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Production and Purification of Single Chain Human Insulin Precursors with Various Fusion Peptides

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Abstract For the production and purification of a single chain human insulin precursor, four types of fusion peptides β-galactosidase (LacZ), maltose binding protein (MBP), glutathione-S-transferase (GST), and $(His)_6$-tagged sequence (HTS) were investigated. Recombinant *E. coli* harboring hybrid genes was cultivated at 37°C for 1 h, and gene induction occurred when 0.2 mM of isopropyl-D-thiogalactoside (IPTG) was added to the culture broth, except for *E. coli* BL21 (DE3) pLysS harboring a pET-BA cultivation with 1.0 mM IPTG, followed by a longer than 4 h batch fermentation respectively. DEAE-Sphacel and Sephadex G-200 gel filtration chromatography, amylose affinity chromatography, glutathione-sepharose 4B affinity chromatography, and a nickel chelating affinity chromatography system as a kind of immobilized metal ion affinity chromatography (IMAC) were all employed for the purification of a single chain human insulin precursor. The recovery yields of the HTS-fused, GST-fused, MBP-fused, and LacZ-fused single chain human insulin precursors resulted in 47%, 20%, 20%, and 18% as the total protein amounts respectively. These results show that a higher recovery yield of the finally purified recombinant peptides was achieved when affinity column chromatography was employed and when the fused peptide had a smaller molecular weight. In addition the pET expression system gave the highest productivity of a fused insulin precursor due to a two-step regulation of the gene expression, and the HTS-fused system provided the highest recovery of a fused insulin precursor based on a simple and specific separation using the IMAC technique

Keywords : fermentation, fusion peptides, gene induction, purification, single chain human, insulin precursor

INTRODUCTION

Insulin is a polypeptide hormone secreted by the β-cells of the pancreas and consists of two polypeptide chains, A and B, which are linked by two inter-chain and one intra-chain disulfide bridges. The hormone is synthesized as a single-chain precursor, proinsulin, and produced by the proteolytic processing of proinsulin in the pancreas [1]. Diabetes mellitus occurs because of the secretion and synthetic disorder of insulin, and a lack of transferring glucose from the blood into cytoplasm. Insulin purified by porcine and bovine pancreas has been used clinically, however, it is limited in production and supply. The heterologous expression of the naturally scarce eukaryotic protein in *E. coli* has provided a more abundant source of this protein for clinical use [2]. It has also been reported that human insulin analog which can reduce the side-effects of the long-term dosages required for diabetes mellitus patients, is being commercially manufactured by a few pharmaceutical companies using a recombinant *E. coli* [3,4].

Human insulin is a polypeptide consisting of 51 amino acids arranged in two chains: A with 21 amino acids, and B consisting of 30 amino acids. The A and B chains are connected by two disulfide bonds. Human insulin has a molecular weight of 5,734 and an isoelectric point of 5.4 [5]. Recently, various types of expression vector systems in recombinant *E. coli* have been developed to express a human insulin precursor containing fusion peptides, such as β-galactosidase, glutathione-S-transferase, a maltose binding protein, and his-tagged sequence with a haxahistidine. In these systems, the gene expression of all the fused peptides is controlled by the *tac* or *lac* promoter. The *tac* promoter, a hybrid promoter of the *lac* promoter and *trp* promoter has been successfully used for the overexpression of heterologous cloned-genes in *E. coli*. Heterologous expression by gene induction in recombinant *E. coli* was already tried with the commonly used chemical inducer, isopropyl-β-D-thiogalactopyranoside (IPTG) [6-8]. In the present study, the heterologous expression of a human insulin precursor was attempted for the produc-

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tion of a single chain insulin analog. The gene for this analog was designed by directly linking the gene for chain B with that for chain A, and by introducing methionine codon at the B^{30} position. The design was made for the development of a much more economical bioprocess for this novel insulin through the simultaneous fermentation and purification of chain A and chain B in the form of a single chain. The human insulin analog obtained after cyanogens bromide cleavage has homoserine instead of threonine at the B^{30} position, yet both amino acids are stereoisomers. This work compared the production and purification processes for four types of single chain human insulin precursors with various fusion peptides in recombinant *E. coli*.

MATERIALS AND METHODS

Bacterial Strains, Media, and Culture Conditions

E. coli JM103[*supE thi-1 endA1 hsdR4 sbcB15 strA Δ (lac⁻proAB)* F'*(traD36 proAB⁺ lacI^q lacZ Δ M15*)] harboring a pKBA plasmid carrying the hybrid gene, was used as a host strain for expression [9]. The culture was maintained in an LB medium (yeast extract 0.5%, tryptone 1.0%, NaCl 1.0%) containing 50 μg/mL of ampicillin. *E. coli* JM109[*recA1 supE44 endA1 hsdR17 gyr96 relA1 thi Δ (lac⁻proAB)* F'*(traD36 proAB⁺ lacI^q lacZ Δ M15*)] harboring pMAL-BA and pGEX-BA plasmids carrying the hybrid gene, was used as a host strain for expression [10]. Fig.1 shows the vector plasmids used in this study [9-11]. The culture with *E. coli* JM 109 and the inserted pMAL-BA plasmid was maintained in an LB medium containing 100 μg/mL of ampicillin, where-as the inserted pGEX-BA plasmid was maintained in a YT-G medium (yeast extract 1.0%, tryptone 1.6%, NaCl 0.5%, glucose 2.0%) containing 100 μg/mL of ampicillin. *E. coli* BL21(DE3)pLysS[F⁻*ompT hsdSB* ($r_B^-m_B^-$) *gal dcm (DE3) pLysS*] harboring a pET-BA plasmid carrying the hybrid gene was used as a host strain for expression. *E. coli* BL21(DE3)pLysS was cultivated in a YT medium (yeast extract 1.0%, tryptone 1.6%, NaCl 0.5%) containing 15 μg/mL of kanamycin. For a flask culture, 1% seed culture of *E. coli* was inoculated into 100 mL of each medium in a 250-mL Erlenmeyer flask and cultivated at 37°C and 150 rpm.

Production of Single Chain Insulin Precursors

The cells were grown in a 5-L fermentor (KFC SY-Series 5 L, Kobiotech Co., Inchon, Korea) with a working volume of 3 L. After the cultivation of *E. coli* harboring plasmids in a flask, 2% cells were inoculated into 3 L of an LB medium containing 50 μg/mL of ampicillin. During fermentation, the pH was controlled at 7.0 ± 0.1 with 6 N NaOH or 5 N HCl. Air was supplied at a rate of 1.0-1.5 vvm and the dissolved oxygen was maintained above a 20% saturation level. The dissolved oxygen was measured using a polarographic oxygen probe (Mettler Toledo, Swiss). The agitation speed was started at 400 rpm and manually adjusted whenever needed. The temperature was controlled at 37°C ± 0.1. The medium components were properly mixed before use. Glucose was autoclaved separately from the other components, and ampicillin, kanamycin, and IPTG were sterilized by filtration through a 0.45 μm membrane filter before use [12]. The IPTG was added after 1 h of cultivation, and the induction was conducted for 4 h. Samples were taken hourly using an automatic sampler (Biomate 2000, Lokas Automation Corp., Taejon, Korea). The optical density of the culture broth sampled was measured at 600 nm. A 20 μL sample was boiled with the same volume of a solvent buffer for 5 min and analyzed on a linear gradient of SDS-PAGE from 10 to 20% to detect the single chain insulin precursors expressed in the *E. coli*. The *E. coli* cell pastes harvested after centrifugation were subjected to sonication for 30 min using an ultrasonic processor (550 Sonic Dismembrator, Fisher Scientific, USA). The single chain insulin precursors with fusion peptides were extracted, centrifuged, and then the precipitates were solubilized in 8 M urea for over 2 h at 4°C [13]. After centrifuging of the solution again, the resulting supernatants were used for further purification.

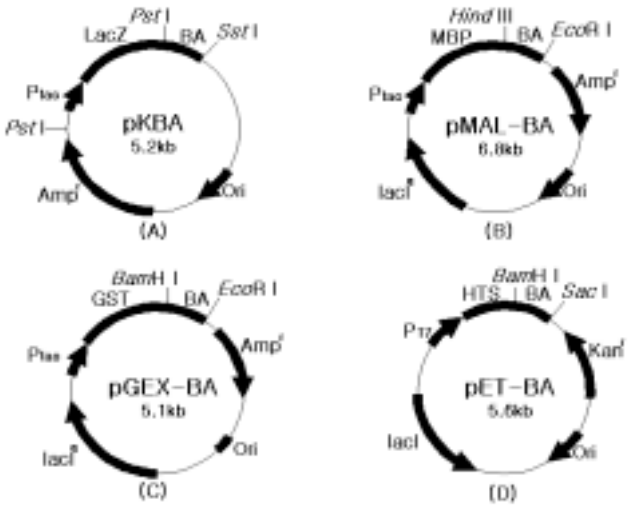

Fig. 1. Recombinant plasmid construction: (A) LacZ, β-galactosidase gene; Ptac, *tac* promoter; (B) MBP, maltose binding protein gene; $lacI^q$, $lacI^q$ repressor protein gene; (C) GST, gluththione-S-transferase gene; (D) P_{T7}, T7 promoter gene; HTS, $(His)_6$-tagged sequence gene; lacI, lacI repressor gene. And BA, single chain human insulin precursor gene; Ori, origin of replication; Amp^r, ampicillin resistance gene; Kan^r, kanamycin resistance gene; *Pst I*, *EcoR I*, *Hind III*, *BamH I*, *Sac I*, restriction enzyme sites, respectively.

Purification of Single Chain Insulin Precursors with Various Fusion Peptides

Following the procedure of Marston's purification method modified [14] for a β-galactosidase (LacZ)-fused fusion protein, the extracted fusion protein in urea was

loaded onto a DEAE-Sphacel ion exchange column (5 × 15 cm) at room temperature. The column was pre-equilibrated with a running buffer (20 mM Tris-HCl, 1 mM EDTA, 50 mM NaCl, 8 M urea, pH 8.0), then the loaded sample was washed with the same buffer. The eluates were fractionated with a linear gradient of sodium chloride from 0 to 0.5 M at 0.6 mL/min. After electrophoresis on a 14% SDS-PAGE, the fractions containing the hybrid protein were collected and concentrated through a Diaflo membrane PM10 (Amicon Co., MA, USA). In the next step, the sample was loaded on Sephadex G-200 column pre-equilibrated with a buffer (20 mM Tris-HCl, 8 M urea, pH 8.0), and eluted with the same buffer at a flow rate 0.1 mL/min. The fractions were also analyzed on a 14% SDS-PAGE.

For the maltose binding protein (MBP)-fused fusion protein, the extracted fusion protein in urea was loaded onto an amylose resin column (2.5 × 10 cm) at room temperature. The column was pre-equilibrated with a running buffer (10 mM phosphate buffer, 0.5 M NaCl, 1 mM NaN_3, 10 mM 2-mercaptoethanol, 1 mM EGTA , pH 7.2), and the loaded sample was then washed with the same buffer. The eluates were fractionated with a running buffer containing 10 mM maltose at a flow rate of 0.5 mL/min. After analyzing using a 12% SDS-PAGE, the fractions containing the fusion protein were collected and concentrated through a Diaflo membrane PM10.

For the glutathione-S-transferase (GST)-fused protein, the extracted fusion protein in urea was loaded onto a glutathione-sepharose 4B column (2.5 × 10 cm) at room temperature. The column was pre-equilibrated with a 1xPBS buffer (0.14 M NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.2), and the loaded sample was then washed with the same buffer. The eluates were fractionated with a glutathione elution buffer (10 mM glutathione in 50 mM Tris-HCl pH 8.0) at a flow rate of 0.5 mL/min. After electrophoresis on a 13% SDS-PAGE, the fractions containing the fusion protein were collected and concentrated through a Diaflo membrane PM10.

For the $(His)_6$-tagged sequence (HTS)-fused fusion protein, the extracted fusion protein in 8 M urea was loaded onto Ni^{2+} affinity sepharose chelating column (2.5 × 10 cm) at room temperature. The column was pre-equilibrated with a binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9), and the loaded sample was then washed with a binding buffer and washing buffer (20 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl pH 7.9) The eluates were fractionated with an elute buffer (1 M imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9) at a flow rate of 1.5 mL/min. After electrophoresis on a linear gradient of SDS-PAGE from 10 to 20%, the fractions containing the fusion protein were collected and concentrated through a Diaflo membrane PM10.

Analytical Procedures

The amounts of protein were determined by measuring the absorbance at 280 nm by Lowry's method [15] for those samples without urea, and by Bradford's method [16]. Bovine serum albumin (BSA) was used as the standard. The gels which used in SDS-PAGE were stained by 0.1% Coomassie Brilliant Blue R-250. The amino acid compositions of the purified fusion proteins were determined using a Biochrom 20 amino acid analyzer (Pharmacia Biotech, Uppsala, Sweden).

RESULTS AND DISCUSSION

Gene Expression of Single Chain Insulin Precursors with Varions Fusion Peptides

In order to investigate the optimum expression conditions of the single chain insulin precursor gene, the effect of the IPTG concentration on gene induction was examined. During the first 4 h after induction, the cell growth was similar in all the fermentations even though different fusion peptides were used (data not shown). However, the level of expression increased as the IPTG concentration increased. The hybrid protein, LacZ-fused single chain insulin precursor (23 kDa) directed by the *tac* promoter, was expressed when the IPTG concentration was higher than 0.05 mM, and the optimal concentration was found to be 0.1 mM (data not shown). Similar results were obtained with the MBP-fused single chain insulin precursor (46 kDa) and GST-fused single chain insulin precursor (29 kDa) (data not shown). An IPTG concentration of 0.1 mM seemed to be enough to saturate the $LacI^q$ repressor protein by the F' factor in both *E. coli* JM103 and JM109 cells. However, for the HTS-fusecd single chain insulin precursor (9 kDa), the optimal IPTG concentration was up to 0.5 mM due to an increased saturation level with the $LacI^q$ repressor. This result implies that the level of the IPTG concentration could be increased for a two-step regulation of gene expression in *E. coli* BL21(DE3)pLysS host cells; i.e. the induction of the *tac* promoter for the production of T7 RNA polymerase in DE3 lysogen and the induction of the T7 promoter for the production of HTS peptides in a pET plasmid.

Purification of Single Chain Insulin Precursors with Various Fusion Peptides

After induction in a batch culture, all hybrid proteins with single chain insulin precursors were accumulated within the cells in the insoluble form of cytoplasmic inclusion bodies. In order to isolate the hybrid proteins from these inclusion bodies, they were dissolved in a 8 M urea solution. Urea and guanidine hydrochloride were successful in solubilizing all the insoluble proteins by breaking the intermolecular and intramolecular non-covalent bondings, such as disulfide bonds. The solubilized proteins were then further purified through different methods with various peptides.

In the case of the LacZ-fusion proteins, DEAE-Sephacel and Sephadex G-200 columns were employed and the elution profiles are shown in Fig. 2, 3 respectively. As

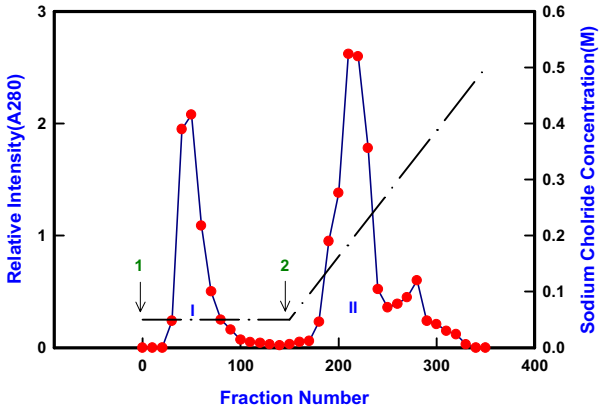

Fig. 2. Elution profile of urea extract of LacZ-fused single chain insulin precursor on DEAE-Sephacel anion exchange column chromatography. The column size was 5.0 × 20 cm, flow rate 0.6 mL/min, and the elution buffer consisted of 20 mM Tris-HCl pH 8.0, 1 mM EDTA, 50 mM sodium chloride, and 8 M urea. Dash-dot line means sodium chloride concentration. The arrows denote a change in the sodium chloride concentration in the buffer when eluted: 1, 0.0 M NaCl; 2, 0.0 to 0.5 M NaCl linear gredient.

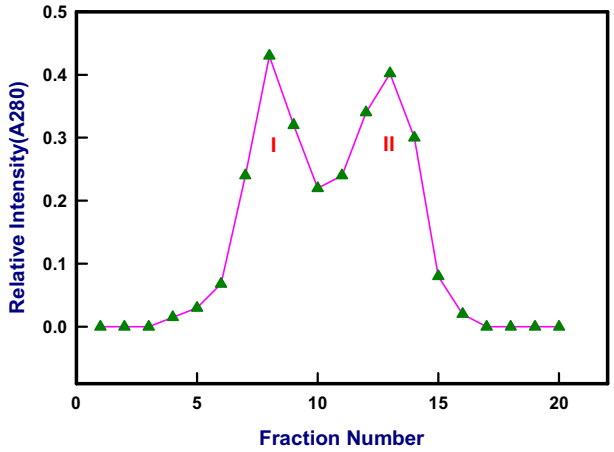

Fig. 3. Elution profile of DEAE-Sephacel eluate on Sephadex G-200 gel filtration column chromatography. The column size was 1.0 × 5.0 cm, flow rate 0.1 mL/min and elution buffer consisted of 20 mM Tris-HCl pH 8.0 and 8 M urea.

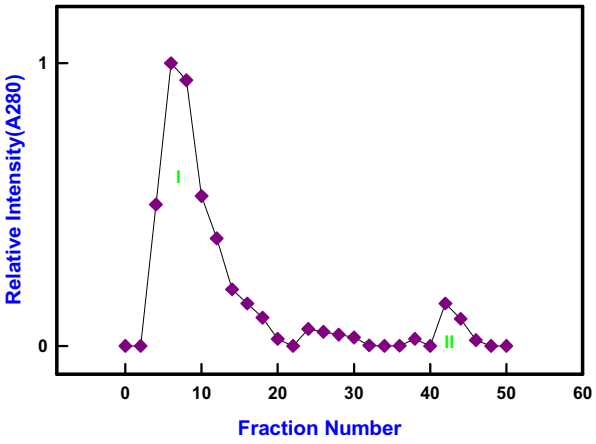

Fig. 4. The elution profile of MBP-fused single chain insulin precursor on amylose affinity column chromatography. The column size was 2.5 × 10 cm, and flow rate 0.5 mL/min. The column was pre-equilibrated with a column buffer (0.5 M NaCl, 1 mM NaN_3, 10 mM mercaptoethanol, 1 mM EGTA, 10 mM phosphate buffer pH 7.2), and eluted with 10 mM maltose in the same buffer and 8 M urea.

shown in Fig 2, the LacZ-fused single chain insulin precursor was eluted with a linear gradient of sodium chloride from 0.0 to 0.5 M. The LacZ-fused single chain insulin precursor was identified as peak II, and exhibited a recovery yield of 51% based on the total protein quantity. The further purification of peak II by a sepahdex G-200 gel filtration column yielded a very pure form of the LacZ-fused single chain insulin precursor on a 14% SDS-PAGE gel. The recovery yield was 36% based on the fusion proteins when using a DEAE-Sephacel anion exchange column. Therefore, the final recovery yield of the LacZ-fused single chain insulin precursor was estimated as 20% of the total protein.

In the case of the MBP-fused single chain insulin precursor, amylose affinity column chromatography was used. The MBP-fused single chain insulin precursor was detected at peak I and II, and separated by a 10 mM maltose solution. After concentrating the fractions at peak I through a Diaflo PM10 ultra membrane, they were lyophilized, and the MBP-fused single chain insulin precursor isolated in a pure form was 18% of the total protein produced by the batch fermentation (Fig. 4).

For the purification of the GST-fused single chain insulin precursor, glutathione sepharose 4B column chromatography was eluted with 10 mM glutathione with 50 mM Tris-HCl (pH 8.0) at a 0.5 mL/min flow rate. The same buffer containing 10 mM glutathione as an eluent was passed through the column in order to displace the bound GST-fused single chain insulin peptide. The isolated GST-fused precursor, as shown in Fig. 5, was concentrated by ultrafiltration and lyophilized to give a pure form with a 20% recovery yield from the total protein.

Finally, immobilized nickel affinity column chromatography was employed to isolate the HTS-fused single chain insulin precursor. As a kind of immobilized metal ion affinity chromatography (IMAC) technique, IMAC is widely used to purify fusion proteins, based on the strong interaction of metal ions with artificial polyhistidine tags either at the N-terminus or C-terminus of the protein of interest [17-20]. After a series pass using this affinity column with a 1X binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl pH 7.9, 8 M urea), the column was washed by a 1× washing buffer (20 mM imidazole, 0.5 M NaCl, 20 mM Tris-Cl pH 7.9, 8 M urea) to eliminate any non-bound proteins at a flow rate of 1.5 mL/min, then a 1X elution buffer (1 M imidazole, 0.5 M NaCl, 20 mM Tris-HCl pH 7.9, 8 M urea) was flowed through the column for the elution of

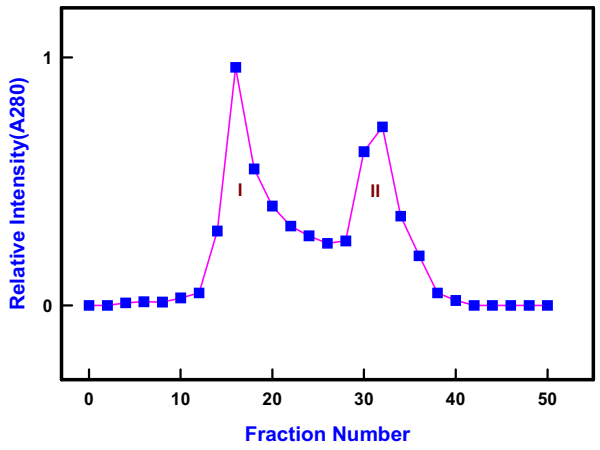

Fig. 5. Elution profile of GST-fused single chain insulin precursor on glutathione sepharose 4B column chromatography. The column size was 2.5 × 10 cm and flow rate 0.5 mL/min. The column was pre-equilibrated with a 1xPBS buffer (0.14 M NaCl, 2,7 mM KCl, 10.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4 pH 7.3), and eluted with 10 mM glutathione in 50 mM Tris-HCl pH 8.0 and 8 M urea.

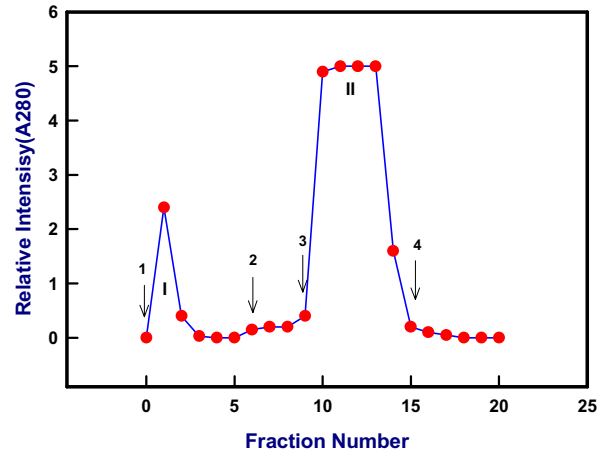

Fig. 6. Elution profile of HTS-fused single chain insulin precursor on Ni^{2+} affinity column chromatography. The column size was 2.5 × 10 cm and flow rate 1.5 mL/min. The arrows denote a change in the buffer as follows: 1, 1X binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl pH 7.9, 8M urea); 2, 1X washing buffer (20 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl pH 7.9, 8M urea); 3, 1X elution buffer (1 M imidazole, 0.5 M NaCl, 20 mM Tris-HCl pH 7.9, 8 M urea); 4, 1X strip buffer (2 M NaCl, 400 mM EDTA, 80 mM Tris-HCl pH 7.9) for resin conditioning.

the metal-bound HTS-fused peptide. The obtained chromatogram is shown Fig. 6. A pure form of the HTS-fused single chain insulin precursor was recovered with 47% of recovery yield from the total protein, after ultrafiltration and lyophilization. The confirmation of the finally purified HTS-fused precursor was confirmed using an amino acid analyzer. As seen in Table 1, the purified HTS-fused single chain precursor showed almost the same amino acid composition as the predicted value, even through there was a slight difference in the content of the Met, Arg, and Lys residues, and cystein amino acids were not detected. Such a difference may have come from some impurities, although only a single band was observed on a 10-20% SDS-PAGE gel (Fig. 7).

Table 1. Amino acid composition of $(His)_6$-tagged human insulin precursor

Amino[a] acid	Value		Amino acid	Value	
	Calculated	Measured		Calculated	Measured
Gly	11	1.50	Ala	4	4.49
Val	5	4.87	Leu	7	6.94
Ile	2	1.93	Pro	3	2.85
Met	6	5.17	Phe	3	2.94
Trp	0	0	Ser	10	10.40
Thr	4	4.22	Cys	6	ND[b]
Tyr	4	4.28	Asp+Asn	3	3.67
Glu+Gln	10	10.85	Lys	1	2.04
Arg	3	4.00	His	9	9.74

[a] Amino acid is written using three letter symbol.
[b] Not detected.

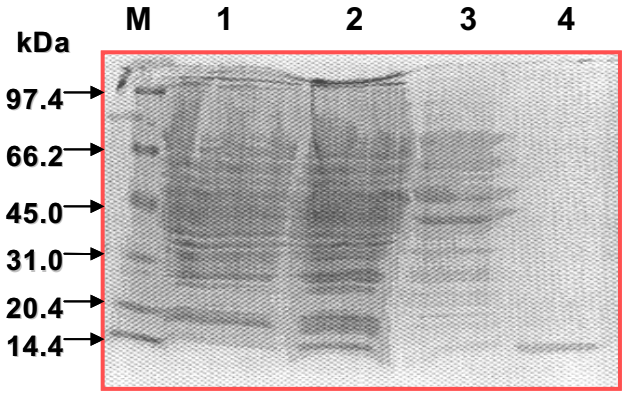

Fig. 7. Identification of single chain insulin precursor from eluates of immobilized Ni^{2+} affinity column chromatography. Lane 1, not induced IPTG; lane 2, 1X binding buffer eluates; lane 3, 1X washing buffer eluates; lane 4, 1X elution buffer eluates. lane M corresponds to the molecular weight marker (14.4 to 97 kDa). The right hand side bold arrow indicates the HTS-fused single chain insulin precursor expressed (9 kDa).

For the development of a more efficient production process for a single chain insulin precursor, fermentative and purification studies were conducted and compared using recombinant host cells which carried four different types of plasmids. As summarized in Table 2, the result obtained showed that a higher recovery yield of the finally purified recombinant peptides was achieved when affinity column chromatography was employed and when the fused peptide had a smaller molecular weight. The pET expression system gave the highest productivity of a fused insulin precursor due to a two-step regulation of the gene expression, and the HTS-

Table 2. Purification process using various fusion BA peptides

Vector	pKBA	pMAL-BA	pGEX-BA	pET-BA
Fusion partner	LacZ	MBP	GST	HTS
Fusion protein molecular weight	23 kDa	46 kDa	29 kDa	9 kDa
Expressed protein form	Inclusion Body	Inclusion Body	Inclusion Body	Inclusion Body
Coupling gel	DEAE-Sephacel	Amylose	Glutathione-Sepharose 4B	Chelating Sepharose
Displacer	NaCl	Maltose	Glutathione	Imidazole
Medium	LB	LB	YT-G	YT
Recovery yield (%)*	18	20	20	47

Abbreviations : LacZ, β-galactosidase; MBP, maltose binding protein; GST, glutathione-S-transferase; HTS, $(His)_6$-tagged sequence.

* Recovery Yield(%) means recovered fusion protein (mg/L) / total protein (mg/L), and LacZ-fused protein, MBP-fused protein, GST-fused protein, and HTS-fused protein of the yield resulted in 32.2/180, 34.5/174, 19.8/100, 70.4/150, respectively.

fused system provided the highest recovery of a fused insulin precursor based on a simple and specific separation using the IMAC technique. In addition, when considering the molecular size of the fusion part with the single chain insulin precursor to be cleaved out, it is worth mentioning that the pET vector with the HTS gene was the best among those tested.

This study clearly demonstrated how the selection of the expression system and purification system is important in the production of recombinant peptides and proteins in *E. coli* host cells.

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REFERENCES

[1] Chang, S. G., D. Y. Kim, K. D. Choi, J. M. Shin, and H. C. Shin (1998) Human insulin production from a novel mini-proinsulin which has high receptor-binding activity. *Biochem. J.* 329: 631-635.

[2] Bae, C. S., M. S. Hong, S. G. Chang, D. Y. Kim, and H. C. Shin (1997) Optimization of fusion proinsulin production by high cell-density fermentation of recombinant *E. coli*. *Biotechnol. Bioprocess Eng*. 2: 27-32.

[3] Michael, R. L. and K. L. Kohlmann (1992) Recombinant human Insulin. *Biotechnol. Prog.* 8: 469-478.

[4] Daniel, C. H., R. R. Browsher, R. L. Brunelle, and J. R. Woodworth (1994) [Lys(B28), Pro(B29)]-Human insulin. A rapidly absorbed analogue of human insulin. *J. Diabetes* 43: 396-402.

[5] Demetri, P., E. Sapidou, and J. Caladranis (1995) Computer-aided process analysis and economic evaluation for biosynthetic human insulin production-A case study. *Biotechnol. Bioeng*. 48: 529-541.

[6] Lee, S. Y., J. H. Ko, M. H. Choi, and D. H. Nam (1996) Fermentation and purification of LacZ-fused single chain insulin precursor for (B^{30}-Homoserine) human insulin. *Biotechnol. Bioprocess Eng*. 1: 9-12.

[7] Wood, T. K. and S. W. Peretti (1991) Effect of chemically-induced, cloned gene expression in *E. coli*. *Biotechnol. Bioeng*. 38: 397-412.

[8] Miao, F. and D. S. Kompala (1992) Over expression of cloned genes using recombinant *E. coli* regulated by a T7 promoter. 1. Batch cultures and kinetic modeling. *Biotechnol. Bioeng.* 40: 787-796.

[9] Nam, D. H., J. H. Ko, and S. Y. Lee (1993) Design and cloning of the a novel insulin analogue, (B^{30}-homoserine) human insulin. *Arch. Pharm. Res*. 16: 271-275.

[10] Kim, S. W. (1995) *Expression and purification of B^{30}-homoserine human insulin analogue in Escherichia coli*. M. S. Thesis, Yeungnam Univ., Daegu, Korea.

[11] Lee, J. S. (1998) *Fermentation of recombinant insulin analogue in Escherichia coli and its purification by affinity chromatography*. M. S. Thesis, Yeungnam Univ., Daegu, Korea.

[12] Kim, J. Y. and D. D. Y. Ryu (1999) Physiological and environmental effects on metabolic flux change caused by heterologous gene expression in *Escherichia coli*. *Biotechnol. Bioprocess Eng*. 4: 170-175.

[13] Marston, F. A. O. (1986) The purification of eucaryotic polypeptides synthesized in *Escherichia coli*. *Biochem. J*. 240: 1-12.

[14] Marston, F. A. O. (1987) *DNA Cloning, A Practical Approach*. vol. 3, pp. 59-88. In: D. M. Glover (ed.). IRL Press, Oxford, UK.

[15] Daniel, M. B. and S. J Edelstein. (1991) *Protein methods*, pp. 50-59, Wiley-Liss,

[16] Bradford, M. M. (1976) A rapid and sensitive methods for the quantization of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72: 248-254.

[17] Grigoriy, C., J. Hopp, and P. Nelson (1999) Immobilized metal ion affinity chromatography on Co^{2+}-carboxymethylaspartate-agarose superflow, as demonstrate by one-step purification of lactate dehydrogenase from chicken breast muscle. *Biotechnol. Appl. Biochem.* 29: 19-24.

[18] Müler, K. M., K. M. Arndt, K. Bauer, and A. Plükthun (1998) Tandem immobilized metal-ion affinity chromatography/immunoaffinity purification of His-tagged proteins-evaluation of two anti-His-tag monoclonal antibodies. *Anal. Biochem.* 259: 54-61.

[19] Vunnum, S., S. Gallant, and S. Cramer (1996) Immobilized metal affinity chromatography: displacer characteristics of traditional mobile phase modifiers. *Biotechnol. Prog.* 12: 84-91.

[20] Cayuela, C., K. Kai, S. Iijima, and T. Kobayashi (1992) Insecticide production by recombinant *B. subtilus* 1A96 in fed-batch culture with control of glucose concentration. *J. Ferment. Bioeng.* 75: 383-386.

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