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Improved Refolding of Recombinant Human Proinsulin from *Escherichia coli* in a Two-Stage Reactor System

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Abstract An improved method of refolding recombinant human proinsulin from *E. coli* was presented. It was based on a two-stage stirred tank reactor in which denatured proinsulin-s-sulfonate was mixed instantaneously with a reaction buffer in the first stage reactor, and then fed to the second stage reactor. The mixture was stirred further for a total of 30 h in the second stage reactor. In this system, unfavorable effects present due to the increase in reaction volume and protein concentration for protein refolding, which becomes significant in a large-scale operation, were avoided. Refolding yields of over 80% was obtained for achieving reaction volume of upto 50 l at a protein concentration of 1 mg/ml. The optimum urea concentration was 1 M. Refolding yield at the 1-l reaction volume and protein concentration of 0.5 mg/ml was increased about 2.5-fold, compared to that in a batch reactor. By increasing protein concentration in a two-stage refolding reaction, the cost for insulin production could be reduced, therefore, making this process economical.

Key words: Proinsulin, proinsulin-s-sulfonate, refolding, two-stage stirred tank reactor

The overexpression of recombinant protein in *E. coli* often results in the formation of insoluble protein aggregates called inclusion bodies [19]. To isolate the protein, the insoluble proteins must be separated from cellular componants mostly by homogenization, centrifugation, and washing with a detergent such as Triton X-100 [7, 15]. The proteins are then solubilized in a denaturant such as urea or guanidine hydrochloride [7, 20]. In most cases, reducing reagents are included to reduce the cysteine residues within the polypeptide to break oxidized disulfide bonds. The reduced, unfolded fusion protein must then be cleaved off chemically or enzymatically in order to remove the fusion protein part [21].

In the industrial process, the proteins containing cysteine residues for the formation of disulfide bridge are sometimes sulfonated with sulfite and tetrathionate to obtain the high efficiency level in refolding and for its stability [3, 9, 11, 28]. The sulfonated proteins must be refolded for the purpose of recovering their biological activities and native structures. The refolding of a polypeptide chain is a spontaneous process which is thermodynamically controlled and driven by hydrophobic interaction [30]. However, low refolding efficiency *in vitro* is the problem in the case of high concentrations of a protein and a large reaction volume, because the formation of insoluble aggregates occurs during refolding of protein due to the intermolecular hydrophobic interaction or intermolecular disulfide bond [5, 27]. Several methods have been used to improve the refolding efficiency by preventing aggregation of a protein. These include the refolding via a binding of proteins to a suitable gel [17, 24, 25], the refolding using polyethylene glycol, arginine, linear dextrins, urea, or guanidine as folding additives [1, 6, 13, 22, 26], the refolding in reversed micelles [12], the refolding by step addition of protein into the refolding solution [2], and the refolding in a plug flow-type reactor [27]. Among these methods, refolding in a flow-type reactor appears to be the method of choice in refolding of proinsulin since uniform mixing might be easily maintained.

To minimize the aggregation of proinsulin caused by high concentration of a protein and a large reaction volume, we have introduced a two-stage reactor in a refolding reaction and improved the efficiency of refolding as well. Human insulin was then successfully generated by a subsequent enzymatic conversion of the refolded proinsulin [14].

MATERIALS AND METHODS

Materials

DEAE-sephacel and HP2MG were purchased from Pharmacia Co. (Uppsala, Sweden) and Mitsubishi Co.

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(Tokyo, Japan), respectively. Cyanogen bromide and RP-chromatography columns were obtained from Acros Co. (New Jersey, U.S.A.) and Rockland Technologies Inc. (Newport, U.S.A.), respectively. Other chemicals including urea and human proinsulin used as a standard were from Sigma Chemical Co. (St. Louis, U.S.A.).

Cell Culture

The plasmid, pHHl, containing the *tac* promoter, modified β-galactosidase gene, and human proinsulin gene for higher expression of proinsulin fusion protein was transformed into *E. coli* JM109. *E. coli* JM109/pHHl was grown in a 30-l fermentor with 15 l of the medium containing 3.5 g NH_4Cl, 7.5 g KH_2PO_4, 7.5 g Na_2HPO_4, 0.17 g $MgSO_4 \cdot 7H_2O$, 7.5 g yeast extract, 5 g glucose, 0.032 g $FeSO_4 \cdot H_2O$, 0.008 g $MnSO_4 \cdot 7H_2O$, 0.0226 g $Al_2(SO_4)_3$, 0.0032 g $CoCl \cdot 6H_2O$, 0.0016 g $ZnSO_4 \cdot 7H_2O$, 0.0016 g $Na_2MoO_4 \cdot 2H_2O$, 0.0008 g $CuCl_2 \cdot 2H_2O$, and 0.0004 g H_3BO_4 per liter [8, 15, 16, 23, 29].

Purification of Proinsulin Fusion Protein

E. coli cells (1.46 kg wet wt.) were disrupted by the homogenization (APV Ranni) in 10 l of 1 mM EDTA, 50 mM phosphate buffer (pH 7.2), 1 mM β-mercaptoethanol. After centrifugation at 8,000 rpm for 30 min, the pellet was washed sequentially in 3 l of 1% Triton-X-100 and 1.5 l of distilled water and centrifuged again at 8,000 rpm for 30 min. The pellet was suspended by stirring in 1 l of 8 M urea, 20 mM Tris (pH 9.0), 1 mM EDTA, 0.4 M sodium sulfite, and 50 mM sodium tetrathionate at 4°C for 12 h. After centrifugation of the suspension at 12,000 rpm for 30 min, the supernatant was diluted ten-fold into cold water and the pH was adjusted to 5.5. The precipitated protein (37 g) was collected by centrifugation at 5,000 rpm for 30 min.

Preparation of Proinsulin-s-Sulfonate

The pellet was treated at 25°C for 12 h with 0.2 M CNBr in 1.5 l of 80% formic acid. After rotary evaporation to dryness, the residue was suspended in 2 l of 7 M urea, 20 mM Tris (pH 8.0), and 1 mM EDTA, and loaded onto a DEAE-sephacel column in the same buffer. The column was eluted using a salt gradient of 0 to 0.5 M NaCl in the same buffer. Proinsulin-s-sulfonate detected by HPLC analysis was eluted from 0.35 M to 0.45 M NaCl. The fractions containing proinsulin-s-sulfonate were pooled.

Refolding Reaction

Refolding in a batch reactor [2, 8]. The eluent containing proinsulin-s-sulfonate was diluted with 50 mM glycine buffer, pH 10.6, to a final protein concentration of 0.5 mg/ml. The pH was adjusted to 10.6 with 1 M NaOH, and β-mercaptoethanol (amount providing 1.5 equivalents of -SH per $-SO_3^-$) was added to the solution. Then, nitrogen gas was purged to remove any traces of oxygen and the solution was stirred at 4°C for 30 h. The refolding yield was monitored by using the HPLC analysis. After the reaction was completed, the solution was acidified with 2 M HCl to pH 3.0.

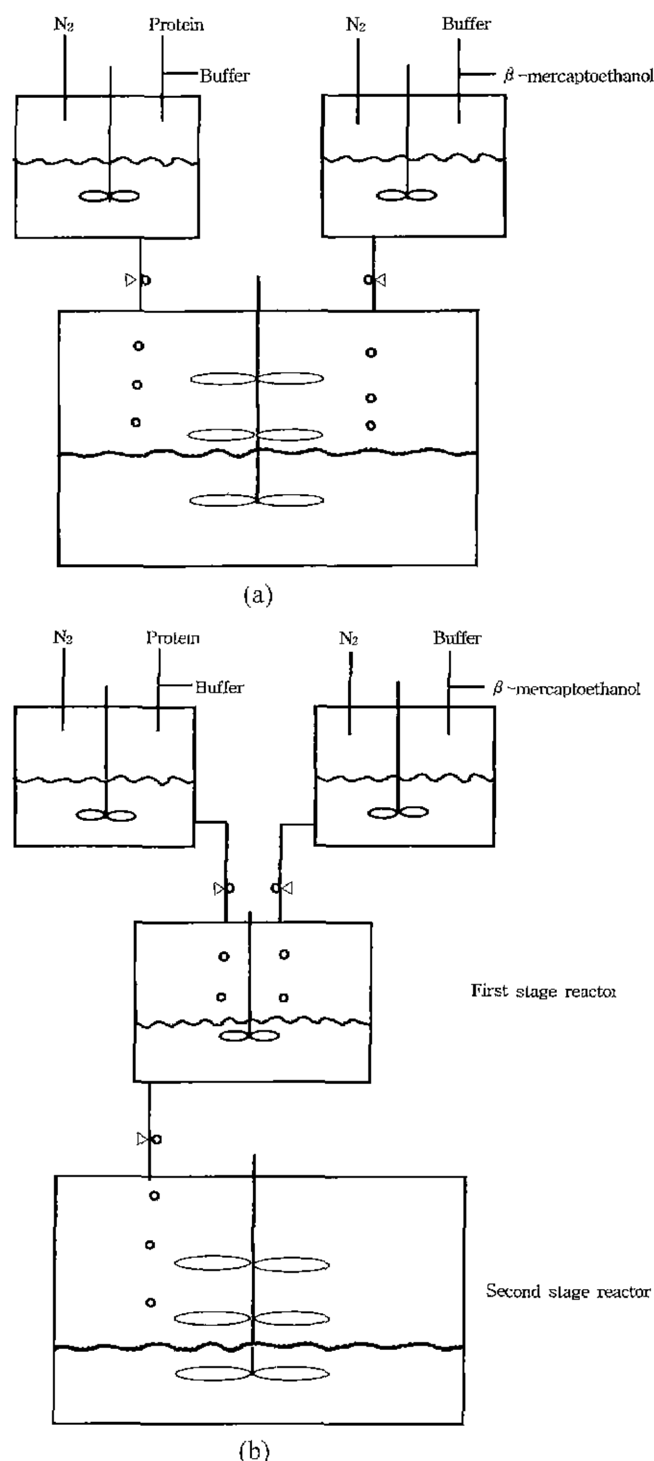

Fig. 1. Diagram of the refolding process.
A, Drop-wise fed-batch reactor; B, Two-stage reactor.

Refolding in a fed-batch reactor. A. Refolding by *using a stepwise system: The stepwise refolding reaction* was carried out using the eluent at a protein concentration of 0.1 mg/ml in the same procedure described above, with an exception of the concentration of proinsulin-s-sulfonate being increased by 0.1 mg every 2 h upto 0.5 mg/ml and this was achieved when proinsulin-s-sulfonate and β-mercaptoethanol were added.

B. Refolding by using a dropwise fed-batch system: The eluent containing proinsulin-s-sulfonate was diluted with 50 mM glycine buffer to a final protein concentration of 1.0 mg/ml and the pH was adjusted to 10.6 with 1 M NaOH. Then, nitrogen gas was purged. β-Mercaptoethanol was added to 50 mM glycine buffer in another feed reservoir. The two solutions were fed into a stirred tank reactor at the same flow rate while being slowly stirred and reacted for a total of 30 h at 4°C (Fig. 1A). The refolding yield was monitored by HPLC analysis. When the reaction was completed, the solution was acidified with 2 M HCl to pH 3.0.

Refolding in a two-stage reactor. The two solutions of the eluent containing proinsulin-s-sulfonate and 50 mM glycine buffer containing β-mercaptoethanol were prepared in the same procedure as described above, unless otherwise specified. The two solutions were fed at the same flow rate into the first-stage reactor while being vigorously stirred. Then, the mixture from the first-stage reactor was *continuously fed into the second-stage reactor (Fig. 1B).* According to the residence time and the reaction volume of the first-stage reactor, the flow rate feeding into the second-stage reactor was different. The mixture was stirred again for as long as 30 h in the second-stage reactor. The refolding yield was monitored by HPLC analysis. After the reaction was completed, the solution was acidified with 2 M HCl to pH 3.0.

Analysis of Proinsulin-s-Sulfonate and Refolded Proinsulin

Proinsulin-s-sulfonate was analyzed by using reversed-phase HPLC (column, Zorbax TMS, 5 µm, 4.6 mm× 250 mm), eluting with a linear gradient from 20 to 29% acetonitrile in 50 mM phosphate buffer, pH 6.0, containing 1% 2-methoxyethanol; flow rate, 1.0 ml/min; detection, at $A_{210\,nm}$ [4]. Refolded proinsulin was analyzed by using reversed-phase HPLC (column, Zorbax TMS 5 µm, 4.6 mm× 250 mm) eluting with a linear gradient from 20 to 29% acetonitrile in 50 mM phosphate buffer, pH 2.0, containing 1% 2-methoxyethanol; flow rate, 1.0 ml/min; detection, $A_{210\,nm}$.

Calculation of Refolding Yield

The yields of refolded proinsulin were calculated based on the amount of proinsulin-s-sulfonate as quantitated by the RP-HPLC peak areas.

Protein Determination

Total protein was determined by the Bio-Rad protein assay kit that was standardized against bovine serum albumin.

RESULTS AND DISCUSSION

Preparation of Proinsulin-s-Sulfonate

During the fermentation process, *E. coli* JM109/pHH1 cells were induced by 0.5 mM isopropyl β-D-thiogalactopyranoside to overexpress the proinsulin fusion protein. The expression level of the fusion protein was about 30% of the total protein, which was analyzed by SDS-PAGE (Fig. 2). After cell disruption, the inclusion bodies of the fusion protein were solubilized with 8 M urea instead of using guanidine HCl in order to minimize the cost, and then sulfonated with sulfite and tetrathionate to simplify the preparative process. The sulfonated fusion protein was chemically cleaved by the CNBr treatment and purified using the anion-exchange chromatography. The fractions containing proinsulin-s-sulfonate were collected. The purity was over 85%.

Refolding of Proinsulin-s-Sulfonate in a Batch Reactor

The refolding reaction was performed in a batch reactor where the reaction volume was varied from 10 ml to 1,000 ml at a molar ratio of -SH to $-SSO_3^-$ of 1.5. When the reaction volume was increased to more than 50 ml, the *refolding yield decreased dramatically* (Fig. 3). *This might* be due to the increase of intermolecular interactions as the reaction volume rises.

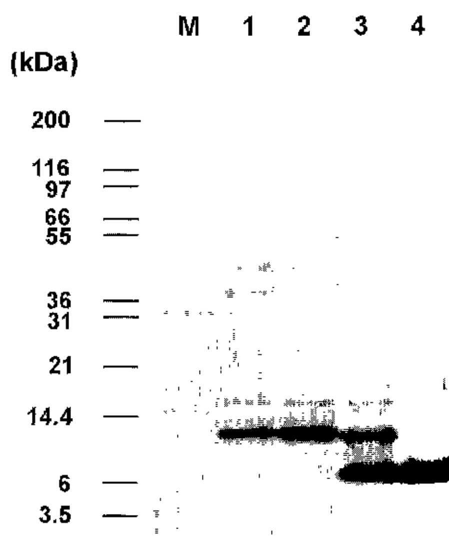

Fig. 2. 10~20% SDS-PAGE analysis of the purification of proinsulin-s-sulfonate.

Lanes: M, molecular-mass marker, 1 whole cell; 2, sulfonated fusion protein, 3, CNBr-cleaved fusion protein; 4, proinsulin-s-sulfonate after ion-exchange chromatography.

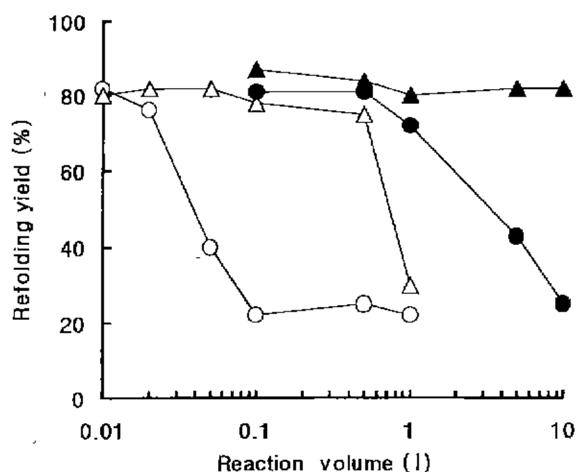

Fig. 3. Effect of reaction volume on refolding yield.
Batch system (○-○), Stepwise system (△-△), Dropwise fed-batch system (●-●), Two-stage system (▲-▲). The final reaction mixture consisted of 0.5 mg/ml protein, 50 mM glycine buffer (pH 10 6) and β-mercaptoethanol (amount providing 1.5 equivalents of -SH per $-SO_3^-$). The reaction mixture was reacted at 4°C for 30 h

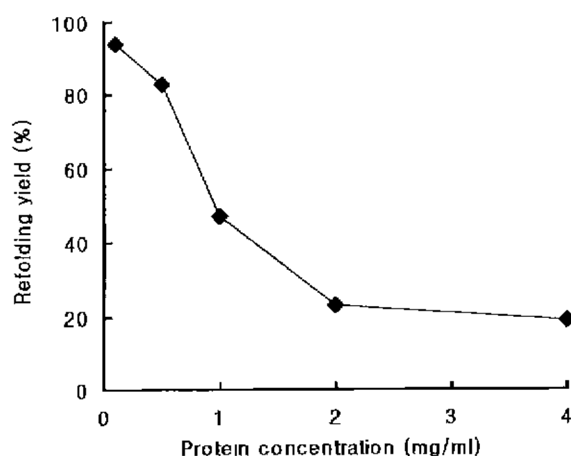

Fig. 4. Effect of protein concentration on refolding yield in a two-stage reactor.
The volume of the first-stage reaction was 10 ml and the residence time was 10 s. The volume of the second-stage reaction was 10 l. The mixture was kept at 4°C for 20 h Proinsulin-s-sulfonate (0 2, 1 2, 4, and 8 mg/ml) and β-mercaptoethanol were mixed at a 1.1 ratio. The final reaction mixture consisted of 0.1, 0.5, 1, 2, and 4 mg/ml protein, 50 mM glycine buffer (pH 10.6), and β-mercaptoethanol (amount providing 1.5 equivalents of -SH per $-SO_3^-$).

Refolding of Proinsulin-s-Sulfonate in a Fed-Batch Reactor

To reduce the protein aggregation caused by increased reaction volume, proinsulin-s-sulfonate and β-mercaptoethanol were added in a step-wise mode every 2 h to gradually increase the protein concentration. By this method, the refolding yield became more than 77% up to 500 ml (Fig. 3). This improvement may be due to the blocking of intermolecular interactions by the gradual increase of the protein concentration in a step-wise mode [2]. However, the refolding yield decreased when the reaction volume exceeded 500 ml. To minimize the aggregation caused by increased volume reaction, the reaction components were continuously added to the reactor as shown in Fig. 1A. Figure 3 shows that the refolding yield is 80% up to a 1 l volume. However, the refolding yield decreased dramatically when the reaction volume increased to more than 1 l (Fig. 3). The low yield that was observed in a large volume reaction might be due to uneven mixing and heterogeneity of refolding [27].

Refolding of Proinsulin-s-Sulfonate in a Two-Stage Reactor System

For avoiding non-uniform mixing that happens as the reaction volume increases, a two-stage reactor system was incorporated in order to mix the reaction components instantaneously in a fast mixing chamber (10 ml), the first-stage reactor, to minimize the mixing time. The mixed solution was then fed into the second-stage reactor (Fig. 1B). According to the result, the refolding yield remained relatively constant even when the reaction volume increased to more than 5 l (Fig. 3). In this two-stage reactor, refolding yield (80%) at a 1-l reaction volume and protein concentration of 0.5 mg/ml was increased by about 2.5-fold, compared to that (23%) in a batch reactor. This yield is comparable to what was reported in the case of lysozyme refolding using a plug flow-type reactor system [27].

The effect of protein concentration on the refolding yield has been shown to be inversely correlated (Fig. 4). The refolding yield sharply decreased as the protein concentration increased. Refolding yield dropped to as much as 30% or less when the protein concentrations were more than 2 mg/ml. The effect of molar ratio of -SH to $-SO_3^-$ on the refolding yield at protein concentration of 0.5 mg/ml and 1 mg/ml is shown in Fig. 5. The maximum refolding yield at 1 mg/ml was at a molar ratio of 1.25, compared to the ratio of 1.5 at 0.5 mg/ml. To minimize protein aggregation and intermolecular interaction at a protein concentration higher than 0.5 mg/ml, the refolding reaction was performed in a two-stage reactor after the chaotropic agent, urea, was added to the feed stream containing glycine buffer (Fig. 6). In addition, the refolding yield increased as the urea concentration increased.

The effects of the mixing volume and residence time of the first-stage reactor for the optimum reaction condition were also examined. The refolding yield remained relatively constant up to 1 l reaction volume, but then decreased thereafter (Fig. 7). This result was similar to that in a drop-wise fed-batch reactor. The refolding yield also decreased as the residence time increased to over 1,000 s (Fig. 8).

Among many factors, protein aggregation by intermolecular hydrophobic interaction or intermolecular disulfide bond formation affects the refolding yield directly [5]. This aggregation can occur as both protein concentration and reaction volume increase in an industrial scale [8, 27]. The

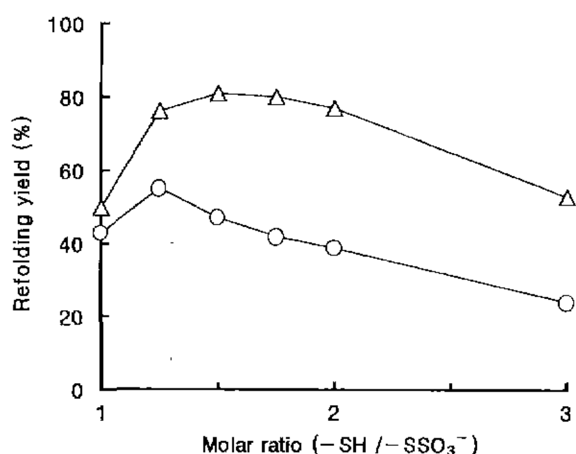

Fig. 5. Effect of -SH:-SSO_3^- molar ratio on refolding yield in a two-stage reactor.

O-O, protein concentration 1 mg/ml; △-△, protein concentration 0.5 mg/ml. The volume of the first-stage reaction was 10 ml and the residence time was 10 s. The volume of the second-stage reaction was 10 l. The mixture was kept at 4°C for 20 h. Proinsulin-s-sulfonate (1, and 2 mg/ml) and β-mercaptoethanol were mixed at a 1:1 ratio. The final reaction mixture consisted of 0.5 or 1 mg/ml protein, 50 mM glycine buffer (pH 10.6), and β-mercaptoethanol

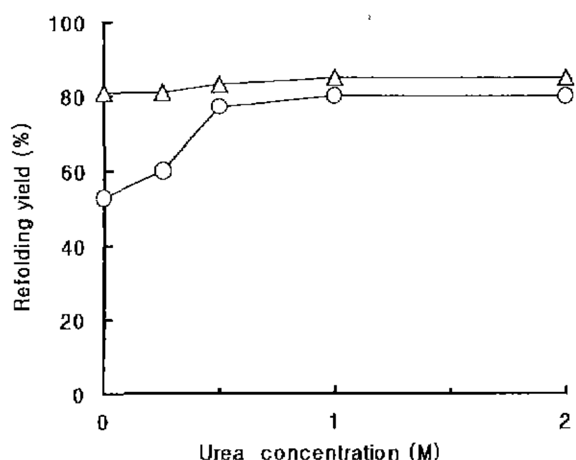

Fig. 6. Effect of urea concentration on refolding yield in a two-stage reactor.

O-O, protein concentration 1 mg/ml; △-△, protein concentration 0.5 mg/ml. The volume of the first-stage reaction was 10 ml and the sample residence time was 10 s. The volume of the second-stage reaction was 10 l. The mixture was kept at 4°C for 20 h. Proinsulin-s-sulfonate (1 and 2 mg/ml) and 0.1 M glycine buffer containing urea and β-mercaptoethanol were mixed at a 1:1 ratio. The final reaction mixture consisted of 0.5 or 1.0 mg/ml protein, 50 mM glycine buffer (pH 10.6), urea (0.25, 0.5, 1, and 2 M), and β-mercaptoethanol (amount providing 1.5 and 1.25 equivalents of -SH per -SSO_3^-).

nonuniform mixing caused by increased reaction volume can be resolved by using a two-stage reactor system that was described in this study. In the case of high protein concentration, urea could be used as a chaotropic agent to interfere with intermolecular hydrophobic interactions, since urea is relatively inexpensive and easy to remove once refolding takes place [10].

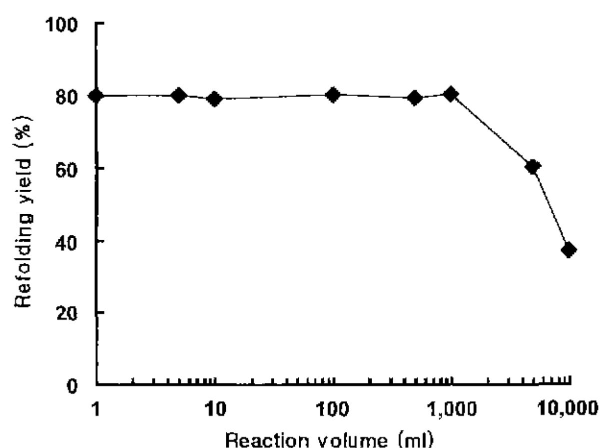

Fig. 7. Effect of reaction volume of the first-stage reactor on refolding yield.

The residence time was 10 s. According to the reaction volume of the first-stage reactor, the flow rate feeding into the second-stage reactor was different. The volume of the second-stage reaction was 10 l and the mixture was kept at 4°C for 20 h. Proinsulin-s-sulfonate (2 mg/ml) and 0.1 M glycine buffer containing urea and β-mercaptoethanol were mixed at a 1:1 ratio. The final reaction mixture consisted of 1 mg/ml protein, 50 mM glycine buffer (pH 10.6), 1 M urea, and β-mercaptoethanol (amount providing 1.25 equivalents of -SH per -SSO_3^-).

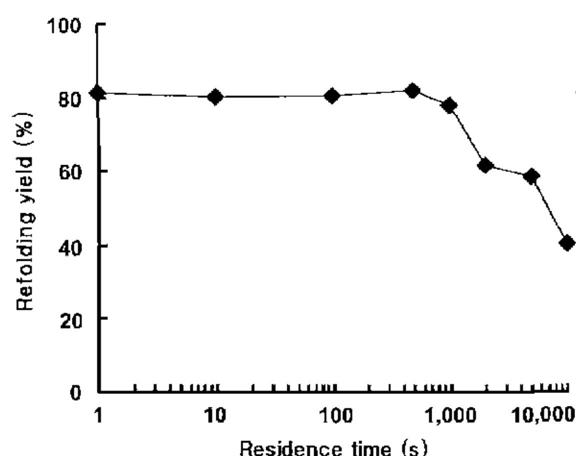

Fig. 8. Effect of residence time of the first-stage reactor on refolding yield.

The volume of the first-stage reactor was 1 ml. The volume of the second-stage reaction was 10 l and the mixture was kept at 4°C for 20 h. Proinsulin-s-sulfonate (2 mg/ml) and 0.1 M glycine buffer containing urea and β-mercaptoethanol were mixed at a 1:1 ratio. The final reaction mixture consisted of 1 mg/ml protein, 50 mM glycine buffer (pH 10.6), 1 M urea, and β-mercaptoethanol (amount providing 1.25 equivalents of -SH per -SSO_3^-).

CONCLUSION

Insulin has been successfully produced from recombinant *E. coli*, preferentially in an insoluble form of proinsulin fusion protein which was solubilized with sulfonation, cleaved chemically with CNBr, refolded, and then enzymatically cleaved to remove C-peptide. Although the refolding reaction is yet to be optimized, our study shows that proinsulin-s-

sulfonate was refolded at a high yield of 80% in a two-stage reactor at a high protein concentration of 1 mg/ml and in a large reaction volume of upto 50 l. In addition, purified insulin was analyzed by several methods, such as amino acid sequence analysis, peptide mapping, NMR, mass spectroscopy, and HPLC after the enzymatic treatment of refolded proinsulin. The analytical data showed that purified insulin was identical to the pancreatic and the standard USP human insulin. Therefore, this process could allow us to produce human insulin effectively and economically.

REFERENCES

1. Arora, D. and N. Khanna. 1996. Method for increasing the yield of properly folded recombinant human gamma interferon from inclusion bodies. *J. Biotech.* **52:** 127-133.
2. Buchner, J., I. Pastan, and U. Brinkmann. 1992. A method for increasing the yield of properly folded recombinant fusion proteins: Single-chain immunotoxins from renaturation of bacterial inclusion bodies. *Anal. Biochem.* **205:** 263-270.
3. Cabilly, S., A. D. Riggs, H. Pande, J. E. Shively, W. E. Holmes, M. Rey, L. J Perry, R. Wetzel, and H. L. Hoyneker. 1984. Generation of antibody activity from immunoglobulin polypeptide chains produced in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **81:** 3273-3277.
4. Chance, R. E., E. P. Kroeff, J. A. Hoffmann, and B. H. Frank. 1981. Chemical, physical, and biologic properties of biosynthetic human insulin. *Diabetes Care* **4:** 147-153.
5. Clark, E. D. B., D. Hevehan, S. Szela, and J. M. Reddy. 1998. Oxidative renaturation of hen egg-white lysozyme. Folding vs aggregation. *Biotechnol. Prog.* **14:** 47-54.
6. Cleland, J. L. and D. I. C. Wang. 1990. Cosolvent assisted protein refolding. *Bio/Technol.* **8:** 1274-1278.
7. Fisher, B., I. Sumner, and P. Goodenough. 1993 Isolation, renaturation, and formation of disulfide bonds of eukaryotic proteins expressed in *Esherichia coli* as inclusion bodies. *Biotechnol. Bioeng.* **41:** 3-13.
8. Frank, B. H. 1984. Process for producing an insulin precursor. United State patent USP 4,430,266.
9. Furman, T. C., J. Epp, H. M. Hsiung, J. A. Hoskins, G. L. Long, L. G. Mendelson, B. Schoner, D. P. Smith, and M. C. Smith 1987. Recombinant human insulin-like growth factor expressed in *Escherichia coli*. *Bio/Technol.* **5:** 1047-1051.
10. Goeddel, D. V., D. G. Kleid, and K. Itakura. 1982. Human proinsulin and analogs thereof and method of preparation by microbial polypeptide expression and conversion thereof to human insulin. European patent EP 0,055,945.
11. Goeddel, D. V., D. C. Kleid, F. Bolivar, H. L. Heyneker, D. G. Yansura, R. Crea, T. Hirose, A. Kraszewski, K. Itakura, and A D. Riggs. 1979. Expression in *Escherichia coli* of chemically synthesized genes for human insulin. *Proc. Natl. Acad. Sci. USA* **76:** 106-110.
12. Hagen, A. J., T. A. Hatton, and D I. C Wang. 1990. Protein refolding in reversed micelles. *Biotechnol. Bioeng.* **35:** 965-995.
13. Hart, R. A., D. M Giltinan, P. M. Lester, D. H. Reifsnyder, J. R. Ogez, and S. E. Bulider. 1994. Effect of environment on insulin-like growth factor I refolding selectivity. *Biotechnol. Appl. Biochem.* **20:** 217-232.
14. Kemmler, W., J. D. Peterson, and D. F. Steiner. 1971. Studies on the conversion of proinsulin to insulin. *J. Biol. Chem.* **246:** 6786-6791.
15. Kim, C. S., C. I. Kim, J. H. Seo, K. H. Choi, and Y. C. Park. 1997. Fed-batch fermentations of recombinant *Escherichia coli* to produce *Bacillus macerans* CGTase. *J. Microbiol. Biotechnol.* **7:** 323-328.
16. Konstantinov, K. B., N. Nishio, and T Yoshida. 1990. Glucose feeding strategy accounting for the decreasing oxidative capacity of recombinant *Escherichia coli* in fed-batch cultivation for phenylalanine production. *J Ferment. Bioeng.* **70:** 253-260.
17. Light, A 1985. Protein solubility, protein modifications, and protein folding. *Biotechniques* **3:** 298-306.
18. Middelberg, A. P. J. 1996. Large-scale recovery of recombinant protein inclusion bodies expressed in *Escherichia coli*. *J. Microbiol. Biotechnol.* **6:** 225-231.
19. Mitraki, A. and J. King. 1989. Protein folding intermediates and inclusion body formation. *Bio/Technol.* **7:** 690-697.
20. Mukhopadhyay, A. 1997. Inclusion bodies and purification of proteins in biologically active forms. *Adv. Biochem. Eng. Biotechnol.* **56:** 61-109.
21. Nygren, P.-A., S. Stahl, and M. Uhlen. 1994. Engineering proteins to facilitate bioprocessing. *TIBTECH.* **12:** 184-188.
22. Orsini, G. and M. E. Goldberg. 1978. The renaturation of reduced chymotrypsinogen A in guanidine HCl. Refolding versus aggregation. *J. Biol. Chem.* **253:** 34-39.
23. Paalme, T., K. Tiisma, A. Kahru, K. Vanatalu, and R. Vilu. 1990. Glucose-limited fed-batch cultivation of *Escherichia coli* with computer-controlled fixed growth rate. *Biotechnol. Bioeng.* **35:** 312-319.
24. Samuelsson, E., H Wadensten, M. Hartmanis, T. Moks, and M. Uhlen. 1991. Facilitated *in vitro* refolding of human recombinant insulin-like growth factor I using a solubilizing fusion partner. *Bio/Technology* **9:** 363-366.
25. Sinha, N. K. and A. Light. 1975. Refolding of reduced, denatured trypsinogen and trypsin immobilized on agarose beads. *J. Biol. Chem.* **250:** 8624-8629.
26. Sivakama, S. C., B. Raman, and D. Balasubramanian. 1999. Artificial chaperoning of insulin, human carbonic anhydrase and hen egg lysozyme using linear dextrin chains. *FEBS Lett.* **443:** 215-219
27. Terashima, M., K. Suzuki, and S. Katoh. 1996. Effective refolding of fully reduced lysozyme with a flow-type reactor. *Process Biochem.* **31:** 341-345.
28. Wetzel, R., D. G. Keid, R. Crea, H. L. Heyneker, D. G. Yansura, T. Hirose, A Kraszewski, A. D. Riggs, K. Itakura, and D. V. Goeddel. 1981. Expression in *Escherichia coli* of a chemically synthesized gene for a "mini-c" analog of human proinsulin. *Gene* **16:** 63-76.
29. Yoon, S. K., W. K. Kang, and T. H. Park. 1996. Regulation of trp promoter for production of bovine somatotropin in recombinant *Escherichia coli* fed-batch fermentation. *J Ferment. Bioeng.* **81:** 153-157.
30. You, J. M. and J. M. Betton. 1991. Protein folding *in vitro* and in the cellular environment. *Biol. Cell* **71:** 17-23.