THE SYNTHESIS AND HYPERGLYCAEMIC ACTIVITY OF THE AMINO ACID SEQUENCE 172-191 OF HUMAN GROWTH HORMONE

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SUMMARY. The COOH-terminal cyclic eicosapeptide of human growth hormone, Lys-Val-Glu-Thr-Phe-Leu-Arg-Ile-Val-Gln-Cys-Arg-Val-Ser-Glu-Gly-Ser-Cys-Gly-Phe, was synthesized by the solid phase methodology. The synthetic peptide was assayed and found to possess significant hyperglycaemic activity in normal rats.

A model has been proposed to explain the diabetogenic action of human growth hormone (hGH) in terms of the endogenous release of a peptide fragment (InG) from the carboxy terminal region of the hGH molecule (1). This growth hormone derived peptide has been shown to possess the in vitro biological effects which can account for the diabetogenic action of hGH (2). Early studies (3) suggested that InG appeared to contain the amino acid residues 167-191 of human growth hormone, and showed that the synthetic hGH 167-191 peptide had in vitro biological effects identical to InG from human growth hormone. Recent evidence indicates that the structure required for hyperglycaemic activity is not an unique one, as peptide sequences containing the same "active core" appear to be capable of producing similar hyperglycaemic effects (4). Structure-activity studies have been directed toward the elucidation of this core sequence. The chemical synthesis and biological study of the shorter derivative, hGH 172-191, are described in this communication.

EXPERIMENTS and RESULTS

The basic procedure for the synthesis of the cyclic peptide hGH 172-191 is shown in Figure 1, and is outlined as follows:

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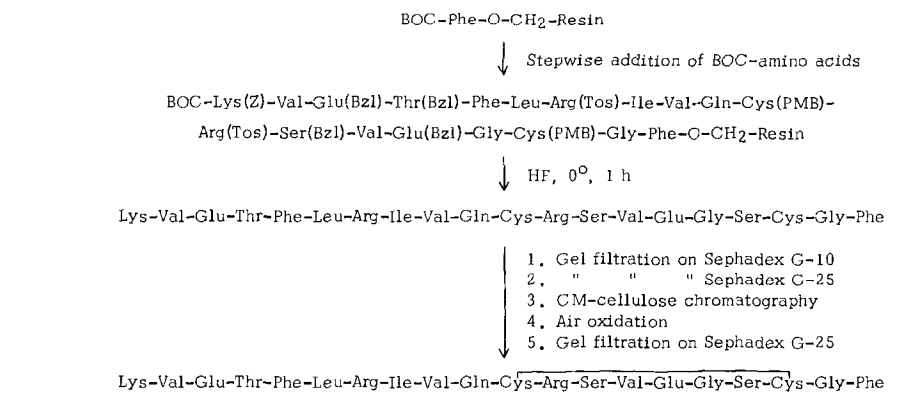

Figure 1

Outline of the synthesis of human growth hormone sequence 172-191 by the solid phase method.

Solid phase Synthesis. BOC-phenylalanine resin (8.0 g, 2.8 mmol Phe) was prepared by the standard triethylamine (TEA) method (5), using 1% cross-linked polystyrene (Bachem Inc., Calif.). The following programme of deprotection, neutralization and coupling was used for the introduction of each amino acid into the peptide:

(i) three washes (60 ml) with CH_2Cl_2; (ii) removal of the BOC group by two treatments (5 and 20 min) with 30% trifluoroacetic acid-CH_2Cl_2; (iii) six washes with CH_2Cl_2; (iv) neutralization by two treatments (2 and 10 min) with 10% TEA-CH_2Cl_2; (v) four washes with CH_2Cl_2; (vi) addition of 8.4 mmol of protected amino acid in 15 ml CH_2Cl_2; (vii) addition of 8.4 mmol of dicyclohexylcarbodiimide in 15 ml CH_2Cl_2 followed by shaking for 60 min; (viii) two washes with CH_2Cl_2; (ix) two washes with dimethylformamide (DMF); (x) two washes with CH_2Cl_2. Steps (vi) to (x) were then repeated.

Abbreviations: BOC, tert-butyloxycarbonyl; PMB, p-methoxybenzyl; Bzl, benzyl; Tos, toluenesulfonyl; Z, carbobenzoxy.

The glutamine residue (11.2 mmol) was incorporated via its p-nitrophenyl ester using DMF as the only solvent, and the coupling was allowed to proceed for 20 hours. All washes were 2 min unless noted otherwise. N^{α}-BOC protection was used with the following side-chain protecting groups: Cys, PMB; Ser, Bzl; Glu, Bzl; Arg, Tos; Thr, Bzl and Lys, Z.

Cyclic Eicosapeptide. Cleavage of the peptide from the solid support, accompanied by simultaneous removal of the side-chain protecting group, was accomplished with hydrogen fluoride (6). A portion of the protected peptide resin (0.94 g) was treated with HF (12 ml) for 45 min at 0° in the presence of anisole (1 ml). After rapid removal of HF and drying in vacuo the resin was washed with ethyl acetate (25 ml), dried, and then extracted with 50% acetic acid (25 ml). The extract was applied to a Sephadex G-10 column equilibrated in 50% acetic acid. Elution with the same solvent gave a major peak as detected by the fluorescamine assay (7) (Fig. 2A). Fractions 26-39 were pooled and made up to 500 ml with 10^{-2} M mercaptoethanol in water. The solution was flushed with N_2 and heated at 60° for 4 h. After freeze drying, one half of this material was submitted to gel filtration on a Sephadex G-25F column (2.5 x 55.0 cm) in 1 N acetic-10^{-2} M mercaptoethanol, which yielded two peaks (Fig. 2B). The peptide material in the major, unsymmetrical peak (elution volume 140-206 ml) as detected fluorometrically was further submitted to chromatography on a column of CM-cellulose (1.0 x 26.5 cm) in ammonium acetate buffer (Fig. 2C). As detected spectrophotometrically at 260 mμ, two peaks were observed after the buffer change. Amino acid analysis showed the two portions of the second, bifurcate peak to be identical, suggesting that some dimerization had occurred. Fractions 64-80 containing the bifurcate peak were pooled and freeze-dried to yield 3.5 mg of purified peptide. Amino acid analysis of

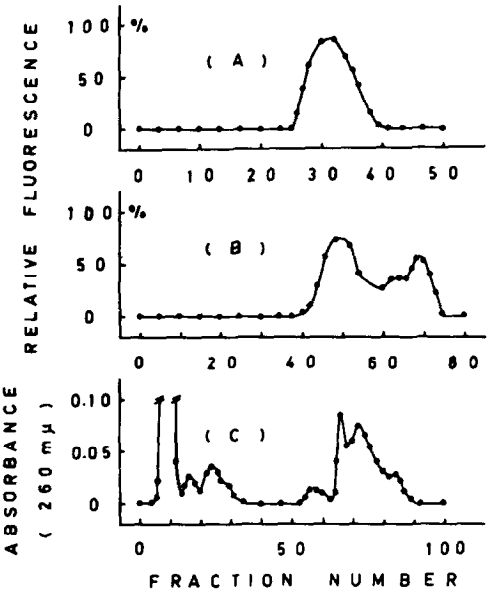

Figure 2

A) Chromatography in 50% acetic acid of crude hGH 172-191 on Sephadex G-10 (void volume 86 ml). Fraction volume 2.8 ml.

B) Exclusion chromatography on Sephadex G-25 (void volume 126 ml) of the peak from Fig. A. Fraction volume 3.5 ml.

C) Ion exchange chromatography on CMC of the front peak from Fig. B. After elution with 145 ml of 0.01 M ammonium acetate, pH 4.5, a gradient was established by the introduction of 50 ml 0.40 M ammonium acetate, pH 4.5, through a mixing chamber containing 50 ml of the starting buffer. Fraction volume 2.8 ml.

an acid hydrolysate (8) gave: Lys, 1.0; Arg, 2.2; Thr, 1.1; Ser, 1.2; Glu, 2.0; Gly, 109; cysteic acid, 0.8; Val, 2.1*; Ile, 0.5*; Leu, 1.1 and Phe, 2.0.

The purified preparation was cyclized by further reduction and air oxidation as follows: After a repeat reduction as previously described, prolonged lyophilization was employed to remove the reducing agent. The reduced peptide (0.5 mg) was dissolved in 0.01 M acetic acid (0.1 μg peptide/ml) and ad-

* Amino acid analysis of a leucine aminopeptidase digest (9) gave Val, 3.0 and Ile, 1.0.

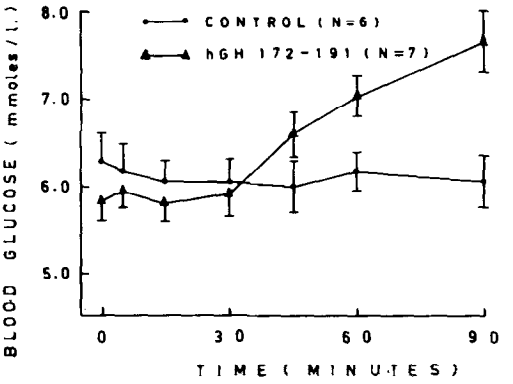

Figure 3

The blood glucose levels (mean ± S.D.) between the control (o) and the test (▲) groups at each time were statistically assessed according to Student t test. Significant differences were observed at 45 min ($p < 0.010$), 60 min ($p < 0.005$) and 90 min ($p < 0.002$) after injection. Each test animal was given intraperitoneally 45 nmol/kg body weight of the synthetic peptide in 0.5 ml saline, and the control animals received an equivalent volume of saline in the identical manner.

justed to pH 8.5 with TEA. Oxygen was bubbled through the solution for 4 hr, the pH adjusted to 3.0 with glacial acetic acid and the solution was then rotary evaporated to a small volume. The Ellman test (10) on an aliquot was negative. In order to prevent inactivation, it is essential to dry the peptide exhaustively and to store in vacuo. The cyclized eicosapeptide when applied to a Sephadex G-25 SF column (1.5 x 63 cm, void volume 48 ml) in 1 M acetic acid yielded a single peak with elution maximum at 55 ml. High voltage paper electrophoresis (1.5 kv, 90 min., Whatman 3 MM) in formic acid-acetic acid buffer, pH 1.9, gave a single ninhydrin positive spot with an Rf value of 0.56 relative to lysine. Thin layer chromatography in 1-butanol-pyridine-acetic acid-water (30:20:6:24, v/v) and ethyl acetate-pyridine-acetic acid-water (5:5:1:3, v/v) gave single ninhydrin positive spots with Rf 0.44 and 0.50 respectively.

Biological Results. The hyperglycaemic action of the synthetic peptide

hGH 172-191 was demonstrated by its effect on the basal levels of blood glucose in normal rats (Fig. 3). A single intraperitoneal injection of hGH 172-191 at a dose of 4.5 nmol per 100 g body weight induced a significant rise of blood glucose in rats fed ad libitum. However, no such biological effect of the synthetic peptide at a similar dose was observed in 36-48 hr fasted rats. This observation is consistent with the previous suggestions (4) that the hyperglycaemic effect of the peptide is a result of both (i) inhibition of glucose uptake by peripheral tissues, and (ii) stimulation of glycogenolysis in liver.

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