Use of *Bacillus brevis* for Synthesis and Secretion of Des-B30 Single-Chain Human Insulin Precursor

Mamoru KOH, Hiroshi HANAGATA,* Shogo EBISU,* Kazuyuki MORIHARA, and Hiroaki TAKAGI*†

Institute for Applied Life Science, University of East Asia, Graduate School, 2-1 Ichinomiya-Gakuen cho, Shimonoseki-shi, Yamaguchi 751-0807, Japan
*R & D Department, Higeta Shoyu Co. Ltd., 2-8 Chuo-cho, Choshi-shi, Chiba 288-8680, Japan

Received November 15, 1999; Accepted January 18, 2000

A synthetic gene encoding a single chain human insulin precursor [B-chain (1-29)-A-chain] linked to the C-terminal lysine of human epidermal growth factor (1-28) (EGF-SCI) was constructed. This gene was expressed using *Bacillus brevis*. EGF-SCI was isolated from the supernatant of the culture broth. Treatment of EGF-SCI with lysyl endopeptidase resulted in the formation of des-B30 human insulin. The identification of the formed des-B30 human insulin was made by the measurement of molecular weight and amino acid analysis. The binding coefficient to anti-human insulin antibody was comparable to that of human insulin.

**Key words:** *Bacillus brevis*; single chain human insulin precursor; human epidermal growth factor (1-28); des-B30-human insulin; anti-human insulin antibody

Human insulin produced by recombinant DNA technology was the first commercial health care product. Insulin obtained in this manner is now called biosynthetic human insulin (BHI), which can be prepared either by combining the two independently synthesized A and B polypeptide chains or converting proinsulin to insulin. An alternative method is the production of single chain insulin precursors, in which post fermentation chemistry can be minimized.

Independent from these works, we planned the efficient production of BHI using *Bacillus brevis* as a host. The characteristics of this system are direct secretion of biologically active proteins into the culture medium and stable accumulation of proteins due to the low proteolytic activity in the culture supernatant.

A preliminary experiment was done on the secretion of des-B30-single-chain human insulin precursor [B-(1-29)-A-chain] using the *B. brevis* system, but the production level was very low. We decided to produce a chimeric protein of the single chain insulin precursor linked to the truncated human epidermal growth factor (EGF) for efficient production. The expression vector, designated pHT117E28CS-SCI (Fig. 1), was constructed from the plasmid pHY100EGF, derived from pH926 as follows. With synthetic linker DNAs, the EcoRI site was inserted into the 5' region of the EGF gene, the HindIII site was introduced downstream of it, and the resulting plasmid was designated pHT117. For the prevention of disulfide bond formation between the Cys residues of EGF and those of insulin, an 80-bp EcoRI-XbaI fragment, in which three Cys residues were replaced by Ser, was synthesized. A 170-bp XbaI-HindIII fragment encoding B-(1-29)-A-chain was obtained from six kinds of synthetic DNAs. These two fragments were cloned into pHT117 between the EcoRI and HindIII sites resulting in pHT117E28CS-SCI (Fig. 1). Transformation was done using electroporation as described previously. *B. brevis* HPD31 was grown in 25L-5E medium. The cultivation was done in a 30-L jar fermenter (201 of broth) at 30°C for 4 days. After centrifugation, the clear supernatant was used for isolation of the product. The content of EGF-SCI was calculated approximately to be 50 mg/l from the immuno-blotting method (Fig. 2).

The culture supernatant was concentrated six-fold using an ultrafiltration membrane (M.W., 3,000; Amicon Inc., U.S.A.). The concentrate (200 ml) was equilibrated with 10 mm Tris-HCl (pH 7.5) and subsequently put on a DEAE-Sephadex A-25 column (3.0×30 cm, Pharmacia Biotech., Sweden) which had been equilibrated with the same buffer. The EGF-SCI fraction was eluted from the column with 600 ml of the buffer solution containing 0.35 M NaCl. The eluted solution was concentrated to 70 ml using ultra-filtration (M.W., 10,000), which was then precipitated with ZnCl₂ (final concentration, 6 mm). The precipitate was collected by centrifugation and dissolved with distilled water (7 ml). The pattern of

---

* To whom correspondence should be addressed.
SDS-polyacrylamide gel electrophoresis indicated that the preparation is homogeneous corresponding to the molecular weight of EGF-SCI (data not shown). The N-terminal 10 amino acid sequence of EGF-SCI identified chemically was identical with that deduced from the DNA sequence (Fig. 1). The concentration of EGF-SCI was measured by liquid chromatography (LC) using porcine insulin solution (1 mg/ml) as a standard. The concentration was 2.1 mg/ml and the yield of EGF-SCI was calculated to be 14.7 mg from 1.21 supernatant.

Five ml of the EGF-SCI solution (2.1 mg/ml) was treated with 0.1 ml lysyl endopeptidase (1 mg/ml) under the following conditions, pH 7.4 with Tris buffer, with overnight incubation at room temperature. Des-B30-human insulin (des-B30-HI) appears to have been generated by the digestion of EGF-SCI. The pattern of the reaction product on LC is shown in Fig. 3, which indicates that the product is identical to des-B30-insulin prepared by digestion of porcine insulin with lysyl endopeptidase.

To confirm that the reaction mixture contains des-B30-HI, a further experiment was done using preparative LC apparatus (column size, 2 × 15 cm). The fractions corresponding to des-B30-HI were collected, dialyzed against distilled water, and lyophilized. The molecular weight was measured as follows. About 1 μg of insulin was dissolved in 1 μl of sinapinic acid matrix and put on a sample plate. The MALDI-TOF mass spectrum was collected in a Voyager DE (Perseptive Biosystems). The result indicated that the molecular weight was 5707, which corresponded to that of des-B30-HI (theoretical value, 5690). Amino acid analysis (theoretical values in parentheses) gave: Asp 3.0 (3), Thr 1.7 (2), Ser 2.5 (3), Glu 7.4 (7), Pro 0.9 (1), Gly 4.2 (4), Ala 1.0 (1), CySO\textsubscript{3} 5.2 (6), Val 3.2 (4), Ile 1.4 (2), Leu 5.8 (6), Tyr 3.7 (4), Phe 2.6 (3), His 1.7 (2), Lys 0.9 (1), Arg 0.9 (1).

The binding activity of insulins to human insulin receptor (HIR) was measured as follows. HIR was isolated from transfected Baby Hamster Kidney cells by solubilization and partial purification on a Wheat Germ Agglutinin column. HIR was incubated with 3 pm Tyrt\textsuperscript{114} (\textsuperscript{125}I)-human insulin and various concentrations of unlabeled insulin in a binding buffer containing 0.1 m Heps, 0.1 m NaCl, 0.01 m MgSO\textsubscript{4}, 0.5% human serum albumin, 0.2% γ-globulin, and 0.025% Triton X-100, pH 7.8, for 48 hrs at 4°C. The
Fig. 3. LC Profile of EGF-SCI Fusion Protein and Its Digest with Lysyl Endopeptidase.

A Shimadzu PepRPC 18 column (0.45 x 15 cm) was used. Linear gradient elution of acetonitrile (from 25% to 60%, 1%/min) was done in the presence of 0.1% trifluoroacetic acid. The flow rate was 1 ml/min, and detection was done at an absorbance of 220 nm. A, EGF-SCI fusion protein isolated by LC; B, digest of A with lysyl endopeptidase; C, des-B30 insulin from porcine insulin; D, porcine insulin.

bound tracer was isolated by precipitation with 400 µl 25% PEG 8000 and washed with 1 ml 15% PEG 8000. The affinities were calculated by fitting the data to a one-site binding model. In this assay, the $K_d$ for the human insulin standard was 15.4 pm and for the sample (des-B30-HI) was 22.2 pm. It indicates that the sample has the usual biological activity, similar to that as seen in human insulin. Human insulin can easily be prepared from the des-B30-HI by an enzymatic method.11

Acknowledgments

We wish to thank Drs. J. Markussen and L. Schaffer of Novo-Nordisk A/S, who made the measurement of molecular weight and bioassay.

References