Arch. Pharm. Res. Vol. 16, No. 4, pp. 271-275, 1993

Design and Cloning of the Gene for a Novel Insulin Analogue, (B^{30}-Homoserine) Human Insulin

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(Received August 6, 1993)

In order to prepare a novel human insulin analogue substituted with homoserine at B^{30} position, (B^{30}-homoserine) human insulin, a synthetic gene was designed by linking directly a gene for B chain with that for A chain. This gene was constructed by enzymatic joining of 10 different synthetic oligonucleotides, and then inserted at the polylinker region of pUC19 plasmid. To achieve a high level of gene expression, the gene fusion technique was employed using amino terminal regions of *lacZ* gene up to *Cla*I or *Hpa*I, and either of them has been located under *tac* promoter. The chemical induction of these fused genes by isopropyl-β-D-thiogalactopyranoside (IPTG) gave a satisfactory level of expression in *Escherichia coli* harboring the constructed plasmids. It was observed that the fused gene product as a single chain insulin precursor was produced more than 30% of total cell protein of *E. coli* as a form of inclusion body.

Key words: Recombinant insulin, Insulin analogue, B^{30}-homoserine insulin, Single chain insulin precursor, Gene fusion

INTRODUCTION

Insulin is a hormone secreted from the beta cell of Langerhan's islet in the pancreas, and its deficiency causes diabetes mellitus and hyperglycemia (Orci *et al.*, 1988). For the treatment of diabetes, animal insulins (bovine and porcine insulins) have been prepared by separation and purification from animal organs, and are widely used clinically. Even though this is one of the most conservative proteins in amino acid sequences, animal insulins cause sensitive allergic reaction on rare occasions, resulting in several chronic complex diseases when applying continuously (Barfoed, 1987).

Recently, the production of human insulin has been attempted by two different ways: one is enzymatic conversion of porcine insulin called 'semisynthetic human insulin' (Obermeier and Geiger, 1976; Inouye *et al.*, 1979; Breddam *et al.*, 1981), and the other is fermentation of genetically engineered microorganisms to produce 'recombinant human insulin' (Barfoed, 1987).

The well-known process for the production of recombinant human insulin is achieved by inserting separately a gene for insulin A chain or insulin B chain into a bacterial plasmid, followed by transformation and expression in *Escherichia coli* (Crea *et al.*, 1978; Goeddel *et al.*, 1979). This human insulin is finally made by chemical reconstitution of A chain and B chain obtained separately by fermentation of genetically engineered microorganisms, to form its intact disulfide bridges (Chance *et al.*, 1981). This reconstitution step is known to be mainly critical in economical assessment of the recombinant human insulin production processes.

This paper describes a novel procedure for the preparation of human insulin analogue, (B^{30}-homoserine) insulin. This novel insulin is designed for the development of much more economical bioprocess by one-step fermentation and purification of a single chain insulin precursor (insulin BA peptide) having A chain and B chain.

MATERIALS AND METHODS

Gene construction for a single chain insulin precursor

In order to design a single chain insulin precursor, the gene for human insulin B chain was directly connected with the gene for A chain, as shown in Fig. 1. At both ends of gene, some recognition sites for restriction enzymes have been introduced for the conve-

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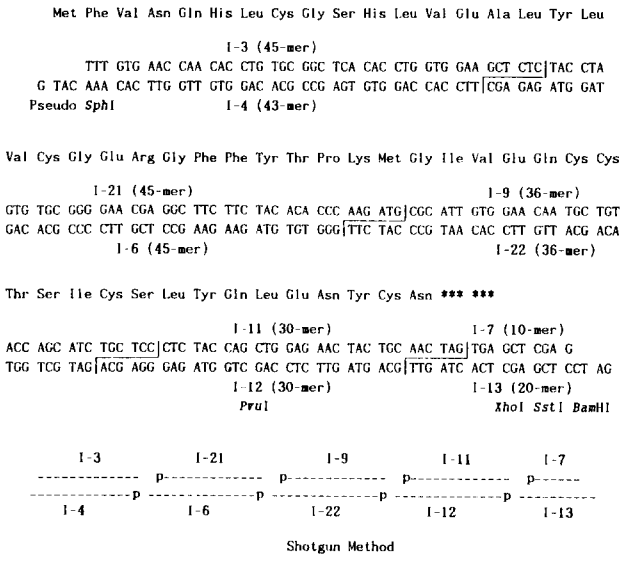

Fig. 1. Design and construction of the gene for B^{30}-(homoserine) human insulin analogue (single chain insulin precursor).

nience in manipulating this gene.

Ten oligonucleotides containing the nucleotide sequences for single chain insulin analogue (BA peptide) was synthesized by Automatic DNA Synthesizer (Beckman Instruments, Inc, CA, USA) using triphosphite method. These oligonucleotide fragments except for 5'-terminal fragments, was labelled by phosphorylation with (γ-P^{32})-ATP in the presence of T4 polynucleotidyl kinase. After kination, the same quantity of each labelled fragments was mixed with the same amount of unlabelled 5'-terminal fragments in one tube, and ligated each other with T4 DNA ligase by shot-gun ligation procedure. Finally, the constructed DNA coding for BA peptide was separated on the 8% polyacrylamide gel denatured with 7 M urea (Szalay *et al.*, 1977), and recovered by eluting the gel pieces of radioactive portions for the DNA sizes of 166 base pairs and 174 base pairs, followed by ethanol precipitation.

Gene cloning in *E. coli* plasmid

The gene for single chain insulin analogue, BA peptide, was inserted into *E. coli* plasmid, pUC19 (Yanisch-Perron *et al.*, 1985). The pUC19 vector digested with *Sph*I and *Bam*HI was ligated with the BA peptide gene, and the resulting plasmid, pIBA, was transformed into *E. coli* JM103 (Fig. 2). After cultivation on Luria-Bertani (LB) agar plate containing 50 μg/ml of ampicillin, 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and 0.004% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), the white colonies were selected. The transformed plasmid was isolated and identified by several restriction digestions (Maniatis *et al.*, 1984).

Construction of expression vectors

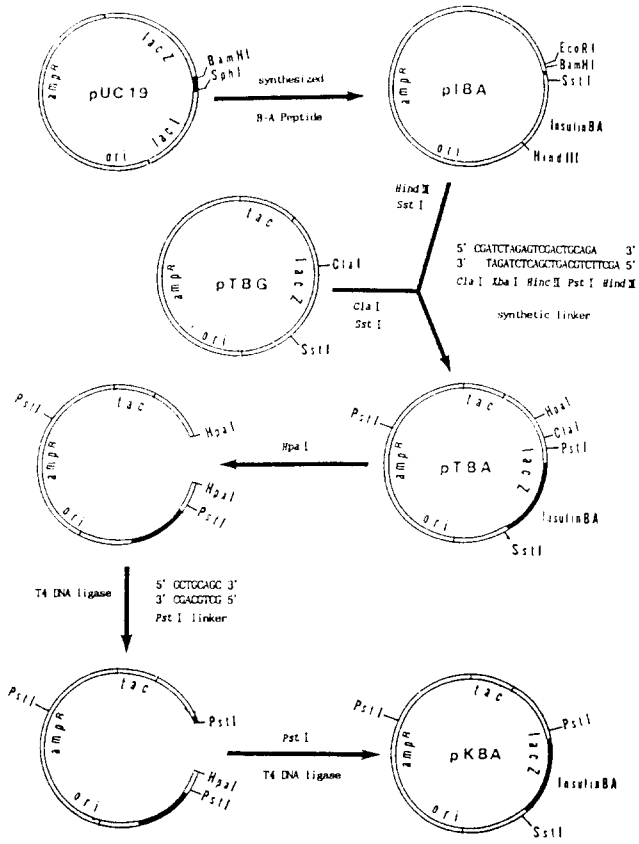

Fig. 2. Cloning strategy of the gene for single chain insulin precursor into expression vectors having *tac* promoter.

As an expression vector, pTBG having *lacZ* gene under *tac* promoter, which was kindly supplied from Dr. Moon H. Han, Genetic Engineering Research Institute, Taejon, Korea, has been employed. As seen in Fig. 2, this plasmid was cut by simultaneous restriction digestion with *Cla*I and *Sst*I, and the large fragments deleted in the middle portion of *lacZ* gene were separated by electroelution (Maniatis *et al.*, 1984). This fragment was ligated with the gene for BA-peptide which was obtained by double digestion of pIBA with *Hin*dIII and *Sst*I, in the presence of synthetic oligonucleotide linker, for the adjustment of reading frame and the introduction of more restriction sites. The ligating product by T4 DNA ligase, pTBA, was transformed into *E. coli* JM103, and the positive clone was selected on LB media containing ampicillin, IPTG and X-Gal as described above.

In order to shorten the portion of β-galactosidase gene fused with BA-peptide, the plasmid pTBA was cut with restriction enzyme *Hpa*I which makes blunt end and ligated with octameric *Pst*I linker, and the resulting plasmid was rejoined with T4 DNA ligase after digested with *Pst*I, to yield a new expression vector, pKBA, as shown in Fig. 2. The clones appeared on LB media containing ampicillin was selected by

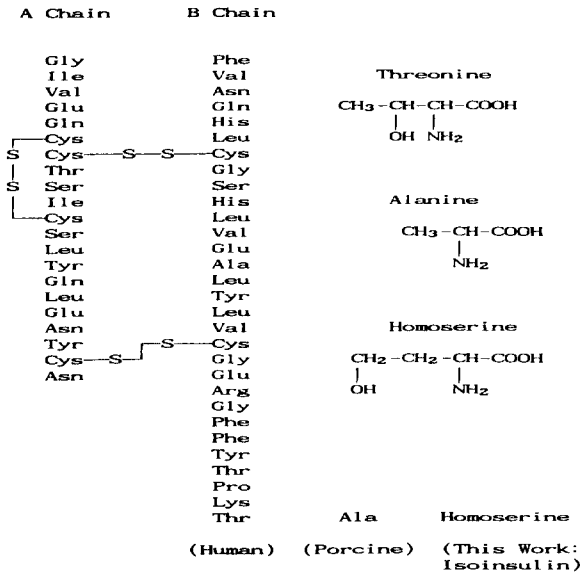

Fig. 3. Amino acid sequences of insulins from different sources and comparison of chemical structure of B^{30} amino acid residues.

testing their plasmids throughout the restriction digestion patterns (Maniatis *et al.*, 1984).

The gene induction of pTBA and pKBA plasmids having insulin BA-peptide gene was carried out by the addition of 1.0 mM IPTG in the cultivation media of *E. coli* harboring these plasmids at the late logarithmic phase.

Analytical procedures

The electophoresis of sodium dodecyl sulfate (SDS)-denatured polyacrylamide gel was performed for the detection of proteins (Laemmli *et al.*, 1970), and the intensity of protein bands was scanned by Laser Gel Densitometer (Pharmacia LKB Biotechnology AB, Uppsala, Sweden). The dideoxy nucleotide sequencing method (Sanger *et al.*, 1977) was employed by using two different universal primers (BRL Life Science, MD, USA).

RESULTS AND DISCUSSION

Design of the gene for a single chain insulin precursor

A novel approach to produce human insulin analogue has been attempted using a DNA sequence for single chain insulin analogue precursor (BA-peptide), rendering B(1-29)-Met-A(1-21) peptide link between B chain and A chain. The finally obtained product after cleavage with cyanogen bromide of gene product will be B^{30}-(homoserine) insulin, which is a structural isomer of human insulin (Fig. 3). This process was designed for the development of more economical method for preparing a human insulin analogue, by the simultaneous fermentation and purification of A chain and B chain peptides from *E. coli* cells and by high yield in chemical reconstitution to afford a correct folding.

First of all, it should be considered whether the final product B^{30}-homoserine insulin will retain the full biological potency of authentic human insulin. During last two decades, many researchers studied on the structure-biological activity relationships of insulin (Carpenter, 1966; Rager *et al.*, 1969; Katsoyannis *et al.*, 1971, 1973, 1974). According their results, the amino acid residues at C-terminal of B chain of insulin does not affect significantly its biological activity, even in the case where these amino acid residues are removed. It is also supported by the fact that there is a great diversity of amino acid residues at B^{30} position even though it is well known to be one of the most conservative proteins in mammalian animals (Smith, 1966). In X-ray crystallographic studies on insulin monomer, it has been revealed that B^{30} amino acid residue is located just outside of insulin molecule, along with making charge interaction between B^{29}-(Lys) and A^4-(Glu) (Blundell *et al.*, 1972). Above all, homoserine is just a structural isomer of threonine, only differing by the attaching site of hydroxyl group (Fig. 3). Kempe *et al.* (1988) attempted to make an active analogue of (homoserine)^{31}-salmon calcitonin I by recombinant DNA and chemical techniques, and they found that their analogue is equipotent to the naturally-occurring salmon calcitonin I in lowering plasma calcium levels by bioassay. Deduced from the previous works, it can be easily concluded that the alteration of amino acid residue of B^{30} threonine in human insulin with homoserine does not make it lose its biological activity in any way.

Another important point to be considered is whether the single chain insulin precursor, insulin BA-peptide, will fold correctly during chemical reconstitution. Markussen *et al.* (1985) synthesized a single chain des-(B30) insulin in such a way that B^{29}-(Lys)-A^1-(Gly) peptide link is formed between the A chain and B chain of insulin. When this single chain insulin was reduced and reoxidized, the yield of correctly folded and reoxidized product was higher than that of porcine proinsulin (Markussen, 1985). On the other hand, Wetzel *et al.* (1981) attempted to shorten the C-peptide to six amino acids (Arg-Arg-Gly-Ser-Lys-Arg), called mini-C peptide, and to connect B chain with A chain of human insulin by recombinant DNA techniques. Their mini-C analogue of human proinsulin could successfully fold in a correct form like proinsulin. From the above results, it can be deduced that our single chain human insulin precursor, BA-peptide, will fold correctly up like proinsulin.

Gene construction and cloning of a single chain insulin precursor

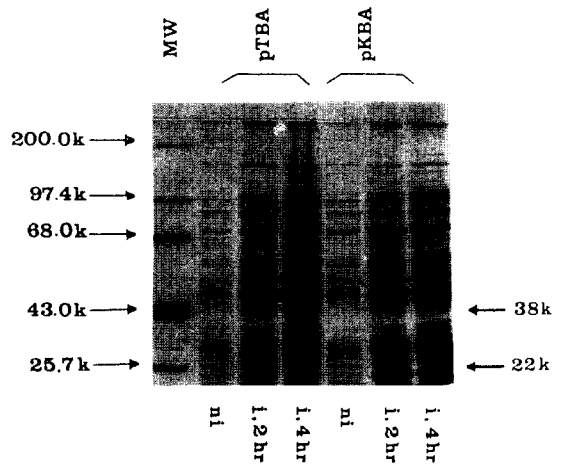

Fig. 4. Induction of gene expression by IPTG in *E. coli* JM103 harboring pTBA and pKBA plasmid. The electrophoretic pattern was obtained from 7% SDS-denatured polyacrylamide gel. ni; not induced by IPTG: i; induced by IPTG at indicated culture time.

The gene for a single chain insulin analogue precursor has been designed by modification of the triplet codon for B^{30}-(Thr) with that for Met and directly connecting the gene for B chain to the gene for A chain peptide, rendering B(1-29)-Met-A(1-21) (BA peptide) (Fig. 1). Ten oligonucleotides consisting this gene were synthesized and connected by shot-gun ligation method. The constructed gene was inserted into pUC19 plasmid in order to amplify the gene copies. The nucleotide sequencing of inserted fragment in the resulting plasmid, pIBA, by dideoxy method proved that the inserted fragment had the same sequences as the gene for insulin BA-peptide as designed.

Gene expression of a single chain insulin precursor

For the expression of this gene in *E. coli* host cell, the cloned insulin BA-peptide gene was introduced into *E. coli* expression vector, pTBG, having *lacZ* gene under *tac* promoter. When introduced at the *Cla*I site of *lacZ* gene (279 amino acids from N-terminus of β-galactosidase) to make an expression plasmid pTBA, a good expression of the fused gene of BA-peptide with *lacZ* was observed. After induction of the fused gene by IPTG, the total protein of *E. coli* cells was subjected to SDS-polyacrylamide gel electrophoresis (Fig. 4). The fraction of the corresponding protein (38 kDa) was found to occupy approximately 30% of total protein, when scanned through Laser Densitometer (Fig. 5). In microscopic examination, the cytoplasmic inclusion bodies in *E. coli* cells induced by IPTG were observed, as reported by Williams *et al.* (1982).

In spite of good expression of this gene for insulin BA peptide in pTBA plasmid, methionine residues up

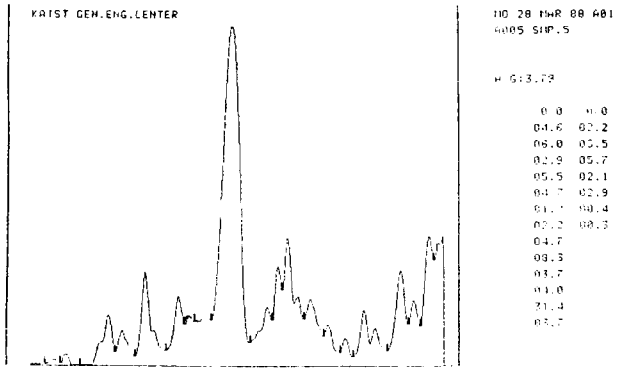

Fig. 5. The scanning pattern of bands on 7% SDS-denatured polyacrylamide gels of the total proteins in *E. coli* JM103 harboring pTBA plasmid.

to six in β-galactosidase prior to the cloned site can make trouble in the purification process after cyanogen bromide cleavage. In order to avoid this problem, the fused part of β-galactosidase was shortened to *Hpa*I site corresponding to 146 amino acids from N-terminus. The resulting plasmid pKBA showed a good expression of the fused insulin BA-peptide gene as much as the pTBA plasmid. When induced this gene by IPTG, *E. coli* cells harboring pKBA plasmid also produced the cytoplasmic inclusion bodies of the proteins (22 kDa), as seen in Fig. 4.

ACKNOWLEDGEMENT

This work was kindly supported as the New Drug Development Program by the Ministry of Health and Social Affairs. The authors would like to give thanks to Dr. Moon Hee Han for his kind gift of expression vector pTBG, and Drs. Dae-Sil Lee and Myoung Hee Kim in Molecular Biology Laboratory, Genetic Engineering Research Institute, Taejon, for their heartful discussions.

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