoter were used for expressing the fusion protein. Recombinant *E. coli* BL21 was grown to the mid-exponential phase (at ∼OD_{600} = 0.6) at 37 °C in 200 ml of LB (Luria broth) medium (5 g l^{-1} yeast extract (Sigma, USA), 10 g l^{-1} bacto-tryptone (Difco, USA), and 10 g l^{-1} NaCl) containing 50 μg ml^{-1} ampicillin (Sigma) using 500 ml flask. These cultures were inoculated (5%, v/v) from 37 °C overnight cultures in the same medium. Recombinant *E. coli* was induced by the addition of 1 mM IPTG

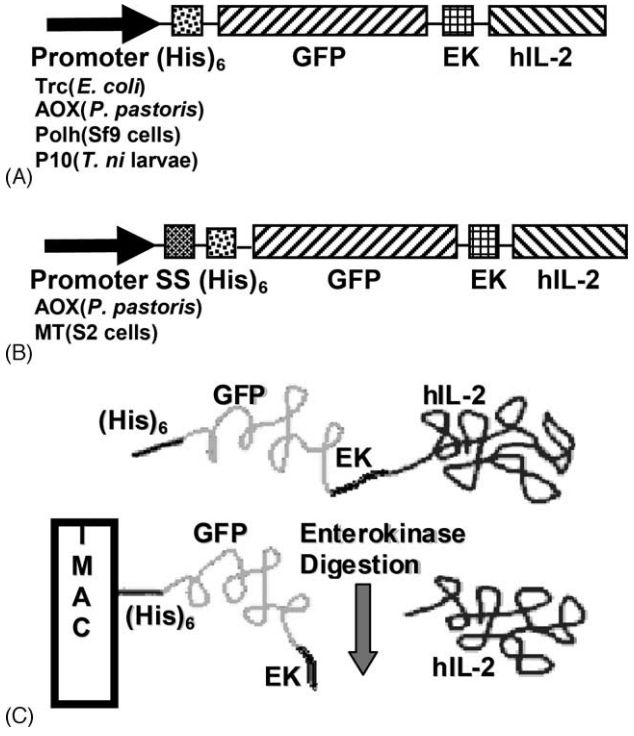

Fig. 1. Structure of the GFP/hIL-2 fusion genes containing histidine affinity ligand $(His)_6$ and enterokinase cleavage site (EK) that are (A) intracellular expressed each by *trc* (Trc) promoter in *E. coli* BL21 [pTH-GFPuv/hIL2], alcohol oxidase (AOX) promoter in *P. pastoris* GS115 [pPIC3.5K-GFPuv/hIL2], polyhedrin (Polh) promoter in insect Sf-9 [*v*PH-GFPuv/hIL2], p10 (P10) promoter in insect *T. ni* [*v*P10-GFPuv/hIL2] and (B) secreted each by alcohol oxidase (AOX) promoter and α-factor signal sequence in *P. pastoris* GS115 [pPIC9K-GFPuv/hIL2] and metallothionein (MT) promoter and BiP signal sequence in insect *Drosophila* S2 cells [pMT/BiP/GFP-hIL2]. (C) Diagram of constructed unique fusion protein. Fusion protein can be purified using $(His)_6$ with IMAC and hIL-2 can be obtained by specific cleavage between GFP and hIL-2 using enterokinase.

(Sigma) to express the fusion protein. The cells were collected and washed with TDTT buffer (50 mM Tris–HCl, pH 7.8, 30 μM DTT, 20 mM EDTA, and 1 mM PMSF). Samples were then resuspended in 300 μl TDTT, sonicated (Fisher Scientific, USA), and then centrifuged at 4 °C to remove cell debris. We used this supernatant as a soluble lysate.

P. pastoris GS115 (*his4*) (Invitrogen, USA) were used for expressing the fusion protein. A DNA fragment containing the hexa histidine tag, gfp_{uv} gene, enterokinase cleavage site, and *hil-2* gene, was amplified by polymerase chain reaction (PCR) amplification (DNA Thermal Cycler; Perkin-Elmer Cetus, USA) from mini-prep (Bio-Rad Lab., USA) purified pBBH-GFPuv/hIL2, a recombinant baculovirus transfer vector [22]. The PCR primers were designed ((5′–3′) CGG AAT TCA CCA TGG CGC GGG GTT CTC ATC ATC ATC and (3′–5′) TTG CGG CCG CTT ATC AAG TTA GTG TTG AGA TGA TGC) to allow cloning of the 1247-bp *Eco*RI and *Not*I-digested amplified product into the *Eco*RI and *Not*I sites of the pPIC3.5K and pPIC9K vectors (Invitrogen) that contain the methanol-regulated *aox1* promoter for over-expression of foreign proteins. Importantly, the pPIC9K vector has an α-factor signal sequence for secretion of desired protein into the culture broth. These vectors were named pPIC3.5K-GFPuv/hIL2 (Fig. 1A) and pPIC9K-GFPuv/hIL2 (Fig. 1B), respectively. Recombinant *P. pastoris* strain GSGI-I22 (for intracellular expression) that has five integrated-gene copies and GSGI-S38 (for secretion) that has six integrated-gene copies were screened and grown at 30 °C in 100 ml of buffered minimal medium (BMM; 100 mM potassium phosphate, pH 6.0, 1.34% (w/v) yeast nitrogen base (YNB), and 4×10^{-5}% (w/v) biotin (Sigma)) after resuspending from harvesting with 50 ml overnight cultures that were grown in minimal glycerol medium (MGY; 1.34% YNB, 1% glycerol, and 4×10^{-5}% biotin). Recombinant *P. pastoris* strains were induced by the addition of 0.5% methanol from the beginning of culture to express the fusion proteins. Cell culture broth was divided into two fractions, intracellular and extracellular, by centrifugation at 10,000 × *g* for 10 min. We used culture medium as an extracellular supernatant. Preparation of soluble lysate consisted of first resuspending the cell pellet in 100 μl breaking buffer (50 mM sodium phosphate, pH 7.4, 1 mM PMSF, 1 mM EDTA, and 50 g l^{-1} glycerol). The resuspended cell pellet was then vortexed after adding an equal volume of 0.5 mm acid-washed glass beads (Sigma). Eight vortex-incubation cycles, 30 s vortexing then 30 s incubation on ice, were performed to lyse the cells. After centrifuging at 10,000 × *g* for 10 min, the supernatant was taken and used as an intracellular soluble lysate.

S. frugiperda Sf-9 cell line (available from ATCC, CRL-1711) and recombinant transfer plasmid pBBH-GFPuv/hIL2 [22] (Fig. 1A) that contains polyhedrin (Polh) promoter were used for expressing the fusion protein. The culture medium was serum-free Sf-900 II SFM (Life Technologies, USA). Experiments were performed on cell cultures divided from a single inoculum grown at 27 °C until the time of infection using the recombinant baculovirus, *v*PH-GFPuv/hIL2

[22]. Infection was performed by inoculating the cell cultures during exponential growth (1×10^6 cells ml^{-1}) with a determined volume of viral solutions to give multiplicity of infection (MOI) of 2. The culture was grown in 500 ml spinner flask with 200 ml working volume. The infected Sf-9 cells were collected approximately 12 h for 5 days, sonicated in phosphate buffered saline (PBS) containing 60 mM dithiothreitol (DTT) and 0.5% Triton X-100 at pH 7.0 for 30 s on ice, and then centrifuged at 4 °C to remove cell debris. We used this supernatant as a soluble lysate.

Cabbage looper, *T. ni* larvae and recombinant transfer plasmid pAcUWH-GFPuv/hIL2 [32] (Fig. 1A) that contains p10 (P10) promoter were used for expressing the fusion protein. The eggs (Entopath, USA) were hatched in Styrofoam cups containing solid food (Entopath) at 30 °C, and the fourth instar larvae (4 days after hatching) were used for infection experiments. The recombinant baculovirus, *v*P10-GFPuv/hIL2 [32], was spread on the media at the virus loading, 5×10^7 pfu per cup. The fourth instar larvae were then placed into the cups (15 larvae per cup). The larvae were allowed to feed on the infected food at 30 °C. For each sample, five infected larvae were collected and homogenized in PBS containing 60 mM DTT and 0.5% Triton X-100 at pH 7.0. The homogenate was then centrifuged at 4 °C to remove large debris. We used this supernatant as a soluble lysate.

D. melanogaster S2 cells (Invitrogen) and recombinant plasmid pMT/BiP/GFP-hIL2 [33] (Fig. 1B) that contains copper sulfate-inducible *Drosophila* metallothionein (MT) promotor and signal sequence of immunoglobulin binding chaperone protein (BiP) to facilitate the secretion were used for expressing the fusion protein. Three 100 mm cell culture dishes containing stably transfected recombinant S2 cells [33] grown to 1×10^6 cells ml^{-1} (over 90% viable) in M3 medium (Shields and Sang M3 insect medium; Sigma) containing 10% insect medium supplement (IMS; Sigma), were transferred into a 500 ml spinner flask containing 150 ml serum-free M3 medium. Cells were incubated at 27 °C with constant stirring (80 rpm) until a cell density of at least 4×10^6 cells ml^{-1} was reached, after which copper sulfate was added (500 μM) to induce secretion of the fusion protein. Cell culture broth was divided into two fractions, intracellular and extracellular, by centrifugation at 10,000 × *g* for 10 min. We used culture medium as an extracellular supernatant. Cell pellets were sonicated in PBS containing 60 mM DTT and 0.5% Triton X-100 at pH 7.0, and then centrifuged at 4 °C to remove cell debris. We used this supernatant as an intracellular soluble lysate.

2.2. Analytical assays

Cell growth for *E. coil* or *P. pastoris* was monitored by optical density (at 600 nm, OD_{600}) on a UV–vis spectrophotometer (Beckman, USA). Total cell counts for suspended Sf-9 and S2 cells were performed with a hemacytometer (Fisher Scientific, USA), and viability was determined by trypan blue (Sigma) exclusion using a 0.4% (w/v) solution. Total protein amount was measured at 595 nm by a UV–vis spectrophotometer using Bradford method. 1.44 mg ml^{-1} of bovine serum albumin (BSA; Bio-Rad, USA) was used as a standard protein. GFP assay was performed by measuring fluorescence intensity using a fluorescence spectrometer (Perkin-Elmer, England or Shimadzu, Japan) at an excitation wavelength of 395 nm and emission at 509 nm. In the case of intracellular-expression from *E. coli*, *P. pastoris*, and Sf-9 cells, GFP fluorescence intensity was measured using whole cell fraction without medium. In the case of intracellular-expression from *T. ni* larva, GFP fluorescence intensity was measured using soluble lysate. In the case of secreted-expression from *P. pastoris* and S2 cells, GFP fluorescence was measured using medium fraction. The quantities of hIL-2 in soluble lysate (intracellular) and medium fraction (secretion) were determined by Western blot analyses using pure recombinant hIL-2 (Life Technologies) from *E. coli* as a calibration standard. Assays of hIL-2 biological activity which require T-cell proliferation were not performed as its use in our laboratory is as an immunodiagnostic reagent.

2.3. Western blot analysis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by mixing a sample with sample buffer (0.5 M Tris–HCl, pH 6.8, 10% glycerol, 5% SDS, 5% β-mercaptoethanol, and 0.25% bromophenol blue), incubating at 100 °C for 3 min, centrifuging for 1 min, and loading onto a 15% slab gel. After electrophoresis, the gel was transferred onto a nitrocellulose membrane (Bio-Rad, USA) with a Bio-Rad Mini-Trans Blot Cell in Bjerrum and Schafer-Nielsen transfer buffer (48 mM Tris, 39 mM glycine, and 20% methanol; pH 9.2) for 20 min at 10 V followed by 20 min at 20 V. The nitrocellulose membrane was probed with 1:2000 dilution of polyclonal anti-hIL-2 antibody (CYTImmune Science, USA), and detected with 1:5000 dilution of goat anti-rabbit IgG conjugated to alkaline phosphatase (Kirkegaard and Perry Laboratories, USA) and 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) color development reagent (Sigma).

3. Results and discussion

3.1. Noninvasive monitoring of GFP/hIL-2 fusion protein expression

We used the *E. coli*, *P. pastoris*, Sf-9 cell, *T. ni* larvae and S2 cell systems to express a fusion protein containing the unique structure of $(His)_6$ preceding the GFP expression cassette, which allowed simple purification by metal immobilized affinity chromatography (IMAC). We could then use the EK site to separate the target hIL-2 from the fusion structure by enterokinase treatment (Fig. 1C). Fig. 2 shows photographs of *P. pastoris* (intracellular expression; Fig. 2A), Sf-9 cells (intracellular expression; Fig. 2B), *T. ni*

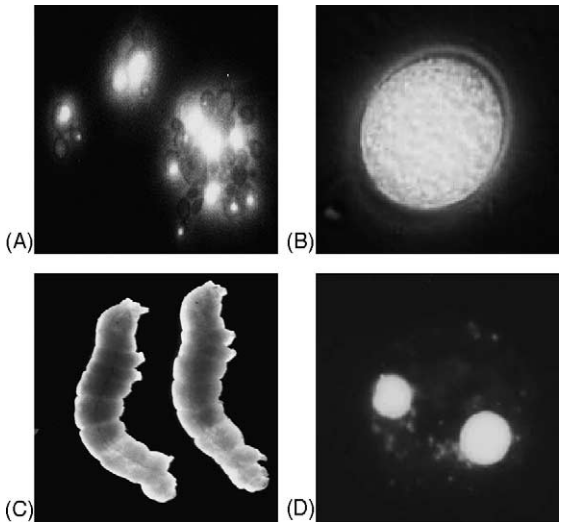

Fig. 2. Green fluorescent recombinant (A) *P. pastoris* GS115 (intracellular expression), (B) insect Sf-9 cells (intracellular expression), (C) insect *T. ni* larvae (intracellular expression), and (D) insect *Drosophila* S2 cells (secretion). Cells in (A), (B) and (D) were photographed using fluorescent microscopy (1000× magnification).

larvae (intracellular expression; Fig. 2C), and S2 cells (secretion; Fig. 2D) expressing the GFP/hIL-2 fusion protein. These demonstrated that the GFP/hIL-2 fusion proteins were successfully expressed in the tested recombinant expression systems, and thus noninvasive monitoring of foreign protein expression was possible using GFP fluorescence. We confirmed fusion protein expression by Western blot analysis of cell lysis supernatants or culture medium fractions (Fig. 3). Probing with a polyclonal anti-hIL-2 antibody revealed that the GFP/hIL-2 fusion protein was expressed at the appropriate molecular weight (∼52 kDa) in intracellular-expressing *P. pastoris* (lane 2), intracellular-expressing Sf-9 cell (lane 3), intracellular-expressing *T. ni* larvae (lane 4), secreted-expressing *P. pastoris* (lane 5), and secreted-expressing S2 cells (lane 6). In contrast, green fluorescence was barely detectable in the *E. coli* system (data not shown), though Western blot analysis revealed the proper fusion band (lane 1 in Fig. 3). This may suggest that the GFP/hIL-2 fusion protein was expressed as inactive aggregates (inclusion bodies) in *E. coli* due to the high insolubility of hIL-2; this would likely interfere with GFP fluorescence. Since the native conformation of hIL-2 requires a disulfide bond that does not form in the cytoplasm of *E. coli*, it has previously been expressed as insoluble aggregates of inactive protein that become biologically active after purification and refolding [35,36].

A hIL-2 band (∼18 kDa) was detected in each sample of *P. pastoris* (lanes 2 and 5 in Fig. 3), Sf-9 cells (lane 3 in Fig. 3), or *T. ni* larvae (lane 4 in Fig. 3). The appearance of the hIL-2 band indicated that a fraction of the fusion protein was unintentionally cleaved at the EK cleavage site during the expression (based on molecular weight similarity). Therefore, we can suspect that *Pichia*, Sf-9, and *T. ni* larvae express an enterokinase or enterokinase-like protease. Detailed investigation of this is underway using enterokinase-specific substrates. Note, lower molecular weight band under the fusion was also found suggesting suffer from proteolysis.

3.2. Correlation between GFP fluorescence and hIL-2 quantity

The profiles of GFP fluorescence intensity and hIL-2 concentration from *P. pastoris*, Sf-9, *T. ni* larvae, and S2 cells are shown in Fig. 4. All hIL-2 measurements were performed from cell lysis supernatants (in cases of intracellular expression) or conditioned media (in cases of secreted expression). Quantification of hIL-2 concentrations was performed by densitometric scanning of Western blots. In the case of intracellular-expressing yeast *P. pastoris*, GFP fluorescence and hIL-2 levels increased similarly with culture time (Fig. 4A); there was a linear relationship between the two when hIL-2 levels were plotted against GFP fluorescence intensity. Based on this relationship, we propose that intracellular hIL-2 levels can be quantified by simple detection of whole cell GFP fluorescence instead of intracellular GFP. This is particularly attractive in yeast, since yeast cell walls are quite resistant to lysis, and invasive monitoring of intracellular protein production can be labor intensive. As shown in Fig. 4B, the profiles of GFP fluorescence and hIL-2 concentration were almost identical in intracellular-expressing

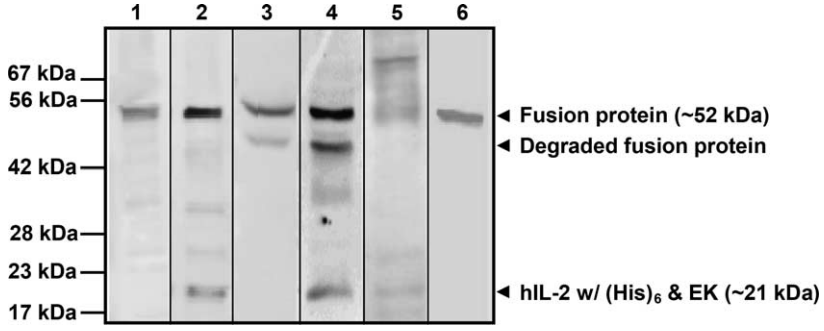

Fig. 3. Western blot analysis of GFP/hIL-2 fusion proteins. Lane 1 is *E. coli* BL21 (intracellular expression), lane 2 is *P. pastoris* GS115 (intracellular expression), lane 3 is insect Sf-9 cells (intracellular expression), lane 4 is insect *T. ni* larvae (intracellular expression), lane 5 is *P. pastoris* GS115 (secretion), and lane 6 is insect S2 cells (secretion).

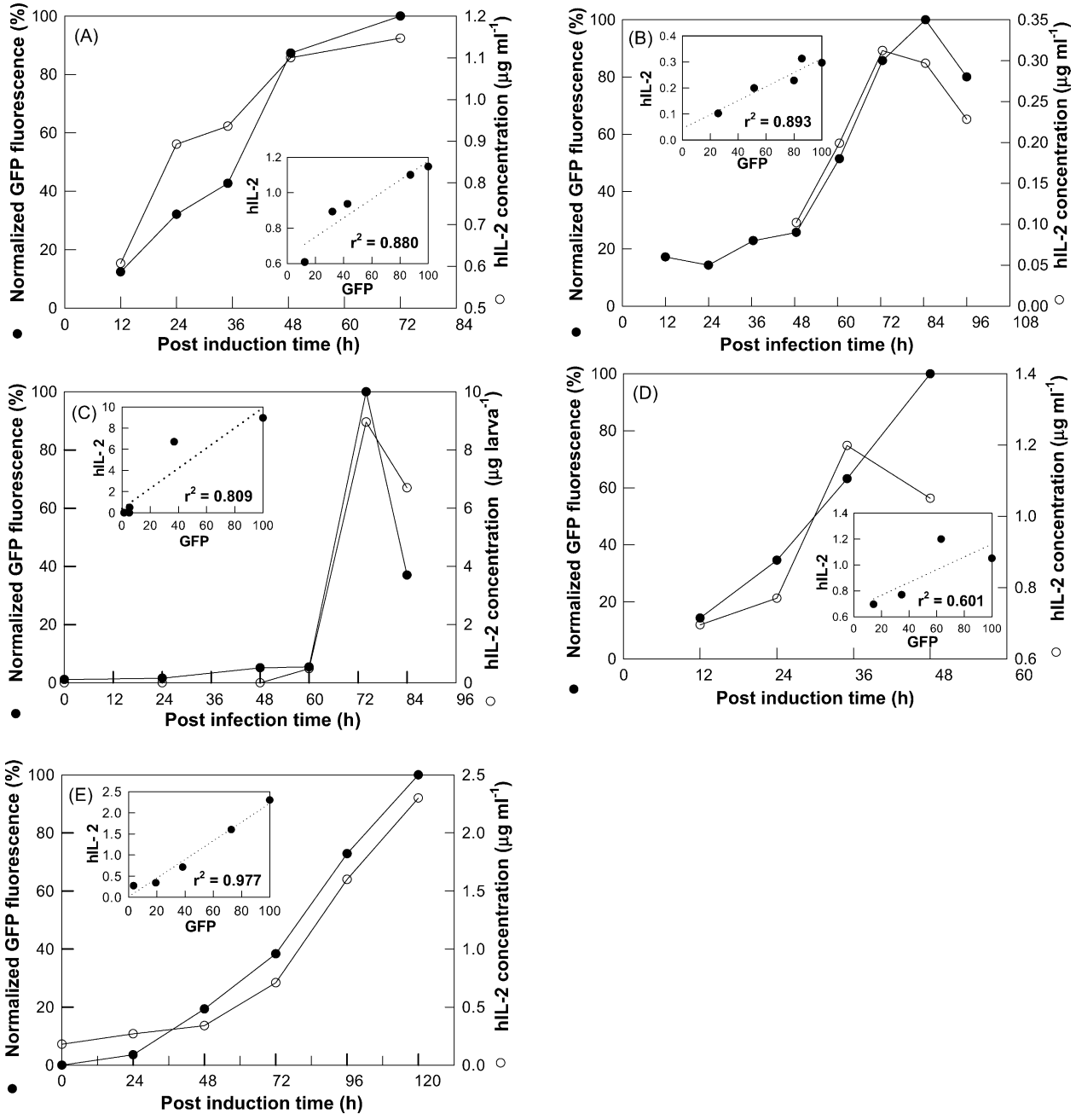

Fig. 4. Time profile of GFP fluorescence intensity and hIL-2 concentration, and correlation between two in recombinant (A) *P. pastoris* (intracellular expression), (B) insect Sf-9 cells (intracellular expression), (C) insect *T. ni* larvae (intracellular expression), (D) secreted *P. pastoris* (secretion), and (E) insect S2 cells (secretion). Quantifications of hIL-2 were performed by Western blot.

insect Sf-9 cells. The hIL-2 levels reached their maximum at 72 h post-infection (hpi), while the fluorescence reached its maximum at 84 hpi. This time difference might be due to high proteolytic sensitivity of fusion-bound or free hIL-2; such proteolysis was confirmed by the protein band pattern revealed by Western blot analysis (lane 3 in Fig. 3). Our previous work has shown that hIL-2 is easily degraded [31,32]. On the other hand, GFP is known for its stability [34]. In the Sf-9 cell system, we again obtained a linear correlation between hIL-2 levels and GFP fluorescence (Fig. 4B). Interestingly, the intracellular-expressing insect *T. ni* larvae had a much sharper correlation pattern compared to that in Sf-9 cells, even though the two utilized almost the same baculovirus system (Fig. 4C). In *T. ni* larvae, GFP fluorescence was insignificant until 60 hpi, increased rapidly to a maximum during the next 14 h, and then rapidly decreased. Importantly, the hIL-2 profile was closely mimicked by the GFP fluorescence intensity, and we almost obtained a linear correlation between hIL-2 and GFP fluorescence in this system. However, this linearity was not seen in the case of the secreted-expressing yeast

Table 2
Summary of human interleukin-2 (as a fusion form) production yield in several recombinant expression systems[a]

	Natural hIL-2 (from T-cell)	Intracellular production				Secreted production	
		E. coli BL21[b]	*P. pastoris* GS115[c]	Insect Sf-9 cells[d]	Insect *T. ni* larvae[e]	*P. pastoris* GS115[c]	Insect S2 cells[f]
Maximum total yield[g] ($\mu g\,ml^{-1}$)	–	6.79	1.70	1.03	22.74	1.50	2.55
Maximum recoverable yield[h] ($\mu g\,ml^{-1}$)	0.000001	0.72	1.15	0.31	4.48 (2 ml/larva)	1.05	2.30
Culture time[i] (h)	–	5	72	144	168	48	192
Total productivity[g] ($\mu g\,ml^{-1}\,h^{-1}$)	–	1.358	0.024	0.007	0.135	0.031	0.013
Recoverable productivity[h] ($\mu g\,ml^{-1}\,h^{-1}$)	–	0.144	0.016	0.002	0.027	0.022	0.012
Solubility[j] (%)	–	10.6	67.5	30.0	19.7	N/A	N/A
Secretion efficiency[k] (%)	–	N/A	N/A	N/A	N/A	70.2	90.2

[a] Each culture condition was not optimized.
[b] Culture in 200 ml LB medium using a 500 ml flask. Induction was performed by 1 mM IPTG at 0.6 OD_{600}.
[c] Culture in 100 ml BMM medium using a 500 ml flask. Induction was performed by 0.5% methanol from initial point.
[d] Culture in 200 ml serum-free Sf-900 II SFM medium using a 500 ml spinner flask. Infection was performed by baculovirus with 2 MOI at 1×10^6 cells ml^{-1}.
[e] Culture (total 15 larvae) in a styroform cup containing solid food. Infection was performed by baculovirus with 5×10^7 pfu per cup at fourth instar larvae.
[f] Culture in 150 ml serum-free M3 medium using a 500 ml spinner flask. Induction was performed by 500 μM copper sulfate at 4×10^6 cells ml^{-1}.
[g] Based on total cell (cell lysis supernatant and cell pellet) for intracellular production and total culture broth (whole cell and culture medium) for secreted production.
[h] Based on cell lysis supernatant for intracellular production and culture medium for secreted production.
[i] Total culture time for maximum yield after transferring into culturing vessel, not including time for seed culture (hatching in case of larvae) and medium preparation.
[j] hIL-2 in cell lysis supernatant divided by total hIL-2 at the culture time for maximum yield.
[k] hIL-2 in culture medium divided by total hIL-2 at the culture time for maximum yield.

P. pastoris system. In this system, secreted hIL-2 peaked at 36 hpi, whereas GFP fluorescence in the conditioned medium continued to increase (Fig. 4D). We believe that this may also be due to the instability of hIL-2. And finally, the secreted-expressing insect S2 cell system showed almost identical profiles and a linear relationship between secreted hIL-2 amount and GFP fluorescence intensity of the conditioned medium (Fig. 4E).

Based on the linear relationships observed in most of the tested expression systems, we conclude that this correlation can be used as potential tool for evaluation of various expression systems: GFP fusion strategy can be used for facile monitoring of target protein levels by simple detection of GFP fluorescence, regardless of whether the protein is intracellular or secreted.

3.3. Comparison of production yield, productivity, solubility, and secretion efficiency

In the tested intracellular expression systems (excluding the *E. coli* system), we found that high levels of the GFP/hIL-2 fusion proteins remained in the cell pellet after lysis, as detected by UV transillumination (data not shown). However, even though the fusion proteins were associated with the cell membranes, they emitted green fluorescence and linear correlations existed between GFP fluorescence and hIL-2 levels in the cell pellet (data not shown). Therefore, this method allowed us to estimate the solubility of the desired protein by simply checking the GFP fluorescence of the cell pellet versus that of the cell lysis supernatant. Also in the case of secretion systems, we were able to monitor hIL-2 fusion protein secretion by measuring GFP fluorescence intensity in whole cells versus that in the culture medium [33].

Table 2 shows the production yields, productivity and solubility (or secretion efficiency) in whole cells and cell lysis supernatants (or culture media). Note that we did not strictly optimize each culture condition presented herein. The baculovirus-based insect *T. ni* larvae system had the highest value maximum production yield (4.48 $\mu g\,ml^{-1}$ for cell lysis supernatant and 22.74 $\mu g\,ml^{-1}$ for total cells). However, even though the fusion proteins were mainly expressed as inclusion bodies, the *E. coli* system showed the highest productivity (0.144 $\mu g\,ml^{-1}\,h^{-1}$ for cell lysis supernatant and 1.358 $\mu g\,ml^{-1}\,h^{-1}$ for total cells), due to a much shorter culture time. The insect S2 secretion system showed better production yield and productivity than did the insect Sf-9/baculovirus intracellular expression system. The yeast *P. pastoris* system had the best intracellular product solubility, though this value was relatively low (67.5%), due to the high hydrophobicity of hIL-2. In general, the insect systems had poor solubility (<30%), even though most of the membrane-associated GFP/hIL-2 fusion proteins showed

GFP functionality. The *E. coli* system had a product solubility of 10.6%, though cell lysis experiments showed that this portion of the total GFP/hIL-2 fusion protein did not represent real solubility, but rather an insoluble fusion protein particle [31]. In the case of secreted expression systems, the higher-level eukaryotic insect S2 cell system showed better (>90%) secretion efficiency than did the lower-level eukaryotic yeast *P. pastoris* system.

4. Conclusions

We showed that we could compare several expression systems under different culture conditions based on the determined linear relationship of the protein product levels and GFP fluorescence. In addition, we were able to easily calculate production yield and productivity from GFP fluorescence measurements. In the specific case of the highly insoluble protein tested here (hIL-2), we could easily check whether the expressed target protein formed inclusion bodies, and whether it showed cell membrane association, which can complicate recombinant protein separation and purification. Because each culture condition was not optimized in this work, we did not make a definitive decision as to which expression system is best for hIL-2 production. However, these results can be used as preliminary criteria for selecting the best recombinant expression systems for certain target proteins.

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