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Synthesis of mini-proinsulin precursors using N-termini of human TNF α as fusion partners in recombinant *Escherichia coli*

CS Shin[1], MS Hong[1], DY Kim[2], J Lee[2] and Y-H Park[2]

[1]*Bioprocess Engineering Laboratory, Hanhyo Institutes of Technology, Yusong, Taejon 305–390, South Korea;*
[2]*Biochemical Process Engineering RU, Korea Research Institute of Bioscience and Biotechnology (KRIBB), PO Box 115, Yusong, Taejon 305–600, South Korea*

Synthesis of human mini-proinsulin precursors was investigated in controlled fed-batch cultures at high cell concentrations of recombinant *Escherichia coli.* Transcription of the recombinant gene was controlled by a T7 promoter system. The human mini-proinsulin was prepared by substituting a C-chain peptide of natural proinsulin with a peptide sequence of only nine amino acids. The reduced size of fusion proinsulin and hence the increased purity of human insulin in the recombinant product may contribute to increasing the fermentation yield of human insulin. Three precursors (T1-, T2-, and T3-M2PI) were constructed by utilizing the N-terminus residues of human tumor necrosis factor α as fusion partners. The T2 precursor was most soluble in the cytoplasm, and exerted the most inhibitory effect on recombinant cell growth. In the production of T2-M2PI, significant amounts of undesirable metabolic by-products (acetate and ammonia) accumulated in the culture broth even at very low specific cell growth rate. The major portion of all synthesized precursors aggregated to insoluble inclusion bodies but the protein aggregates were easily converted to monomers in the presence of the anionic detergent (SDS) without using any reducing agent. With the expression of T1-M2PI, growth inhibition was minimal, and the maximum volumetric yield of mini-proinsulin (M2PI) in fermentation cultures was at the highest level among the synthesized precursors.

Keywords: *Escherichia coli*; mini-proinsulin; N-terminus residues; human tumor necrosis factor α; fusion partners

Introduction

One potential disadvantage of direct expression of foreign proteins in *Escherichia coli* cytoplasm is that synthesis of the desired proteins is initiated with methionine which is not efficiently processed *in vivo*. To overcome this problem, several methods have been developed, including the secretion of heterologous protein [5,6,10,11,15] and enzymatic removal of an appropriately designed N-terminal fusion partner [3,4,9,14]. Sometimes protein stability is a problem in the production of small heterologous proteins or peptides such as human proinsulin because of their short half-life in the host cell [16]. In order to increase the stability of expressed protein in *E. coli*, expression in fusion proteins has also been widely employed. Although bacterial intracellular fusion systems are frequently characterized by production of insoluble inclusion bodies [17], the choice of fusion partner sometimes significantly determines the solubility, toxicity to host cell, and/or yield (or concentration) of expressed fusion protein, which in turn affects the efficiency of complex downstream processes [2,7,8,12]. At the same time, there has not been the same physiological background for the development of fermentation processes producing the desired recombinant proteins with high stability as well as high yield, where the biomass concentration is increased to high levels (>25 g L^{-1}). Therefore, the characteristics of the fusion expression system in protein stability, yield, and bacterial physiology need to be investigated in detail for development of optimal fermentation processes.

Human insulin derived from rDNA technology was the first marketed human health-care product, commercialized by Eli Lilly and Company in the United States in 1982. Since the bulk market of human insulin requires large-scale fermentation ($>10\,000$ L) producing human proinsulin, even small improvements in protein concentration significantly affect productivity of the fermentation process. Therefore, it is of great importance to develop an efficient and stable expression system for a high-yield fermentation process.

The present investigation is concerned with the synthesis of recombinant fusion human proinsulin in fed-batch cultures of *E. coli.* Human proinsulin was prepared by replacing the natural C-chain peptide (35 amino acids) with a new peptide sequence consisting of only nine amino acids. This mutant human proinsulin has been named as 'mini-proinsulin' [13]. The fusion mini-proinsulin produced can be converted to authentic human insulin by removing the fusion partner and the pro-sequence by *in vitro* treatment with cyanogen bromide and proteolytic enzymes, respectively [1]. In the present work, the effects of various fusion partners (derived from N-terminus residues of tumor necrosis factor α) on cell growth and gene expression were investigated in well-controlled fed-batch cultures at high cell concentrations. Some important characteristics in solubility and aggregation of synthesized recombinant precursors were demonstrated.

Correspondence: Dr J Lee, Biochemical Process Engineering RU, Korea Research Institute of Bioscience and Biotechnology (KRIBB), PO Box 115, Yusong, Taejon 305–600, South Korea
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Materials and methods

Recombinant E. coli strains and precursor design

The host-plasmid systems used in this study consist of the structural genes for mini-proinsulin (M2PI) precursors carried on the recombinant T7-based pET expression plasmids (pET-3aT1M2, pET-3aT2M2, and pET-3aT3M2) with the *E. coli* strain BL21(DE3) (F^- *ompT* $hsdS_B(rB^-\ mB^-)$) as the host. The preparation of the mini-proinsulin, the shuttle vector, and the host strain were described in detail by Shin *et al* [13]. The mini-proinsulin precursors [T1-M2PI (13 kDa), T2-M2PI (8.6 kDa), and T3-M2PI (8.9 kDa)] were expressed using N-terminus residues of hTNFα (human tumor necrosis factor α) as fusion partners (Figure 1). The following peptide consisting of 54 amino acids (ie N-terminus 8th (Pro) to 61st (Arg) residues of hTNFα) was used as the fusion partner T1: MPSDKPVAHVVANPQAEGQLQWLNRRANALLANGVELRDNQLVVPIEGLFLIYSR. Another fusion partner, T2, consists of the 15-residue peptide, or N-terminus 8th to 12th residues of hTNFα plus ten histidine residues (H_{10}). Finally, N-terminus 8th to 22nd residues of hTNFα were used as the fusion partner T3. The same spacer sequence, Ser-Ser-Gly-Ser-Met, was added to the C-terminus of T1, T2, and T3 above, and all fusion partners were removed by *in vitro* treatment with cyanogen bromide (CNBr) using the last methionine as the cleavage site. All recombinant *E. coli* strains producing M2PI precursors were kindly donated by the Protein Engineering Laboratory at Hanhyo Institutes of Technology, Yusong, South Korea.

Cultivation media and bioreactor operation

The preparation of stock cultures and inoculum for each bioreactor experiment was as described in our previous report [13]. The synthetic medium used for initial batch cultures contained per liter: 13 g KH_2PO_4, 4 g $(NH_4)_2HPO_4$, 1.7 g citric acid, 20 ml trace metal solution (containing per liter: 0.42 g EDTA, 0.125 g $CoCl_2{\cdot}6H_2O$, 0.75 g $MnCl_2{\cdot}4H_2O$, 0.11 g $CuSO_4{\cdot}5H_2O$, 0.15 g H_3BO_3, 0.125 g $Na_2MoO_4{\cdot}2H_2O$, 0.85 g $ZnSO_4{\cdot}7H_2O$, and 5 g $FeCl_3{\cdot}6H_2O$), 0.1 g thiamine-HCl, 1.2 g $MgSO_4{\cdot}7H_2O$, and 20 g glucose. The $MgSO_4$ and glucose were autoclaved separately, and 200 mg ampicillin was added per liter through a 0.2-μm syringe filter. In the growth-phase fed-batch operation, the glucose-salt synthetic medium (containing per liter: 800 g glucose, 2 g $MgSO_4{\cdot}7H_2O$, 20 ml trace metal solution) was used as a feed with the addition of ammonium hydroxide as both nitrogen source and pH regulator. The feed media used for recombinant protein synthesis contained per liter: 1.5 g $(NH_4)_2SO_4$, 211 g yeast extract, 1 g $MgSO_4{\cdot}7H_2O$, and 274 g glucose. All feed media contained 1 g ampicillin per liter. The $MgSO_4$ and glucose were autoclaved separately.

All batch and fed-batch bioreactor experiments were conducted in 5-L Bioflo III laboratory fermenters (New Brunswick Scientific, NJ, USA). In the growth (or preinduction) phase of fed-batch operation, all recombinant strains were grown at a specific growth rate, 0.2 h^{-1} via exponential feeding of glucose-salt defined media. Ammonium hydroxide was added to the culture as both nitrogen source and pH regulator only in the growth phase. When the cell concentration reached 65–70 g dry weight per L culture, the recombinant gene expression was initiated with the addition of IPTG (0.03 mmoles per g dry cell weight), and then the postinduction medium was continuously added at a constant feed rate, 74 ml h^{-1}, until the end of postinduction phase. Unless otherwise mentioned, the bioreactors were operated at pH 6.75 (only in the growth phase) and 37°C. Dissolved oxygen was controlled above 40% air saturation to avoid oxygen limitation. Data acquisition and control of various operating parameters (eg temperature, dissolved oxygen concentration, pH, rpm, and the medium flow rate) were effectively achieved by using the Bioflo III's electronic control module and the software, Advanced Fermentation System.

Analytical methods

The glucose concentration was measured by a YSI glucose analyzer (model 2300 Stat Plus, Yellow Springs Instruments, Yellow Springs, OH, USA). The acetic acid and ammonia concentrations were measured by employing acet-

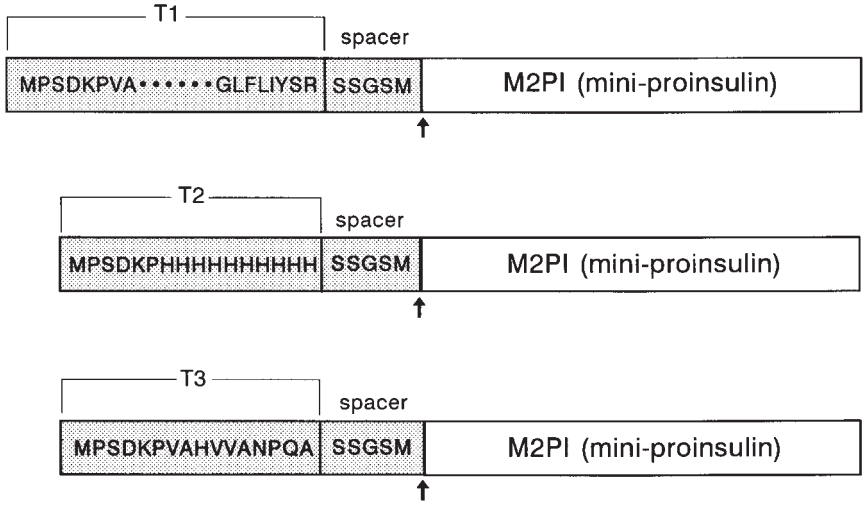

Figure 1 Schematic presentation of mini-proinsulin precursors. Arrows indicate the cleavage site for CNBr.

ate (Boehringer Mannheim, Mannheim, Germany) and ammonia (Sigma, St Louis, MO, USA) assay reagents as per the procedures suggested by the supplier. The optical density of each culture sample was measured at 600 nm (OD_{600}) using a Pharmacia Ultrospec III spectrophotometer. The dry weight concentration of recombinant cells (DCW, g L^{-1}) was then obtained using a previously developed correlation between optical density and dry cell mass concentration, ie DCW = 0.417 OD_{600}.

Expression levels of M2PI precursors were measured by subjecting samples taken from the fermenter to denaturing gradient sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (10–20% tricine gel, Novex, San Diego, CA, USA). Bovine serum albumin (BSA, 3 μg) was separately loaded on each gel with culture samples as standard for protein quantification [13]. The resulting protein bands were scanned with a laser densitometer (Ultroscan XL, Pharmacia LKB Biotechnology, Uppsala, Sweden). From this analysis, the percentage of recombinant protein from the total cellular protein was determined and the amount of human mini-proinsulin produced was also calculated from a previously developed correlation between BSA concentration and the measured band area. Non-reducing SDS-PAGE with inclusion bodies separated from cell lysates was also conducted to estimate the molecular interaction of recombinant protein aggregates.

Results and discussion

Expression of three M2PI precursors in high cell density cultures

As discussed in the earlier report [13], even a small amount of the fusion mini-proinsulin (T2-M2PI) inhibits host cell growth, and therefore the time and extent of plasmid gene expression were controlled using the inducible T7 promoter system. For the purpose of developing the fusion expression partner with which low growth inhibition and high M2PI

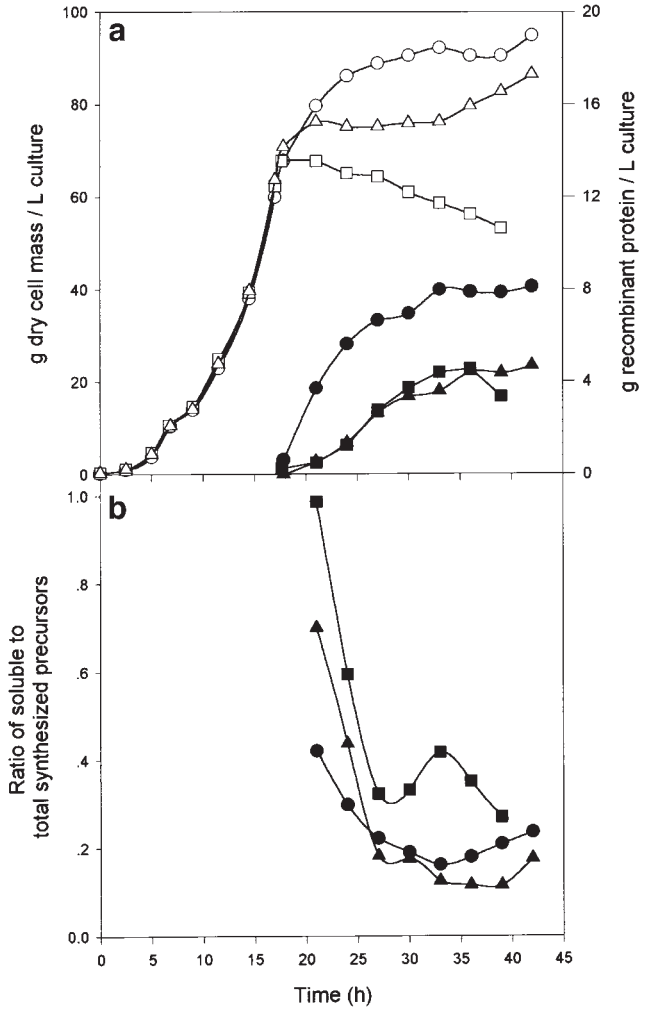

Figure 2 (a) Cell growth (open symbols) and production of M2PI precursors (closed symbols) (T1-M2PI (○, ●), T2-M2PI (□, ■), and T3-M2PI (△, ▲)) in recombinant *E. coli*. (b) Ratio of soluble to total synthesized precursors in the postinduction phase: ●, T1-M2PI; ■, T2-M2PI; ▲, T3-M2PI.

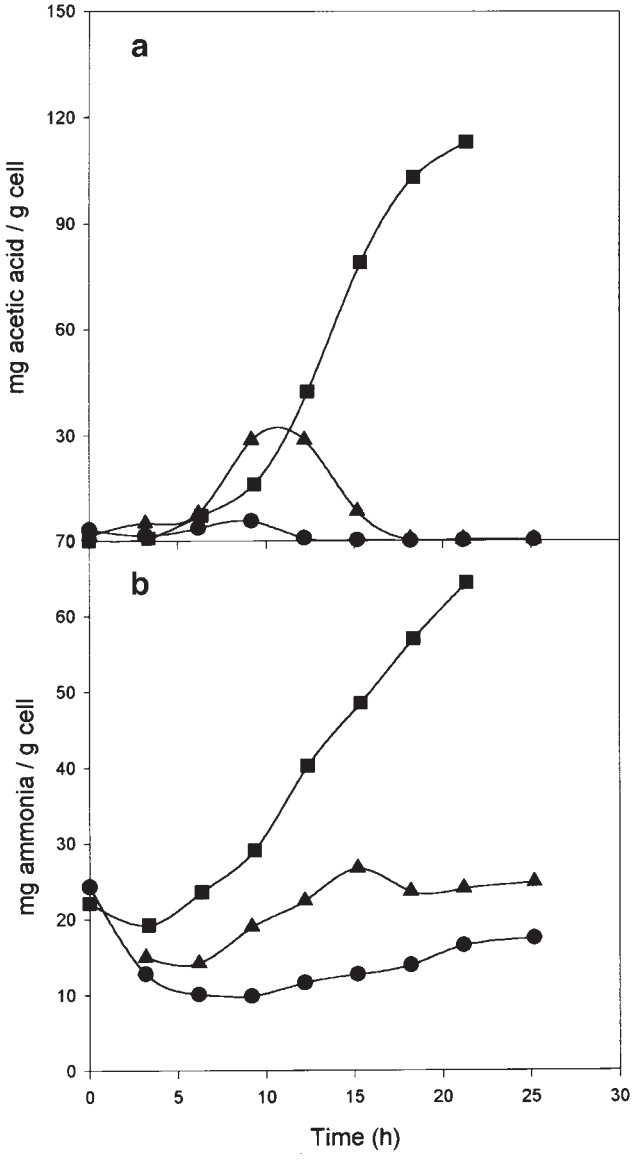

Figure 3 Production of (a) acetate and (b) ammonia in recombinant *E. coli* cultures under gene expression during postinduction phase: ●, T1-M2PI; ■, T2-M2PI; ▲, T3-M2PI.

yield can be obtained, three precursors of mini-proinsulin consisting of different fusion partners were synthesized using the same host-vector system. The growth of recombinant *E. coli* during the postinduction phase was most significantly inhibited with the expression of T2-M2PI, whereas the growth inhibition was effectively circumvented with the expression of T1 precursors (Figure 2a). The maximum concentrations of synthesized T1-, T2-, and T3-M2PI (g recombinant protein per L culture) are 8.1 g L^{-1} (0.62 mM), 4.6 g L^{-1} (0.53 mM), and 4.7 g L^{-1} (0.53 mM), respectively, where the highest expression level of T1 precursor might result from the largest molecular size of T1-M2PI. From the maximum molar concentrations above, the volumetric yield of mini-proinsulin (M2P1) in the fermentation cultures was highest with the expression of T1 precursor. Figure 2b shows that almost all synthesized T2-M2PI was initially soluble in the cytoplasm, and the soluble portion of T2-M2PI was maintained during the whole postinduction period at an apparently higher level than that of the other two precursors.

Recombinant gene expression in *E. coli* leads mainly to aggregation of the synthesized proteins into insoluble inclusion bodies, but sometimes (especially at low expression levels) all or some of the expressed proteins exist in soluble form in the cytoplasm. The inclusion bodies may cause distension of recombinant cell walls [17] and make the cells more sensitive to hypo-osmolarity of the culture environment [18] by reducing the integrity of the cell membrane, which in turn inhibits recombinant cell growth. However, it is generally accepted that the heterologous soluble proteins inhibit host cell growth more significantly than the aggregated proteins, either due to the toxicity of protein itself or because it competes with an essential growth factor of the host cell.

The growth of recombinant *E. coli* producing T2-M2PI was most repressed immediately after promoter induction (Figure 2a). This may be related to the fact that the T2 precursor was initially all soluble protein (Figure 2b). The key factors determining the solubility of precursors are not clear, but the higher solubility of T2-M2PI may be somewhat associated with the high hydrophilicity of the T2 fusion partner (ie percentages of hydrophilic amino acids in T1, T2, and T3 are 49, 88, and 38%, respectively).

Accumulation of the metabolic by-products (acetate and ammonia)

As shown in Figure 3, significant amounts of acetate and ammonia were accumulated in the induced culture broths of cells producing the T2-M2PI. In the postinduction phase, acid or base as pH regulator were not added to the culture, hence the accumulated acetate and ammonia are cellular metabolic by-products. In the production of T1 and T3 precursors, the accumulation of acetate and ammonia was apparently less than in the production of T2 precursor.

The overproduction of T2-M2PI may hamper metabolic activities and thereby result in the unbalanced rates of glycolysis and the oxidation of metabolites due to saturation of the respiration capacity. Provided that glucose overflow occurs during unbalanced cellular metabolism, the extra glucose and pyruvate could be released as the inhibitory by-product (acetate) even at a very low specific cell growth rate as shown in Figure 3a. A significant amount of ammonia is also accumulated in every recombinant culture broth (Figure 3b). With the expression of T2-M2PI, the cell growth rate decreased to nearly zero after recombinant gene expression (Figure 2a), while at the same time the recombinant protein yield drastically increased due presumably to the drastic increase of plasmid copy number with the decrease of cell growth rate, from the results of our earlier report [13]. Since glucose did not accumulate in the culture broth, probably the increased nutrient requirement for cellular maintenance and protein synthesis caused glucose starvation and hence ammonia accumulation in the culture broth.

Analysis of synthesized protein aggregates

Although most of the expressed recombinant protein was present as intracellular soluble protein at the beginning of the postinduction phase, the soluble proteins were progressively aggregated into inclusion bodies (Figure 2b). Inclusion body formation was estimated by reducing and non-reducing SDS-PAGE with the total cell lysates (Figure 4). Even in the non-reducing conditions of analysis, all synthesized precursors appeared at the same monomer position, which suggests that the protein aggregation was not mediated by a strong molecular interaction such as covalent disulfide linkage and hence that the insoluble aggregates of

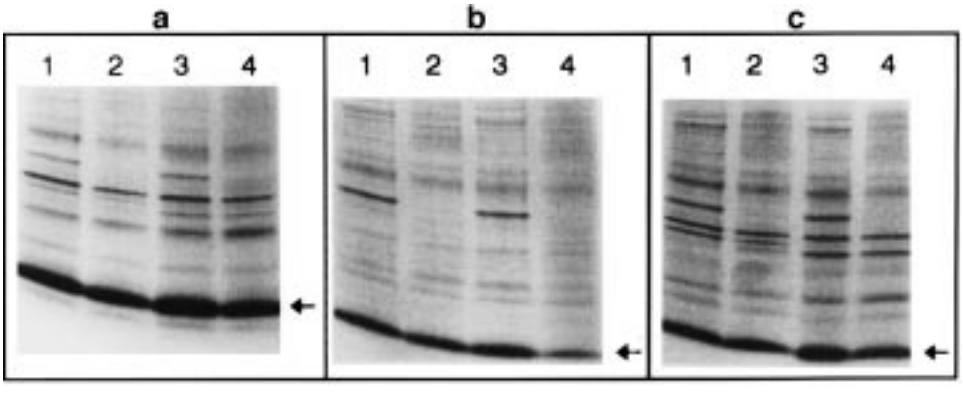

Figure 4 SDS-PAGE analysis of total cell lysate samples from induced recombinant *E. coli* cultures producing (a) T1-M2PI, (b) T2-M2PI, and (c) T3-M2PI. Lane 1: boiled samples under reduced conditions; lane 2: non-boiled samples under reduced conditions; lane 3: boiled samples under non-reduced conditions; lane 4: non-boiled samples under non-reduced conditions. Arrows indicate the M2PI precursor in each recombinant culture.

all M2PI precursors can be easily converted to the monomer form in the presence of anionic detergent without using other denaturing agents (Figure 4). Intracellular ammonia production may have some influence on the formation of reduced protein aggregates. The intracellular pH shift to a more alkaline condition would decrease the intracellular redox potential, and hence the *E. coli* cytoplasm would be under more reducing conditions, which might provide a cellular environment protecting the recombinant protein from aggregation by covalent disulfide linkage.

In the production of T1-M2PI, the growth inhibition effect was minimal, and the maximum volumetric yield of M2PI in fermentation culture was highest. Increased protein turnover, decreased metabolic efficiency, and hence growth inhibition of *E. coli* at high cell density cultures are often responsible for fermentation failures, and consequently, it seems possible that the N-terminus T1 fragment of human TNFα is an adequate fusion partner for high-level expression of mini-proinsulin as well as for stable bioreactor control.

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