J. Microbiol. Biotechnol. (2015), 25(10), 1742–1750
http://dx.doi.org/10.4014/jmb.1504.04062

jmb

Large-Scale Refolding and Enzyme Reaction of Human Preproinsulin for Production of Human Insulin

Chang-Kyu Kim[1], Seung-Bae Lee[1*], and Young-Jin Son[2*]

[1]*Division of Animal Resources and Life Science, Sangji University, Wonju 220-702, Republic of Korea*
[2]*Department of Pharmacy, Sunchon National University, Jeonnam, Suncheon 540-742, Republic of Korea*

Received: April 23, 2015
Revised: June 27, 2015
Accepted: June 29, 2015

First published online
July 2, 2015

*Corresponding authors
Y.-J.S.
Phone: +82-61-750-3755;
Fax: +82-61-750-3028;
E-mail: sony@sunchon.ac.kr
S.-B.L.
Phone: +82-33-730-0542;
Fax: +82-33-730-0503;
E-mail:sblee@sangji.ac.kr

pISSN 1017-7825, eISSN 1738-8872

Human insulin is composed of 21 amino acids of an A-chain and 30 amino acids of a B-chain. This is the protein hormone that has the role of blood sugar control. When the recombinant human proinsulin is expressed in *Escherichia coli*, a serious problem is the formation of an inclusion body. Therefore, the inclusion body must be denatured and refolded under chaotropic agents and suitable reductants. In this study, H27R-proinsulin was refolded from the denatured form with β-mercaptoethanol and urea. The refolding reaction was completed after 15 h at 15°C, whereas the reaction at 25°C was faster than that at 15°C. The refolding yield at 15°C was 17% higher than that at 25°C. The refolding reaction could be carried out at a high protein concentration (2 g/l) using direct refolding without sulfonation. The most economical and optimal refolding condition for human preproinsulin was 1.5 g/l protein, 10 mM glycine buffer containing 0.6 M urea, pH 10.6, and 0.3 mM β-mercaptoethanol at 15°C for 16 h. The maximum refolding yield was 74.8% at 15°C with 1.5 g/l protein. Moreover, the refolded preproinsulin could be converted into normal mature insulin with two enzymes. The average amount of human insulin was 138.2 g from 200 L of fermentation broth after enzyme reaction with H27R-proinsulin. The direct refolding process for H27R-proinsulin was successfully set up without sulfonation. The step yields for refolding and enzyme reaction were comparatively high. Therefore, our refolding process for production of recombinant insulin may be beneficial to the large-scale production of other biologically active proteins.

Keywords: Preproinsulin, refolding, enzyme reaction, pilot scale

Introduction

Inclusion body is a dense, amorphous protein deposit that can be found in both the cytoplasmic and periplasmic spaces of bacteria [2]. The formation of inclusion bodies has some advantages commercially [14]. First, the isolation step of target protein is comparatively simple using collecting inclusion bodies. Second, the aggregated inclusion bodies can escape from cleavage by intracellular proteases [8]. Despite of these advantages, we need additional steps in order to recover the activity of the inclusion body [1]. After cell disruption and centrifugation to collect the inclusion body, it is solubilized by denaturants such as guanidine hydrochloride or urea [9, 19, 30, 31]. After inclusion body solubilization, a sulfonation step is used to increase the yield of refolding [30, 33]. During the refolding process, reducing agents such as β-mercaptoethanol, dithiothreitol, and dithioerythritol are used to cut disulfide bonds between cysteine residues and to prevent the misfolded form of the target protein [9, 18]. The unfolded and reduced forms of target proteins are refolded in order to recover their biological activity, using the refolding process [5, 9, 18, 34].

The synthesis of human insulin using recombinant DNA technology is achieved by using *Escherichia coli* or *Saccharomyces cerevisiae* as a host cell [6, 32]. When human insulin is produced in recombinant *E. coli*, a proinsulin fusion protein is usually expressed as an inclusion body to increase the stability and the level of expression in the cell. The proinsulin fusion protein is the precursor of active

human insulin and must be refolded to be transformed into an active form. *In vitro* refolding of the proinsulin fusion protein is accompanied by the formation of the correct disulfide bonds. Oxidative folding of proinsulin is a widely used process with high yield [7, 16]. Human proinsulin contains three disulfide bonds that are essential for its native conformation. The formation of the native disulfide bonds is the rate-determining step during proinsulin folding [23]. During consecutive purification steps, the proinsulin fusion protein is converted into the mature form of insulin using an *in vitro* enzyme reaction with two enzymes (carboxypeptidase B and trypsin) [3, 15, 22, 27].

In this study, we describe the protocols of refolding and the enzyme processes for preproinsulin protein. A direct refolding process has many advantages. Using this process, we can shorten the overall production process and increase the production yield. Our results suggested that the direct refolding process of proinsulin fusion protein may be informative for recombinant protein production in *E. coli.*

Materials and Methods

Reagents

The main reagents and restriction enzymes were purchased from Takara (Takara Korea, Seoul, Korea). QUICK-Clone cDNA was ordered from BD Science (Clontech 7156-1; Franklin Lakes, NJ, USA), and *E. coli* JM109 was purchased from Stratagene (La Jolla, CA, USA) as a bacterial host strain. All other reagents were purchased from Sigma (St. Louis, MO, USA).

Bacterial Strain and Plasmid Construction

E. coli JM109 (*endA*1 *recA*1 *gyrA*96 *hsdR*17 *relA*1*supE*44*thiΔ*(*lac-proAB*) *F'* [*traD*36*proAB*$^+$*lacI*^{q}*lacZΔM*15]) purchased from Stratagene was used for the expression of proinsulin. Plasmid pPT was composed of the P2 promoter [20] and *lac* operator, T7 ribosome binding site, ColE1 origin of replication, ampicillin resistance gene, and a multiple cloning site. Plasmid pPT-H27Rpi was constructed from plasmid pPT to express the preproinsulin gene in *E. coli* [13]. The plasmid pPT-H27Rpi expressed H27R-preproinsulin with a leader peptide containing a 28-amino-acid sequence, Met-Thr-Met-Ile-Thr-Asn-Ser-Pro-Glu-Ile- Ser-His-His-His-His-His-His-His-His-His-His-Gln-Leu-Ile-Ser-Glu-Ala-Arg, and proinsulin.

Fed-Batch Fermentation

The medium used for stock and seed culture of recombinant *E. coli* strain was composed of 10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl, 100 mg/l ampicillin, and 0.1 g/l antifoaming agent. The 500 ml seed culture was grown overnight at 30°C and 200 rpm in a rotary shaker. The nutrient sources were three types of solutions, including initial production medium and feeding solutions I and II. The production medium for fed-batch fermentation contained (per liter) 6 g glucose, 4 g Yeast extract, 4 g KH_2PO_4, 8 g $Na_2HPO_4 \cdot 12H_2O$, 3 g $MgSO_4 \cdot 7H_2O$, 0.2 g $FeSO_4 \cdot 7H_2O$, 0.075 g $MnSO_4 \cdot 5H_2O$, 0.013 g $CoCl_2 \cdot 6H_2O$, 0.075 g $ZnSO_4 \cdot 7H_2O$, 0.013 g $CuCl_2 \cdot 2H_2O$, 100 mg ampicillin, 0.013 g $Na_2MoO_4 2H_2O$, and 0.2 g antifoaming agent (Silicon fluid SAG-471; Union Carbide, USA). Feed solution I contained (per liter) 600 g glucose, and solution II contained (per liter) 150 g yeast extract, 20 g $MgSO_4$, 0.2 g $FeSO_4 \cdot 7H_2O$, 0.075 g $MnSO_4 \cdot 5H_2O$, 0.013 g $CoCl_2 \cdot 6H_2O$, 0.075 g $ZnSO_4 \cdot 7H_2O$, 0.013 g $CuCl_2 \cdot 2H_2O$, 0.013 g $Na_2MoO_4 \cdot 2H_2O$, and 1 g antifoaming agent.

Fed-batch culture was performed in a 300 L fermenter (B. Braun Biotech International, Germany) with a working volume of 200 L. The dissolved oxygen concentration was maintained at 20% air saturation by modulation of the agitation speed up to 700 rpm and the aeration rate up to 2 vvm, and by a manual change of internal pressure. The pH of the culture broth was controlled at 6.8 using ammonia water. The substrate feeding strategy was the glucose concentration control method based on pH-stat and glucose concentration in the culture broth, which was maintained below 0.05%. The culture temperature was initially 30°C and then changed to 37°C. Elevation of culture temperature was carried out as previously reported [29]. For the three-step process, the culture temperature was raised from 30°C to 37°C by three shifts of the same interval. Each increment of temperature took 10 min and was maintained for 1 h. The temperature shifting was started at the optical density of 30 at 600 nm (OD_{600} = 30).

Solubilization and Sulfitolysis for Refolding of S-Sulfonated Preproinsulin

The cells were harvested by centrifugation at 12,000 rpm with a continuous centrifuge (Tomoe, Japan). The cells were resuspended in resuspension buffer (10% sucrose, 0.1 M Tris, 50 mM ethylenediamine tetraacetic acid (EDTA), 0.2 M sodium chloride, pH 7.9). The cells were lysed under 13,000 psi with a high-pressure homogenizer (Rannie, Denmark). The disrupted cells were centrifuged at 12,000 rpm. The supernatant was discarded and the precipitate was collected. The collected inclusion bodies containing preproinsulin were washed with inclusion body washing solution of various compositions of washing solution that consisted of Tris, EDTA, Triton X-100, lysozyme, and urea [10]. The washed inclusion bodies were collected by centrifugation at 12,000 rpm. The inclusion bodies containing preproinsulin were solubilized and converted to their S-sulfonated forms by oxidative sulfitolysis. The inclusion bodies were suspended in 20 mM Tris–HCl, 1 mM EDTA, and 8 M urea at a protein concentration of 10–15 mg/ml. To enhance solubility, the pH was adjusted to 11 and then readjusted to 9.5. The protein concentration was analyzed by the Bradford assay method after resolving the collected inclusion body. To start the sulfitolysis reaction, 0.2 M sodium sulfite and 20 mM sodium tetrathionate were added and the mixtures were incubated with stirring for 4 h at 25°C. HPLC analysis of S-sulfonated preproinsulin (SSPPI) production was used to optimize

the reaction time. The reaction was stopped by diluting 10-fold with water and adjusting the pH to 4.5 for SSPPI precipitation. The pellets were centrifuged at 6,000 rpm, suspended in 20 ml of deionized water, and solubilized by raising the pH to 10.6. The SSPPI solution was diluted in refolding buffer (0.6 M urea, 10 mM glycine, pH 10.6) containing β-mercaptoethanol. The diluted solution was mixed gently for 16 h at 15°C and 25°C.

Direct Refolding of Preproinsulin and Acid Precipitation

The washed inclusion bodies were solubilized in solubilization buffer (4 M urea, 10 mM glycine, pH 10.6). The solution was diluted with refolding buffer (0.6 M urea, 10 mM glycine, pH 10.6) to final protein concentrations of 0.5, 1.0, 1.5, and 2.5 g/l. Refolding was performed with various protein concentrations at various β-mercaptoethanol concentrations. The solution was incubated for 16 h at 15°C and 25°C. The refolding kinetics were analyzed at 15°C and 25°C. After the refolding reaction ended, the pH of the solution was adjusted to 5.5 with 5 N HCl and the sample was centrifuged at 12,000 rpm. The supernatant containing the refolded preproinsulin was collected and the precipitate was removed.

Bioconversion of Preproinsulin into Mature Insulin Using Recombinant Carboxypeptidase B and Trypsin

Purified preproinsulin (98% purity) was obtained as described previously [27]. The refolded preproinsulin in the supernatant was collected and used as a substrate for enzymatic modification at 15°C for 16 h. Then 0.45 unit trypsin and 0.2 unit recombinant carboxypeptidase B (CPB) [11] per 0.5 mg of the refolded preproinsulin were added into the enzyme reaction mixture. After incubation of the refolded preproinsulin at pH 8.5 for 2 h with citraconic anhydride, trypsin and CPB were added into the reaction mixture containing hydrogen peroxide. HPLC analysis was performed as described in a previous report [28].

Analytical Methods

Cell growth was monitored by measuring the absorbance of the culture broth at 600 nm (OD_{600}) using a spectrophotometer (Shimadzu UV-265, Japan). The glucose concentration was measured by a glucose analyzer (YSI 2700 STAT, OH, USA). Protein concentration was measured by the Bradford method.

The GRAVY (grand average of hydropathy) value was calculated by adding the hydropathy value for each residue and dividing by the length of the sequence, at http://www.bioinformatics.org/sms2/protein gravy.html. We obtained the hydrophilicity plot of the expressed protein based on the Kyte-Doolittle-Hydropathy method using ExPASy ProtScale [12].

Refolded preproinsulin and insulin were analyzed on an HPLC system (Alliance 2695; Waters, MA, USA) equipped with a Protein & Peptide C18 analytical column (250 mm × 4.6 mm i.d., particle size 5 μm; Vydac, USA) heated at 40°C. Solvent A was prepared with 0.5 M sodium dihydrogen phosphate and 0.1 M sodium perchlorate (pH 2.5) and solvent B with 100% acetonitrile. The flow rate was 1 ml/min and the gradient condition was as follows: starting with 56% solvent A and 44% solvent B mobile phase, the gradient was increased linearly to 60% solvent B in 27 min. After injection of 20 μl samples, absorbance at 214 nm wavelength was monitored by a UV detector (Waters, USA).

The human insulin preparation was lyophilized and subjected to amino acid analysis. Amino acid composition analysis and N-terminal amino acid sequencing were carried out with technical assistance from Korea Basic Science Institute (Daejeon, Korea). The amino acid composition of human insulin was analyzed by the Pico-Tag method (Waters) after hydrolysis in constant boiling with 6 M HCl containing 0.5% (w/v) phenol at 110°C for 24 h. For cysteine content analysis, cysteine residues were oxidized to cysteic acid with a mixture of formic acid and hydrogen peroxide (19:1 (v/v)). In the case of tryptophan analysis, lyophilized human insulin was hydrolyzed in constant boiling with 4 M methane-sulfonic acid at 110°C for 24 h. The N-terminal amino acid sequences of each chain of human insulin were analyzed using an automated Edman degradation method with a Procise 491 Protein sequencing system (Applied Biosystems, CA, USA).

We performed peptide mapping analysis to confirm the identity of purified human insulin. The high-performance liquid chromatography system was equipped with a 214 nm detector and a 4.6 mm × 150 mm column that contained packing C18 reverse phase resin (Grace Vydac, USA). The flow rate was 1 ml/min. The column temperature was maintained at 40°C. Solution A (10% acetonitrile, 20% sulfate buffer, 70% water) and Solution B (40% acetonitrile, 20% sulfate buffer, 40% water) were prepared and used as mobile phases. Elution was carried out with a linear gradient of the two mobile solutions. The reference human insulin and purified recombinant human insulin were resolved with 0.01 N hydrochloric acid and transfered into a clean vial. Then 10 mM HEPES (*N*-2-hydroxyethyl-piperazine-*N'*-2-ethane sulfonic acid) buffer and enzyme solution (*Staphylococcus aureus* V-8 protease) were added into the samples and incubated at 25°C for 6 h. Sulfate buffer (2.0 M ammonium sulfate, 0.5 M sulfuric acid) was added to quench the reaction. Then these samples were analyzed and chromatograms of the samples were compared.

Results

Purification and Washing of Inclusion Bodies Containing H27R-Preproinsulin

The fermentation broth was centrifuged in order to collect the *E. coli* cells. The fermentation graph is depicted in Fig. 1. The cells were resuspended in buffer solution and lysed with a high-pressure homogenizer [26]. The inclusion bodies were collected, and soluble proteins and cell debris were removed by centrifugation [17]. The separated inclusion bodies containing H27R-preproinsulin were washed with a washing solution in order to remove other components of the cell wall and membrane. The cell wall of *E. coli* comprises phospholipid, protein, peptidoglycan, *etc*.,

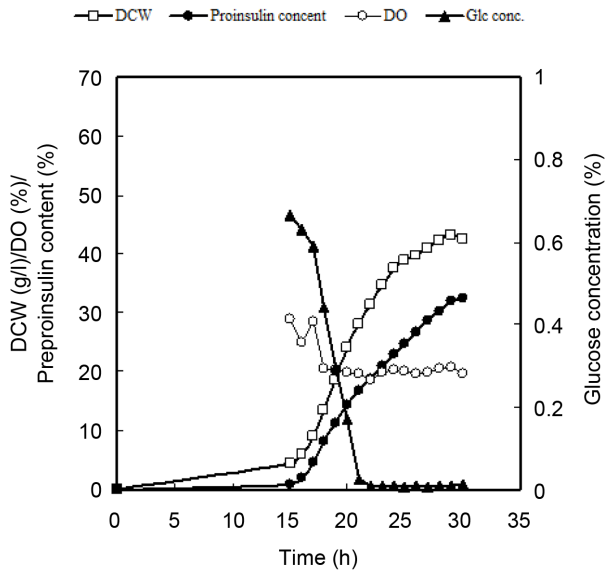

Fig. 1. Profiles of cell growth and preproinsulin production in fed-batch fermentation of *E. coli* JM109/pPT-H27Rpi.

so Triton X-100, lysozyme, and EDTA were used as washing reagents [4]. Triton X-100 can remove phospholipid [21], and lysozyme can cleave the backbone of peptidoglycan. The action site of EDTA is the site of insertion of lipopolysaccharide (LPS) into the outer membrane [25].

The inclusion bodies were washed with various compositions of washing solution. The washing solution was composed of Tris, EDTA, Triton X-100, lysozyme, and urea. As shown in Table 1, inclusion bodies were washed with five different washing solutions. The unfolded protein was refolded and analyzed in order to compare the efficiency of each washing solution. The refolding yield was 74.8% when the inclusion bodies were washed with washing solution 2 and refolded at protein concentration 1.5 g/l with 0.3 mM β-mercaptoethanol. The composition of washing solution 2 was 20 mM Tris, 1 mM EDTA, 0.02% lysozyme, 1% Triton X-100, and 0.5 M urea. After washing the inclusion bodies with washing solution, the samples were centrifuged at 12,000 rpm to collect the inclusion bodies containing H27R-preproinsulin.

Table 1. Comparison of washing efficiency for inclusion body.

Washing solution	1	2	3	4	5[d]
Buffer	TE[a]	TE	TE	TE	-
Lysozyme (%)	0.02	0.02	0.02	0.02	-
Tx-100[b] (%)	1	1	1	-	-
Urea (M)	2	0.5	-	-	-
Refolding yield[c] (%)	71.0	74.8	69.0	61.4	67.1

[a]TE, 20 mM Tris buffer (1 mM EDTA, pH 7.0).

[b]Tx-100, detergent Triton X-100.

[c]Refolding yield was calculated after pH precipitation process of refolding.

[d]Washing solution 5 was distilled water.

Direct Refolding of Preproinsulin

The washed inclusion body was solubilized in solubilization buffer (4 M urea, 10 mM glycine, pH 10.6). Then the solution was diluted with refolding buffer (0.6 M urea, 10 mM glycine, pH 10.6) and β-mercaptoethanol was added [24]. The solution was incubated for 16 h at 15°C in order to refold unfolded H27R-preproinsulin. The refolding reaction was successfully completed without sulfonation. The results of refolding with various protein concentrations at different β-mercaptoethanol concentrations are summarized in Table 2. When the refolding was carried out at 1.5 g/l of protein with 0.3 mM β-mercaptoethanol, the refolding yield was 74.2%. The optimal refolding condition of H27R-proinsulin could be optimized through many experiments. As shown in Fig. 2, there was no difference in the peak of oxidative refolding with sulfonation and that of direct refolding based on the RP-HPLC chromatogram. Moreover, there was no difference between oxidative refolded and direct refolded preproinsulins that were subsequently converted to insulin using trypsin and recombinant carboxypeptidase B (Fig. 3). Thus, the direct refolding method was successfully performed.

The refolding kinetics were examined while the refolding reaction was carried out at 15°C and 25°C. When the refolding was performed at 15°C and 25°C, the refolding yields were 75.3% and 62.8%, respectively (data not shown). An analysis sample was collected from the direct refolding reactor at every 1.5 h over 20 h, and the results are shown in Fig. 4A. When the effect of temperature on the refolding

Table 2. Comparison of refolding yield with various protein concentrations at various β-mercaptoethanol concentrations.

Protein concentration at refolding	β-Mercaptoethanol concentration at refolding	Refolding yield[a] (%)
0.5 g/l	0.1 mM	53.6
1.0 g/l	0.1 mM	61.8
1.5 g/l	0.1 mM	68.0
2.0 g/l	0.1 mM	57.7
1.0 g/l	0.2 mM	66.0
1.5 g/l	0.3 mM	74.2
2.0 g/l	0.4 mM	66.0

[a]Refolding yield was calculated after pH precipitation process of refolding.

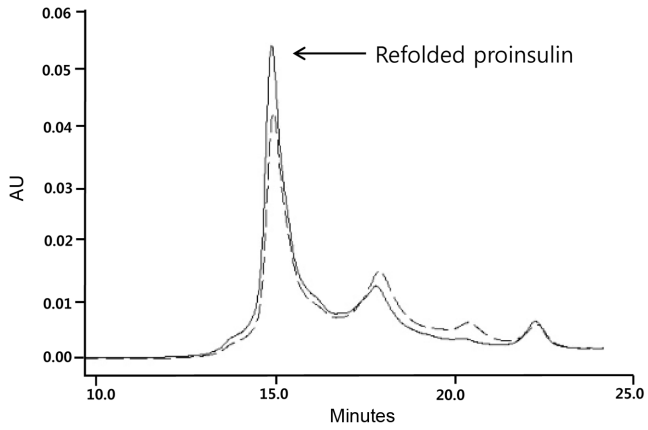

Fig. 2. Comparison of refolded proinsulin RP-HPLC chromatograms between oxidative refolding (dashed line) and direct refolding (solid line).
The arrow indicates the refolded proinsulins.

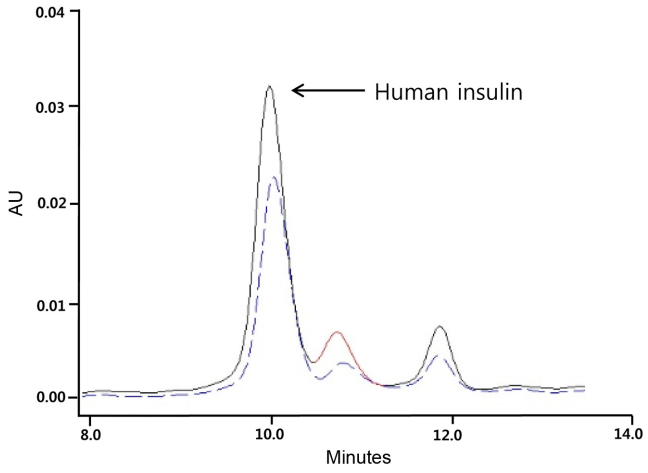

Fig. 3. Reverse phase HPLC chromatogram of human insulin from preproinsulin after enzymatic conversion.
Human insulin from the oxidative refolded preproinsulin (dashed line) and the direct refolded preproinsulin (solid line). The arrow indicates the human insulin.

process was analyzed, the refolding yield at 15°C was 17% higher than that at 25°C but the elapsed time for refolding at 25°C was faster than that at 15°C. The refolding process could successfully be performed even at high concentration of protein (2 g/l). The RP-HPLC chromatograms of refolding kinetics are shown in Figs. 4B and 4C for 15°C and 25°C. The refolded preproinsulin (RPI), misfolded preproinsulin (peak 1), and unfolded preproinsulin (peak 2) were separated at 15.4, 18.6, and 21.0 min, respectively. We found that the peak of refolded preproinsulin at 15°C was

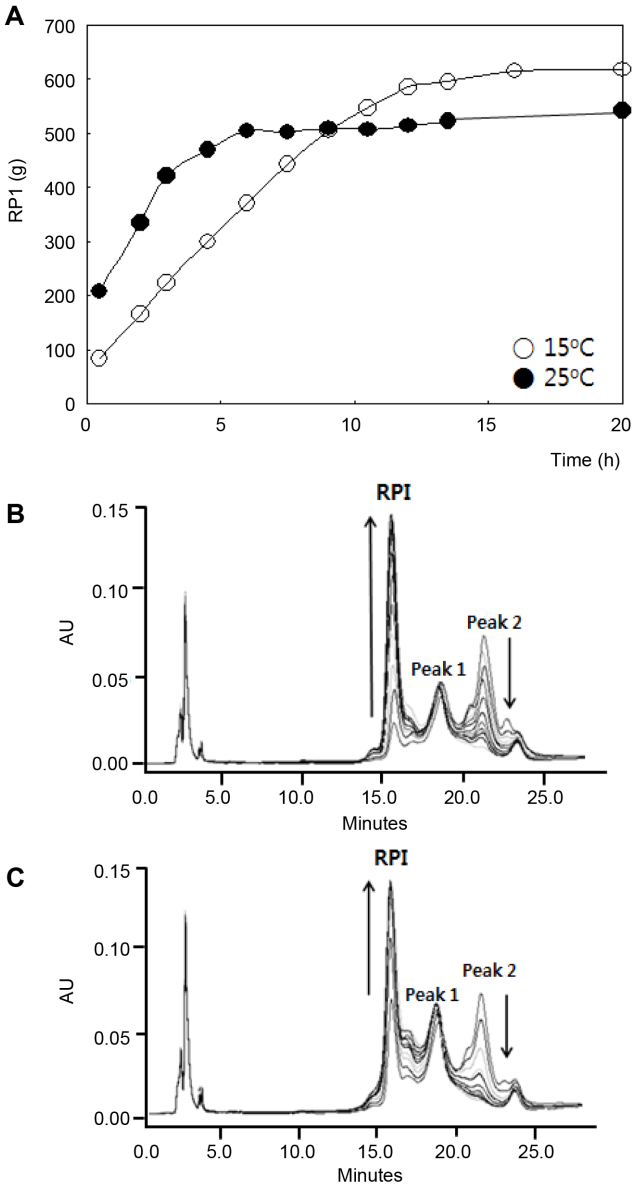

Fig. 4. Refolding kinetics of the preproinsulin.
(**A**) Refolding kinetics of the direct refolded preproinsulin at 15°C and 25°C. Closed circles indicate the refolding at 25°C, and open circles indicate that at 15°C. (**B**) RP-HPLC chromatogram of refolding kinetics at 15°C. (**C**) RP-HPLC chromatogram of refolding kinetics at 25°C. The refolded preproinsulin (RPI), misfolded preproinsulin (Peak 1), and unfolded preproinsulin (Peak 2) were separated at retention times 14.4, 18.5, and 21 min, respectively. The arrow indicates the changing heights of peaks.

sharper and higher than that of refolded preproinsulin at 25°C. This indicates that refolding at 15°C was better than that at 25°C.

After the refolding reaction, the refolding solution was

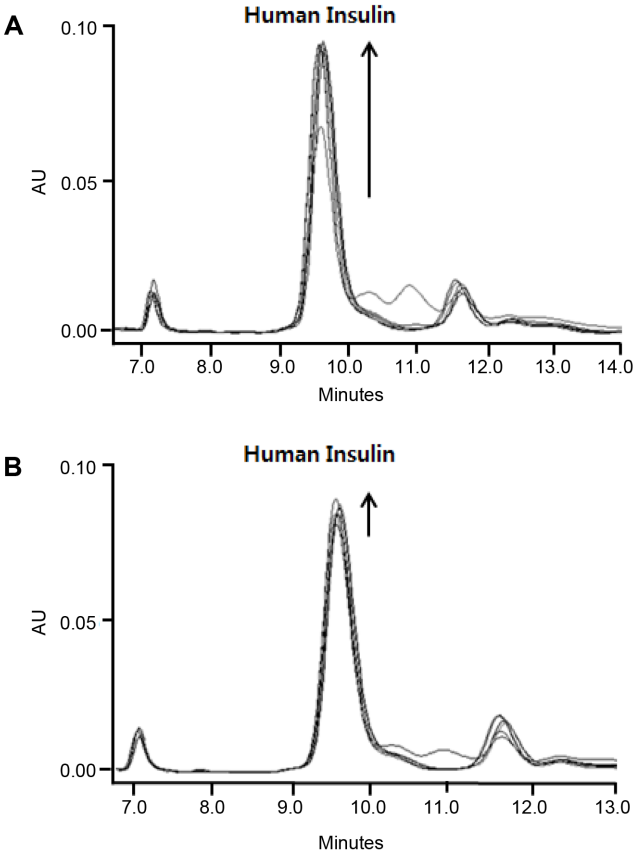

Fig. 5. Enzyme reaction kinetics of refolded preproinsulin.
Human insulin was separated at 9.6 min. (**A**) The human insulin peak from the refolded preproinsulin at 15°C and (**B**) the human insulin peak from the refolded preproinsulin at 25°C. The arrow indicates the changing heights of peaks.

adjusted to pH 5.5. The misfolded preproinsulin and other impurities were removed through precipitation and centrifugation, and the supernatant containing refolded preproinsulin was collected.

Effect of Leader Peptide on Refolding of the Fused Preproinsulin

The hydrophilicity analysis for H27R-proinsulin and Met-proinsulin were performed with amino acid sequences for two recombinant proteins (data not shown). The hydropathic results showed that the leader peptide of H27R proinsulin was hydrophilic. The GRAVY value for H27R-proinsulin was –0.36, whereas that for Met-proinsulin was –0.05. A negative value in the hydropathic score indicates a hydrophilic protein, whereas a positive value indicates a hydrophobic protein [12]. Thus H27R-proinsulin was more hydrophilic than Met-proinsulin.

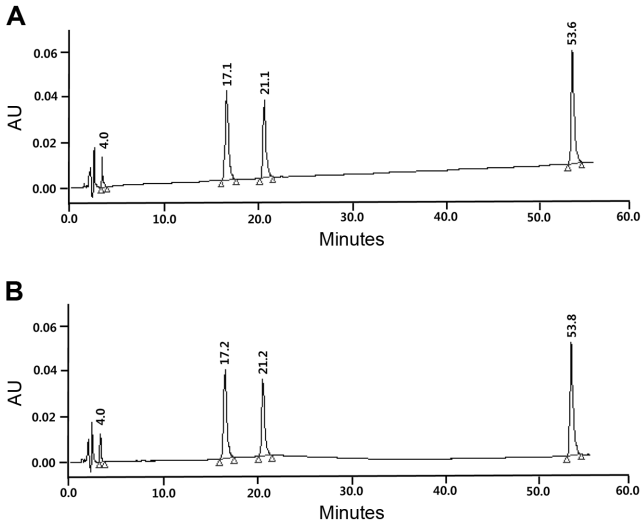

Fig. 6. Peptide map chromatograms of (**A**) standard and (**B**) purified human insulin by Glu-C protease.
Fragment I (retention time (RT) 53 min): amino acids A5 to A17 and B1 to B13. Fragment II (RT 21 min): amino acids A18 to A21 and B14 to B21. Fragment III (RT 17 min): amino acids B22 to B30. Fragment IV (RT 4 min): amino acids A1 to A4.

Conversion of Preproinsulin into Insulin Using Recombinant Carboxypeptidase B and Trypsin

The refolded recombinant preproinsulin was produced by the *E. coli* JM109/pPT-H27Rpi transformant. Recombinant carboxypeptidase B and trypsin were used in the conversion of H27R-proinsulin into insulin. The H27R (28 amino acids), leader peptide, and C-peptide were enzymatically removed from the H27R-proinsulin fusion protein. Before the enzyme reaction, H27R-proinsulin was citraconylated as described previously [28]. The products were analyzed by RP-HPLC after enzyme reaction with H27R-proinsulin. Fig. 5 shows the RP-HPLC chromatogram of enzyme reaction kinetics of H27R-proinsulins refolded at 15°C and 25°C. The mature insulin, the intermediate, and the misfolded preproinsulin peaks appeared at retention times (RT) of approximately 9.6, 10.8, and 11.5 min, respectively. The average step yields of the nine batches are summarized in Table 3. In nine pilot-scale batches, the average step yields for refolding, acid precipitation, and enzyme reaction were 69.7%, 99.3%, and 56.6%, respectively. The average amount of human insulin after enzyme reaction was 138.2 g from 200 L of culture broth.

Comparison with Human Insulin Standard and Purified Human Insulin

The amino acid composition data showed that the

Table 3. Average step yields from several pilot scale batches with H27R preproinsulin.[a]

Process steps	Total mass (gram/Mean ± SD[b])	Yield	Remark weight
Washed inclusion bodies	5733 ± 839	-	Amount of water content: 67%
Refolding	539.8 ± 75.1	69.7%	RP-HPLC
Acidic precipitation	536.1 ± 69.3	99.3%	RP-HPLC
Enzyme reaction	138.2 ± 14.5	25.8% (56.6%)[c]	RP-HPLC

[a]Based on treatment of 200 L culture broth.

[b]Each value represents the mean of nine performances ± SD.

[c]The value of conversion to refolded preproinsulin (51 amino acids / 112 amino acids).

composition of purified recombinant human insulin was in agreement with the theoretical composition and the composition of human insulin standard (Table 4).

Peptide mapping analysis is usually used to compare the protein structure of protein product with that of a reference material. The peptide map chromatogram of purified human insulin was performed by cleaving the subunits into some number of small fragments by cutting with endopeptidase Glu C, which specifically digests the protein at the C-terminus of glutamate. The cutting samples were separated on a RP-HPLC column with on-line UV monitoring. Fig. 6 shows chromatograms of human insulin standard and purified human insulin. Four fragments were detected in cutting of the standard and purified human insulins with endopeptidase Glu C. Fragment I contains the peptides from A5 to A17 and from B1 to B13. Fragment II contains the peptides from A18 to A21 and from B14 to B21. Fragment III contains the peptide from B22 to B30. Fragment IV contains the peptide from A1 to A4. The peptide map chromatogram of purified human insulin was found to be in agreement with that of the human insulin standard.

Table 4. Comparison of amino acid composition of purified human insulin, reference human insulin, and theoretical pancreatic human insulin.

Amino acid residue	Purified human insulin	Human insulin standard	Theoretical value
Asp	3.00	3.00	3
Thr	3.39	3.08	3
Ser	2.50	2.40	3
Glu	7.03	6.84	7
Pro	1.13	1.07	1
Gly	3.63	3.63	4
Ala	1.03	1.14	1
Cys	5.39	5.08	6
Val	3.55	3.63	4
Ile	1.72	1.72	2
Leu	6.49	6.63	6
Tyr	3.70	3.89	4
Phe	3.28	3.28	3
His	2.05	2.08	2
Lys	0.95	0.93	1
Arg	1.06	1.05	1

Discussion

This experiment was performed in order to decrease the refolding volume and increase the refolding yield. To slow down the aggregation process, refolding is usually performed at low protein concentrations, in a range of 10–100 μg/ml [14]. Before the optimal refolding condition was established in this study, the refolding reaction was performed with 0.5 g/l of protein and 0.1 mM β-mercaptoethanol. The demerit of performance of refolding at 0.5 g/l of protein was that the volume of refolding solution was very large. However, when the refolding was carried out at 1.5 g/l of protein with 0.3 mM β-mercaptoethanol, it was possible to considerably decrease the volume of refolding solution. In addition to the decrease of the volume of refolding solution, the refolding yield was increased by optimization of the refolding conditions for H27R-proinsulin. It is difficult to set up the refolding reaction conditions for specific proteins, because the refolding reaction is very dynamic. In this study, we set the optimal refolding conditions for H27R-proinsulin and confirmed the identity of refolded H27R-proinsulin and mature human insulin after enzyme reaction using RP-HPLC. We checked the hydropathic score of the expressed preproinsulin compared with proinsulin. A negative hydropathic score indicates a hydrophilic protein, whereas a positive value indicates a hydrophobic protein. The

addition of the leader peptide, H27R, on the proinsulin resulted in a more hydrophilic expressed preproinsulin. It is possible to make H27R-proinsulin more soluble and for it to be refolded without sulfonation. Using the direct refolding method, it is possible to decrease the total volume of refolding solution, to decrease the number of process steps for production of human insulin, and to increase the total production yield. Therefore, this production process may be a more viable process for human insulin production. After the enzyme reaction, 138.2 g of human insulin was produced from 200 L of fermentation broth using the production process. Thus, 0.691 g of human insulin was produced from 1 L of fermentation broth. It was confirmed that the purified recombinant human insulin was the same as human insulin standard.

In conclusion, the optimal refolding condition for H27R-proinsulin was 1.5 g/l of protein, 0.3 mM β-mercaptoethanol, 0.6 M urea, 10 mM glycine, and pH 10.6 at 15°C for 16 h. The leader peptide of H27R-proinsulin was more hydrophilic than Met-proinsulin. Therefore, direct refolding of H27R-proinsulin was possible without sulfonation. The average amount of produced human insulin was 138.2 g from a 200 L pilot-scale fermentation broth.

Acknowledgments

This study was financially supported by the Ministry of Education(MOE) and National Research Foundation of Korea(NRF) through the Human Resource Training Project for Regional Innovation(2015H1C1A1035828) and Sangji University Research Fund, 2014.

References

1. Bailey SM, Meagher MM. 2000. Separation of soluble protein from inclusion bodies in *Escherichia coli* lysate using crossflow microfiltration. *J. Membr. Sci.* **166:** 137-146.
2. Bowden GA, Paredles AM, Georgiou G. 1991. Expression vectors based on the *rac* fusion promoter. *Gene* **42:** 97-100.
3. Castellanos Serra LR, Hardy E, Ubieta R, Vispo NS, Fernandez C, Besada V, *et al*. 1996. Expression and folding of an interleukin-2-proinsulin fusion protein and its conversion into insulin by a single step enzymatic removal of the C-peptide and the N-terminal fused sequence. *FEBS Lett.* **378:** 171-176.
4. Clark EDB. 1998. Refolding of recombinant proteins. *Curr. Opin. Biotechnol.* **9:** 157-163.
5. Clark EDB. 2001. Protein refolding for industrial processes. *Curr. Opin. Biotechnol.* **12:** 202-207.
6. Cousens LS, Shuster JR, Gallegos C, Ku L, Stempien MM, Urdea MS, *et al*. 1987. High level expression of proinsulin in the yeast *Saccharomyces cerevisiae*. *Gene* **61:** 265-275.
7. Cowley DJ, Mackin RB. 1997. Expression, purification and characterization of recombinant human proinsulin. *FEBS Lett.* **402:** 124-130.
8. Enfors SO. 1992. Control of *in vivo* proteolysis in the production of recombinant proteins. *TIBTECH* **10:** 310-315.
9. Fischer BH, Sumner I, Goodenough P. 1993. Isolation, renaturation, and formation of disulfide bonds of eukaryotic proteins expressed in *Escherichia coli* as inclusion bodies. *Biotechnol. Bioeng.* **41:** 3-13.
10. Gu Z, Weidenhaupt M, Ivanova N, Pavlov M, Xu B, Su ZG, Janson JC. 2002. Chromatographic methods for the isolation of, and refolding of proteins from *Escherichia coli* inclusion bodies. *Protein Exp. Purif.* **25:** 174-179.
11. Kim CK, Lee SM, Jeong SM. 2010. High cell density cultivation of recombinant *Escherichia coli* for production of rat procarboxypeptidase B. *Food Sci. Biotechnol.* **19:** 1627-1633.
12. Kyte J, Doolittle RF. 1982. A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.* **157:** 105-132.
13. Lee SY, Oh SJ, Kim CK, Son YJ, Park KH, Min CK, *et al*. 2006. Plasmids expressing human insulin and the preparation method for human insulin thereby. United States Patent Application Publication US 2006, 0035316 A1.
14. Lilie H, Schwarz E, Rudolph R. 1998. Advances in refolding of proteins produced in *E. coli*. *Curr. Opin. Biotechnol.* **9:** 497-501.
15. Linde S, Welinder BS, Nielsen JH. 1993. Analysis of proinsulin and its conversion products by reversed-phase high-performance liquid-chromatography. *J. Chromatogr. B* **614:** 185-204.
16. Markussen J. 1985. Comparative reduction/oxidation studies with single chain des-(B30) insulin and porcine proinsulin. *Int. J. Pept. Protein Res.* **25:** 431-434.
17. Middelberg APJ, O'Neill BK. 1991. Monitoring the centrifugal recovery of recombinant protein inclusion bodies. *Aust. J. Biotechnol.* **5:** 87-92.
18. Middelberg APJ. 2002. Preparative protein refolding. *Trends Biotechnol.* **20:** 437-443.
19. Mukhopadhyay A. 1997. Inclusion bodies and purification of proteins in biologically active forms. *Adv. Biochem. Eng. Biotechnol.* **56:** 61-109.
20. Murray HD, Appleman JA, Gourse RL. 2003. Regulation of the *Escherichia coli rrnB* P2 promoter. *J. Bacteriol.* **185:** 28-34.
21. 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.
22. Nilsson J, Jonasson P, Samuelsson E, Stahl S, Uhlen M. 1996. Integrated production of human insulin and its C-peptide. *J. Biotechnol.* **48:** 241-250.
23. Qiao ZS, Guo ZY, Feng YM. 2001. Putative disulfide-forming pathway of porcine insulin precursor during its refolding *in vitro*. *Biochemistry* **40:** 2662-2668.
24. Rudolph R, Lilie H. 1996. *In vitro* folding of inclusion body

proteins. *FASEB J.* **10:** 49-56.
25. Schnaitman CA. 1971. Effect of ethylene diamine tetraacetic acid, Triton X-100, and lysozyme on the morphology and chemical composition of isolated cell walls of *Escherichia coli. J. Bacteriol.* **108:** 553-563.
26. Schutte H, Kula MR. 1990. Pilot- and process-scale techniques for cell disruption. *Biotechnol. Appl. Biochem.* **12:** 599-620.
27. Son YJ, Kim CK, Choi BT, Park YC, Seo JH. 2008. Effects of β-mercaptoethanol and hydrogen peroxide on enzymatic conversion of human proinsulin to insulin. *J. Microbiol. Biotechnol.* **18:** 983-989.
28. Son YJ, Kim CK, Kim YB, Kweon DH, Park YC, Seo JH. 2009. Effects of citraconylation on enzymatic modification of human proinsulin using trypsin and carboxypeptidase B. *Biotechnol. Prog.* **25:** 1064-1070.
29. Son YJ, Park KH, Lee SY, Oh SJ, Kim CK, Choi BT, *et al*. 2007. Effect of temperature shift strategies on human preproinsulin production in the fed-batch fermentation of recombinant *E. coli. Biotechnol. Bioprocess Eng.* **12:** 556-561.
30. Tikhonov RV, Pechenov SE, Belacheu IA, Yakimov SA, Klyushnichenko VE, Boldireva EF, *et al*. 2001. Recombinant human insulin VIII. Isolation of fusion protein-S-sulfonate, biotechnological precursor of human insulin, from the biomass of transformed *Escherichia coli* cells. *Protein Exp. Purif.* **21:** 176-182.
31. Tsumoto K, Ejima D, Kumagai I, Arakawa T. 2003. Practical considerations in refolding proteins from inclusion bodies. *Protein Exp. Purif.* **28:** 1-8.
32. Walsh G. 2005. Therapeutic insulins and their large-scale manufacture. *Appl. Microbiol. Biotechnol.* **67:** 151-159.
33. Wang XM, Guo ZY. 2013. Recombinant expression, different downstream processing of the disulfide-rich anti-tumor peptide Ranpirnase and its effect on the growth of human glioma cell line SHG-44. *Biomed. Rep.* **5:** 747-750.
34. Yon JM, Betton JM. 1991. Protein folding *in vitro* and in the cellular environment. *Biol. Cell* **71:** 17-23.