(A Tribute to Dr. Sun Ho Park)

Production of human insulin analogue using recombinant *Escherichia coli*

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Abstract

For the production of B^{30}-homoserine insulin analog as a novel anti-diabetic drug, the fermentative study was attempted for the maximal gene expression of HTS-fused B^{30}-homoserine insulin precursor in the recombinant *Escherichia coli* cells. In a batch fermentation, the maximal production of insulin precursor as much as 38.95 mg/L-h, which occupied more than 12.8% of total cell protein. was achieved when the gene expression was induced by 0.5 mM IPTG at the middle logarithmic growth phase. The HTS-fused B^{30}-homoserine insulin precursor was recovered from a batch culture through the processes of cell harvest, collection of insoluble fraction after sonication and purification by nickel affinity column chromatography. The isolated insulin precursor was 14 mg/L with a recovery yield of 35.9% of expressed gene product. The insulin A and B chain mixture was recovered after the insulin precursor was subjected to CNBr cleavage and purified by nickel affinity column chromatography. The isolated insulin chains were then sulfitolyzed with sodium thiosulfat and sodium tetrathionate, and reconstituted to insulin analog with β -mercaptoethanol, followed by purification with CM-Sepharose C-25 column chromatography.

Construction of an Expression Vector

For the production of a novel human insulin analog, (B^{30}-homoserine) insulin, the gene for insulin B chain was directly connected with the gene for insulin A chain, and then inserted into an *E. coli* expression vector, pET-28(a), to construct pET-BA plasmid (Fig. 1). This vector was transformed into *E. coli* BL21(DE3)pLysS [F *ompT hsd* $S_B(r_B^- m_B^-)$

gal dcm (DE3) pLysS] for the expression of the cloned gene.

Fermentative Production of Insulin Precursor

The recombinant *E. coli* cells harboring pET-BA plasmid was grown for 2 hrs on LB broth having 15㎍/㎖ of kanamycin, and then gene induction was performed by 4-hr cultivation after the addition of 0.5 mM IPTG. Throughout the fermentation study, it was found that the maximal gene expression was achieved when cultured for longer than 4 hr after supplement- ation of higher than 0.5 mM IPTG. And the amount of insulin precursor produced was determined as 39 ㎎/L, which corresponded to 12.8% of total cell protein on the SDS- PAGE gel. From fermentation broth, the *E. coli* cells were harvested by centri- fugation at 5,000 rpm for 10 min, and sonicated 10 times for 30 sec after suspending in 1/50 volumn of binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-Cl, pH 7.9). After centrifuging the resulting solution at 15,000 rpm for 20 min, most of insulin BA precursor peptides were found in insoluble part of inclusion body, as shown in Fig. 2. Thus, the inclusion body was extracted from precipitates with 3 volume of binding buffer containing 6 M urea.

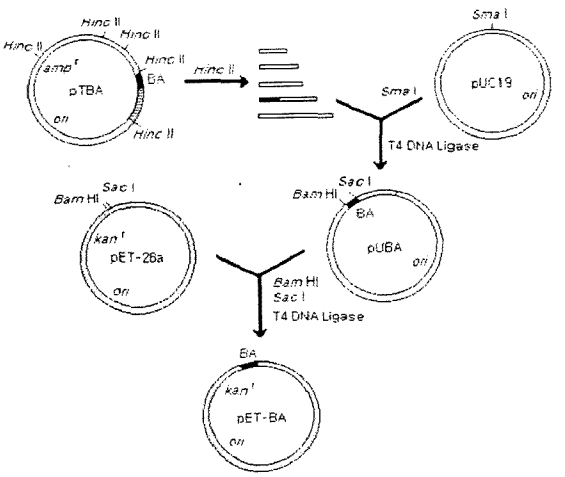

Fig. 1. Construction of Expression Vector for B^{30}-Homoserine Insulin Analog.

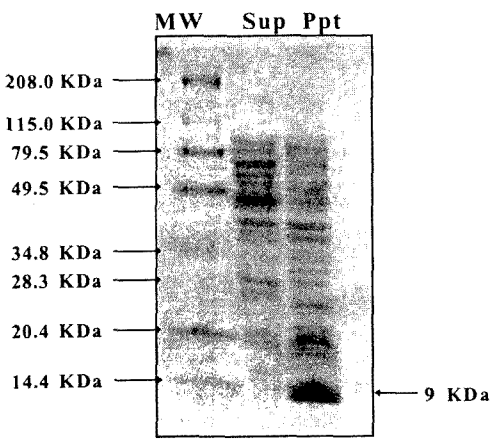

Fig. 2. Identification of Insulin Precursor After Sonication.

Purification of Insulin Precursor

Since insulin precursor was produced in the form of a fus- ion protein with His-tag, a

nickel-affinity column chromatography was employed for the purification of precursor protein. The urea extract of inclusion body was loaded on nickel-affinity column (2.5×10cm), and washed with 10 volume of the same buffer and 6 volume of washing buffer (20 mM imidazole, 0.5 M NaCl, 20 mM Tris-Cl, pH 7.9) having 6 M urea. The insulin precursor was eluted from column by passing elute buffer (1 M imidazole, 0.5 M NaCl, 20 mM Tris-Cl, pH 7.9) containing 6 M urea (Fig. 3), and dialyzed against binding buffer having 6 M urea. The amount of isolated insulin precursor was 12 ㎎ /L, with the purity above 86% on SDS-PAGE gel. By amino acid analysis, it was confirmed that the purified insulin analog pre- cursor had nearly the same amino acid composition as expected.

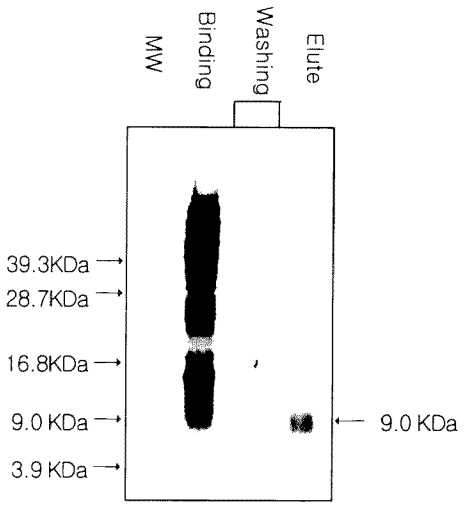

Fig. 3. Purification of Insulin Precursor by Ni-Affinity Column Chromatography.

Cleavage of Insulin Precursor with Cyanogen Bromide

In order to release insulin A chain and B chain, the purified insulin precursor was subjected to CNBr cleavage. For this, insulin precursor (10 ㎎) was reacted with 5 ㎎ of sodium thiosulfate for 15 min in 5 ㎖ of 88% formic acid, and then treated with 25 ㎎ of CNBr at room temperature for 12 hr. After reaction, formic acid in reaction mixture was removed by rotary evaporation and followed by freeze- drying. Finally, the reactant was dissolved in binding buffer having 6 M urea, and loaded on nickel-affinity column (2.5×10cm). The fraction not bound to nickel-affinity resin was isolated as mixture of insulin A and B chains, because both chains were believed to be free from His-tag (Fig. 4). The recovered insulin chains was as much as 1.4 ㎎/L of culture broth.

Chemical Reconstitution to Insulin Analog

In order to make the proper conformation of insulin, two insulin chains were refolded by chemical reconstitution. First, the isolated mixture of insulin chains in 6 M urea was subjected to oxidative sulfitolysis under nitrogen atmosphere by reacting with 3% sodium

sulfite and 1.5% sodium tetrathionate dihydrate at pH 8.9~9.2 and 37℃ for 6 hr. When the amount of free SH group was determined by DTNB, the color was almost diminished by disappearance of SH group in insulin chains through oxidative sulfitolysis. The resulting peptide solution was dialyzed against 20 mM acetate buffer (pH 4.4) containing 6 M urea, and two insulin chains were reconstituted by the action of β-mercaptoethanol (18 ㏖/1 ㏖ insulin) in 0.1 M glycine buffer (pH 10.5) under nitrogen gas at 4℃ for 18 hr. After completing the reaction by lowering pH to pH 2.5 with acetic acid, the reaction mixture was loaded on CM-Separose column. The reconstituted insulin analog was eluted by 0~0.5 M NaCl gradient with 20 mM acetic acid (pH 4.0) buffer having 6 M urea. The amount of insulin analog obtained finally was 0.6 ㎎ from 1 L of fermentation broth. In order to confirm the conformation of insulin analog, it was subjected to MonoQ column of FPLC after digesting with Glu-C. The elution pattern of digests was the same as that from authentic human insulin.

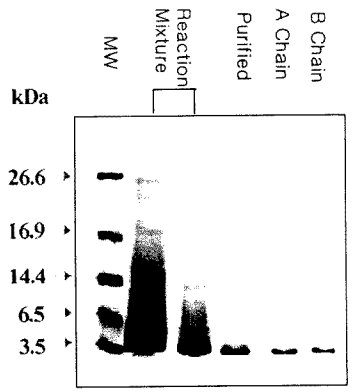

Fig. 4. CNBr Cleavage of Insulin Precursor to Release Insulin Chains, Followed by Purification through Ni-Affinity Column.

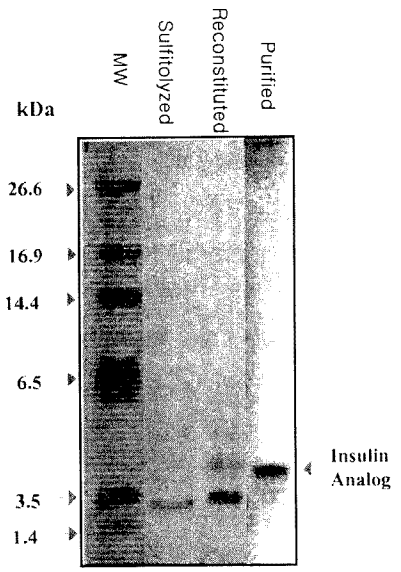

Fig. 5. Chemical Reconstitution of Two Insulin Chains to Insulin Analog, Followed by Purification through CM-Separose column.

References

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