ISOLATION AND PARTIAL CHARACTERIZATION OF THE Asp-B10, Tyr-B25-des-(B26-B30)-PROINSULIN ANALOG FROM INCLUSION BODIES IN Escherichia coli

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Summary
A proinsulin analog constructed by site-directed mutagenesis was expressed as a fusion protein that formed inclusion bodies inside the cells. It was purified from the isolated inclusion bodies and proinsulin was obtained by trifluoro-acetic acid, dimethyl sulfoxide and hydrochloric acid cleavage. The released proinsulin analog was confirmed by its molecular weight as determined by SDS-PAGE.

Introduction
Methods for the production of bovine and porcine insulins were devised immediately after insulin’s discovery by Banting and Best, in 1921 (Banting and Best, 1922). Although the purity of these early insulin products was low, giving rise to toxic effects, their existence drastically improved the quality of life of diabetics.

Until recently, commercial insulins for therapeutic use were obtained from bovine or pork pancreas. The use of bovine insulin, that differs from the human hormone by three aminoacid residues, Ala-A8, Val-A10 and Ala-B30, even when highly purified, might elicit antibodies in some patients. On the other hand, pork insulin, which differs from the human hormone by only one residue, Ala-B30, has been a successful substitute for the bovine protein.

The development of the recombinant DNA technology has allowed for the preparation of human insulin, human insulin analogs with altered pharmacokinetic properties (Bristow, 1993), as well as other polypeptides in heterologous organisms, with applications in biotechnology or in therapeutics. Significant problems in the production of recombinant proteins are: protein degradation by host bacterial proteases, protein recovery in an active and soluble form, as well as its final purification, free from host proteins. Different approaches have been adopted to facilitate recovery of recombinant proteins. One such is a expression as a chimera. This strategy is prone to success because fusion proteins occur as inclusion bodies, insoluble aggregates usually being more resistant to cytoplasmic proteases (Hellebust et al., 1989). The isolation and purification of fusion proteins is a complex process due to their localization, difficulties in dissolution and the presence of other bacterial proteins (Sassenfeld and Brewer, 1984). This paper describes the isolation and purification of a proinsulin analog Asp-B10, Tyr-B25, des-(B26-B30) from its inclusion bodies in E. coli.
Material and Methods

**Bacterium and recombinant plasmid:** The insulin mutant gene was prepared by site-directed mutagenesis (Sambrook *et al.*, 1989), in which codons for histidyl and phenylalanyl residues at positions 10 and 25 in the B-chain, respectively, were substituted by codons for aspartyl and tyrosyl residues. Another modification involved deletions of codons in positions B-26 to B-30.

The gene coding for mutant human proinsulin was synthesized and expressed as fusion with the initial part of DNA polymerase I (mini-Klenow fragment) plus six C-terminal arginyl residues. The mutant gene was cloned in the expression vector containing the p1 promoter; the resulting plasmid, called pEBIMTA72.0, was used to transform *E. coli*, strain N4830-1 (Sambrook *et al.*, 1989). The gene sequence was confirmed by dideox-DNA sequencing (Sambrook *et al.*, 1989).

**Fusion protein expression in *E. coli***: A single colony on agar 2YT plate containing ampicillin (100 μg/ml) was inoculated in 2YT medium with the respective selective antibiotic. The culture was incubated at 30°C with agitation (150 rpm) for 24 hours. Following the culture (corresponding 8% of the medium volume fermentation) was transfer to nine litres of 2YT medium containing ampicillin (100 μg/ml) and was incubated at 30°C with agitation (600 rpm), aeration (1 vol/vol/min) to a reading at A540 of 20. The induction of the fusion protein expression, under control by the p1 promoter from lambda phage was started by raising temperature to 40°C. For that purpose, glucose (0.5%), and ampicillin (100 μg/ml) were now added and the culture incubated at 40°C for two hours. The cells were collected by centrifugation at 5000 x g for 10 min at 40°C.

**Inclusion body solubilization:** The inclusion bodies were solubilized in treatment solution (100 mM Tris-HCl, 8 M urea, pH 8.0) and kept under agitation for 16 hours, at room temperature. The suspension was then centrifuged, the supernatant dialyzed against water and precipitated by adding 0.1 N HCl to pH 4.5. The precipitate was centrifuged and the sediment suspended in water and freeze-dried.

**Inclusion body purification:** After solubilization, the fusion protein was dissolved (5 mg/ml) in 20 mM NaH2PO4/Na2HPO4 buffer, 1 mM EDTA, 8 M urea, at pH 7.0, containing 100 mM beta-mercaptoethanol and applied to a Q-Sepharose FF column (30 x 18 cm). Elution took place under a flux of 200 ml/min, at room temperature, with the same buffer as above for one column volume. Next, one and a half column volume of the same buffer containing an additional 200 mM NaCl was passed through and, finally, one column volume of the buffer with 1.0 M NaCl. Absorption at 280 nm was used to collect the fusion protein peak tubes that were pooled, dialyzed and freeze-dried.

**Fusion protein cleavage:** Fusion protein cleavage was done according to a method modified from Savage and Fontana (1977). Peak B in the Q-Sepharose FF chromatography, that corresponds to the fusion protein, was dissolved to 1.0 mM in anhydrous trifluoro-acetic acid (TFA), with added dimethyl sulfoxide (DMSO 0.25 M) and hydrochloric acid (HCl 0.10 M), the mixture was incubated at 40°C for 24 hours. The protein was then precipitated from water and centrifuged. The sediment was suspended in 20 mM Tris-HCl, 2 mM EDTA, 8 M urea with 500 mM beta-mercaptoethanol.

**Protein purification after cleavage:** The protein was applied to a 15 x 5 cm column of Q-Sepharose FF equilibrated with 20 mM Tris-HCl, 2 mM EDTA, 8 M urea and 100 mM beta-mercaptoethanol, pH 8.0. The sample, at 5 mg/ml, was eluted as follows: one column volume with equilibration buffer, followed by two column volumes of the buffer plus 100 mM NaCl and, finally, with one column volume of buffer with 1.0 M NaCl, at a flux of 10 ml/min, at room temperature. The peak corresponding to proinsulin, as identified by gel filtration in Superose 12, was dialyzed and freeze-dried.

**Protein analysis by Superose 12 gel filtration:** Throughout the purification process fractions obtained were analyzed using a Superose 12 column (1 x 30 cm) together with the FPLC system of Pharmacia LKB Biotechnology. The sample, 50 to 1000 μl, was eluted with 20 mM Tris-HCl buffer, 2 mM EDTA, 200 mM NaCl, 8 M urea, at pH 8.0 and a flux of 0.5 ml/min, at room temperature. For the identification of the proteins of interest, the column was calibrated with standards of known molecular weight.

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