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Fermentation and Purification of LacZ-Fused Single Chain Insulin Precursor for (B^{30}-Homoserine) Human Insulin

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In order to produce the single chain precursor of a novel human insulin analogue, (B^{30}-homoserine) insulin, the fermentative behaviors of *Escherichia coli* JM103 were studied, which harbors pKBA plasmid carrying a hybrid gene in which the gene for a single chain precursor was fused with *lacZ* gene under *tac* promoter. The maximal induction of gene expression was achieved when more than 0.05 mM of isopropyl-β-D-thiogalactopyranoside (IPTG) was supplemented to fermentation medium after 4 h cultivation of *E. coli*, and followed by longer than 2-h fermentation. The hybrid protein of the single chain insulin precursor was isolated from cytoplasmic inclusion bodies by dissolving in 8 M urea solution, and purified through DEAE-Sephacel and Sephadex G-200 column chromatographies with a recovery of 35%. The finally purified hybrid protein showed a single band on sodium dodecyl sulfate-polyacrylamide gel.

Key words: recombinant insulin, insulin analogue, (B^{30}-homoserine) insulin, single chain insulin precursor, gene induction, *tac* promoter, inclusion body

INTRODUCTION

The *tac* promoter, a hybrid promoter of *lac* promoter and *trp* promoter [1-2], is widely used for the overexpression of heterologous cloned-genes in *Escherichia coli*. Due to its construction by fusing -35 region of the *trp* promoter with -10 region of the *lac* promoter, the *tac* promoter contains the *lac* operator region. The strength of this promoter can be regulated in *E. coli* hosts overproducing *lac* repressor, and the gene induction can be achieved by the commonly used chemical inducer of the *lac* promoter, isopropyl-β-D-thiogalactopyranoside (IPTG) or lactose itself [3].

Nevertheless, the cloned-gene products under strong promoters are, in general, likely to be aggregated as the form of insoluble inclusion body in *E. coli* cells [4]. In spite of overproduction of gene products by controlling the strong promoters, the aggregates in host cells make it difficult to recover the biologically active form of proteins having a right conformation. Several approaches have been made for the purification of the biologically active proteins from the insoluble inclusions in *E. coli*, by the way of solubilization in denaturants and refolding by the exchange of denaturants with proper buffers [5-7].

Recently, we developed a novel procedure for the preparation of human insulin analogue, (B^{30}-homoserine) insulin [8]. This novel insulin was designed for the development of much more economical bioprocess, due to the simultaneous fermentation and purification of A chain and B chain as a single chain insulin precursor (insulin BA peptide).

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Using the expression system for insulin BA peptide, of which the gene was fused with *lacZ* gene under *tac* promoter (*lacZ*-fused insulin BA peptide gene), as a model system, the fermentative characteristics were studied with inducing the gene expression by IPTG. The purification of hybrid protein, LacZ-fused insulin BA peptide, from inclusion bodies in *E. coli* was also attempted.

MATERIALS AND METHODS

Fermentative Analysis of LacZ-fused BA Peptide in *E. coli*

E. coli JM103 (*supE thi-1 endA1 hsdR4 sbcB15 strA* Δ*(lac-proAB)* F'[*traD36 proAB*$^+$ *lacI*^{q} *lacZ*Δ*M15*]) harboring pKBA plasmid which carries the hybrid gene, *lacZ*-fused BA peptide gene [8] was used throughout this work. The culture was maintained in Luria-Bertani (LB) broth with 50 μg/ml of ampicillin. For the examination of gene induction by IPTG [9], 1% seed culture of *E. coli* was inoculated into 100 mL of LB broth in 500 mL Erlenmeyer flask and cultivated at 37°C with shaking at 150 rpm on a rotary shaker. The different concentrations of IPTG were supplied into culture broth at a given culture time, and continue to cultivate until sampling. One mL of culture broth was harvested by centrifugation and diluted with 0.1 M phosphate buffer (pH 7.0) to give 0.15 of the optical density at 600 nm. The samples (20 μL) were boiled with 20 μL of denaturing dyes for 3 min and analyzed by 14% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for the detection of total proteins in *E. coli* [10].

Purification of LacZ-fused BA Peptide from *E. coli*

Five L of LB broth containing 50 μg/ml of ampicillin was fermented with *E. coli* JM103 having pKBA plasmid at 37°C with stirring at 300 rpm in a 10 L jar fermentor (Korea Fermentor Company, Ltd.). The inducer, IPTG was added after 4-h cultivation, and followed by 2-h cultivation for the induction of gene expression. The harvested *E. coli* cell pastes by centrifugation was subjected to sonication for 9 min by using Ultrasonic Processor (XL2010, Heat Systems Inc., NY, USA). The hybrid protein, LacZ-fused insulin BA peptide, in insoluble inclusion bodies was isolated as precipitates by centrifugation and then solubilized in 8 M urea [5-6]. After centrifuging the solution again, the supernatant was recovered as solubilized hybrid protein.

Following to the procedure of Marston *et al.* [7], the isolated proteins in urea were loaded on DEAE-Sephacel column (5×15 cm) at room temperature. The column was pre-equilibrated with running buffer (20 mM Tris, 1 mM ethylenediamine tetraacetate (EDTA), 50 mM sodium chloride, 8 M urea, pH 8.0), and the loaded sample was first washed with the same buffer. The eluents was fractionated with a linear gradient of sodium chloride to 0.5 M (flow rate; 0.6 ml/min). After analyzing by 14% SDS-PAGE, the fractions containing hybrid protein was collected and concentrated through Diaflo membrane PM10 (Amicon Co., MS, USA).

In next step, Sephadex G-200 column chromatography was employed. The sample was loaded on the column which was pre-equilibrated with a buffer (20 mM Tris, 8 M urea, pH 8.0), and eluted with the same buffer at the flow rate of 0.1 ml/min. The fractions were also analyzed by 14% SDS-PAGE.

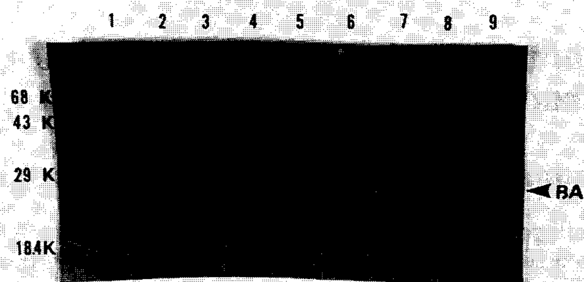

Fig. 1. Comparison of gene expression level of LacZ-fused insulin BA peptide by the supplementation of IPTG at different growth phases.
1.0 mM of IPTG was supplemented to the broth of *E. coli* JM103 harboring pKBA plasmid at different culture times, and *E. coli* was further cultured for 2 h. As described in Materials and Methods section, total proteins of *E. coli* were analyzed by 14% SDS-PAGE. Lane 1; high molecular weight marker, lane 2; not supplementing IPTG to 2 h-culture broth, lane 3; supplementing IPTG to 2 h-culture broth, lane 4; not supplementing IPTG to 4 h-culture broth, lane 5; supplementing IPTG to 4 h-culture broth, lane 6; not supplementing IPTG to 6 h-culture broth, lane 7; supplementing IPTG to 6 h-culture broth, lane 8; not supplementing IPTG to overnight culture broth, lane 9; supplementing IPTG to overnight culture broth. ◄ BA indicates the position of the hybrid protein.

Analytical Procedures

The amounts of protein in samples were determined by measuring the absorbance at 280 nm, and quantitatively by Lowry's method [11] with bovine serum albumin as standard. The molecular standards used in SDS-PAGE were β-lactoglobin (18.4 kDa), chymotrypsinogen A (25 kDa), carbonic anhydrase (29 kDa), ovalbumin (43 kDa), albumin from hen egg (45 kDa), albumin from bovine serum (68 kDa), and phosphorylase b (97.4 kDa). The gels were stained by 0.1% Coomassie Brilliant Blue R-250.

RESULTS AND DISCUSSION

Gene Induction for LacZ-fused Insulin BA Peptide by IPTG during Cultivation

In order to determine the supplementation time of IPTG for gene induction, 1 mM IPTG was added to culture broth of *E. coli* JM103 harboring pKBA plasmid at different culture time and the *E. coli* strain was further cultivated for 2 h. As seen in Fig. 1, the hybrid protein, LacZ-fused insulin BA peptide (22 kDa), was observed on SDS-PAGE gel, when IPTG was added after longer than 2 h-cultivation. The highest gene expression level was achieved when 4 h-culture broth was treated with IPTG. However, the gene was not induced by IPTG in case of overnight culture broth, presumably due to ceasing the cell growth. From this, it could be concluded that the chemical inducer like IPTG should be supplied to culture broth in the late logarithmic growth phase for the maximal formation of gene products.

The time for gene induction after the addition of IPTG was also examined. After 1 mM IPTG was supplemented to 4-h culture broth, the gene expression was observed after 2 h or longer time of gene induction (Fig. 2). Any significant increment of hybrid protein was not detected on SDS-PAGE gel, in case of longer than 2 h-cultivation. It is a similar result to Goulding's [12] that the production of β-galactosidase is linearly increased for 2 h after the induction of *lac* operon by IPTG or lactose.

The effect of IPTG concentration on gene induction was also tested (Fig. 3). Higher than 0.005 mM of IPTG could induce hybrid gene expression of LacZ-fused insulin BA peptide directed by *tac* promoter, and the optimal concentration for gene induction was found to be 0.05 mM. Above this concentration, any more increased amounts of hybrid protein were not seen on SDS-PAGE gel. This result shows that 0.05 mM IPTG could almost saturate the $LacI^Q$ repressor protein by F' factor in *E. coli* JM103 cell. According to the result of Wood and Peretti [3], this repressor was nearly titrated by 1.0 mM IPTG, in case $lacI^q$ gene was introduced in the plasmid containing lacZ gene under *tac* promoter with the copy number of 84. This difference might be attributed to the copy numbers of $lacI^q$ gene in *E. coli* cells.

Purification of LacZ-fused Insulin BA Peptide

The hybrid protein for a single chain insulin precursor was accumulated in *E. coli* host cell as insoluble form of cytoplasmic inclusion bodies, as confirmed by microscopic examination. In order to isolate the hybrid protein from this inclusion bodies after sonication, it

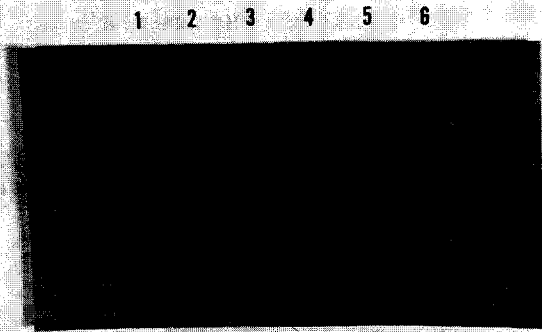

Fig. 2. The time course of gene induction of LacZ-fused insulin BA peptide by IPTG.
1.0 mM of IPTG was supplemented to 4 h-cultivated broth of *E. coli* JM103 harboring pKBA plasmid, and *E. coli* was further cultured for gene induction. As described in Materials and Methods section, total proteins of *E. coli* were analyzed by 14% SDS-PAGE. Lane 1; without supplementation of IPTG, lane 2; 2 h-cultivation after IPTG addition, lane 3; 4 h-cultivation, lane 4; 6 h-cultivation, lane 5; overnight cultivation, lane 6; low molecular weight marker. BA ▸ indicates the position of the hybrid protein.

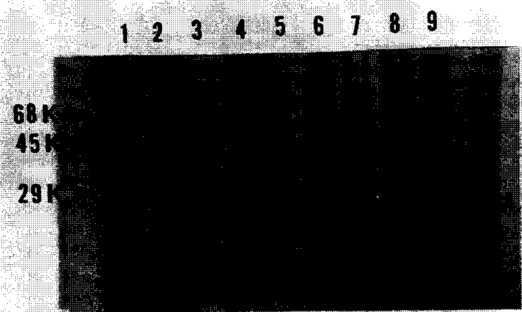

Fig. 4. The solubilization pattern of LacZ-fused insulin BA peptide in inclusion bodies by urea.
After sonication, the inclusion bodies in precipitates were extracted with different concentrations of urea, and the soluble fractions were analyzed by 14% SDS-PAGE. Lane 1; low molecular weight marker, lane 2; without dissolving in urea solution, lane 3; dissolved in 2 M urea solution, lane 4; 3 M urea solution, lane 5; 4 M urea solution, lane 6; 5 M urea solution, lane 7; 6 M urea solution, lane 8; 7 M urea solution, lane 9; 8 M urea solution. ◂ BA indicates the position of the hybrid protein.

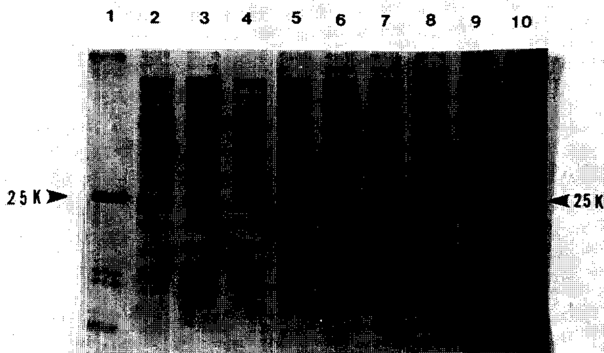

Fig. 3. Effect of the concentrations of IPTG on the gene expression of LacZ-fused insulin BA peptide.
Different concentrations of IPTG was supplemented to 4 h-cultured broth of *E. coli* JM103 harboring pKBA plasmid, and further cultured for 2 h. As described in Materials and Methods section, total proteins of *E. coli* were analyzed by 14% SDS-PAGE. Lane 1,10; low molecular weight marker, lane 2; without supplementation of IPTG, lane 3; with supplementation of 0.001 mM IPTG, lane 4; 0.005 mM IPTG, lane 5; 0.01 mM IPTG, lane 6; 0.05 mM IPTG, lane 7; 0.1 mM IPTG, lane 8; 0.5 mM IPTG, lane 9; 1.0 mM IPTG. ◂ BA indicates the position of the hybrid protein.

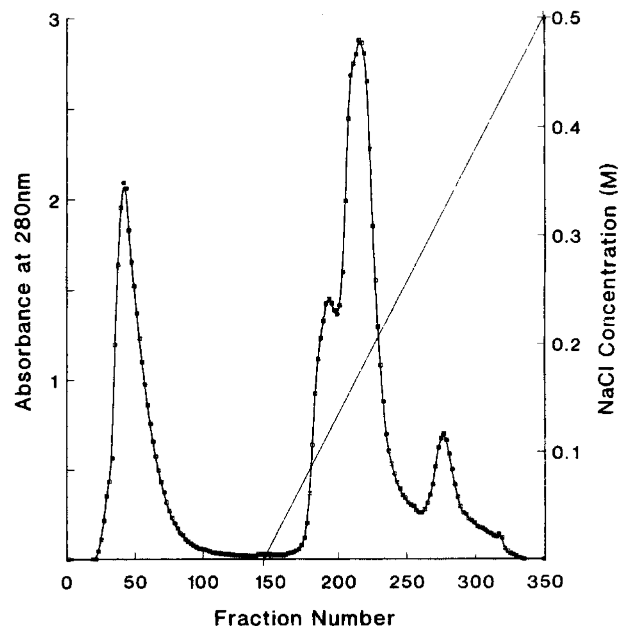

Fig. 5. The elution profile of urea extract of LacZ-fused insulin BA peptide on DEAE-Sephacel anion exchange column.
Column size; 5.0× 20 cm, flow rate; 0.6 ml/min, elution buffer; 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 50 mM sodium chloride and 8 M urea.

was tried to extract LacZ-fused insulin BA peptide by dissolving in urea solution. Urea and guanidine hydrochloride as a denaturant are commonly used to solubilize the insoluble proteins by breaking the intermolecular and intramolecular noncovalent bondings such as disulfide bridges [5-6]. The solubilization test of this protein by using urea up to 8 M revealed that above 6 M urea could dissolve out the hybrid protein from inclusion bodies (Fig. 4).

The extract of 8 M urea was further purified through DEAE-Sephacel and Sephadex G-200 column chromatography. In DEAE-Sephacel column eluted by linear gradient of sodium chloride, LacZ-fused insulin BA peptide was separated around 0.15 M of sodium chloride with 51% of the recovery yield (Fig. 5). After concentrating through Diaflo PM10 ultramembrane, the eluents was subjected to Sephadex G-200 column chromatography. Finally the hybrid protein was isolated in a pure form on SDS-PAGE gel with 36% of the recovery yield (Figs. 6 and 7).

In further experiment, the purified hybrid protein

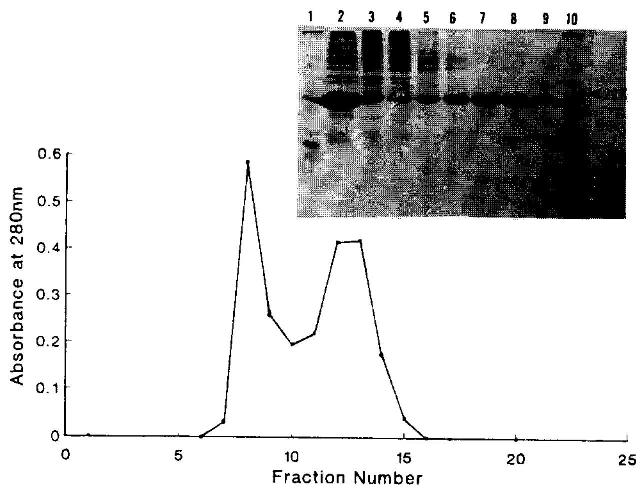

Fig. 6. The elution profile of DEAE-Sephacel eluate of LacZ-fused insulin BA peptide on Sephadex G-200 gel filtration column.
Column size; 1.0× 50 cm, flow rate; 0.1 ml/min, elution buffer; 20 mM Tris-HCl, pH 8.0 and 8 M urea. Each fractions was analyzed by 14% SDS-PAGE. Lane 1; low molecular weight marker, lane 2; DEAE-Sephacel eluate, lane 3; fraction 8, lane 4; fraction 9, lane 5; fraction 10, lane 6; fraction 11, lane 7; fraction 12, lane 8; fraction 13, lane 9; fraction 14, lane 10; molecular weight marker. BA ▶ indicates the position of the hybrid protein.

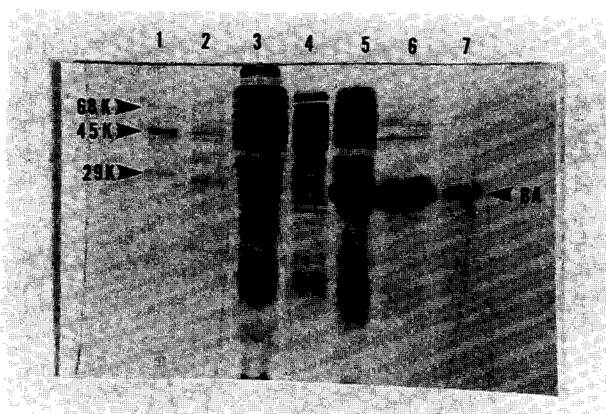

Fig. 7. The electrophoretic patterns of LacZ-fused insulin BA peptide at each purification steps on 14% SDS-PAGE. Lane 1; low molecular weight marker, lane 2; total cell protein of *E. coli* JM103, lane 3; supernatant after cell lysis, lane 4; supernatant after wahing with Triton X-100, lane 5; 8 M urea extracts of insoluble inclusion bodies, lane 6; fractions of DEAE-Sephacel column, lane 7; fractions of Sephadex G-200 column. ◀ BA indicates the position of the hybrid protein.

containing a single chain precursor of human insulin analogue, (B^{30}-homoserine) insulin, should be subjected to the chemical reconstitution to form the proper disulfide bridge, and to chemical cleavage of peptide by cyanogen bromide to yield a human insulin analogue.

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