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Recombinant Glargine Insulin Production Process Using *Escherichia coli*

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Glargine insulin is a long-acting insulin analog that helps blood glucose maintenance in patients with diabetes. We constructed the pPT-GI vector to express prepeptide glargine insulin when transformed into *Escherichia coli* JM109. The transformed *E. coli* cells were cultured by fed-batch fermentation. The final dry cell mass was 18 g/l. The prepeptide glargine insulin was 38.52% of the total protein. It was expressed as an inclusion body and then refolded to recover the biological activity. To convert the prepeptide into glargine insulin, citraconylation and trypsin cleavage were performed. Using citraconylation, the yield of enzymatic conversion for glargine insulin increased by 3.2-fold compared with that without citraconylation. After the enzyme reaction, active glargine insulin was purified by two types of chromatography (ion-exchange chromatography and reverse-phase chromatography). We obtained recombinant human glargine insulin at 98.11% purity and verified that it is equal to the standard of human glargine insulin, based on High-performance liquid chromatography analysis and Matrix-assisted laser desorption/ionization Time-of-Flight Mass Spectrometry. We thus established a production process for high-purity recombinant human glargine insulin and a method to block Arg (B31)-insulin formation. This established process for recombinant human glargine insulin may be a model process for the production of other human insulin analogs.

Keywords: Diabetes, glargine, fed-batch fermentation, citraconylation, Arg (B31)-insulin

Introduction

Diabetes mellitus (DM) is a group of metabolic diseases of hyperglycemia resulting from defects in insulin secretion and/or insulin action [26]. DM is classified into two types: type 1 DM (T1DM) and type 2 DM (T2DM) [1, 15]. T1DM is a chronic disease that results from the autoimmune destruction of insulin-producing beta cells in the pancreas; several autoantibodies such as glutamic acid decarboxylase antibody, islet cell antibody, and IA-2 antibody are considered specific to T1DM [2, 7, 9, 12, 24]. T2DM is a common endocrine disorder that accompanies pluralistic mechanisms. These mechanisms involve resistance to insulin action, increased production of hepatic glucose, and a defect in insulin secretion, all of which contribute to the growth of obvious hyperglycemia [13]. All patients with T1DM require insulin therapy, and a large percentage of T2DM patients will ultimately require insulin therapy as well [14].

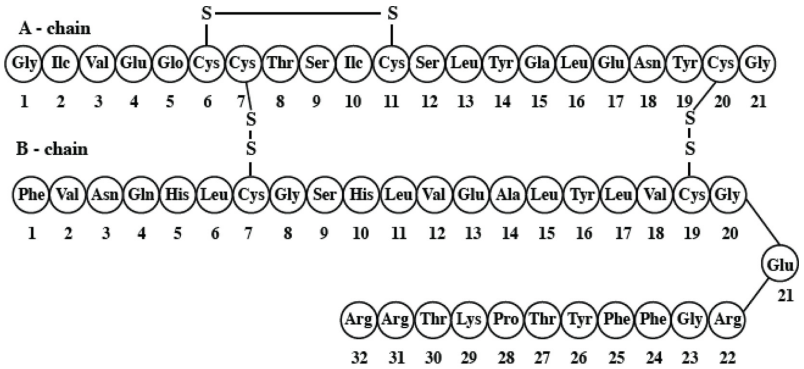
In mammals, insulin is produced in beta cells located in the Langerhans islets of the pancreas [19]. Insulin regulates the metabolism of carbohydrates and fats to maintain

blood glucose levels by elevating the uptake of glucose from the blood to muscles and fatty tissue [42]. Human insulin is composed of two polypeptide chains, the A- and B-chains, which are linked together by disulfide bonds; it has a molecular mass of 5,808 Daltons, consisting of 51 amino acids. Insulin was originally extracted from bovine and porcine pancreata for use in patients with diabetes. Nowadays, biosynthetic human insulin is manufactured by recombinant DNA technology, which has led to the availability of insulin analogs such as glargine insulin [34].

All insulin analogs are produced in *Escherichia coli* or yeast and modified from the human insulin gene using genetic engineering techniques. In commercial production, insulin lispro, glulisine, and glargine are expressed in *E. coli*, and insulin aspart and detemir are expressed in *Saccharomyces cerevisiae* [3]. Amino acid substitution in insulin analogs such as glargine insulin and insulin detemir allows a longer duration and a slower onset of action [33, 46]. Insulin glargine has two additional arginine residues (two positive charges) and one amino acid modification from Asn to Gly, as compared with insulin. These modifications result in a change of the isoelectric point from a pH of 5.4 (human insulin) to 6.7 (human insulin glargine). They make the protein less soluble in the physiological pH of subcutaneous tissue and more soluble in slightly acidic condition. Insulin glargine is a clear solution at pH 4.0, but it forms a precipitate at physiological pH 7.0 after subcutaneous injection. The physical structure of the insulin hexamer with zinc ion can influence the mechanism of precipitation and the kinetics of its absorption and dissolution. As a result, the subcutaneous injection of insulin glargine is seen as a delayed and prolonged absorption from the injection site [17]. Glargine insulin, a long-acting insulin analog (Lantus; Aventis Pharma), was approved for use in patients with T1DM and T2DM by the European Agency for the Evaluation of Medicinal Products in June 2000, and by the US Food and Drug Administration in April 2000 [6]. It is similar to human insulin in structure, except for the addition of two arginine (RR) residues at the B-chain C-terminus and the replacement of A21 amino acid from asparagine to glycine in the A-chain (Fig. 1). It has a 24 h duration of action with no peak and shows good glycemic control. It reduces the incidence of nocturnal hypoglycemia [35]. The commercial manufacture of glargine insulin employs two recombinant microbial hosts, namely *E. coli* and *Pichia pastoris*. Trypsin cleaves the C-terminus of both lysine and arginine residues [33]. Since the glargine B-chain has several internal arginine and lysine residues, a number of impurities are generated following trypsin treatment [45].

E. coli is the most widely used host for producing recombinant proteins and for metabolic engineering, because it is easy to handle and incurs less incubation costs. In addition, *E. coli* genes can be easily manipulated. High cell density fermentation of recombinant *E. coli* is an effective process for enhancing the final concentration and production of the protein product. In the high cell density fermentation, cells are placed in adverse conditions such as nutrient depletion, exhaustion of dissolved oxygen, elevated osmotic pressure, and by-product formation. To resolve these problems, the fed-batch fermentation has applied feeding strategies, including constant feeding, exponential feeding, and indirect feeding such as pH-stat and DO-stat [22, 25, 39, 41].

Citraconylation is the additive of one or more citraconyl groups into a material by acylation reaction [40]. Citraconic anhydride is usually used to citraconylate primary amino



Formula: $C_{267}H_{404}N_{72}O_{78}S_6$
Mol. mass: 6063 g/mol

Fig. 1. Structure of glargine insulin.

groups and free lysine residues of the target protein. The citraconylated lysine residues are not recognized by trypsin, and the adjoining peptide bonds are able to withstand trypsin hydrolysis [5, 20, 31].

In this study, we focused on developing a fermentation and purification process for high-purity recombinant glargine insulin in *E. coli*. Accordingly, we performed the fed-batch fermentation for high-level expression of recombinant glargine insulin, and we identified the major impurity, Arg (B31)-insulin, and used citraconylation to minimize the amount of impurities in order to obtain high-purity glargine insulin.

Materials and Methods

Construction of pPT-GI Vector Expressing Glargine

The pPT-GI vector was constructed to express the prepeptide fusion glargine insulin in *E. coli*. The coding region for glargine insulin was amplified with human insulin c-DNA in our previous report [43] by using the polymerase chain reaction (PCR). Primer sequences are as follows: Forward primer: 5′-TAT ACA TAT GAC CAT GAT TAC GAA TTC C-3′; Reverse primer: 5′-GGG TAC TCG AGC ACT CAG CCG CAG-3′. PCR products were cleaved with restriction enzymes NdeI (Elpis Biotech, Korea) and XhoI (Elpis Biotech, Korea) and then electrophoresed on 1% agarose gel to isolate a gene segment of about 300 bp. (The respective restriction site sequences for NdeI and XhoI are underlined.) The isolated cDNAs were subcloned between the NdeI and XhoI sites of the pPT vector. The constructed pPT-GI vectors were transformed into *E. coli* JM109 (RBC Bioscience, Taiwan).

Expression of Prepeptide Fusion Glargine with Fermentation

The transformed *E. coli* cells containing pPT-GI vector were cultured at 37°C for 19 h in 10 ml of Luria-Bertani (LB) medium supplemented with 50 μg/ml ampicillin (Sigma-Aldrich, USA), as a seed for the main culture for glargine insulin. The cultured cells were inoculated into 1 L of fermentation medium (tryptone 10 g/l, yeast extract 5 g/l, NaCl 5 g/l, K_2HPO_4 5 g/l) in an aerated 2.5 L fermentor (Sartorius-Stedim Biotech, USA). After 12 h culture at 25°C, the temperature was raised to 30°C. The fermentation temperature was then raised from 30°C by about 2.3°C every 2 h when the absorbance was at least 10 and finally *E. coli* cells were cultured at 37°C. During the fermentation, cells were fed glucose and NH_4OH as nutrients and to maintain the pH at 6.9, and pO_2 was kept at 30%.

Purification and Washing of Inclusion Bodies Containing Prepeptide Fusion Glargine

The cells were harvested by centrifugation at 20,000 ×*g* for 30 min. The cells were resuspended in 20 ml of resuspension buffer (10% sucrose, 0.1 M Tris, 50 mM ethylenediaminetetraacetic acid (EDTA), 0.2 M sodium chloride, pH 7.9) per 1 g of cell weight. Resuspended cells were disrupted by sonication (Sonics & Materials Inc., USA) and centrifuged at 20,000 ×*g*, 4°C for 30 min to collect inclusion bodies. The inclusion bodies were collected, and soluble proteins and some of the cell debris were removed by centrifugation [27]. After centrifugation, the supernatant was discarded and the precipitate was collected. The collected inclusion bodies containing prepeptide fusion glargine insulin were washed with 30 ml of inclusion body washing solution (20 mM Tris, 1 mM EDTA, 0.02% lysozyme, 1% Triton X-100, 0.5 M urea) per 1 g of precipitate, and the washed inclusion bodies were collected by centrifugation at 20,000 ×*g*, 4°C for 30 min [17]. Lastly, the inclusion bodies were washed with 30 ml of deionized water per 1 g of precipitate and collected by centrifugation at 20,000 ×*g*, 4°C for 30 min.

Direct Refolding of Prepeptide Fusion Glargine

The washed inclusion body was solubilized with solubilization buffer solution (8 M urea, 10 mM glycine, pH 10.6). The inclusion body solution was diluted with refolding buffer solution (0.6 M urea, 10 mM glycine, pH 10.6) to a final protein concentration of 0.5 mg/ml. The refolding buffer containing inclusion body was incubated in 0.1 mM β-mercaptoethanol at 4°C for 24, 48, 72, and 96 h. After the refolding reaction, the refolding solution was adjusted to pH 4.5 with 5 N HCl and centrifuged at 20,000 ×*g*, 4°C for 30 min. The supernatant containing the refolded peptide fusion glargine was collected and the precipitate was removed.

Conversion into Active Glargine Insulin by Protease Cleavage with Citraconylation

Borate (20 mM) was added to the refolded peptide fusion glargine solution. The pH of the solution was adjusted to 8.5, and citraconic anhydride (Thermo Fisher, USA) was added (4.26 g per 1 g protein). The solution was incubated with stirring at 25°C. After 2 h, 9 units of trypsin (Sigma-Aldrich, USA) was added per 1 mg protein and the solution was incubated at 25°C for 5 h. The pH of the enzyme-reacted solution was then adjusted to 8.5 with 10 N NaOH. For deacylation, the acidity of the glargine insulin solution was changed to pH 2.5 by adding glacial acetic acid and then the solution was incubated at 25°C for 5 h.

Zinc and pH Precipitation

Zinc chloride solution (18%) was added to the converted glargine insulin to a final concentration of 0.1%. The glargine insulin solution was adjusted to pH 6.1 and incubated at 4°C for 16 h and then centrifuged at 20,000 ×*g* for 30 min. The supernatant was removed and the precipitate containing glargine insulin was collected. The pellet containing human glargine insulin was resolved with sample buffer (7 M urea, 0.25 M acetic acid, pH 2.5) for ion-exchange chromatography.

Cation-Exchange Chromatography

We performed cation exchange chromatography with the soluble glargine insulin in the sample buffer using a cation column packed with Sp Sepharose Fast Flow resin (GE Healthcare Bio-Sciences,

USA) into XK 16 columns (GE Healthcare Bio-Sciences, USA) on an AKTA avant (GE Healthcare Bio-Sciences). The protein solution was loaded at a flow rate of 1 ml/min on a 50 ml SP Sepharose column equilibrated at a flow rate of 5 ml/min with 10 CV (column volume) equilibration buffer (7 M urea and 0.25 M acetic acid, pH 2.5). The column was washed at a flow rate of 5 ml/min with 10 CV elution buffer A (7 M urea and 0.25 M acetic acid, pH 2.5), and then bound proteins were eluted at a flow rate of 5 ml/min with 6 CV elution buffers A and B (7 M urea, 0.25 M acetic acid, and 1 M sodium chloride, pH 2.5) by application of a linear gradient (0–1 M NaCl). The eluent was monitored at 280 nm and each peak was collected in fraction tubes. The collected fractions were analyzed by HPLC with a Protein & Peptide C4 analytical column.

Preparative High-Performance Liquid Chromatography (Prep-HPLC)

The fractions collected by cation-exchange chromatography that contained more than 60% purity of glargine insulin were pooled based on the purity of HPLC analysis. Prep-HPLC was carried out on an Agilent 1200 system (USA) equipped with a C8 prep HT column (21.2 mm × 150 mm, particle size 5 μm) (Agilent Technologies, USA). Solvent A was prepared with 0.25 M acetic acid and 15% acetonitrile (ACN) and solvent B was prepared with 0.25 M acetic acid and 45% ACN. The column was equilibrated at a flow rate of 3 ml/min with 10 CV solvent A and the collected protein was loaded onto the column at a flow rate of 1 ml/min. After washing at a flow rate of 3 ml/min with 10 CV solvent A, bound proteins were eluted at a flow rate of 3 ml/min with 6 CV solvent A and B by application of a linear gradient (0%–70% solution B). The eluent was monitored at 280 nm and each peak was collected.

Condition for Analytical HPLC and MALDI-TOF Mass Spectrometry

Glargine insulin was analyzed by the HPLC system (Waters, USA) equipped with a Protein & Peptide C4 analytical column (250 mm × 4.6 mm, particle size 5 μm) (Grace Vydac, USA). Buffer A was prepared with 50 mM $NaH_2PO_4 \cdot H_2O$, 0.1 M $NaClO_4$, pH 2.5, and buffer B with ACN. The flow rate was 1 ml/min and the gradient condition was as follows: the gradient started with 90% buffer A and 10% buffer B mobile phase and then linearly increased to 80% buffer B in 30 min. After injection of 20-μl samples, absorbance at 214 nm was monitored by a UV detector (Waters, USA).

Purified glargine insulin was analyzed by MALDI-TOF/MS with technical assistance from Gyenggi Bio Center (Korea), using a Ultraflex III-MALDI TOF mass spectrometer (Bruker Daltonik Inc., USA).

Results and Discussion

Construction of pPT-GI Vector and Expression of Prepeptide Fusion Glargine

To express the recombinant prepeptide fusion glargine in *E. coli*, pPT-GI vector contains the P2 promoter; its transcriptional activity is controlled in response to growth rate, amino acid starvation, and rRNA gene dose [29, 36] and the *lac* operator, which regulates the activity of the structural genes of the *lac* operon. Recombinant prepeptide fusion glargine was successfully expressed in the fed-batch fermentation of recombinant *E. coli* JM109/pPT-GI with temperature shift processes from 30°C to 37°C [44].

The final optical density at 600 nm reached 50 after 28 h of fed-batch fermentation. The total dry cell mass of cultured cells was 18 g from 1 L culture broth (Fig. 2A). We performed SDS-PAGE to determine the time course of expression during cell culture; a sample was collected at each time point after raising the temperature. The SDS-

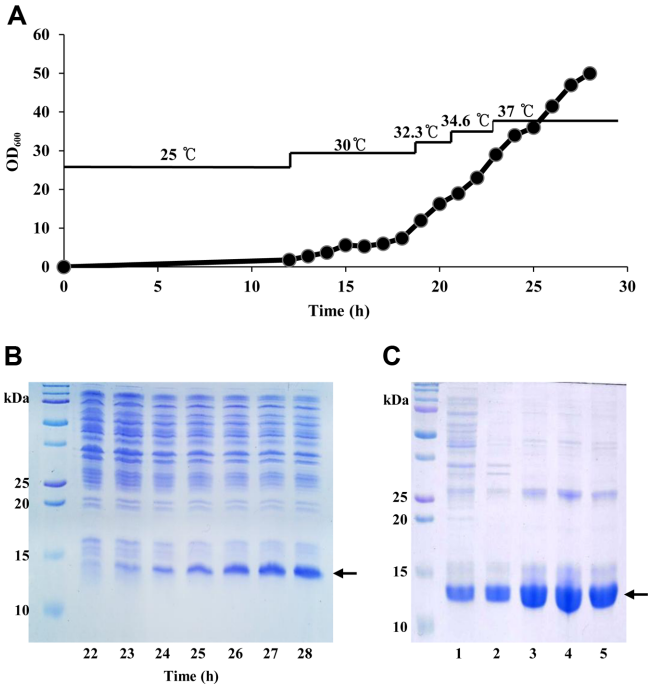


Fig. 2. Cell culture and expression of prepeptide fusion glargine.

(**A**) The temperature-shift fermentation curve of prepeptide fusion glargine. The temperature was increased to 37°C at 23 h in order to express prepeptide fusion glargine. (**B**) SDS-PAGE analysis of the expressed prepeptide fusion glargine insulin by culture time. (**C**) SDS-PAGE analysis of prepeptide fusion glargine insulin during inclusion body washing. Lane 1: total cell lysate of induced prepeptide fusion glargine insulin, Lane 2: 1st inclusion body washing with washing buffer, Lane 3: 2nd inclusion body washing with washing buffer, Lane 4: 1st inclusion body washing with deionized water, Lane 5: 2nd inclusion body washing with deionized water, Lane 6: 2nd inclusion body washing with deionized water. Arrows indicate the prepeptide fusion glargine.

PAGE analysis showed that the expression of prepeptide fusion glargine was significantly increased after raising the temperature to 37°C (Fig. 2B). The content of the prepeptide fusion glargine was 38.52% of the total protein. The molecular mass of the expressed prepeptide fusion glargine was around 12 kDa.

Washing of Inclusion Bodies Containing Prepeptide Fusion Glargine

The separated inclusion bodies containing prepeptide fusion glargine were washed with washing solution in order to remove the components of the cell wall and membrane. The cell wall of *E. coli* is constituted of phospholipid, protein, peptidoglycan, *etc*. Thus, Triton X-100, lysozyme, and EDTA were used as washing reagents [10]. Triton X-100 can remove phospholipid [28], and lysozyme can cleave the backbone of peptidoglycan. The action site of EDTA is the site of insertion of lipopolysaccharide into the outer membrane [38]. The results of SDS-PAGE analysis during inclusion body washing are shown in Fig. 2C. The purity of inclusion bodies containing prepeptide fusion glargine gradually increased during the inclusion body washing.

Direct Refolding of Prepeptide Fusion Glargine

In vitro folding of the inclusion body was performed with denaturant, a reducing agent for appropriate disulfide bond formation [23]. The washed inclusion body was solubilized with solubilization buffer solution. The inclusion body solution was diluted with refolding buffer solution to a final protein concentration of 0.5 mg/ml. The refolding yield was 82.1%, and it was best at 48 h based on the experiments in various reaction times (data not shown). The optimal refolding time was similar to that of human insulin. After the refolding reaction, the refolding solution was adjusted to pH 4.5 and centrifuged to remove misfolded protein, and the supernatant containing the refolded prepeptide fusion glargine was collected. The optimal pH for removing misfolded analog shifted from 5.5 for insulin to 4.5 for insulin glargine. The recovery of insulin glargine was 92.3% and the content of the impurity was a minimum at pH 4.5.

Conversion into Active Glargine Insulin by Protease Cleavage with Citraconylation

Trypsin is one of the most specific proteolytic enzymes and is most frequently used in protein modification. It is a member of the serine endopeptidase family S1, which cleaves peptide bonds specifically at the C-terminal side of arginine and lysine residues. The substrate-binding site of trypsin has a negative charge due to the Asp-189 residue (in bovine trypsin), which specifically binds to positively charged amino acid side chains of the substrate via ionic interaction [11,16]. Since trypsin only cuts peptide bonds behind positively charged residues, trypsin cleavage sites can be modified by the addition or deletion of positive charges [20]. The hydrophobicity analysis for prepeptide fusion glargine was performed. The GRAVY value for prepeptide fusion glargine was -0.39, and the prepeptide fusion insulin was -0.41. That meant the prepeptide fusion glargine was more hydrophobic than the prepeptide fusion insulin [37]. Therefore prepeptide fusion glargine had different characteristics for refolding and protease cleavage compared with human insulin. Citraconylation is the adoption of one or more citraconyl groups into a substance by the acylation reaction. Citraconic anhydride is often used to citraconylate free arginine and lysine residues and the target protein's primary amino groups [43]. Citraconic anhydride modifies the protein's primary amino groups at pH higher than 8, whereas incubation at pH 4 or lower leads to complete removal of citraconyl groups [18, 31]. Modification of arginine residues on the protein surface with citraconic anhydride changes the positive charge of arginine residues to negative, which protects them from the attack of trypsin enzyme [20, 28].

The major impurity, Arg (B31)-insulin, is formed by the trypsin-mediated elimination of Arg 32 in the B-chain during the production of glargine insulin. Arg (B31)-insulin is very similar to glargine insulin in physicochemical characteristics, so it is hard to separate during the purification process. Therefore, blocking Arg (B31)-insulin formation during the enzymatic modification is requisite in order to develop a cost-effective process for glargine insulin production. To minimize the amount of Arg (B31)-insulin, we performed citraconylation and deacylation during trypsin cleavage of recombinant prepeptide fusion glargine.

Citraconylation of prepeptide fusion glargine by the addition of citraconic anhydride before trypsin treatment substantially reduced the content of Arg (B31)-insulin. To verify the effect of citraconylation on blocking the formation of Arg (B31)-insulin, the enzyme reactions were conducted without and with citraconic anhydride (Fig. 3). The percentages of Arg (B31)-insulin formed were 72.5% without citraconylation and 12% with citraconylation in the enzyme reaction. The formation of Arg (B31)-insulin was higher than that of insulin glargine without citraconylation. The reason for the high formation level of Arg (B31)-insulin was caused by the sequence of amino acid. The B33 amino

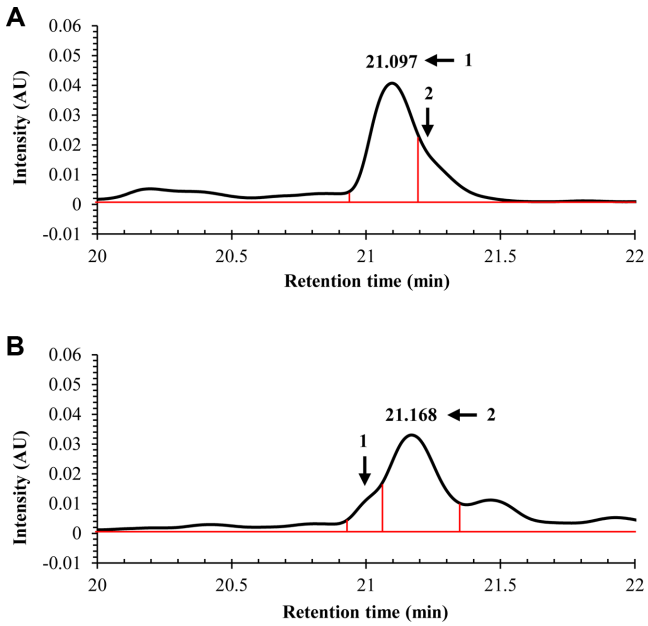


Fig. 3. HPLC chromatogram of peptide fusion glargine digested by trypsin in the absence (**A**) and presence (**B**) of citraconic anhydride.
Arrows indicate Arg (B31)-insulin (1) and glargine insulin (2).

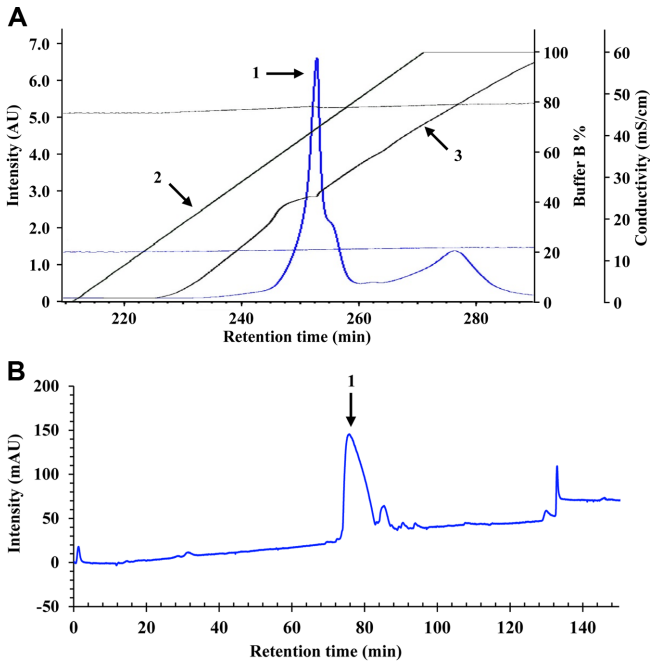


Fig. 4. Cation-exchange chromatography with an SP Sepharose resin column (**A**) and preparative high-performance liquid chromatography with a C8 prep HT column (**B**).
Arrows indicate human glargine insulin (1), concentration of buffer B (%) (2), and conductivity (3).

acid was Glu for the prepeptide fusion glargine. It is considered that the cleavage substrate 1 for trypsin, peptide 1 – Arg – Arg – peptide 2 was more sensitive than the cleavage substrate 2 for trypsin, peptide 1 – Arg – Glu – peptide 2. However, the modification of prepeptide fusion glargine with citraconylation changed the substrate sensitivity, so formation of the impurity was decreased dramatically. The adequate amount of addition of citraconic anhydride was 4.26 g per 1 g protein.

After the enzyme reaction, citraconylated glargine insulin has to be transformed into the native form. The optimal pH of deacylation was 2.5. When the deacylation was performed at 25°C, it was completed in 5 h. Using deacylation, the natural glargine insulin was recovered from modified glargine insulin after the enzyme reaction. Because of citraconylation, the formation of Arg (B31)-insulin was reduced and the enzymatic conversion yield of glargine insulin increased by 3.2-fold compared with that without citraconylation.

Purification of Glargine Insulin on Cation-Exchange and Reverse-Phase Chromatography

Glargine insulin transformed by trypsin was precipitated with zinc at pH 6.1 and then obtained by centrifugation. The collected glargine insulin precipitate was resolved with sample buffer (7 M urea, 0.25 M acetic acid, pH 2.5). Ion-exchange chromatography is commonly used to remove protein derivatives and soluble aggregates during recombinant protein purification [4, 8]. Thus, we carried out cation-exchange chromatography with the resolved glargine insulin in sample buffer. Elution was accomplished with a linear gradient, ranging from 0 to 1 M sodium chloride, and then the major peak of glargine insulin was eluted from around 0.6 to 0.75 M NaCl. Fig. 4A shows a cation-exchange chromatogram from the SP Sepharose resin column and the result of HPLC analysis of the fraction. The fractions of glargine insulin were pooled at above 60% purity on the chromatogram.

To increase the purity of glargine insulin, the pooled glargine insulin solution prepared from the cation-exchange chromatography was loaded onto the reverse-phase chromatography column. Elution was accomplished with a linear gradient, ranging from 15% to 36% ACN. Fig. 4B displays the preparative HPLC chromatogram from the C8 prep HT column and the results of the HPLC analysis of the collected glargine insulin fraction. The main peak of glargine insulin eluted between 28% to 30% ACN. The high purity (over 95%) of the main peak was pooled.

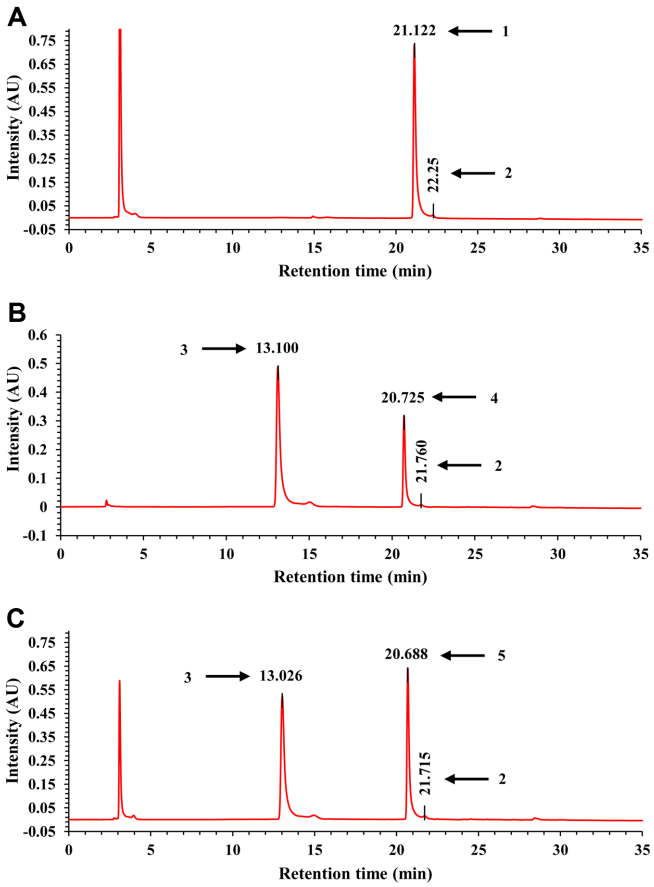


Fig. 5. HPLC chromatogram for recombinant human glargine insulin (**A**), the standard of human glargine insulin (Lantus) (**B**), and the mixture of recombinant human insulin and standard (**C**).

Arrows indicate human glargine insulin (1), desamido glargine insulin (2), excipients of Lantus (3), Lantus (4), and glargine insulin + Lantus (5). Glargine insulin: 98.11% (included 1.96% desamido glargine insulin).

The purity of the final glargine insulin product was 98.11% (included 1.96% desamido insulin) based on the HPLC chromatogram from a Protein & Peptide C4 analytical column (Fig. 5A). In addition, we compared the chromatogram of glargine insulin product with that of Lantus (Sanofi Aventis, FR), as a standard. The retention time was 21.12 min for the purified recombinant glargine insulin, 20.72 min for Lantus, and 20.68 min for the mixture (Fig. 5). Even though the retention times of the purified recombinant glargine insulin and Lantus (as a standard) were not consistent, we considered that the final purified glargine insulin was equal to the standard because the

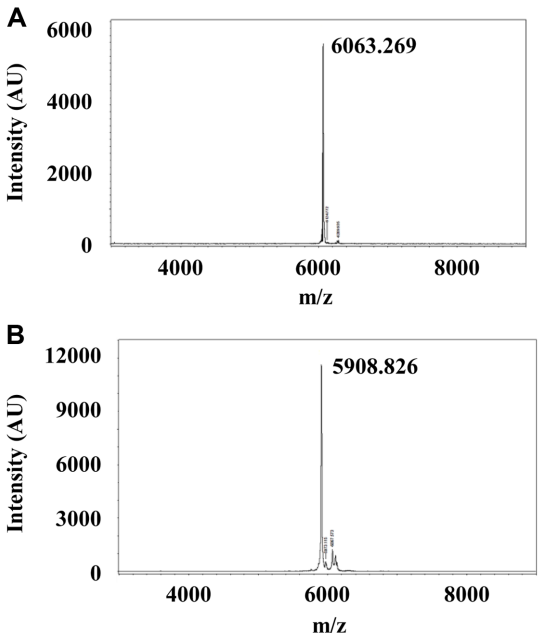


Fig. 6. Mass spectrum of purified recombinant glargine insulin (**A**) and Arg (B31)-insulin (**B**).

chromatogram of the mixture showed one peak (Fig. 5C). Other peaks were the desamido glargine insulin, some impurities, and the excipient.

To identify the molecular weights of the obtained products, the purified glargine insulin and Arg (B31)-insulin, we analyzed them by Matrix-assisted laser desorption/ionization Time-of-Flight Mass Spectrometry(MALDI-TOF/MS). Each of the graphs shows mass ions at 6063.26 and 5908.82 *m/z*, which were identical to the theoretical values for glargine insulin and Arg (B31)-insulin, respectively (Fig. 6).

Finally, we summarize the production process for recombinant glargine insulin. After fermentation, we performed cell lysis, refolding, citraconylation, and bioconversion. Then we carried out two-step chromatography, cation-exchange and reverse-phase chromatographies, to obtain the high-purity recombinant human glargine insulin.

The purpose of this study was to establish a production process for high-purity recombinant human glargine insulin and a method to block Arg (B31)-insulin formation. We expressed a large amount of prepeptide fusion glargine insulin in *E. coli* using the fed-batch culture method. We also successfully reduced the main impurity, Arg (B31)-insulin, by using the citraconylation method. The established process for recombinant human glargine insulin production may be a model process for the production of other human insulin analogs.

Acknowledgments

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References

1. Alberti KG, Zimmet PZ. 1998. Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: diagnosis and classification of diabetes mellitus provisional report of a WHO consultation. *Diabet. Med.* **15:** 539-553.
2. Baekkeskov S, Aanstoot HJ, Christgau S, Reetz A, Solimena M, Cascalho M, *et al*. 1990. Identification of the 64K autoantigen in insulin-dependent diabetes as the GABA-synthesizing enzyme glutamic acid decarboxylase. *Nature* **347:** 151-156.
3. Baeshen NA, Baeshen MN, Sheikh A, Bora RS, Ahmed MM, Ramadan HA, *et al*. 2014. Cell factories for insulin production. *Microb. Cell Fact*. **13:** 141.
4. Bhambure R, Rathore AS. 2013. Chromatography process development in the quality by design paradigm I: establishing a high-throughput process development platform as a tool for estimating "characterization space" for an ion exchange chromatography step. *Biotechnol. Prog.* **29:** 403-414.
5. Bindels J, Misdom L, Hoenders H. 1985. The reaction of citraconic anhydride with bovine α-crystallin lysine residues. Surface probing and dissociation-reassociation studies. *Biochim. Biophys. Acta* **828:** 255-260.
6. Bolli G, Di Marchi R, Park G, Pramming S, Koivisto VA. 1999. Insulin analogues and their potential in the management of diabetes mellitus. *Diabetologia* **42:** 1151-1167.
7. Bonifacio E, Genovese S, Braghi S, Bazzigaluppi E, Lampasona V, Bingley PJ, *et al*. 1995. Islet autoantibody markers in IDDM: risk assessment strategies yielding high sensitivity. *Diabetologia* **38:** 816-822.
8. Borg N, Brodsky Y, Moscariello J, Vunnum S, Vedantham G, Westerberg K, Nilsson B. 2014. Modeling and robust pooling design of a preparative cation-exchange chromatography step for purification of monoclonal antibody monomer from aggregates. *J. Chromatogr. A* **1359:** 170-181.
9. Bottazzo GF, Florin-Christensen A, Doniach D. 1974. Islet-cell antibodies in diabetes mellitus with autoimmune polyendocrine deficiencies. *Lancet* **2:** 1279-1283.
10. Clark EDB. 1998. Refolding of recombinant proteins. *Curr. Opin. Biotechnol.* **9:** 157-163.
11. Evnin LB, Vasquez JR, Craik CS. 1990. Substrate specificity of trypsin investigated by using a genetic selection. *Proc. Natl. Acad. Sci. USA* **87:** 6659-6663.
12. Expert Committee on the Diagnosis and Classification of Diabetes Mellitus. 2003. Report of the expert committee on the diagnosis and classification of diabetes mellitus. *Diabetes Care* **26 Suppl 1:** S5-S20.
13. Ferrannini E. 2012. Physiology of glucose homeostasis and insulin therapy in type 1 and type 2 diabetes. *Endocrinol. Metab. Clin. North Am.* **41:** 25-39.
14. Galloway JA, Chance RE. 1994. Improving insulin therapy: achievements and challenges. *Horm. Metab. Res.* **26:** 591-598.
15. Gavin III JR, Alberti K, Davidson MB, DeFronzo RA. 1997. Report of the expert committee on the diagnosis and classification of diabetes mellitus. *Diabetes Care* **20:** 1183.
16. Graf L, Jancso A, Szilagyi L, Hegyi G, Pinter K, Naray-Szabo G, *et al*. 1988. Electrostatic complementarity within the substrate-binding pocket of trypsin. *Proc. Natl. Acad. Sci. USA* **85:** 4961-4965.
17. Gu Z, Weidenhaupt M, Ivanova N, Pavlov M, Xu B, Su Z, Janson J. 2002. Chromatographic methods for the isolation of, and refolding of proteins from, *Escherichia coli* inclusion bodies. *Protein Expr. Purif.* **25:** 174-179.
18. Habeeb A, Atassi MZ. 1970. Enzymic and immunochemical properties of lysozyme. Evaluation of several amino group reversible blocking reagents. *Biochemistry* **9:** 4939-4944.
19. Haist RE, Best CH. 1940. Factors affecting the insulin content of pancreas. *Science* **91:** 410.
20. Kadlčík V, Strohalm M, Kodíček M. 2003. Citraconylation — a simple method for high protein sequence coverage in MALDI-TOF mass spectrometry. *Biochem. Biophys. Res. Commun.* **305:** 1091-1093.
21. Khajeh K, Naderi-Manesh H, Ranjbar B, Moosavi-Movahedi A, Nemat-Gorgani M. 2001. Chemical modification of lysine residues in *Bacillus* α-amylases: effect on activity and stability. *Enzyme Microb. Technol.* **28:** 543-549.
22. Korz DJ, Rinas U, Hellmuth K, Sanders EA, Deckwer WD. 1995. Simple fed-batch technique for high cell density cultivation of *Escherichia coli*. *J. Biotechnol.* **39:** 59-65.
23. Kyte J, Doolittle RF. 1982. A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.* **157:** 105-132.
24. Lan MS, Wasserfall C, Maclaren NK, Notkins AL. 1996. IA-2, a transmembrane protein of the protein tyrosine phosphatase family, is a major autoantigen in insulin-dependent diabetes mellitus. *Proc. Natl. Acad. Sci. USA* **93:** 6367-6370.
25. Lee SY. 1996. High cell-density culture of *Escherichia coli*. *Trends Biotechnol.* **14:** 98-105.
26. Loghmani E. 2005. Diabetes mellitis: type 1 and type 2, pp. 167-182. *In* Stang J, Story M (eds.). *Guidelines for Adolescent Nutrition Services*. Center for Leadership, Education and Training in Maternal and Child Nutrition, Division of Epidemiology and Community Health, School of Public Health, University of Minnesota, Minneapolis, MN.
27. Middelberg AP, O'Neill BK. 1991. Monitoring the centrifugal recovery of recombinant protein inclusion bodies. *Aust. J. Biotechnol.* **5:** 87-89.

28. Mossavarali S, Hosseinkhani S, Ranjbar B, Miroliaei M. 2006. Stepwise modification of lysine residues of glucose oxidase with citraconic anhydride. *Int. J. Biol. Macromol.* **39:** 192-196.
29. Murray HD, Appleman JA, Gourse RL. 2003. Regulation of the *Escherichia coli rrnB* P2 promoter. *J. Bacteriol.* **185:** 28-34.
30. Naglak TJ, Wang HY. 1990. Recovery of a foreign protein from the periplasm of *Escherichia coli* by chemical permeabilization. *Enzyme Microb. Technol.* **12:** 603-611.
31. Nieto MA, Palacián E. 1983. Effects of temperature and pH on the regeneration of the amino groups of ovalbumin after modification with citraconic and dimethylmaleic anhydrides. *Biochim. Biophys. Acta* **749:** 204-210.
32. Olsen JV, Ong SE, Mann M. 2004. Trypsin cleaves exclusively C-terminal to arginine and lysine residues. *Mol. Cell. Proteomics* **3:** 608-614.
33. Owens DR. 2002. New horizons — alternative routes for insulin therapy. *Nat. Rev. Drug Discov.* **1:** 529-540.
34. Owens DR, Zinman B, Bolli GB. 2001. Insulins today and beyond. *Lancet* **358:** 739-746.
35. Poon K, King AB. 2010. Glargine and detemir: safety and efficacy profiles of the long-acting basal insulin analogs. *Drug Healthc. Patient Saf.* **2:** 213-223.
36. Rasmussen LJ, Lobner-Olesen A, Marinus MG. 1995. Growth-rate-dependent transcription initiation from the *dam* P2 promoter. *Gene* **157:** 213-215.
37. Rudolph R, Lilie H. 1996. In vitro folding of inclusion body proteins. *FASEB J.* **10:** 49-56.
38. Schnaitman CA. 1971. Effect of ethylenediaminetetraacetic acid, Triton X-100, and lysozyme on the morphology and chemical composition of isolate cell walls of *Escherichia coli*. *J. Bacteriol.* **108:** 553-563.
39. Seo MJ, Choi HJ, Chung KH, Pyun YR. 2011. Production of a platelet aggregation inhibitor, salmosin, by high cell density fermentation of recombinant *Escherichia coli*. *J. Microbiol. Biotechnol.* **21:** 1053-1056.
40. Shetty JK, Kinsella JE. 1980. Ready separation of proteins from nucleoprotein complexes by reversible modification of lysine residues. *Biochem. J.* **191:** 269-272.
41. Shiloach J, Fass R. 2005. Growing *E. coli* to high cell density – historical perspective on method development. *Biotechnol. Adv.* **23:** 345-357.
42. Sonksen P, Sonksen J. 2000. Insulin: understanding its action in health and disease. *Br. J. Anaesth.* **85:** 69-79.
43. Son Y, Kim C, Kim YB, Kweon D, Park Y, Seo J. 2009. Effects of citraconylation on enzymatic modification of human proinsulin using trypsin and carboxypeptidase B. *Biotechnol. Prog.* **25:** 1064-1070.
44. Son Y, Park K, Lee S, Oh S, Kim C, Choi B, Park Y, Seo J. 2007. Effects of temperature shift strategies on human preproinsulin production in the fed-batch fermentation of recombinant *Escherichia coli*. *Biotechnol. Bioprocess Eng.* **12:** 556-561.
45. Sreenivas S, Krishnaiah SM, Govindappa N, Basavaraju Y, Kanojia K, Mallikarjun N, *et al*. 2015. Enhancement in production of recombinant two-chain insulin glargine by over-expression of Kex2 protease in *Pichia pastoris*. *Appl. Microbiol. Biotechnol.* **99:** 327-336.
46. Yadav S, Parakh A. 2006. Insulin therapy. *Indian Pediatr.* **43:** 863-872.