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Production of Human Insulin in an *E. coli* System with Met-Lys-Human Proinsulin as the Expressed Precursor

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ABSTRACT

The construction of a gene encoding Lys-human proinsulin, its direct expression in *E. coli*, and the simple purification procedure are described here. The temperature inducible promotor was employed for induction in a very short time. The expression level could reach 20–30%. After simple downstream processing and only one step of Sephadex G50 purification, 150 mg recombinant Lys-human proinsulin with a purity of up to 90% could be obtained easily from 1 L of high density fermentation medium. The obtained product is in the form of Met-Lys-human proinsulin because of the failure of the bacterial host to remove the initiator methionine residue. The Lys-human proinsulin could be changed into human insulin by trypsin and carboxypeptidase B treatment in later steps. After separation with DEAE-Sephadex A25, human insulin with expected amino acid composition and full native biological activity could be obtained with a yield of 50 mg/L of fermentation medium.

Index Entries: Human insulin; expression; *E. coli*; purification.

INTRODUCTION

The use of insulin in the treatment of diabetes has been one of the most significant successes of pharmaceutical science. In the past decade, human insulin produced by recombinant DNA technology turned out to be more and more dominant in insulin manufacture and marketing (*1*).

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The major problem in the production of small proteins or peptides by recombinant DNA techniques is their short half-life in the host cell (*2,3*). The half-life for rat proinsulin has been reported to be only 2 min in *E. coli* (*2*). In order to increase the stability of expressed product in *E. coli*, the expression in fusion protein was widely employed. Although the expression level of the fusion protein is quite good, the final yield of recombinant human insulin is still not satisfactory because of the complicated purification procedures (*4–8*). No report has been presented with an acceptable yield in intact human proinsulin expressed in *E. coli* (*3,6*) until recently an inverted A-C-B human proinsulin molecule, with the carboxyl terminus of A-chain connected to the amino terminus of B-chain by a connecting peptide, thus leaving the critical N-terminal of GlyA1 residue free. This has been reported to be expressed with an expression level of 10% (*9*). However, the downstream process is still complicated with sulfitolysis, dialysis, anion-exchange chromatography, and reversed-phase high-performance liquid chromatography that are quite expensive and time-consuming from the manufacturing view. Meanwhile, the transformation of A-C-B human proinsulin to human insulin is more difficult than that of human proinsulin to human insulin because of the possible problem of steric hindrance for the enzymatic cleavage in the AsnA21 region. Furthermore, the N-terminus of the expressed A-C-B human proinsulin product is not homogeneous, with one-fifth of A-C-B proinsulin and four-fifths of Met-A-C-B proinsulin, because of the failure of the host cell to remove the N-terminal initiator methionine residue.

In our previous work, the direct expression of the gene encoding human proinsulin was achieved with an expression level of 20–30% under the control of a P_RP_L promoter in a very short induction time (*10*). However, the expressed product was in the form of Met-proinsulin, not acceptable for the production of human insulin. So did our other directly expressed proinsulin analogs (*11–13*). In the present work, the gene encoding human proinsulin was modified by adding a code for lysine between the initiator ATG and the code for N-terminal PheB1 residue of proinsulin by polymerase chain reaction (PCR) mutagenesis. Thus, the methionine could be eliminated during the conversion of Lys-human proinsulin to human insulin. Furthermore, this newly constructed gene also can be used for human insulin analogs production in *E. coli* system. The direct high level expression of Lys-proinsulin gene in *E. coli* and the simplified way of the target product purification is described.

MATERIALS AND METHODS

Modification of the Gene Encoding Human Proinsulin

The plasmid pBCA containing synthetic gene encoding human proinsulin was a gift from Tongjian Shen of the Institute of Biophysics, Academia Sinica, originally provided by Ray Wu (*14*). The proinsulin gene

was cleaved with *Eco*RI and *Bam*HI and cloned into M13mp19. The mutagenesis procedure was mainly according to the PCR method described elsewhere (*15*), with single-strand DNA as a template. Insertion mutagenesis primer (26-mer): 5′-GGAATTCCGGATGAAGTTTGTCAATC-3′ was used with the addition of AAG (encoding Lys) between ATG (initiator Met) and TTT (encoding PheB1), meanwhile there was an *Eco*RI site at the 5′-terminus. The other oligonucleotide primer was M13 primer 1211 (Biolabs, 17-mer): 5′-GTAAAACGACGGCCAGT-3′. After cloned into the expression vector, the mutation was confirmed by DNA sequencing.

Construction of Expression Vector

The 3.66-kb expression vector pBV220, which contains a P_RP_L promoter, cIts857 gene, multiple cloning sites, and two strong transcription terminators, was a gift from Yunde Hou of the Institute of Virology, Chinese Academy of Preventive Medicine (*16*). When an exogenous gene is inserted into the multiple cloning sites with a translation start codon ATG, nonfusion protein can be expressed by temperature induction. The direct expression plasmid pVK100 for Lys-human proinsulin was then constructed by the insertion of the gene encoding Lys-human proinsulin obtained by PCR and treated with *Eco*RI and *Bam*HI into the multiple cloning sites of pBV220. The constructed plasmid pVK100 was used to transform *E. coli* strain DH5α.

Cell Culture

As for the culture of the recombinant *E. coli* strain in a tube, one bacterial colony was inoculated into 300 μL of LB medium containing 50 μg/mL of ampicillin (Amp), shaken vigorously at 30°C overnight. After transfering into 3 mL LB, the cell culture was kept at 30°C for 4 h, then the temperature was increased to 42°C for another 4 h for the induction. The scale-up production of cells for subsequent processing in a 5-L fermentor was as follows. A single bacterial colony was transferred into 3 mL of LB medium (Amp+), vigorously shaken at 30°C for 12 h, then transferred into 300 mL of LB (Amp+). Another 12 h later, the bacterial culture was put into the fermentor containing 3 L of M9 minimal medium (Amp+) with 18 g of tryptone and 12 g of yeast extract. One hundred fifty grams of yeast extract and 150 g of glucose in 750 mL water was added linearly during the later fermentation. When OD_{600} reached 40, the temperature was raised to 42°C to initiate the induction for 1 h. The wet cell pellet was harvested by 80 g/L and was kept at −20°C.

Purification and Processing of Recombinant Lys-Human Proinsulin

The expressed human proinsulin could be simply purified as follows. Wet cell pellet was thawed in 5 vol of lysis buffer (0.05*M* Tris-HCl, 5% Triton, 8% sucrose, 0.05*M* EDTA, pH 8.0) and lysed by sonication. The

lysed pellet was collected by centrifugation at 10,000*g* and suspended by stirring overnight at 10°C with 4 vol of 8*M* urea (or 6*M* guanidine-HCl), 1 m*M* DTT, in 0.1*M* Tris-HCl, pH 8.0. After centrifugation, DTT was added to the supernatant to give a concentration of 10 m*M* and the mixture was incubated at 37°C for 1 h. Then the mixture was diluted five times with cold water, with the pH adjusted to 4.5, and placed at 4°C for 2 h. The precipitate was collected by centrifugation, and quickly dissolved in 50 mL of cold water with the pH adjusted to 10–12. Then the solution was diluted with 2 L of 0.05*M* Gly-NaOH buffer, pH 10.8 and kept at 4°C overnight for refolding. After ultrafiltration, the sample was loaded onto a Sephadex G50 (fine) column (4 × 100 cm), and eluted with the same buffer. The Lys-human proinsulin fraction was pooled, ultrafiltrated, and lyophilized. The yield was up to 150–200 mg/80 g wet cell pellet. For conversion of Lys-human proinsulin to human insulin, 100 mg of Lys-proinsulin was treated with 1 mg of trypsin and 300 μg of carboxypeptidase B in 10 mL of 0.08*M* Tris-HCl buffer, pH 7.5 at 37°C for 30 min (*17,18*). Seven milliliters of propanol was added and the mixture immediately was loaded onto a DEAE-Sephadex A25 column (1.6 × 20 cm) and eluted with 400 mL of 0.05*M* Tris-HCl, 40% propanol, pH 7.5, with a linear NaCl gradient from 0–0.1*N*. Human insulin fraction was pooled. After evaporation of propanol, solid NaCl was added to a concentration of 25%, and the pH was adjusted to 2.0 with 4*N* HCl. The pellet was collected with a recovery of up to 50% of human insulin protein. The amino acid composition analysis was done as described previously (*19*). Receptor binding (*19*) and insulin radioimmunoassays were employed for the activity determination of the recombinant human insulin with porcine insulin as the standard.

RESULTS AND DISCUSSION

Construction of Expression Plasmid

It has been shown previously in our laboratory that the directly expressed human proinsulin in *E. coli* has an additional methionine at its N-terminus requiring further treatment with CNBr (*10*). In order to overcome this problem, Lys-human proinsulin gene was constructed for the easy processing of trypsin on Met-Lys-human proinsulin to produce human insulin with correct N-terminus. Figure 1A shows the PCR product of the mutant gene. After filling in by T4 DNA polymerase and cutoff by *Eco*RI and *Bam*HI, the Lys-human proinsulin gene was cloned into an expression vector under the control of a P_RP_L promoter. This Lys-human proinsulin gene also can be used for the construction of other human insulin analogs in the *E. coli* expression system.

Expression of Target Product

Much previous work with Lac, Trp, or Tac promoters required a long period of time for the induction, usually more than 10 h (*2,3,6,7*). The

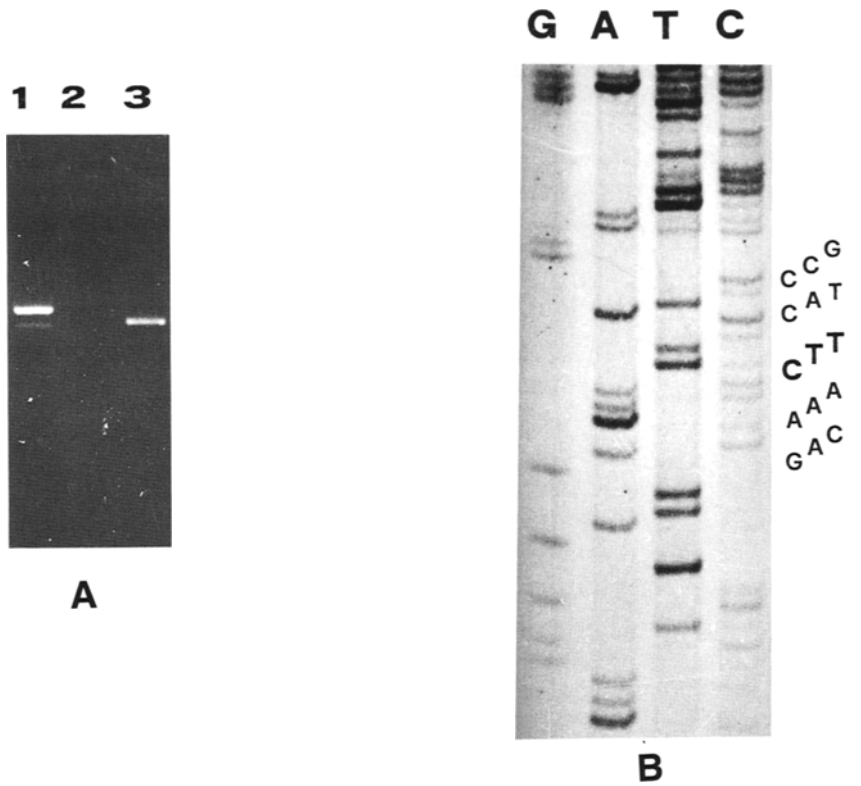

Fig. 1. (**A**) 1.2% agarose gel analysis of PCR product. (1) Shows the PCR product, (2) as a PCR control with no template DNA, and (3) molecular weight marker of the gene encoding human proinsulin cleaved from pBCA by *Eco*RI and *Bam*HI. (**B**) DNA sequence analysis of the mutant gene for Lys-human proinsulin. Bold bases indicate the mutation.

existence of expressed protein in living cells for such a long time may be the crucial reason for the failure to obtain the protein of interest. Figure 2 shows the SDS-polyacrylamide gel electrophoresis (PAGE) analysis of our expressed product for small-scale fermentation. According to densitometric scanning of the gel, the expressed Lys-human proinsulin material could constitute 20–30% of the total cellular protein. It is supposed that the major factor for the high yield of proinsulin is the short induction time with temperature inducible promoter, as Heath et al. have found (*9*). After sonication and centrifugation the expressed product was obtained with a purity of 60–80% in the pellet, indicating that the directly expressed Lys-human proinsulin is in insoluble form. The formation of inclusion body may be another factor for the stability of the product, by preventing enzymatic digestion. The formation of an inclusion body is also helpful to simplify the purification steps by centrifugation to separate inclusion bodies from soluble proteins and other soluble cellular materials to reach a purity of up to 60%. The high density fermentation gives the expression

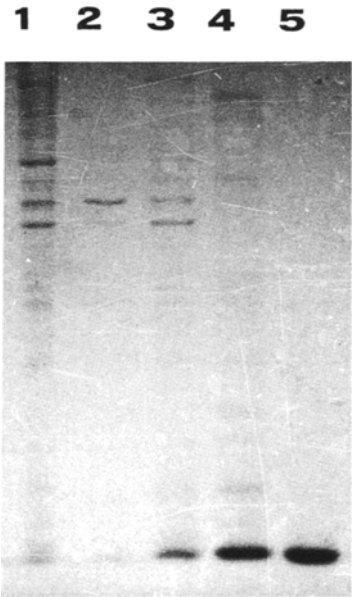

Fig. 2. Electrophoretic analysis of the expressed product. The cell was spun down and the expressed proteins were analyzed on 15% SDS-polyacrylamide gel. (1) Total cellular proteins of the cell transformed with pBV220, (2) pellet of the cell as (1) by sonication and centrifugation, (3) total cellular proteins of the cell transformed with pVK100, (4) pellet of the cell as (3) by sonication and centrifugation, and (5) purified crude lys-human proinsulin by Sephadex G50.

level of 10%, which is a little lower than that of the fermentation in the tube. However, the significant result is that just 1 h induction is enough for the high level of the target product.

Purification of Recombinant Lys-Human Proinsulin

Our procedure for the purification and processing of expressed Lys-human proinsulin is quite superior to any other published papers on the production of human insulin in the *E. coli* system (*4,6,9*). One single gel filtration chromatography step was used to separate the refolded proinsulin from its dimers, oligomers, and other large molecules, as shown in Fig. 3, and there was no detergent in the eluting buffer. One hundred fifty to two hundred milligrams crude recombinant product with a purity of up to 90% (Fig. 4) could be obtained easily from 1 L of fermentation medium. The procedure makes it unnecessary to transform the target proinsulin into S-sulfonated form, which would need several column chromatography steps for the purification before and after refolding, require large amount of detergent in the eluting buffer, and is time-consuming. The result of the amino acid composition analysis of the crude

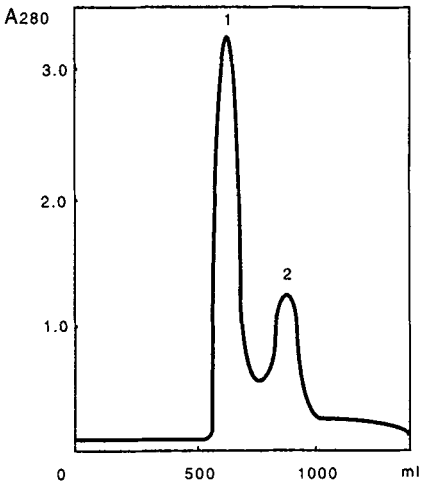

Fig. 3. Gel filtration separation of the recombinant Lys-human proinsulin. The reconstituted sample was loaded onto a Sephadex G50 (fine) column (4 × 100 cm), and eluted with 0.05*N* Gly-NaOH buffer (pH 10.8). The second peak was pooled, ultrafiltrated, and lyophilized.

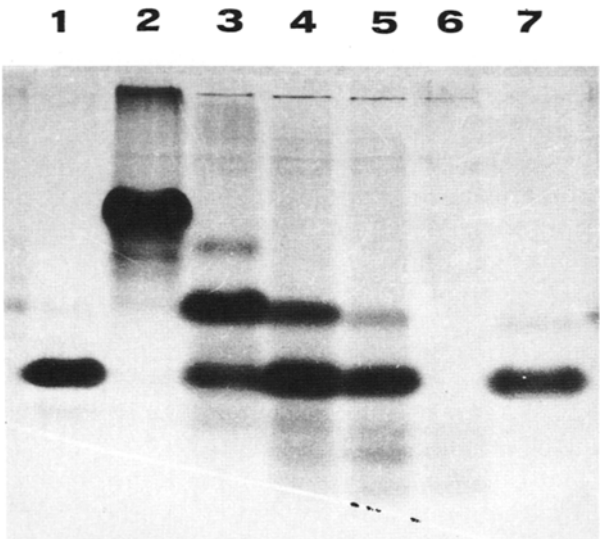

Fig. 4. Electrophoretic analysis of the purified Lys-human proinsulin and its products after treatment with trypsin and carboxypeptidase B on 10% polyacrylamide gel. (1) crystalline porcine insulin, (2) crude Lys-human proinsulin, (3), (4), (5), and (6) the products of (2) (250 μg of Lys-proinsulin) after treatment with 3 μg of carboxypeptidase B and increasing amounts of trypsin (1, 4, 16, 64 μg, respectively). (7) Purified recombinant human insulin after DEAE-Sephadex A25 separation.

Table 1
The Amino Acid Composition Analysis
of Recombinant Lys-Human Proinsulin

	Expected	Found
Asx	4	3.9
Thr	3	2.7
Ser	5	4.1
Glx	15	13.8
Pro	3	3.6
Gly	11	10.7
Ala	4	4.6
Val	6	5.8
Met	0	1.0
Ile	2	1.7
Leu	12	11.9
Tyr	4	3.8
Phe	3	3.0
Lys	3	3.0
His	2	2.0
Arg	4	4.0

product was shown in Table 1. As could be seen, the data are quite in agreement with that expected, except for one more methionine, suggesting the failure of the host cell to remove the N-terminal initiator methionine residue. As also shown in our previous reports for direct expression of human proinsulin (*10*), proinsulin mutant (*11,13*), and double-C-peptide proinsulin (*12*), and by Heath et al. (*9*) for expression of A-C-B human proinsulin, many publications have demonstrated the same phenomenon with a methionine residue at the N-terminus of the recombinant product. One explanation is that the residues near the N-terminus cause steric hindrance and thus affect the efficiency of methionine removal (*20*).

Conversion of Lys-Human Proinsulin to Human Insulin

The crude Met-Lys-human proinsulin could be changed directly into human insulin by treatment with trypsin and carboxypeptidase B under conditions that cleavage of trypsin at B22Arg and B29Lys could be reduced greatly (*17,18*). Figure 4 shows PAGE analysis of our transformed products. The byproducts increased with the increasing amounts of trypsin. Under appropriate conditions human insulin could be obtained with a conversion of up to 50% of the insulin materials. Figure 5 shows the result of anion-exchange chromatography on DEAE-Sephadex A25 of the enzyme-treated products with human insulin as the predominant product. The obtained recombinant human insulin was analyzed by PAGE to show

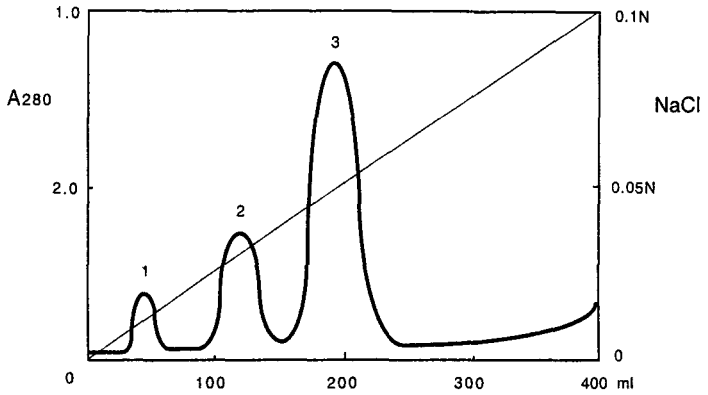

Fig. 5. DEAE-Sephadex A25 separation of the digested products of Lys-human proinsulin by trypsin and carboxypeptidase B. The column (1.6 × 20 cm) was eluted with 0.05*M* Tris-HCl, 40% propanol, pH 7.5, with a linear NaCl gradient of 0–0.1*N* in a total volume of 400 mL.

Table 2
The Amino Acid Composition Analysis
of Recombinant Human Insulin

	Expected	Found
Asx	3	2.7
Thr	3	2.8
Ser	3	3.5
Glx	7	3.9
Pro	1	0.8
Gly	4	4.1
Ala	1	1.0
Val	4	4.2
Met	0	0.03
Ile	2	1.8
Leu	6	6.2
Tyr	4	3.1
Phe	3	2.9
Lys	1	1.2
His	2	2.1
Arg	1	1.0

a purity of up to 97% (Fig. 4). The result of amino acid composition analysis of this recombinant human insulin was given in Table 2. It is worth mentioning that the found value is in good agreement with that of expected, and especially, that there was almost no *methionine* residue, indicating the complete removal of N-terminal Met-Lys-dipeptide from the Met-Lys-human proinsulin by trypsin cleavage. The receptor binding (*19*)

Table 3
Receptor Binding Analysis of Recombinant Human Insulin

[C] ng/mL	1000	300	100	30	10	3
Porcine, CPM	630	809	772	964	1365	1424
Human, CPM	620	729	799	1024	1285	1405
Total CPM, 6584, Control CPM: 1595						

Table 4
Radioimmunoassay Assay of Recombinant Human Insulin

[C] ng/mL	100	30	10	3	1
Porcine, CPM	361	355	448	695	1313
Human, CPM	331	375	524	725	1296
Total CPM, 5426, Control CPM: 1479					

and radioimmunoassays were employed for the activity determination of the recombinant human insulin (shown in Tables 3 and 4). Also, the potency of lowering rabbit blood glucose by this recombinant human insulin was the same as that of porcine insulin (our unpublished data).

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