Solid Phase Synthesis of a 31-Residue Fragment of Human Glucose-Dependent Insulinotropic Polypeptide (GIP) by the Continuous Flow Polyamide Method

Mats Carlquist

Department of Biochemistry II, The Karolinska Institute, Box 60 400, S-104 01 Stockholm, Sweden

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A fragment, GIP_{1-31}, of the human Glucose-dependent Insulinotropic Polypeptide (GIP_{1-42}) Tyr-Ala-Glu-Gly-Thr-Phe-Ile-Ser-Asp-Tyr-Ser-Ile-Ala-Met-Asp-Lys-Ile-His-Gln-Gln-Asp-Phe-Val-Asn-Trp-Leu-Leu-Ala-Gln-Lys-Gly has been synthesized by solid phase methodology under continuous flow conditions. The peptide was assembled on a polydimethylacrylamide kieselguhr support by using 9-fluorenylmethoxycarbonyl amino acid pentafluorophenyl esters. The crude peptide, obtained after trifluoroacetic acid cleavage, was purified by gel filtration and by reverse-phase HPLC.

This synthetic replicate, corresponding to human GIP_{1-31}, retains the ability of naturally occurring GIP to stimulate insulin release.

GIP (Gastric Inhibitory Polypeptide) was originally isolated from pig intestine on the basis of its inhibitory effect on gastric acid secretion.[1] Later investigations into the biological role(s) of GIP suggested, however, that the major physiological action of this polypeptide might be to stimulate the release of insulin at elevated glucose concentrations, rather than the enterogastrone effect.[2] As a consequence of this incretin effect[3] it has been renamed Glucose-dependent Insulinotropic Polypeptide, thus retaining the original acronym GIP.

GIP is composed of 42 amino acid residues[4] and is structurally related to the glucagon-secretin family of polypeptides[5] (Fig. 1). Subsequently, the bovine[6] and human[7] forms of GIP have also been isolated.

In contrast to most of the members of the

	10 20 30 40
Human GIP	YAEGTFISDYSIAMDKIHQQDFVNWLLAQKGKKNDWKHNITQ
Bovine GIP	YAEGTFISDYSIAMDKIRQQDFVNWLLAQKGKKSDWIHNITQ
Porcine GIP	YAEGTFISDYSIAMDKIRQQDFVNWLLAQKGKKSDWKHNITQ
Human/bovine/porcine VIP	HSDAVFTDNYTRLRKQMAVKKYLNSILN$_a$
Human/bovine/porcine glucagon	HSQGTFTSDYSKYLDSRRAQDFVQWLMNT
Human secretin	HSDGTFTSELSRLREGARLQRLLQGLV$_a$
Bovine/porcine secretin	HSDGTFTSELSRLRDSARLQRLLQGLV$_a$
Human PHI	HADGVFTSDFSKLLGQLSAKKYLESLM$_a$
Bovine PHI	HADGVFTSDYSRLLGQLSAKKYLESLI$_a$
Porcine PHI	HADGVFTSDFSRLLGQLSAKKYLESLI$_a$

Fig. 1. Amino acid sequences of members of the glucagon-secretin group of peptides. a: amidated residue. Underlined residues indicate identity in type and position with human GIP.

glucagon-secretin family, the C-terminal residue in GIP is not amidated. An interesting characteristic common to all known GIP structures is the -Gln-Lys-Gly-Lys-Lys- sequence (positions 29–33 in Fig. 1). It has been shown in many cases, although not all, that proteolytic cleavage of peptide hormone precursors occurs at pairs of basic amino acid residues[8] and that in precursors to C-terminally α-amidated peptides the amino acid residue that is amidated in the product peptide is followed in the precursor molecule by a glycine residue.[9,10] This might indicate a potential cleavage site in GIP, which should result in a peptide not only structurally related, but also similar in size, to the other members of the glucagon-secretin family. However, no such shorter amidated form of GIP has been detected, and further, no mammalian peptide with an amidated C-terminal lysine residue has been found. This, together with the fact that two of the three known amino acid substitutions (species variations) are located after position 33, indicates the possibility that shorter forms of GIP might retain the ability to stimulate insulin release. Recently, GIP_{1-39} has been obtained after enzymatic treatment of naturally occurring GIP and shown to be able to stimulate insulin release.[11] In order to obtain other C-terminally truncated forms of GIP, organic chemical peptide synthesis is an alternative strategy.

This paper describes the solid phase synthesis of human GIP_{1-31} by the 9-fluorenylmethoxycarbonyl (Fmoc) – polyamide method.[12]

Results and discussion

The present synthesis is based on the methodology described by Atherton and Sheppard.[13] Esterification of the C-terminal Fmoc-glycine residue to the polyamide resin utilized the preformed symmetrical anhydride, with 4-dimethylaminopyridine as catalyst. Amino acid analysis indicated ca. 60 % incorporation after 1 h based on the internal norleucine standard. This low initial coupling yield was due to low solubility of the newly-formed symmetrical anhydride in dichloromethane. Thus, a portion of the activated amino acid was lost when the dicyclohexylurea was eliminated by filtration. However, residual hydroxy groups do not interfere with subsequent amino acylation reactions in the absence of basic catalysts.[14]

The subsequent acylation reactions were performed using activated esters, pentafluorophenyl

Table 1. Amino acid analyses. Values are molar ratios without correction for destruction, incomplete hydrolysis or impurity. In parentheses are nearest integers.

Amino acid	Resin-bound peptide	Crude preparation after gel-filtration	HPLC-purified peptides			Human GIP_{1-31}
			A	B	C	
Trp	n.d.	+[a]	+[a] (1)	+[a] (1)	+[a] (1)	1
Lys	2.1	2.2	2.0 (2)	2.0 (2)	2.0 (2)	2
His	0.9	1.0	1.0 (1)	1.0 (1)	1.0 (1)	1
Asp	4.1	3.9	3.9 (4)	3.9 (4)	3.9 (4)	4
Thr	1.0	0.9	1.0 (1)	1.0 (1)	1.0 (1)	1
Ser	2.0	1.9	2.0 (2)	2.0 (2)	1.9 (2)	2
Glu	4.2	4.2	4.0 (4)	4.0 (4)	4.0 (4)	4
Gly	2.2	2.2	2.1 (2)	2.1 (2)	2.0 (2)	2
Ala	3.1	3.0	3.0 (3)	3.0 (3)	3.0 (3)	3
Val	0.9	1.0	1.0 (1)	0.8 (1)	1.0 (1)	1
Met	0.9	0.9	0.9 (1)	0.9 (1)	0.9 (1)	1
Ile	2.4	2.5	2.0 (2)	2.9 (3)	3.0 (3)	3
Leu	2.1	2.2	2.0 (2)	2.1 (2)	2.0 (2)	2
Tyr	1.9	1.9	2.0 (2)	1.9 (2)	2.0 (2)	2
Phe	2.1	2.0	2.0 (2)	2.0 (2)	2.0 (2)	2
Total			30	31	31	31

[a]Tryptophan was detected by the method of Ehrlich.[20]

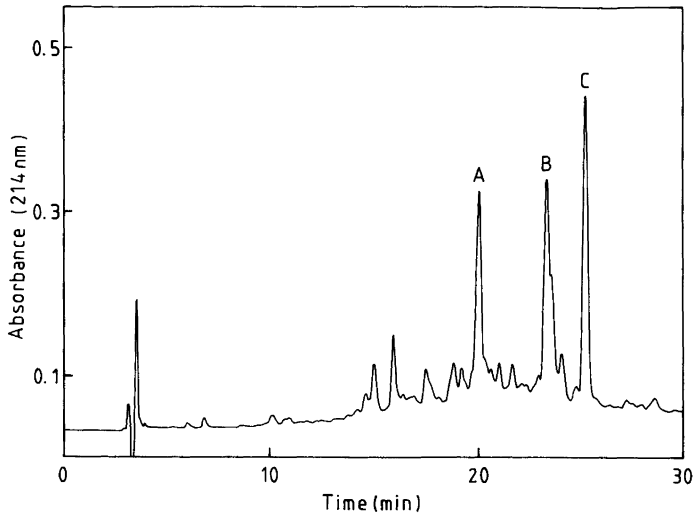

Fig. 2. Reverse-phase HPLC of synthetic GIP_{1-31}. Load: 50 μg of the material obtained after gel filtration on Sephadex G-25. Column: Vydac 218TP54 (4.6 × 250 mm). Solvent system: 0.1 % trifluoroacetic acid with a linear gradient of acetonitrile (25–36 % in 30 min). Flow rate: 1 ml min^{-1}.

and oxo-benzotriazine derivatives. The double coupling of the serine residues was a consequence of previously low coupling yields for this ester.

An aliquot of the completed entriacontapeptide, with the total composition shown in Table 1, was cleaved from the resin with 90 % trifluoroacetic acid in the presence of an excess of methionine and tryptophan as scavengers. Amino acid analysis of the residual resin showed that peptide detachment was 70 % complete.

The cleavage product, obtained after the trifluoroacetic acid was evaporated, was not completely soluble in 0.2 M acetic acid. The acetic acid was therefore removed by lyophilization and

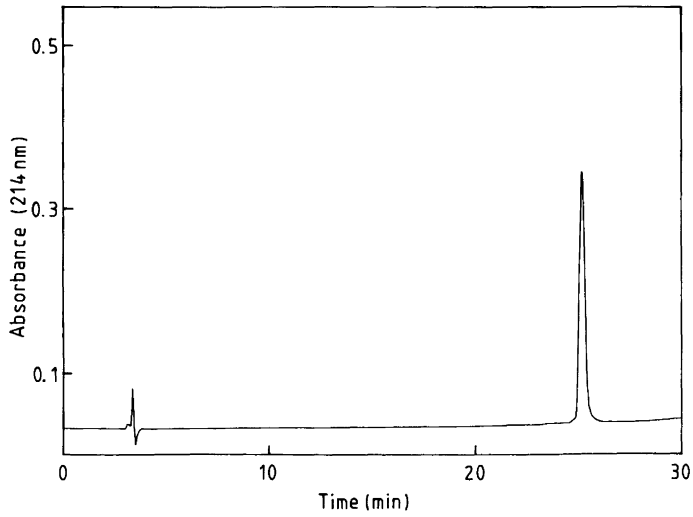

Fig. 3. HPLC of 10 μg of purified GIP_{1-31} (corresponding to peak C in Fig. 2). For chromatographic system, see Fig. 2.

the material was dissolved in trifluoroacetic acid. Peptides were then precipitated by the addition of ether and dried *in vacuo*. After this treatment the peptide material was soluble in 0.2 M acetic acid and was chromatographed on Sephadex G-25 (fine). The product obtained after the gel chromatography (18 mg) amounted to ca. 50 % of that expected, and the amino acid composition is shown in Table 1. The high performance liquid chromatography (HPLC) profile (shown in Fig. 2) of this material revealed peaks for three principal components: A, B and C. Both B and C have an amino acid composition, determined after both acid and enzymatic hydrolysis, consistent with the corresponding human GIP_{1-31} sequence, whereas the isoleucine residue at position 7 is absent in A (Table 1).

Component C was found to be the desired product, human GIP_{1-31}, while the N-terminal tryptic fragment of B does not co-elute with the corresponding porcine fragment in HPLC analysis,[6] the reason for this discrepancy being as yet unknown.

Totally 4.5 mg of GIP_{1-31} was obtained after the HPLC step, corresponding to an overall yield of 5 % calculated by comparison with the internal norleucine reference. This material, which gave a single spot in thin-layer chromatography, binds to a GIP-specific receptor, stimulates production of cyclic AMP and stimulates insulin release in the same manner as naturally occurring GIP_{1-42}.[15,16] The HPLC profile of this material is shown in Fig. 3.

Experimental

The Fmoc amino acids, Fmoc amino acid pentafluorophenyl esters and Fmoc amino acid oxobenzotriazine esters were purchased from Cambridge Research Biochemicals. Side-chain protection was provided by *tert*-butyl groups for aspartic acid, glutamic acid, serine, threonine and tyrosine. The ε-amino group of lysine and the imidazole ring of histidine were protected by *tert*-butoxycarbonyl groups. The polydimethylacryl amide-kieselguhr composite resin (Pepsyn KA) with an internal norleucine residue was obtained from Cambridge Research Biochemicals. *N,N*-Dimethylformamide (DMF) ("AnalaR"), obtained from BDH Chemicals Ltd., was shaken occasionally with 4Å molecular sieves (Merck) for 24 h to remove water and dimethylamine, whereafter the DMF was filtered through a sintered-glass filter. Other solvents and reagents were of analytical grade. Natural porcine GIP_{1-42} was prepared in the laboratory. Acid hydrolysis was performed in evacuated tubes with 6 M HCl, containing 0.5 % phenol, at 110 °C for 22 h. Digestions with trypsin, chymotrypsin and aminopeptidase M were performed in 1 % ammonium bicarbonate at 37 °C for 6–20 h. Total amino acid compositions were determined on a Beckman 121 M instrument. Thin-layer chromatography was carried out on silica plates (Riedel-de Haën) with the solvent system: 1-butanol – acetic acid – pyridine – water (30:6:20:24, *v/v*)[17] and peptide spots were visualized with ninhydrin. HPLC was carried out in 0.1 % trifluoroacetic acid with a gradient of acetonitrile on a Vydac 218TP54 reverse-phase column using an instrument from Waters Associates.

Synthesis of the peptide. The peptide was synthesized by the continuous flow polyamide method,[12] using a manual instrument from Cambridge Research Biochemicals set at 3.3 ml min^{-1}. The reaction column was packed with 0.8 g of resin (ca. 0.1 mequiv OH per g) suspended in DMF. An eight-fold molar excess of the C-terminal Fmoc-glycine residue (0.64 mmol) was activated by pre-forming the symmetrical anhydride using dicyclohexylcarbodiimide (0.32 mmol) in dichloromethane. The dicyclohexylurea was removed by filtration and the filtrate was evaporated. Esterification of the C-terminal residue was performed in DMF with 4-dimethylaminopyridine (0.032 mmol) as catalyst.[18] The acylation reaction was performed during recirculation of the activated residue for 1h. Excess reagents were washed out with DMF (10 min), and the α-amino protecting (Fmoc) group was removed by washing the column with 20 % piperidine in DMF for 10 min. The base was eliminated by a second DMF treatment (20 min). The subsequent 30 step-wide coupling reactions were performed using a five-fold molar excess of Fmoc-amino acid pentafluorophenyl esters, with the exceptions that serine and threonine were coupled as their Fmoc-amino acid oxo-benzotriazine ester derivatives, in DMF with a coupling time of 45 min.[13] The two serine residues were double coupled. The progress of the coupling and deprotection reactions, as well as the washing steps, was

continuously monitored using a flow-cell in a UV spectrophotometer set at 308 nm.[19]

After the final piperidine treatment, the resin was transferred to a sintered-glass funnel, washed thoroughly with DMF, dichloromethane and ether, and dried *in vacuo* (yield 0.968 g). A sample of the dried peptide resin was hydrolyzed and subjected to amino acid analysis.

The completed 31-residue peptide was liberated from the resin (yield 305.1 mg) and deprotected in a round-bottom flask with 20 ml 90 % trifluoroacetic acid containing a 15-fold molar excess of methionine and tryptophan. After 3 h at room temperature the content of the flask was filtered through a sintered-glass funnel and the filtrate was collected. The reaction flask was rinsed three times with small portions of trifluoroacetic acid. Each rinse was transferred to the glass funnel to wash the resin. The solvent was removed from the combined filtrate using a rotary evaporator. The residue was dissolved in 0.2 M acetic acid, but since it did not dissolve completely the acetic acid was eliminated by lyophilization and the material was dissolved in trifluoroacetic acid (5 ml), whereafter the peptides were precipitated with ether (75 ml). The precipitate was collected, washed with ether and dried *in vacuo*. This material was then chromatographed on a Sephadex G-25 (fine) column (2.5 × 90 cm) in 0.2 M acetic acid and was finally purified by HPLC (for details see legend to Fig. 2).

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