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Secretion of Human Interleukin-2 Fused With Green Fluorescent Protein in Recombinant *Pichia pastoris*

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Abstract

Methylotrophic yeast *Pichia pastoris* is convenient for the expression of eukaryotic foreign proteins owing to its potential for posttranslational modifications, protein folding, and facile culturing. In this work, human interleukin (hIL)-2 was successfully produced as a secreted fusion form in recombinant *P. pastoris*. By employing green fluorescent protein (GFP) as a monitoring fusion partner, clear identification of fusion protein expression and quantification of intracellular hIL-2 were possible even though there was no correlation between culture supernatant fluorescence and secreted hIL-2 owing to high media interference. Importantly, by the addition of casamino acids in basal medium, we were able to enhance threefold amount of secreted hIL-2, which was present both as a fusion and as a clipped fragment.

Index Entries: Human interleukin-2; green fluorescent protein; *Pichia pastoris*; fusion protein; secretion.

Introduction

Human interleukin (hIL)-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 *(1–3)*, and it has also

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been shown to enhance the immune function in HIV$^+$ individuals *(4)*. 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 posttranslational modification of this protein in vivo. However, this glycosylation is not critical for the protein's in vivo biological activity *(5)*, which means that hIL-2 can be produced in *Escherichia coli*. However, hIL-2 has previously been expressed as insoluble aggregates of inactive protein because its native conformation requires a disulfide bond that does not form in the cytoplasm of *E. coli (6,7)*. Therefore, production of active hIL-2 in *E. coli* would require extra refolding steps for the inclusion body.

In order to solve the problems arising from *E. coli*, yeast expression systems were employed *(8–10)*. In the present work, we have employed methylotrophic yeast *Pichia pastoris* system for hIL-2 production. Unlike conventional yeasts (*Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*), methylotrophic yeasts (e.g., *P. pastoris*, *Hansanula polymorpha*, *Candida boidinii*, etc.) have the ability to use methanol as a sole carbon source *(11)*. Adaptation to growth on methanol is associated with induction of alcohol oxidase (AOX) (also referred to as methanol oxidase [MOX]), which is virtually absent in glucose-grown cells *(12)*, but can account for more than 30% of the cell protein in methanol-grown cells *(13)*. Among the many *Pichia* species, *P. pastoris* is the most common for foreign protein expression *(14,15)*. *P. pastoris*, like *S. cerevisiae*, is well suited for fermentative growth, having the ability to reach high cell density levels, which may improve overall protein yield. Specifically, the *P. pastoris* expression system is convenient for the expression of eukaryotic foreign proteins *(16)* because of its potential for posttranslational modifications and protein folding, while being as easy to manipulate as *E. coli* or *S. cerevisiae*. Also, *P. pastoris* secretes products with high purity because very few native proteins are secreted *(17)*.

In the present work, we performed secreted expression of hIL-2 fusion via the α-factor signal sequence in *P. pastoris* system. We designed fusion protein to facilitate hIL-2 detection using green fluorescent protein (GFP) as a fusion partner. Unique attributes of GFP as a protein marker are: it requires no co-factors 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 *(18)*. In our previous work, we demonstrated the use of GFP (specifically an ultraviolet [UV] variant, GFPuv) as a quantitative marker of protein level in several expression system including insect *Trichoplusia ni* larvae *(19)*, insect *Sf*-9 cells *(20)*, insect *Drosophila* S2 cells *(21)*, yeast *P. pastoris* (intracellular expression; 22), and *E. coli (7)*. Although GFP fusions for bioprocess monitoring have been demonstrated in other hosts, this is the first indication in secreted *P. pastoris*. In other recent reports, GFP has been used to elucidate trafficking of proteins within cell organelles *(23–25)*. Also, Braspenning et al. *(26)* showed that the pho1$^+$ leader sequence enabled secretion of GFP to the medium from *Schizosaccharomyces pombe*. Further, Raemakers et al. *(27)* showed that a modified α-factor signal

sequence and phytohemagglutinin-E signal sequence both enable secretion of GFP from *P. pastoris*.

Materials and Methods

Strains and Media

E. coli TOP10 (*F⁻ mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ*80lacZ* Δ*M15* Δ*lacX74 deoR recA1 araD139* Δ(*ara-leu*)*7697 galU galK rpsL* (*StrR*) *endA1 nupG*) (Invitrogen, Carlsbad, CA) was used for constructing the recombinant plasmid containing gfp_{uv} and *hil-2* genes. *P. pastoris* GS115 (*his4*) (Invitrogen) was used for protein expression. Recombinant *P. pastoris* strain was initially grown overnight in 50 mL of MGY (Minimal Glycerol Medium; 1.34% YNB, 1% glycerol, and 4×10^{-5}% biotin) after which culture was resuspended and grown at 30°C in 500-mL flask with 100 mL of Buffered Minimal Methanol (BMM: 100 m*M* potassium phosphate, pH 6.0, 1.34% [w/v] yeast nitrogen base [YNB; Difco, Detroit, MI], 4×10^{-5}% [w/v] biotin [Sigma, St. Louis, MO], and 0.5% methanol) or BMMC (BMM with 1% casamino acids [Sigma]) medium.

Construction of Recombinant Plasmid

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, Norwalk, CT) from mini-prep (Prep-A-Gene DNA Purification systems; Bio-Rad, Hercules, CA) purified pBBH-GFPuv/hIL2, a recombinant baculovirus transfer vector *(20)*. The PCR primers were designed ([5'-3'] CGGAATTCACCATGGCG CGGGGTTCTCATCATCATC and [3'-5'] TTGCGGCCGCTTATCAAGTTAGTGTTGAGAT GATGC) 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 pPIC9K vector (Invitrogen) that contain the methanol-regulated *aox1* promoter for overexpression of foreign proteins and an α-factor signal sequence for secretion of desired protein into the culture broth. This vector was named pPIC9K-GFPuv/hIL2 (Fig. 1). We confirmed the correct open reading frame of gfp_{uv} and *hil-2* gene fusion by DNA sequencing (model 373A DNA sequencer; Perkin-Elmer Applied Biosystems, Foster City, CA) using 5' *aox1* and 3' *aox1* primers.

Transformation of Constructed Recombinant Plasmid Into Pichia

To integrate the constructed recombinant plasmid into the *P. pastoris* chromosome, the plasmid was linearized by *Bgl*II. For transformation of linearized plasmid into the chromosome, a spheroplast/PEG 1000 method was used as per manufacturer's instructions (Multi-Copy *Pichia* Expression Kit; Invitrogen). Among many the His^+ transformants produced, the G418 resistance method was used to screen for multiple inserts. Briefly, G418 (Invitrogen) is an antibiotic and G418 hyper-resistant colonies typi-

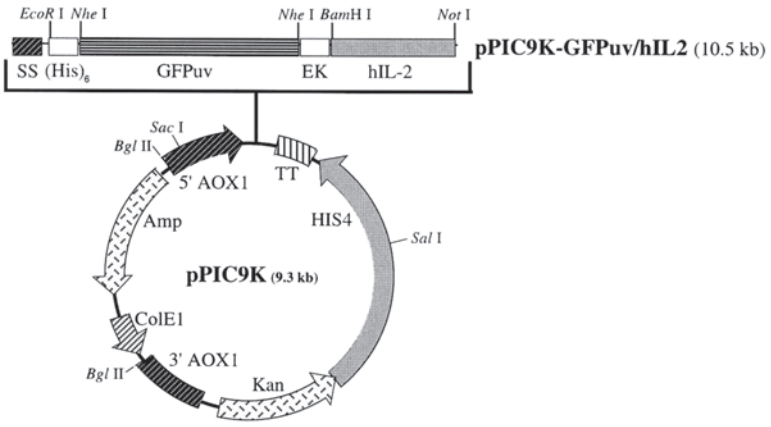

Fig. 1. Gene map of recombinant plasmid pPIC9K-GFPuv/hIL2. Abbreviation: AOX1, alcohol oxidase gene; Amp, ampicillin resistant gene; ColE1, *E. coli* replication origin; Kan, kanamycin resistant gene; HIS4, histidine coding gene; TT, transcription termination sequence; SS, secretion signal sequence; $(His)_6$, histidine affinity ligand; EK, enterokinase cleavage site.

cally have multiple inserts, as selective pressure leads to more resistance (no. inserts). After G418-resistance screening, quantitative dot blots were performed to determine the gene copy number for multiple integrants *(16)*. Genomic DNA was isolated from several multiple integrants and equivalent DNA levels were spotted directly onto nitrocellulose paper. DNA was fixed to the membrane using UV induced crosslinking. Both the gfp_{uv} and *hIL-2* gene fragments were used as probes. Specifically, probes were prepared using the PCR DIG Probe Synthesis Kit (Boehringer Mannheim, Indianapolis, IN) and detection was performed using a DIG detection kit (Boehringer Mannheim). The stained membrane was scanned, and the digitized image was analyzed by NIH image software (NIH Image). Finally, a strain with six gene copies was found and named GSGI-S38 (data not shown). Several reports regarding protein expression in *Pichia* recommend *(28)* that either Mut^+ (Methanol utilization plus) or Mut^S (Methanol utilization slow) cells can be used for secretion purpose. Mut^+ enables utilization of methanol be used as a carbon source because it has both *aox1* and *aox2* genes. Mut^S strains are unable to utilize methanol due to the lack of an *aox1* gene. In this work, GSGI-S38 is a Mut^+ strain.

Sample Preparation and Analytical Assays

The samples were taken at several times during the culture and then measured optical density at 600 nm (OD_{600}) on a UV-vis spectrophotometer (DU640, Beckman, Fullerton, CA). Remaining samples were centrifuged (10,000*g*, 10 min), then immediately frozen at –70°C until they were ready for total protein, GFP, and hIL-2 assays. For "extracellular supernatant," culture supernatant was taken after centrifuging. Preparation of soluble lysates consisted of first resuspending the cell pellet in 100 µL breaking

buffer (50 m*M* sodium phosphate, pH 7.4, 1 m*M* PMSF [phenylmethylsulfonyl fluoride], 1 m*M* EDTA, and 5% 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 of vortexing then 30 s of 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." The total protein assay was performed using a protein assay kit (Bio-Rad) with bovine serum albumin as a standard. The quantity of hIL-2 was determined using pure recombinant hIL-2 expressed in *E. coli* (Life Technologies, Gaithersburg, MD) as a calibration standard on Western blots. 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. The GFP fluorescence intensity was measured using a fluorescence spectrometer (LS-3B; Perkin-Elmer, Buckinghamshire, England) at an excitation wavelength of 395 nm and emission at 509 nm. Fluorescence intensities of whole broth, extracellular supernatant, whole cell, and intracellular soluble lysate stand for GFP fluorescence intensity values from the samples containing both intact cells and medium fraction, only medium fraction, only intact cells, and supernatant fraction after cell disruption, respectively.

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) with a Bio-Rad Mini-Trans Blot Cell in Bjerrum and Schafer-Nielsen transfer buffer (48 m*M* Tris, 39 m*M* 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, College Park, MD), and detected with 1:5000 dilution of goat anti-rabbit IgG conjugated to alkaline phosphatase (Kirkegaard and Perry Laboratories, Gaithersburg, MD) and BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium) color development reagent (Sigma). The stained membranes were scanned and the digitized images were analyzed by National Institutes of Health image software. Pure recombinant hIL-2 concentrations of 0.025 and 0.05 μg were loaded for Western blot calibration.

Results and Discussion

Expression Patterns of GFP and hIL-2 Fusion Protein

Western blot assay was used to evaluate the expression pattern of GFP/hIL-2 fusion protein from recombinant *P. pastoris* (Fig. 2). A polyclonal anti-hIL-2 antibody was used for detection. Lane 1 is the intracellular

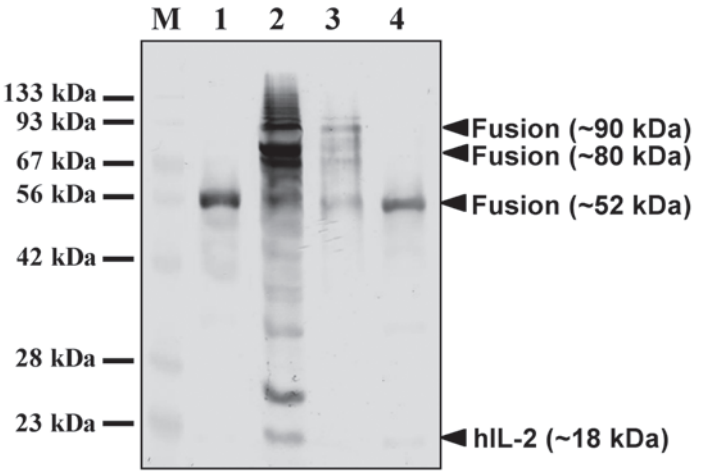

Fig. 2. Western blot analysis of GFP and hIL-2 fusion protein. Lane M, prestained molecular weight marker; lane 1, intracellular soluble lysate sample from intracellular expression strain; lane 2, intracellular soluble lysate sample from secretion strain; lane 3, extracellular supernatant sample from secretion strain; lane 4, intracellular soluble lysate sample from *E. coli*. A polyclonal anti-hIL-2 antibody was used for immunological detection.

soluble lysate from the intracellular expression *Pichia* strain GSGI-I22 *(22)*; a band of approx 52 kDa was obtained as expected for the fusion product. This band was at the same molecular weight as that of a fusion band from expression in *E. coli* (Lane 4) *(7)*. Lane 2 is the intracellular soluble lysate sample from the secretion expression Mut$^+$ strain, GSGI-S38. A prominent band of approx 80-kDa and a faint band of approx 52-kDa fusion protein were obtained. Interestingly, this large protein was not seen in lane 3 of secreted supernatant sample from the secretion expression Mut$^+$ strain GSGI-S38. Instead, a smear from approx 90 kDa to the correct 52 kDa was seen. We have no explanation for the large intracellular protein in the secretion strain other than a hypothesis suggesting a bottleneck in the signal pathways and post-translational processing such as hyper glycosylation noted by Raemakers et al. *(27)* and Vuorela et al. *(29)*. When secreted, a heterogeneous smear that included a presumable non-glycosylated fusion protein of the correct size in the extracellular supernatant.

Expression Profiles of GFP and hIL-2 Fusion Protein

Because GFP was employed as a monitoring fusion partner, the GFP fluorescence profiles were investigated for the secretion expression *Pichia* Mut$^+$ strain GSGI-S38 grown in BMM medium (Fig. 3A). Whole broth fluorescence increased monotonically. However, in the whole cell samples, a maximum was observed at 24 h, followed by a decrease until 48 h when the fluorescence increased again. The fluorescence of the extracellular supernatant also began to increase significantly at around 48 h. Importantly, the

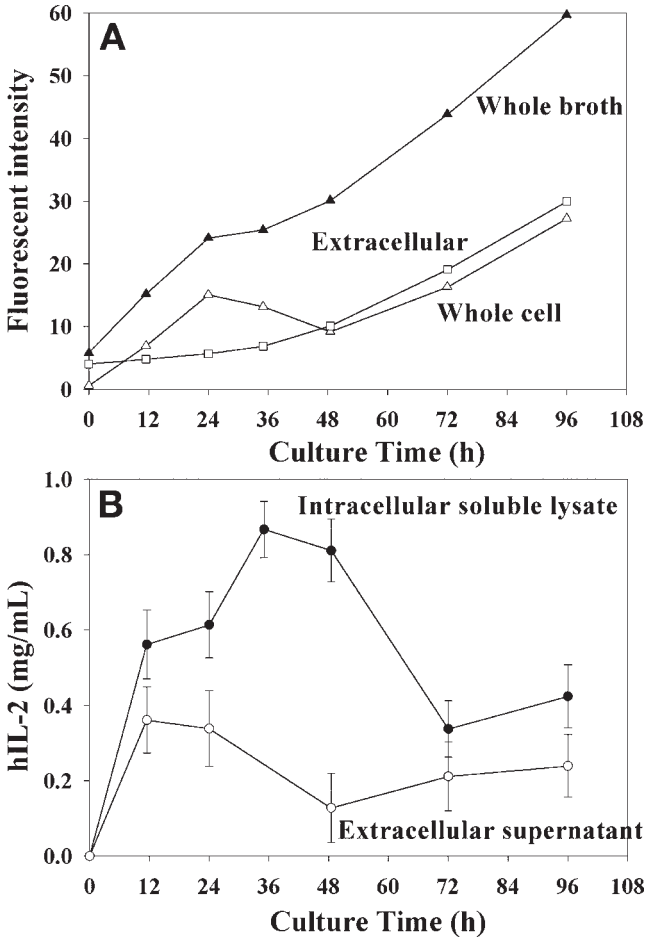

Fig. 3. Time profiles of **(A)** GFP fluorescence intensities (▲, whole broth; △, whole cell; □, extracellular supernatant) and **(B)** hIL-2 mass (●, intracellular soluble lysate; ○, extracellular supernatant) for secretion Mut$^+$ strain GSGI-S38 culture in BMM medium.

increase in fluorescence seen in the extracellular fractions of the secretion strain after 48 h was not the result of the GFP fluorescence. Instead, we found the color of the culture broth changed progressively to yellow during this time (data not shown), leading to a false indication. Also, a portion of the fluorescence signal was because of an increase in cell density. In fluorescent microscope observations, the cells showed fluorescence after 20 h, but the fluorescence progressively declined to none at 48 h (data not shown).

hIL-2 concentrations were analyzed for both intracellular and extracellular fractions (Fig. 3B). Owing to the fluorescence background in the culture broth and the disparity between fluorescence and hIL-2 level, likely because of secretion processing, fluorescence based monitoring of the GFP/hIL-2 fusion protein expression and secretion was not good. That is, after 24 h and before 48 h, the intracellular hIL-2 level increased whereas the

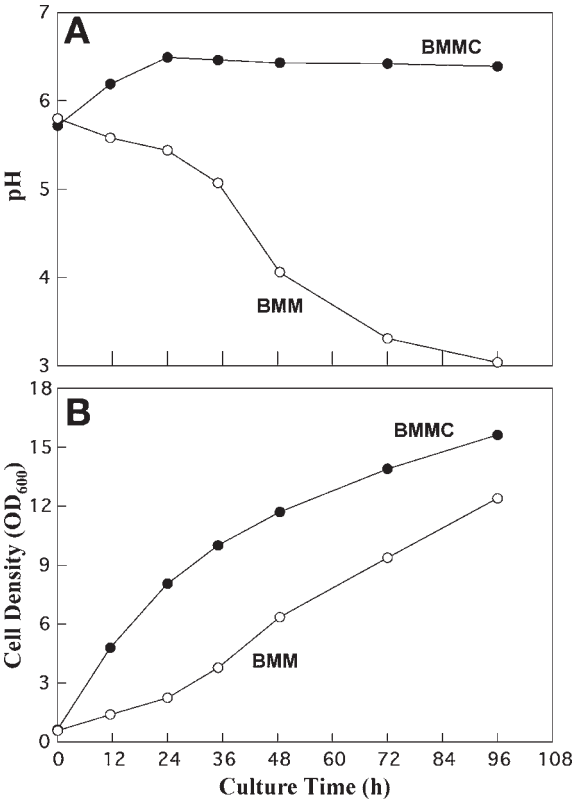

Fig. 4. Time profiles of **(A)** pH and **(B)** cell growth for secretion Mut$^+$ strain GSGI-S38 cultures in BMM (○) and BMMC media (●).

whole cell fluorescence decreased. Moreover, the extracellular hIL-2 level also decreased during this time. These peculiar results could be explained by secretion of GFP/hIL-2 after 24 h and extracellular degradation of hIL-2 fraction. Note, the hIL-2 levels are corrected for the total culture volume. Thus, the secretion efficiency can be directly compared and there was generally more hIL-2 inside the cells.

The culture fluorescence from 12 to 48 h and beyond was examined in more detail. Interestingly, the pH declined through the course of the fermentation in the case of BMM medium (Fig. 4A). In particular, the pH dropped rapidly during the time the intracellular fluorescence disappeared (24–48 h). Also, as the broth fluorescence increased quickly during this time (recall Fig. 3A), we suspected the pH drop in the BMM medium might have contributed to the low level of GFP/hIL-2 fusion protein after 24 h. As this work was performed in pH unregulated shake flasks containing BMM media, we ran the cultures with the secretion expression Mut$^+$ stain GSGI-S38 in BMMC media (BMM media with 1% casamino acids), where the pH drop was not observed. In fact, a slight increase in pH until 24 h was observed, after which the pH was steady. Cell growth was also higher in BMMC than in BMM medium (Fig. 4B).

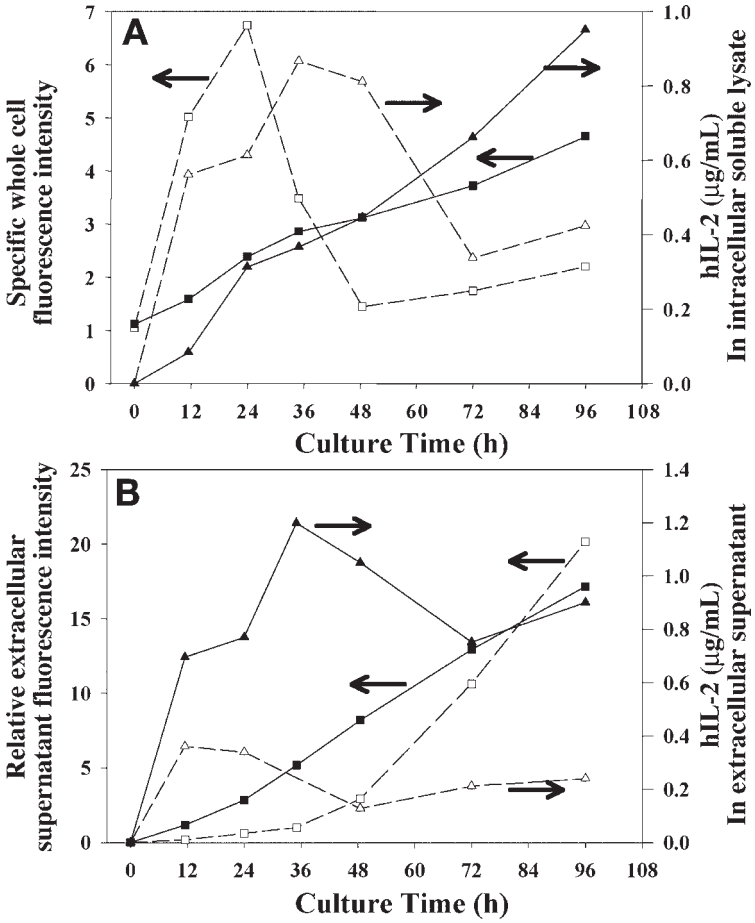

Fig. 5. Effects of medium on GSGI-S38 *P. pastoris* expression and secretion. BMM media cultures indicated by dotted lines, BMMC media cultures indicated by solid lines. **(A)** ■, specific whole cell fluorescence intensity in BMMC media; □, specific whole cell fluorescence intensity in BMM media; ▲, intracellular hIL-2 concentration in BMMC; △, intracellular hIL-2 concentration in BMM. **(B)** ■, extracellular supernatant fluorescence intensity in BMMC media; □, extracellular supernatant fluorescence intensity in BMM media; ▲, extracellular hIL-2 concentration in BMMC; △, extracellular hIL-2 concentration in BMM.

Interestingly, in BMMC medium, the specific whole cell fluorescence increased steadily as shown in Fig. 5A. There was no disappearance of fusion protein after 24 h. Using a fluorescence microscope, we confirmed that the cell fluorescence continually increased (data not shown). The intracellular hIL-2 concentration in BMMC also increased steadily like specific whole cell fluorescence profile. The extracellular supernatant fluorescence was plotted as a relative (difference) value in Fig. 5B. This was the result of the high initial fluorescence background of BMMC from the casamino acids. Thus, the relative fluorescence intensity was calculated by subtracting initial fluorescence of each medium. It should be noted that the fluorescence

increased for the Mut^S control culture in BMM media, which does not express GFP (data not shown). Interestingly, when BMMC medium was used, the color of the culture broth did not change to yellow (data not shown). The slope of fluorescence increase in BMMC media was lower than that of BMM media even though the fusion protein still emitted fluorescence. However, the supernatant fluorescence still increased more than we would have expected by secretion of GFP. When the secreted extracellular hIL-2 amounts were assayed, a higher enhancement (threefold) of hIL-2 secretion was seen in the BMMC medium (Fig. 5B). The profile of secreted hIL-2 concentration was similar to that of extracellular supernatant florescence until 48 h. However, high fluorescence background of BMMC medium made readings of extracellular fluorescence inaccurate as an indicator of hIL-2, particularly after 48 h.

Importantly, by adding casamino acids to the BMM basal minimal medium, stable expression and high secretion of the fusion protein was obtained. We cannot explain exact mechanism for effect of casamino acid addition yet. However, we can surmise that casamino acid might enhance production of fusion protein directly due to altered nutritional regulation of the *aox1* gene promoter. We found that pH decreased significantly and production of fusion protein was very low in minimal BMM medium. Because, after casamino acid addition, pH drop was prevented and production of fusion protein was highly enhanced, we can also surmise that pH is sensitive in the production of fusion protein and casamino acid might play a role as a buffering agent to prevent pH decrease during the culture.

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