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Modified penicillin acylase signal peptide allows the periplasmic production of soluble human interferon-γ but not of soluble human interleukin-2 by the Tat pathway in *Escherichia coli*

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Abstract

Production of periplasmic human interferon-γ (hINF-γ) and human interleukin-2 (hIL-2) by the Tat translocation pathway in *Escherichia coli* BL21-SI was evaluated. The expression was obtained using the pEMR vector which contains the Tat-dependent modified penicillin acylase signal peptide (mSP*pac*) driven by the T7 promoter. The mSP*pac*-hINF-γ was processed and the protein was transported to periplasm. Up to 30.1% of hINF-γ was found in the periplasmic soluble fraction, whereas only 15% of the mSP*pac*-hIL-2 was processed, but hIL-2 was not found in the periplasmic soluble fraction.

Introduction

Escherichia coli is the most common host used in recombinant DNA technology for over-expression of proteins that do not require glycosylation. It has a periplasmic space where recombinant proteins can be transported and purified. Production of recombinant proteins into periplasm has advantages over cytoplasm production such as, *in vivo* cleavage of the signal peptide yields the authentic N-terminus, disulfide bonds are formed easily due to oxidizing environment, there are fewer proteases in the periplasm compared to the cytoplasm, and recombinant proteins can be released by osmotic shock, facilitating the purification process (Makrides 1996). The general secretor (Sec) system is the main route to transport unfolded periplasmic and outer membrane proteins in *E. coli*. The Sec signal peptides such as OmpT, OmpA, PhoA and MalE have been used to transport recombinant proteins to the bacterial periplasm (Ignatova et al. 2003; Hytonen et al. 2004; Xu et al. 2002; Pratap and Dikshit 1998). The twin arginine translocation (Tat) system was identified as a transport mechanism for a specific group of periplasmic proteins (Palmer et al. 2005). The Tat system is a complex composed of TatABCE proteins with a relative mass of 600 kDa. Proteins transported by Tat pathway require a signal peptide with the consensus sequence S/TRRXFLK (Palmer et al. 2005). The TorA is the most studied Tat signal peptide (Thomas et al. 2001). There are some exceptions of Tat-canonical signal peptides, for example *E. coli* penicillin acylase signal peptide (SP*pac*) has a small and unusual Tat signal peptide with an asparagine separating the twin arginine motif (Ignatova et al. 2002) and *Salmonella enterica* tetrathionate reductase signal peptide (TtrB) contains only a single arginine residue (Hinsley et al. 2001). Previous work

showed that the SP*pac* drives translocation of pre-propenicillin acylase specifically by the Tat pathway and it is not substrate for Sec machinery (Ignatova et al. 2002).

The Tat machinery presents advantages over the Sec pathway. First, the Tat pathway can transport complex proteins containing FeS, Ni-Fe, molybdopterin center (Berks 1996; Palmer et al. 2005), heme group (Sturm et al. 2006) and multidisulfide bonds (Kim et al. 2005). Second, under aerobic conditions, few proteins use the Tat pathway, making this system readily available (Robinson and Bolhuis 2004). Proteomic analysis and bioinformatics indicate that only 5-8% of periplasmic proteins are transported by the Tat pathway (Berks 1996). Third, unlike the Sec route, the Tat pathway is not essential, thus the Tat complex can be modified for transport of specific proteins (Bogsch et al. 1998).

In this work, we evaluated an expression system driven by the T7 promoter and a modified SP*pac* for the transport of human interferon-γ (hINF-γ) and human interleukin-2 (hIL-2) to the periplasmic space using the Tat pathway in *E. coli* BL21-SI.

Materials and Methods

Construction of pEMR

An expression/transport vector was constructed using a pBR322 origin, the *bla* gene and the modified SP*pac* under control of the T7 promoter (Fig. 1). To facilitate the cloning of the target genes, we introduced restriction sites in the wild-type SP*pac*. The changes were: 1) the insertion of CAT at position 1 generated a *Nde*I site (CATATG) right in front of the start codon (ATG). 2) The change of G for A at position 60 generated a

*Hin*dIII cloning site (AAGCTT) and changed the amino acid sequence from tryptophan for a serine. It has been reported that this mutation improves the transport of penicillin acylase (Monroy-Lagos et al. 2006). 3) The changes of A for G at position 72 and G for A at position 75, both generated a *Nhe*I site (GCTAGC), but did not change the aminoacid sequence. The mutations were carried out using the Quick-change site-directed mutagenesis kit (Stratagene) and were confirmed by sequencing. The modified penicillin acylase signal peptide (mSP*pac*) let the cloning of genes encoding proteins with biotechnological interest. The resultant plasmid was identified as pEMR (Mexican patent pending No. NL/a/2006/000069).

Bacterial strains and plasmids

E. coli DH5α was used for routinely plasmid screening and was grown at 37 °C in Luria-Bertani medium (LB). The pCR4-516 and pCR4-556 vectors containing the synthetic hINF-γ and hIL-2 genes, respectively, were purchased from Entelechon GmbH (Regensburg, Germany). The *Hin*dIII and *Bam*HI sites were added to hINF-γ synthetic gene by PCR using pCR4-516 as template and the primers sense 5'-AAGCTTACCTGCACTGCCTCAGGACCCATACGTGAAA-3' and anti-sense 5'-GGATCCTTACTGACTTGC-3'. The *Hin*dIII-hINF-γ-*Bam*HI amplified fragment was cloned in the pEMR vector to construct pEMR-hINF-γ, then *E. coli* BL21-SI (GIBCO) was transformed by heat-shock method with the pEMR-hINF-γ plasmid and transformant clones were selected on LBON medium (Luria-Bertani salt free) with 100 µg ampicillin ml^{-1}. For the construction of pEMR-hIL-2, the same protocol described above was used, but using the pCR4-556 as template and the primers sense 5'-

AAGCTTACCTGCACTGGCTGCCCCAACCTCCTCC-3' and anti-sense 5'-GGATCCATTAAGTTAAGGTGGA-3' for the PCR.

Media and culture conditions

E. coli BL21-SI/pEMR-hINF-γ and *E. coli* BL21-SI/pEMR-hIL-2 were grown in minimum medium containing per liter: 5 g glucose, 3.5 g $(NH_4)_2HPO_4$, 3.5 g KH_2PO_4, 1 g $MgSO_4$, 40 µg thiamine, 100 mg ampicillin and 3 ml trace elements solution (containing per liter: 27 g $FeCl_3$, 2 g $ZnCl_2$, 2 g $CoCl_2 \cdot 6H_2O$, 2 g $Na_2MoO_4 \cdot 2H_2O$, 1 g $CaCl_2 \cdot 2H_2O$, 1 g $CuCl_2 \cdot 2H_2O$, 0.5 g H_3BO_3 and 100 ml HCl). The pH was adjusted to 7.4 with NaOH prior to sterilization (15 min at 121 °C). Batch cultures were carried out in 500 ml Erlenmeyer flasks with 100 ml minimum medium inoculated to initial absorbance at 620nm (Abs_{620nm}) of 0.2 and grown at 37 °C and 250 rpm. The cultures were induced with 0.3M NaCl when they reached an $Abs_{620nm}$of 0.6 and post-induction temperatures were 20, 28 and 37 °C. For all experiments, pre-inocula in minimum medium plus 5 g yeast extract l^{-1} (Difco Labs. Frankling Lakes, NJ) were grown overnight at 37 °C and 250 rpm.*

Cell fractionation

Periplasmic soluble (PS), cytoplasmic soluble (CS) and insoluble membrane (IM) fractions were obtained as described previously by Robbens et al. (2006). The IM fraction contains membrane proteins and both the cytoplasmic and periplasmic inclusion bodies, since these are associated to the cell-inner membrane (Betton & Hofnung 1996). The proteins were separated in 4-20% sodium dodecyl sulphate polyacrylamide gel gradient

electrophoresis (SDS-PAGE), transferred into nitrocellulose membranes and analyzed by western blot as described below.

Analytical methods

Biomass concentration was determined turbidimetrically at 620 nm and converted to dry cell weight (DCW) using a standard curve. The concentration of glucose was measured by a high sensitivity evaporative light scattering detector (Eurosep Inst. DDL 31) after separation by HPLC with a Shodex SP0810 column (Waters). Total protein concentration was quantified by Lowry method. For Western blot analysis of hINF-γ or hIL-2, respective rabbit polyclonal antibodies (Peprotech) were used at 0.2 µg ml^{-1}. Goat anti-rabbit immunoglobulin conjugated with alkaline phophatase (Bio-Rad) diluted to 1:3000 was used as secondary antibody. The blot was developed with *p*-nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate, sodium salt (Amersham Biosciences). Densitometry of nitrocellulose membranes was carried out using the Quantity One^{TM} v 4.5 software (Bio-Rad). Recombinant hINF-γ and hIL-2 (Preprotech) were used as standards. The concentration of soluble hIL-2 and hINF-γ were quantified by ELISA (R&D systems).

Results and Discussion

Expression of hINF-γ and hIL-2

Typical batch cultures of *E. coli* BL21-SI/pEMR-hINF-γ and *E. coli* BL21-SI/pEMR-hIL-2 are shown in the Fig. 2 and 3 respectively. The specific growth rate and the

maximum values of biomass, recombinant protein and total protein concentrations are summarized in the table 1. The analysis by Western blot showed that mSP*pac*-hINF-γ was efficiently cleaved, and all recombinant hINF-γ was found as processed form with a molecular weight of 17 kDa (Fig. 4A), whereas only 15 % of mSP*pac*-hIL-2 was processed and two bands were observed with molecular weight of 17.5 and 15.5 kDa corresponding to the precursor and processed form, respectively (Fig. 4B). It has been reported that the processing efficiency and translocation depends of aminoacid sequence and intrinsic folding characteristics of each protein (Koshland & Botstein 1980).

Cell fractionation analysis

For the cultures of *E. coli* BL21-SI/pEMR-hINF-γ with post-induction at 37 °C, the hINF-γ was found in the PS, CS and IM fractions at 5.3, 2.9 and 91.8 %, respectively (Fig. 5A). However, for the cultures at 28 and 20 °C, the fractions of hINF-γ in the PS increased to 14.3 and 30.1%, respectively (Table 2). These results demonstrated that mSP*pac* let the transport of hINF-γ. It has been reported that the use of low post-induction temperatures (20-28°C) increases solubility of the recombinant proteins transported to periplasm of *E. coli* (Robbens et al. 2006). Kim et al., (2005) reported the TorA signal peptide is capable of transporting complex eukaryotic proteins such as the human tissue plasminogen activator to the bacterial periplasm by the Tat pathway. For the case of hIL-2, the processed protein was detected in both, the CS and IM fractions at 9.8 and 7.3 %, respectively, and as precursor in the CS and IM fractions at 3.6 and 79.3 %, respectively (Fig. 5B). Similar to the results obtained here, Sturm et al. (2006), found the procesed and non-procesed YcdB protein, a Tat-dependent substrate, in the CS and

IM fractions. They concluded that the Tat system is rapidly saturated and the signal sequences of accumulating Tat substrates are often sensitive to proteases, resulting in a digestion of the signal sequence while the mature protein remains intact (Yahr & Wickner 2001; Sturm et al. 2006). The hIL-2 was not found in the PS fraction, probably because it could form periplasmic inclusion bodies associated to cell-membrane (Betton & Hofnung 1996). Similar to the results obtained here, Denèfle et al. (1989) found that hIL-1β transported to periplasm using OmpA and PhoA signal sequences, led the formation of periplasmic inclusion bodies associated to cell-membrane. The post-induction temperature had not effect in the processing of mSP*pac*-hIL-2 (data non-shown). We found that the mSP*pac*-hIL-2 was partially processed, whereas van Kimmenade et al. (1989) reported that hIL-2 fused to OmpA signal sequence could not be processed.

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Table 1. Summary of the parameters measured in the cultures of *E. coli* producing hINF-γ or hIL-2.

Parameter	BL21-SI/pEMR-hINF-γ	BL21-SI/pEMR-hIL-2
Specific growth rate (h^{-1})	0.22	0.25
Biomass conc. (g_{DCW} l^{-1})	1.46	1.22
Recombinant protein conc. (mg l^{-1})	41.6	282
Total protein conc. (mg ml^{-1})	0.45	0.40

Table 2. Effect of the post-induction temperature on the transport of the hINF-γ to the periplasm of *E. coli.*

Post-induction temperature (°C)	**Amount of total hINF-γ (mg l^{-1})**	**Percentage of hINF-γ in the periplasmic soluble fraction (%)**
37	42	5.3
28	49	14.3
20	55	30.1

Figure legends

Figure 1. Expression vector pEMR. Abbreviation, *bla*: β-lactamase gene, *ori*: origin of replication pBR322, mSP*pac*: modified penicillin acylase signal peptide, T7: T7 promoter.

Figure 2. Growth kinetics of *E. coli* BL21 SI/pEMR-hINF-γ in 500 ml flask containing 100 ml of minimal medium. (●) Biomass concentration, (❍) Glucose concentration, (□) hINF-γ concentration, (▲) Total protein concentration. Arrow shows induction time with NaCl.

Figure 3. Growth kinetics of *E. coli* BL21 SI/pEMR-hIL-2 in 500 ml flask containing 100 ml of minimal medium. (●) Biomass concentration, (❍) Glucose concentration, (■) mSP*pac*-hIL-2 concentration, (□) hIL-2 concentration, (▲) Total protein concentration. Arrow shows induction time with NaCl.

Figure 4. Western blot analysis for the hINF-γ and hIL-2 obtained in *E. coli* BL21-SI cultures. Total cells lysate of *E. coli* BL21-SI/pEMR-hINF-γ (A) and *E. coli* BL21-SI/pEMR-hIL-2 (B). Lanes 1-5 show samples from different time points of the culture during 22.5 h after induction. Lane 6: standard of recombinant hINF-γ (A) and hIL-2 (B).

Figure 5. Cell fractionation analysis of hINF-γ and hIL-2. The plots show the fraction of recombinant hINF-γ (A) and hIL-2 (B) in periplasmic soluble (PS), cytoplasmic soluble (CS) and insoluble membrane (IM) fractions.

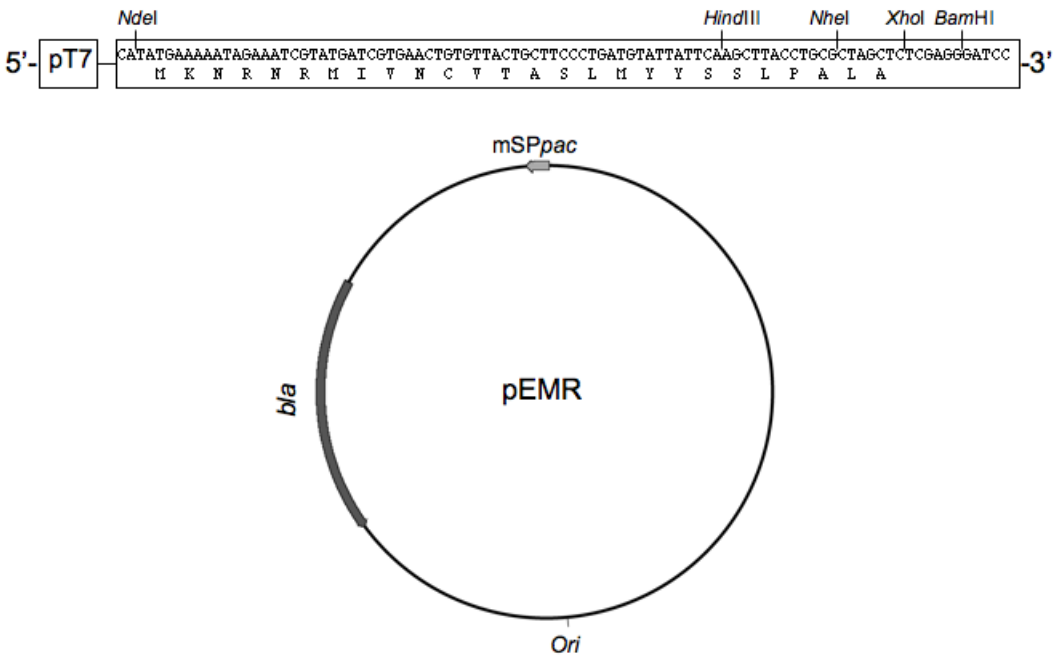

NdeI
HindIII
NheI
XhoI
BamHI
5'-
pT7
CATATGAAAAATAGAAATCGTATGATCGTGAACTGTGTTACTGCTTCCCTGATGTATTATTCAAGCTTACCTGCGCTAGCTCTCGAGGGATCC
M K N R N R M I V N C V T A S L M Y Y S S L P A L A
-3'
mSPpac
bla
pEMR
Ori

Figure 1.

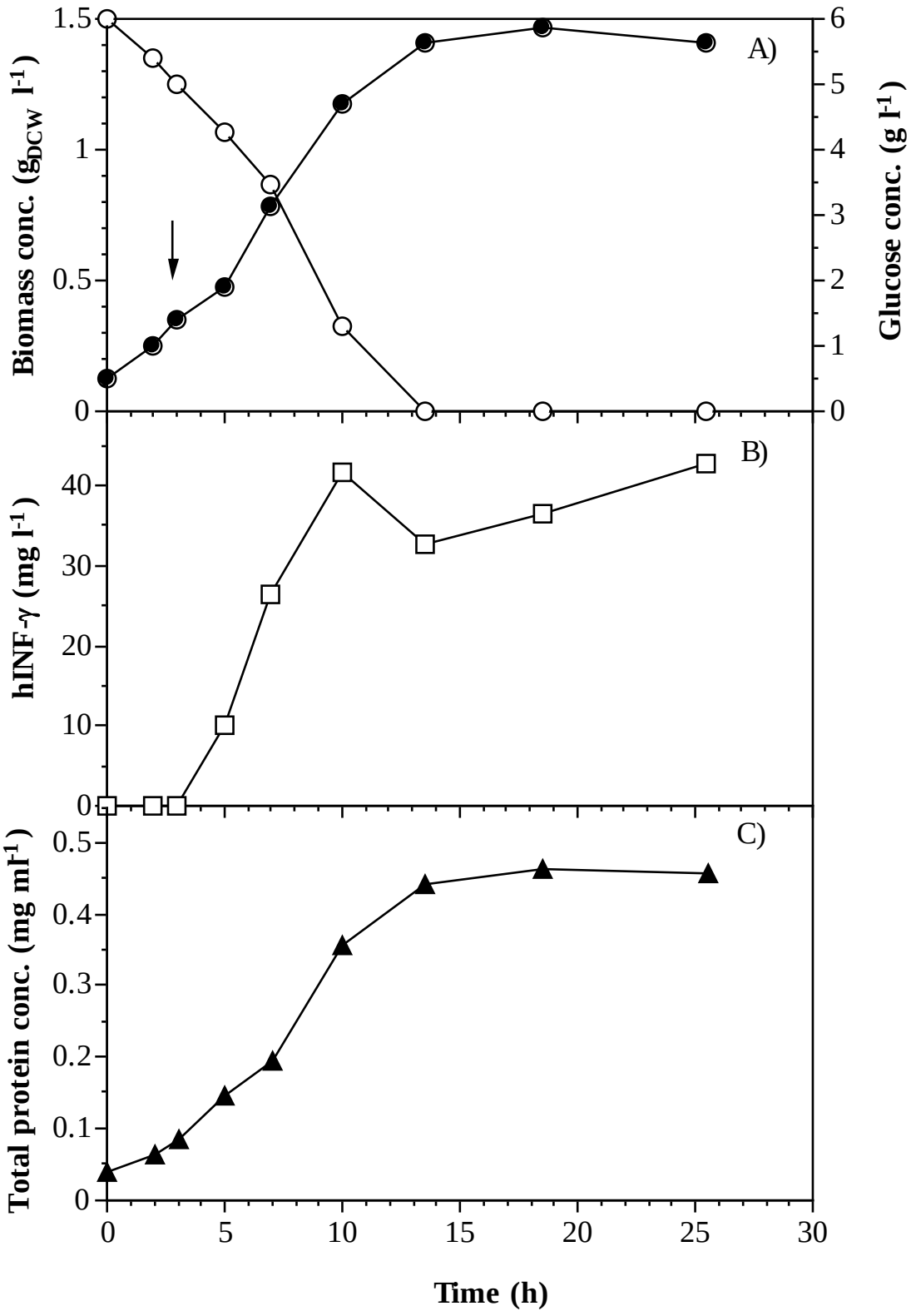
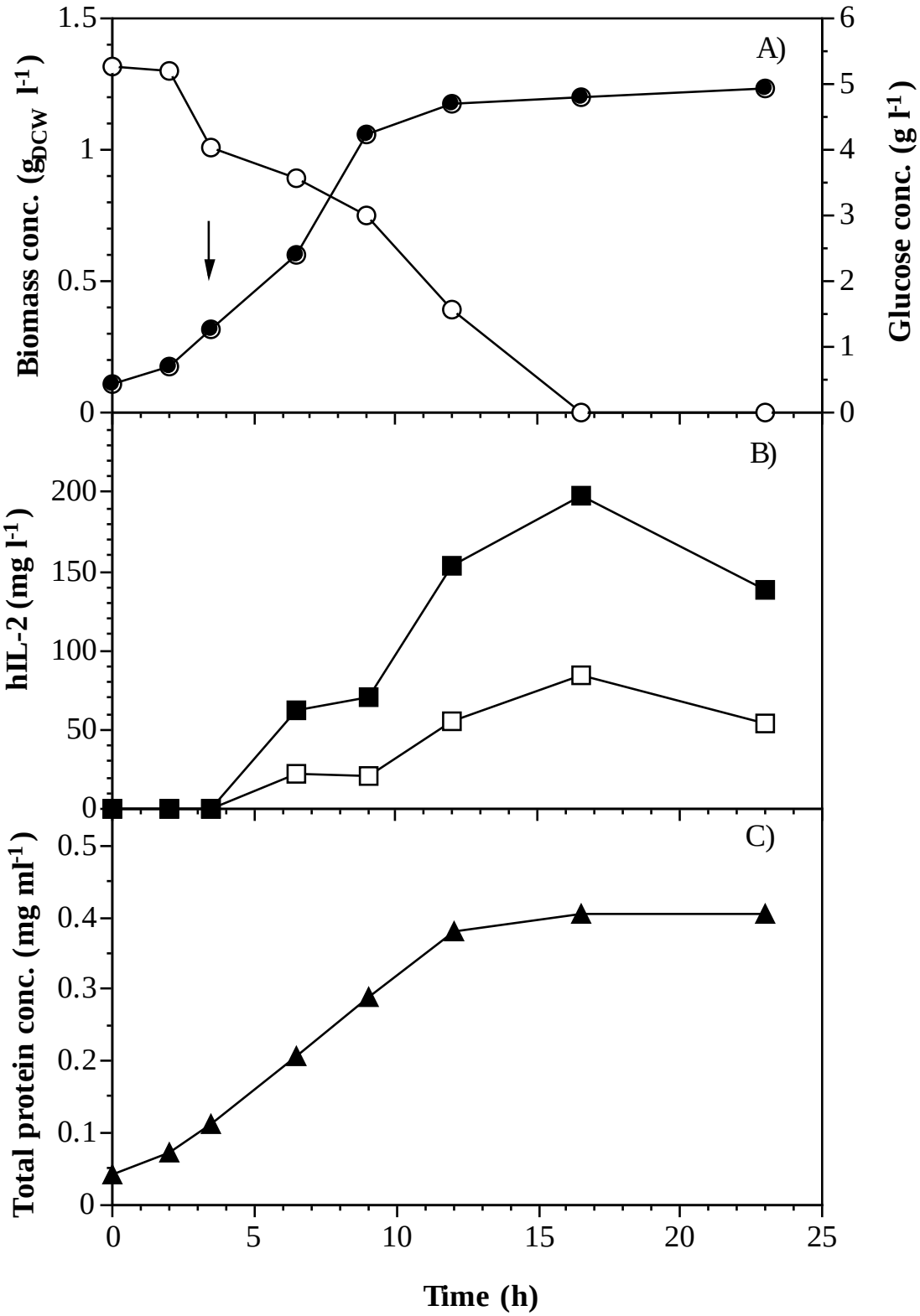

A)
B)
C)
Biomass conc. (g_{DCW} l^{-1})
Glucose conc. (g l^{-1})
hINF-γ (mg l^{-1})
Total protein conc. (mg ml^{-1})
Time (h)
0
0.5
1
1.5
2
3
4
5
6
10
20
30
40
0.1
0.2
0.3
0.4
15
25

Figure 2.

1



2
3
4 Figure 3.

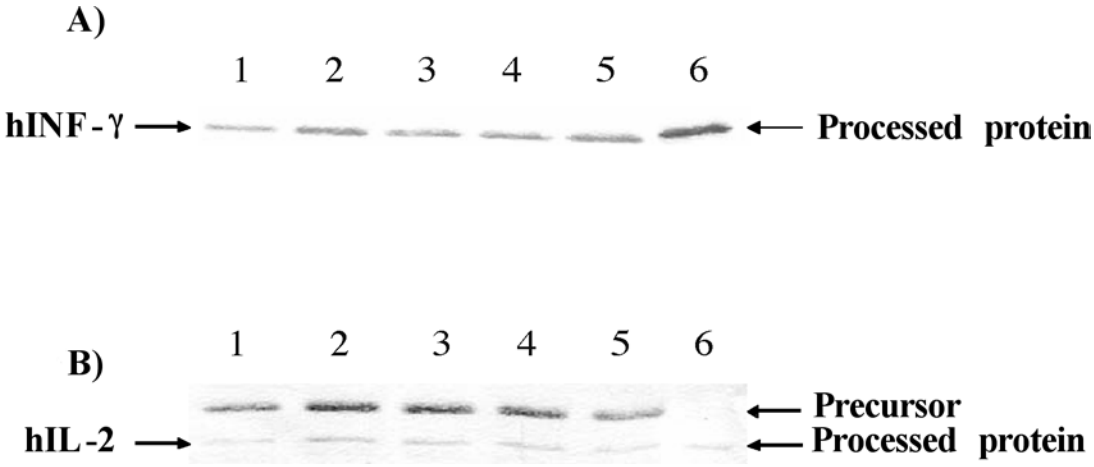

A)
1 2 3 4 5 6
hINF-γ
Processed protein
B)
1 2 3 4 5 6
hIL-2
Precursor
Processed protein

Figure 4.

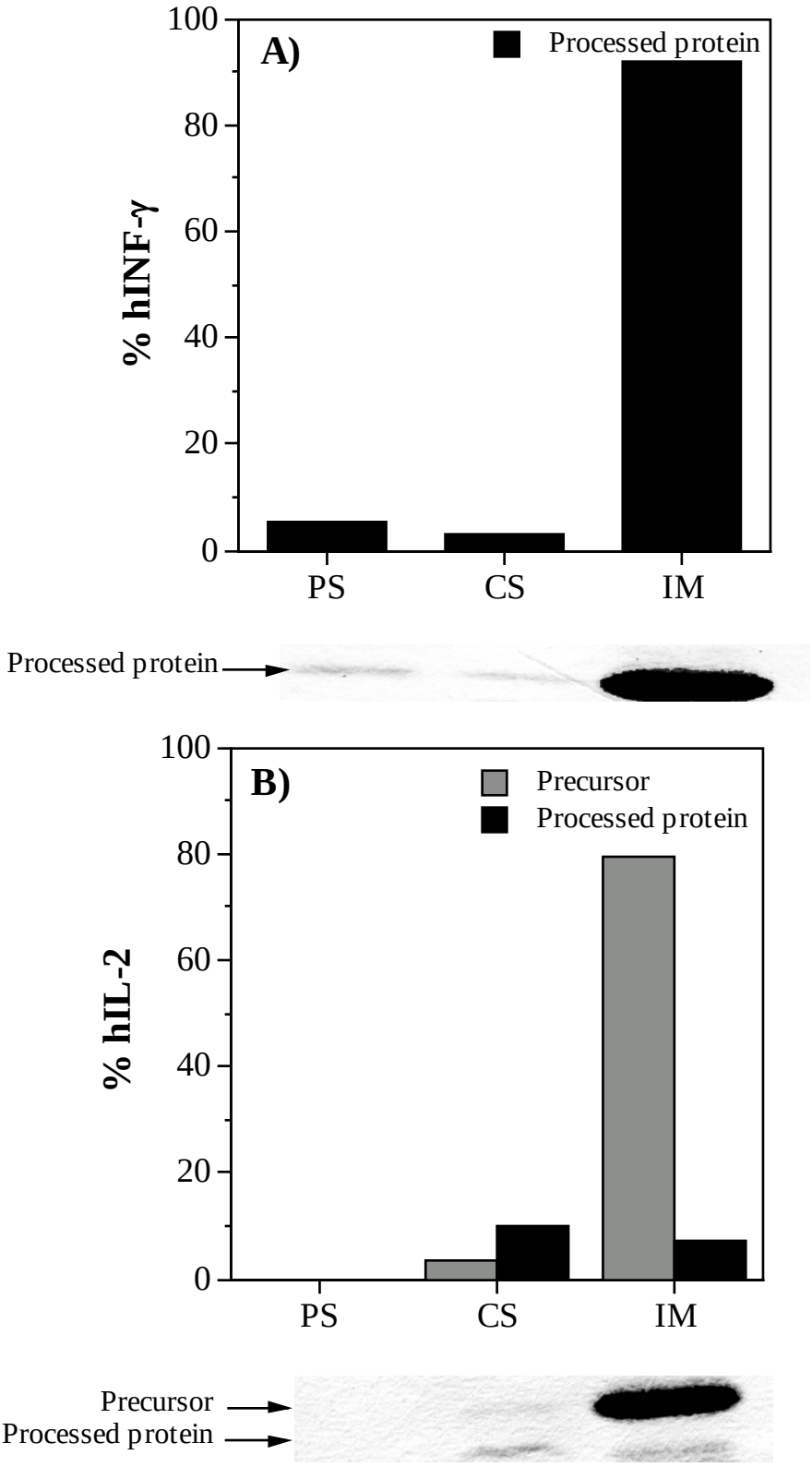

A)
Processed protein
% hINF-γ
100
80
60
40
20
0
PS
CS
IM
Processed protein
B)
Precursor
Processed protein
% hIL-2
100
80
60
40
20
0
PS
CS
IM
Precursor
Processed protein

Figure 5.