Hoppe-Seyler's Z. Physiol. Chem.
Bd. 356, S. 1305 - 1312, August 1975

Studies on Polypeptides, VI[1,*]

Synthesis, Circular Dichroism and Immunological Studies of Tyrosyl C-Peptide of Human Proinsulin

*Vinod K. Naithani**, Mathias Dechesne**, Jan Markussen***, Lise G. Heding*** and Ulla D. Larsen****

(Received 12 May 1975)

Dedicated to Professor Dr. Dr. Günther Weitzel on the occasion of his 60th birthday

Summary: The synthesis of tyrosyl human C-peptide, a sequence of 32 amino acids, by the fragment condensation of the *N*-terminal octapeptide and *C*-terminal tetracosapeptide is described. The *t*-butyl protecting groups were removed by trifluoroacetic acid to obtain *N*-benzyloxycarbonyltyrosyl C-peptide. The hydrogenolytic debenzyloxycarbonylation of this derivative proceeded to an extent of only 85 - 90%, and tyrosyl C-peptide was purified by preparative electrophoresis. This purified tyrosyl C-peptide led to an improved sensitivity of the radioimmunoassay. The synthetic tyrosyl C-peptide in an immunoassay using anti human b-component serum reacted slightly differently from the synthetic human C-peptide. After labelling tyrosyl C-peptide with ^{125}I and then purifying the radioactive product, we observed that 80% of the radioactivity could be bound when reacted with an excess of the serum. The circular dichroism spectrum of tyrosyl C-peptide is very similar to that of synthetic human C-peptide. An analysis of the spectrum indicates that 3 - 7 amino acids are in the β-structure and the rest in random coil conformation.

Untersuchungen an Polypeptiden, VI: Synthese des Tyrosyl-C-Peptids von Human-Proinsulin. Circulardichroismus- und immunologische Untersuchungen

Zusammenfassung: Das Tyrosyl-C-Peptid von Human-Proinsulin, eine Sequenz von 32 Aminosäuren, wurde durch Kondensation des *N*-terminalen Octapeptids mit dem *C*-terminalen Tetracosapeptid synthetisiert. Nach Entfernung der *t*-Butyl-Schutzgruppen erhielt man das *N*-Benzyloxycarbonyltyrosyl-C-Peptid. Die hydrogenolytische Debenzyloxycarbonylierung lief nur zu 85 - 90% ab; das Tyrosyl-C-Peptid wurde durch präparative Elektrophorese gereinigt. Dieses Produkt zeigte erhöhte Empfindlichkeit im Radioimmuntest. Im Immuntest mit Antiserum gegen Human-b-Komponente reagierte es kaum anders als synthetisches Human-C-Peptid. Nach Markierung des Tyrosyl-C-Peptids mit ^{125}J und Reinigung des radioaktiven Produkts ließen sich 80% der Radioaktivität bei Reaktion mit überschüssigem Serum binden.
Das Circulardichroismus-Spektrum des Tyrosyl-C-Peptids ist dem des synthetischen Human-C-

---

* 104th Paper on Peptides. For 103rd see ref.[1].

** *Address:* Dr. V. K. Naithani, Deutsches Wollforschungsinstitut an der TH Aachen, D-51 Aachen, Veltmanplatz 8.
*** Novo Research Institute, Novo Alle, DK-2880 Bagsvaerd.

Abbreviations: Bu^t = *t*-butyl ether, OBu^t = *t*-butyl ester, ONSu = *N*-hydroxysuccinimide ester, Z = benzyloxycarbonyl, CD = circular dichroism.

Peptids sehr ähnlich. Eine Analyse des Spektrums zeigte, daß 3 - 7 Aminosäuren in der β-Struktur vorliegen, die übrigen in „random coil"-Konformation.

In human proinsulin, the connecting peptide of 35 amino acids links the amino terminus of the A-chain with the carboxyl terminus of the B-chain[2,3]. The proteolytic cleavage of proinsulin in the β cells generates insulin and C-peptide, which are released in equimolar concentration into the circulation[4-7]. The C-peptide is defined as the connecting peptide minus the four basic residues. Human C-peptide has been isolated from pancreas and sequenced[2,3]. Due to autolysis, it is extremely difficult to isolate it pure and in substantial amounts.

The human C-peptide immunoassay has been used for the estimation of circulating C-peptide in serum[8-11]. Since human C-peptide does not contain tyrosine (Fig. 1), coupling of this amino acid to the amino group of C-peptide prior to labelling is necessary. Three semisynthetic methods utilise this principle to tyrosylate the C-peptide[8,11,12]. On account of the extremely limited availability of the native human C-peptide, there is a need for a pure synthetic tyrosyl human C-peptide as a tracer in immunological studies. Recently Kaneko et al.[13] have reported the immunoassay of C-peptide using the labelled synthetic tyrosyl human connecting peptide as a tracer. The details of their synthesis, however, have not yet been published.

This paper describes the synthesis and immunological properties of tyrosyl human C-peptide, preparation and purification of ^{125}I-labelled tyrosyl C-peptide, and its immunological properties. The circular dichroism spectrum of tyrosyl human C-peptide is analysed for structural elements.

$$\overset{33}{\text{H-Glu-Ala-Glu-Asp-Leu-Gln-Val-}}\overset{40}{\text{Gly-Gln-Val-Glu-}}$$
$$\text{-Leu-Gly-Gly-Gly-Pro-}\overset{50}{\text{Gly-Ala-}}\text{Gly-Ser-Leu-Gln-Pro-}$$
$$\text{-Leu-Ala-Leu-}\overset{60}{\text{Glu-Gly-}}\text{Ser-Leu-}\overset{63}{\text{Gln-OH}}$$

Fig. 1. Amino acid sequence of human C-peptide. Numbers refer to the human proinsulin sequence. In the synthetic studies, tyrosine is introduced at position 32 (not shown).

Results

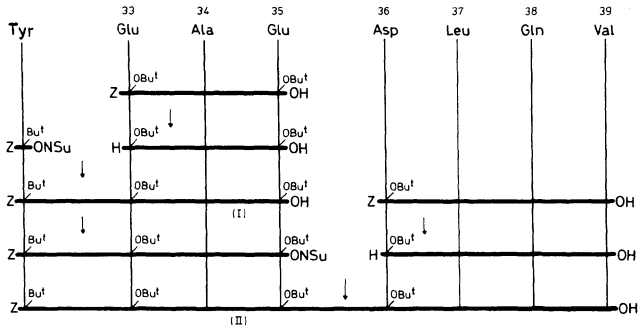
Synthesis

Tyrosyl human C-peptide is assembled from two fragments: octapeptide (Tyr-33-39) and tetracosapeptide (40 - 63). The preparation of the protected tetracosapeptide, which was used in the human C-peptide synthesis, was described earlier[1]. The amino terminus of the octapeptide was protected with a benzyloxycarbonyl group and the side chains, as in the case of tetracosapeptide, with *t*-butyl protection.

Scheme 1 describes the synthesis of the *N*-terminal octapeptide. *N*-Benzyloxycarbonyl-*O*-*t*-butyltyrosine *N*-hydroxysuccinimide ester[14] was reacted with the tripeptide, γ-*t*-butoxyglutamyl-alanyl-γ-*t*-butoxyglutamic acid, to yield the desired tetrapeptide (I). The purification of I was achieved by counter-current distribution. Another tetrapeptide, benzyloxycarbonyl-β-*t*-butoxyaspartyl-leucyl-glutaminyl-valine[15], also an intermediate used in the human C-peptide synthesis, was debenzyloxycarbonylated and condensed with the *N*-hydroxysuccinimide ester of the *N*-terminal tetrapeptide (I) to yield the octapeptide (II). The purification of the octapeptide was effected by column chromatography on Sephadex LH-20 in dimethylformamide.

The tetracosapeptide (sequence 40 - 63, Scheme 2) was debenzyloxycarbonylated by catalytic hydrogenation and the ensuing amine was coupled to the *N*-terminal octapeptide (II) with dicyclohexylcarbodiimide/1-hydroxybenzotriazole to yield the protected dotriacontapeptide (protected tyrosyl human C-peptide, III). The chromatographic purification over Sephadex LH-20 in dimethylformamide removed most of the unreacted octapeptide (II) from the reaction mixture.

The *t*-butyl protecting groups were selectively removed by trifluoroacetic acid treatment. The major purification of the *N*-benzyloxycarbonyltyrosyl human C-peptide (IV), by virtue of its insolubility, was achieved by extracting it with a mixture of ethanol/water (4 : 1) containing 3mM HCl. Amino acid analysis of the insoluble product



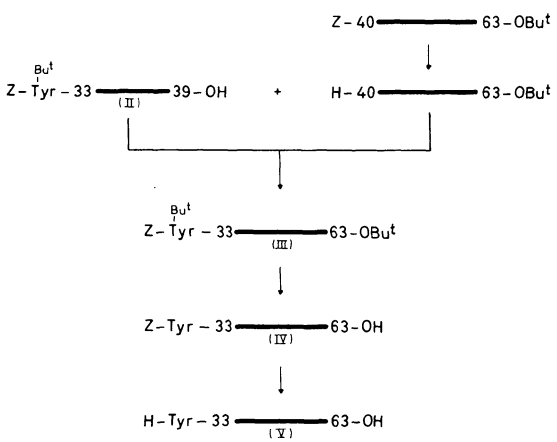
Scheme 1. Synthesis of the octapeptide.

gave theoretically expected values for tyrosyl human C-peptide.

N-Benzyloxycarbonyltyrosyl human C-peptide was catalytically hydrogenated and the resulting product, crude tyrosyl human C-peptide (V), was purified by preparative electrophoresis in pyridine acetate, pH 6.5. The strip containing tyrosyl human C-peptide was eluted by 0.05M NH_4HCO_3 solution and lyophilised. The final product was chromatographed over Sephadex G-25 in 0.05M NH_4HCO_3 solution to yield pure tyrosyl human C-peptide (V). The amino acid composition of the product agreed with the expected values. Tyrosyl human C-peptide was shown to be homogeneous by thin-layer chromatography and by electrophoresis.

Immunological studies

Fig. 2 shows the standard curve of tyrosyl C-peptide as compared to C-peptide[1]. At concentrations higher than 0.1 pmol/ml, tyrosyl C-peptide competed more effectively with ^{125}I-tyrosyl C-peptide for the antibodies than synthetic human C-peptide.



Scheme 2. Synthesis of the tyrosyl human C-peptide.

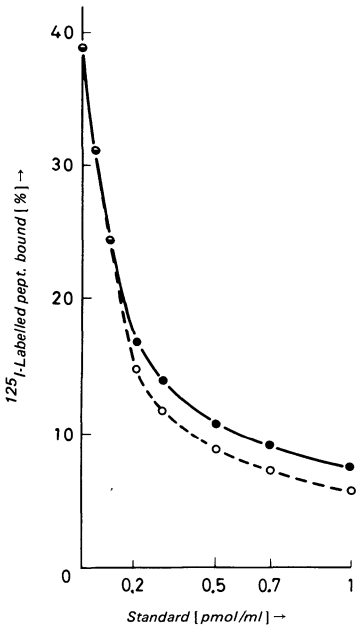


Fig. 2. Standard curves of synthetic tyrosyl C-peptide and synthetic C-peptide.
●——●, Synthetic human C-peptide. ○– – –○, Synthetic human Tyr-C-peptide.

The purification of crude tyrosyl C-peptide to pure tyrosyl C-peptide increased the yields of incorporation of ^{125}I, in two different iodination experiments, from 27 and 34% to 58 and 78%, respectively. The iodination was carried out by the chloramine-T method[16], modified as described in the experimental part. The other iodination procedure employed in the case of semi-synthetic tyrosyl C-peptide[11] using potassium iodate as an oxidising agent failed completely to incorporate ^{125}I into the crude synthetic tyrosyl C-peptide. The crude tyrosyl C-peptide apparently contains an impurity, possibly from the catalyst, that destroys the iodate.

When synthetic ^{125}I-tyrosyl C-peptide was incubated with an excess of an anti-human b-component serum, 80% of the radioactivity could be bound to the antibodies, both with the crude and pure tyrosyl C-peptide. However, the standard curve obtained with the ^{125}I-tyrosyl C-peptide prepared from the purified substance was the steepest (Fig. 3), thereby giving a more precise analysis of C-peptide. The coprecipitation of ^{125}I-tyrosyl C-peptide in the absence of antibodies was 4.1% ± 0.18% (mean of 10 ± SEM).

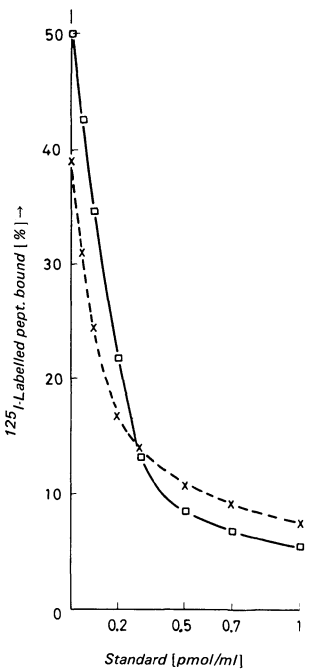


Fig. 3. Standard curves obtained with ^{125}I-tyrosyl C-peptides from the crude and purified tyrosyl C-peptide, respectively.
x– – –x, Crude ^{125}I-Tyr-C-peptide. □——□, Purified ^{125}I-Tyr-C-peptide.

Circular dichroism studies

Fig. 4 shows the CD spectrum of the pure tyrosyl C-peptide. The analysis of the spectrum according to the nonlinear regression procedure[17] estimates 6 - 7 amino acids in β-structure and the rest in random coil conformation, with the coefficients of Greenfield and Fasman[18], and 3 amino acids in β-structure and the rest in random coil with the coefficients of Chen et al.[19]. The CD spectra of ox, pork, duck[17], synthetic human[1] and synthetic tyrosyl human C-peptides were included in the analyses. The synthetic tyrosyl human C-peptide spectrum is very similar to that of human C-peptide[1].

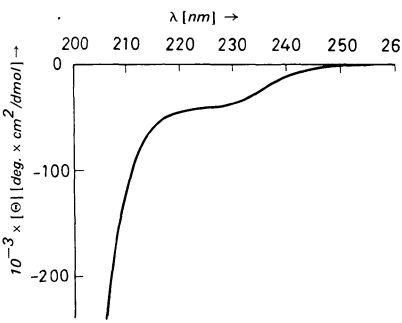


Fig. 4. CD spectrum of synthetic tyrosyl C-peptide. Peptide conc. ≈ 479 μM, l = 0.05 cm. [Θ] = molar circular ellipticity.

Discussion

The fully synthetic tyrosyl C-peptide, like the semisynthetic preparations, has the tyrosyl moiety at the amino terminus of the C-peptide. The fragment condensation of the octapeptide and tetracosapeptide proceeded to the protected tyrosyl human C-peptide. *N*-benzyloxycarbonyltyrosyl human C-peptide was obtained in good purity after a simple extraction procedure.

The catalytic hydrogenation of *N*-benzyloxycarbonyltyrosyl human C-peptide in acetic acid under 6 atmospheres pressure for 24 h failed to remove the benzyloxycarbonyl group quantitatively. The blocked product amounted to about 10 - 15% in the reaction mixture. Preparative electrophoresis in pyridine acetate was employed to separate the blocked derivative and a trace of fast moving component from the tyrosyl C-peptide.

The optical rotation and the CD spectra of human C-peptide[1] and tyrosyl human C-peptide were very similar, indicating that the addition of a tyrosyl residue to the *N*-terminus of the C-peptide has little or no effect upon the overall conformation.

Tyrosyl C-peptide displaced ^{125}I-tyrosyl C-peptide more effectively than C-peptide (Fig. 2). This indicates that the tyrosyl residue has either effected a minor conformational change in the C-peptide which favors a higher affinity toward the antibodies, or that the tyrosyl moiety non-specifically binds to the antibody molecules and thereby increases the binding constant.

The semisynthetic ^{125}I-tyrosyl C-peptide[11] prepared from natural human C-peptide could be bound by an excess of these antibodies to an extent of only 62%, as compared to 80% for the pure synthetic ^{125}I-tyrosyl C-peptide.

The improved sensitivity of the immunoassay (Fig. 3) obtained with iodinated pure synthetic tyrosyl C-peptide is probably due to removal of substances having lower affinity to the antibodies, e.g. tetracosapeptide (sequence 40 - 63) or octapeptide (sequence Tyr-33-39). The higher specific activity obtained with the pure synthetic tyrosyl C-peptide as well as the higher binding of the synthetic ^{125}I-tyrosyl C-peptide with the antibodies have thus considerably increased the sensitivity and precision of the human C-peptide radioimmunoassay.

Experimental

All optically active amino acids are of L-configuration. The melting points are uncorrected. Thin-layer chromatography (Kieselgel G, Merck) of protected intermediate fragments was performed in the solvent systems (v/v): 2-butanol/formic acid/water (75 : 13.5 : 11.5), 2-butanol/-10% ammonia (85 : 15), chloroform/methanol/acetic acid (95 : 5 : 3) and n-heptane/pyridine/*t*-butanol (75 : 15 : 15). The solvent systems n-butanol/acetic acid/pyridine/water (30 : 6 : 20 : 24) and pyridine acetate, pH 6.5 were used for thin-layer chromatography (DC-Allufolian Cellulose, thickness 0.1 mm, Merck) and paper electrophoresis, re-

spectively, of tyrosyl human C-peptide. Spots were developed with the ninhydrin, ninhydrin/collidine, chlorine/toluidine reaction and the Pauly reagent[20]. Amino acid analyses of the samples were carried out after hydrolysis with 6N HCl containing 1% phenol in the sealed evacuated tubes for 20 h at 110 °C. Values for serine and tyrosine are uncorrected. The column chromatography of the protected sequences was done on Sephadex LH-20 in dimethylformamide (3 × 96 cm).

Circular dichroism spectra were recorded on a Roussel-Jouan Dichrograph Model 185 in cells with a light path of 0.05 cm, as described earlier[17]. Pure tyrosyl C-peptide was dissolved in a 0.1M NaCl and 0.01M Na_2HPO_4 buffer, adjusted to pH 7.4 with HCl, in a concentration of 479 μM.

Immunological tests were performed as described earlier[11]. The iodination of crude and purified tyrosyl C-peptide was performed as follows: 10 μl of a solution containing 20 μg of synthetic tyrosyl human C-peptide was pipetted into a small test tube and mixed with 25 μl of 0.4M phosphate buffer (pH 7.2) and 10 μl (1.0 mCi) of a carrier-free solution of ^{125}I (Radiochemical Centre, Amersham, Code No. IMS 3). The following reagents (all in 0.04M phosphate buffer, pH 7.4) were then added with stirring: 5 μl of 0.4% chloramine T, 20 s later 20 μl of 0.24% $Na_2S_2O_5$, then 50 μl of 1% KI. The mixture was diluted to 2 ml with distilled water and then subjected to anion exchange chromatography on a column (*d* = 0.7 cm) containing 100 mg of QAE-Sephadex A-25 (Pharmacia) equilibrated with 0.1M histidine, 0.05M HCl buffer, pH 6.1. The application of the iodination mixture was performed at a low rate (2 ml in the course of 20 min) to ensure complete adsorption of the tyrosyl human C-peptide. To remove unreacted ^{125}I, the column was eluted with the histidine buffer (at a rate of about 1 ml/min) and 2-ml fractions were collected until the radioactivity reached and remained at a low level (after about 20 fractions). The ^{125}I-tyrosyl human C-peptide was then eluted with 1% human albumin (Behringwerke, trocken, reinst) in 0.02N HCl. The solution was diluted with 1% human albumin soution to make 20 ml, corresponding to a calculated concentration of 1 μg of labelled and unlabelled tyrosyl human C-peptide per ml. Finally, the pH was adjusted to 7 with 1N NaOH.

Benzyloxycarbonyl-O-t-butyltyrosyl-γ-t-butoxyglutamyl-alanyl-γ-t-butoxyglutamic acid (I)

Z-Glu(OBu^t)-Ala-Glu(OBu^t)-OH* (2.96 g, 5 mmol) was hydrogenated with 1 g Pd in 90 ml methanol for 4 h. The catalyst was removed by filtration and the filtrate was concentrated in vacuo to dryness. The residue was dissolved in 100 ml dioxane/water (1 : 1, v/v) containing $NaHCO_3$ (1.26 g, 15 mmol). To it Z-Tyr(Bu^t)-ONSu[14] (2.34 g 5 mmol) was added and the reaction mixture was stirred overnight. Dioxane was removed in vacuo and the aqueous phase was extracted with ethyl acetate after acidification with dilute H_2SO_4 solution. The organic phase was washed with water, dried over Na_2SO_4 and evaporated to dryness. The crude tetrapeptide derivative was purified by counter-current distribution (chloroform/carbon tetrachloride/methanol/water, [5:5:8:2], $K = 0.31$).

Yield: 2.8 g (69%), m.p. 103 - 107 °C; $[\alpha]_D^{22}$: − 8.6° (*c* = 0.83, in dimethylformamide)

$C_{42}H_{60}N_4O_{12}$ (812.97)

Calcd. C 62.05	H 7.43	N 6.89
Found C 62.10	H 7.56	N 6.83

Amino acid analysis:
2(2)Glu, 0.94(1)Ala, 0.99(1)Tyr

Benzyloxycarbonyl-O-t-butyltyrosyl-γ-t-butoxyglutamyl-alanyl-γ-t-butoxyglutamyl-β-t-butoxyaspartyl-leucyl-glutaminyl-valine (II)

Z-Asp(OBu^t)-Leu-Gln-Val-OH[15] (1.66 g, 2.5 mmol) was hydrogenated with 1 g Pd in 100 ml dimethylformamide containing 0.42 ml 6N HCl. After 8 h, a drop of pyridine was added and the solvent was concentrated to dryness in vacuo. A solution of I (2.03 g, 2.5 mmol) and *N*-hydroxysuccinimide (0.29 g, 0.25 mmol) in 20 ml dimethylformamide was reacted with dicyclohexylcarbodiimide (0.51 g, 0.25 mmol) and the reaction mixture was stirred for 4 h at room temperature. Dicyclohexylurea was filtered and the filtrate was added to the amine (obtained by hydrogenolysis of Z-Asp(OBu^t)-Leu-Gln-Val-OH) and *N*-methylmorpholine (0.5 ml, 0.5 mmol) in 30 ml dimethylformamide. After the reaction mixture had been stirred overnight, the solvent was removed in vacuo, treated with water and filtered. The purification was effected by column chromatography over LH-20 using dimethylformamide as elution solvent.

Yield: 1.4 g (42%), m.p. 216 - 218 °C (decomp.)
$[\alpha]_D^{22}$ − 18.8° (*c* = 0.41 in dimethylformamide)

$C_{66}H_{101}N_9O_{19} \cdot 1\ H_2O$ (1342.62)

Calcd. C 59.04	H 7.73	N 9.39
Found C 58.70	H 7.46	N 9.35

Amino acid analysis
1(1)Asp, 2.99(3)Glu, 0.96(1)Ala, 1.05(1)Val, 1.01(1)Leu, 0.92(1)Tyr

Benzyloxycarbonyl-O-t-butyltyrosyl-γ-t-butoxyglutamyl-alanyl-γ-t-butoxyglutamyl-β-t-butoxyaspartyl-leucyl-glutaminyl-valyl-glycyl-glutaminyl-valyl-γ-t-butoxy-glutamyl-leucyl-glycyl-glycyl-glycyl-prolyl-glycyl-alanyl-glycyl-O-t-butylseryl-leucyl-glutaminyl-prolyl-leucyl-alanyl-leucyl-γ-t-butoxyglutamyl-glycyl-O-t-butoxyseryl-leucyl-glutamine-t-butyl ester (III)

* The optical rotation of this compound is + 2.8° (*c* = 2.5 in chloroform). Due to typographical error it was reported[15] as − 2.8°.

A solution of Z-Gly-Gln-Val-Glu(OBu^t)-Leu-Gly-Gly-Gly-Pro-Gly-Ala-Gly-Ser(Bu^t)-Leu-Gln-Pro-Leu-Ala-Leu-Glu(OBu^t)-Gly-Ser(Bu^t)-Leu-Gln-OBu^t[15] (700 mg, 0.264 mmol) was hydrogenated in 150 ml methanol with 1 g Pd in presence of 0.264 ml 1N HCl. After 8 h a drop of pyridine was added, the catalyst was filtered off, and the filtrate was concentrated to dryness. The residue was dissolved in 10 ml dimethylformamide containing octapeptide II (536 mg, 0.4 mmol), 1-hydroxybenzotriazole (59 mg, 0.44 mmol) and *N*-methylmorpholine (0.285 ml, 0.264 mmol) and cooled to 0 °C. To it a solution of dicyclohexylcarbodiimide (82 mg, 0.4 mmol) in 1 ml dimethylformamide was added. The reaction mixture became jelly-like after overnight stirring at room temperature. The product was precipitated by the addition of ether, filtered and purified over Sephadex LH-20 using dimethylformamide for column development.

Yield: 580 mg (59%), mp.. 220 - 222 °C (decomp.)
$[\alpha]_D^{23}$: − 29.9° (*c* = 0.44 in dimethylformamide).

Amino acid analysis:
0.88(1)Asp, 1.93(2)Ser, 8.20(8)Glu, 2.23(2)Pro, 7(7)Gly, 3.09(3)Ala, 1.89(2)Val, 5.98(6)Leu, 0.80(1)Tyr

Benzyloxycarbonyltyrosyl-glutamyl-alanyl-glutamyl-aspartyl-leucyl-glutaminyl-valyl-glycyl-glutaminyl-valyl-glutamyl-leucyl-glycyl-glycyl-glycyl-prolyl-glycyl-alanyl-glycyl-seryl-leucyl-glutaminyl-prolyl-leucyl-alanyl-leucyl-glutamyl-glycyl-seryl-leucyl-glutamine (IV)

The product III (368 mg, 0,098 mmol) was treated with 5 ml trifluoroacetic acid for 15 min. The solvent was removed in vacuo to dryness, the residue was triturated with ether, washed twice with ether by decantation and dried in vacuo over NaOH pellets. The residue was triturated with 40 ml of a mixture of ethanol/water (v/v, 4:1) containing 3mM HCl. The insoluble product was isolated by centrifugation and dried in vacuo over KOH.

Yield: 196 mg (60%); $[\alpha]_D^{23}$: − 76.5° (*c* = 0.4 in formic acid).

Amino acid analysis:
1.01(1)Asp, 1.83(2)Ser, 8.37(8)Glu, 2.01(2)Pro, 7.11(7)Gly, 2.92(3)Ala, 2.03(2)Val, 6.01(6)Leu, 0.92(1)Tyr

Tyrosyl-glutamyl-alanyl-glutamyl-aspartyl-leucyl-glutaminyl-valyl-glycyl-glutaminyl-valyl-glutamyl-leucyl-glycyl-glycyl-glycyl-prolyl-glycyl-alanyl-glycyl-seryl-leucyl-glutaminyl-prolyl-leucyl-alanyl-leucyl-glutamyl-glycyl-seryl-leucyl-glutamine (V)

The hydrogenolytic debenzyloxycarbonylation of the product IV (32 mg, 9.7 μmol) was performed in 130 ml 50% acetic acid under 6 atmospheres pressure with 56 mg Pd. The catalyst was removed and the solvent was concentrated in vacuo at 20 °C to dryness. The residue was dissolved in 1.5 ml pyridine/acetate, pH 6.5, and applied to Whatman 3MM paper (35 × 40 cm). The electrophoresis was carried out at a potential of 3 V/cm for 24 h. The strip containing tyrosyl C-peptide was cut and eluted with 0.05M NH_4HCO_3 solution and lyophilised. The product was chromatographed on Sephadex G-25 (2.5 × 21 cm) and eluted with 0.05M NH_4HCO_3 solution. The peptide was located by absorbance at 280 nm (LKB-Uvicord-II) and the desired fractions were pooled and lyophilised.

Yield: 17 mg (56%); $[\alpha]_D^{23}$: − 96.5° (*c* = 0.27 in 0.05M NH_4HCO_3 solution).

Amino acid analysis:
1.01(1)Asp, 1.81(2)Ser, 8.15(8)Glu, 2.19(2)Pro, 6.72(7)Gly, 2.80(3)Ala, 1.99(2)Val, 6.58(6)Leu, 0.85(1)Tyr.

Tyrosyl C-peptide was shown to be homogeneous by thin-layer chromatography and by electrophoresis.

The authors are grateful to Professor *H. Zahn*, Director of the Deutsches Wollforschungsinstitut, Aachen, for his interest and constant encouragement, to Dr. *H.-G. Gattner* for helpful suggestions, to Dr. *J. Föhles* and Mr. *M. Moris* for amino acid analysis, to Mrs. *L. Drube* and Miss *M. Knudsen* for their excellent technical assistance, to Mrs. *D. Naithani* for reading the manuscript, to Professor *F. Woldby* of the Technical University of Denmark for access to the dichrograph and to *L. Penzien* for measuring the CD-spectra.

One of the authors, VKN, received financial assistance from the *Deutsche Forschungsgemeinschaft*.

Literature

[1] V. Commun.: Naithani, V. K., Dechesne, M., Markussen, J. & Heding, L. G. (1975) *this J.* **356**, 997 - 1010.
[2] Ko, A.S.C., Smyth, D. G., Markussen, J. & Sundby, F. (1971) *Eur. J. Biochem.* **20**, 190 - 199.
[3] Oyer, P. E., Cho, S., Peterson, J. D. & Steiner, D. F. (1971) *J. Biol. Chem.* **246**, 1375 - 1386.
[4] Rubenstein, A. H., Clark, J. L., Melani, F. & Steiner, D. F. (1969) *Nature (London)* **224**, 697 - 699.
[5] Kemmler, W., Peterson, J. D. & Steiner, D. F. (1971) *J. Biol. Chem.* **246**, 6786 - 6791.
[6] Yip, C. C. (1971) *Proc. Nat. Acad. Sci. U.S.A.* **68**, 1312 - 1315.
[7] Steiner, D. F., Cho, S., Oyer, P. E., Terris, S., Peterson, J. D. & Rubenstein, A. H. (1971) *J. Biol. Chem.* **246**, 1365 - 1374.
[8] Melani, F., Rubenstein, A. H., Oyer, P. E. & Steiner, D. F. (1970) *Proc. Nat. Acad. Sci. U.S.A.* **67**, 148 - 155.
[9] Block, M. B., Mako, M. E., Steiner, D. F. & Rubenstein, A. H. (1972) *Diabetes* **21**, 1013 - 1026.

[10] Heding, L. G. & Rasmussen, S. (1975) *Diabetologia*, in press.
[11] Heding, L. G., Larsen, U. D., Markussen J., Jørgensen, K. H. & Hallund, O. (1974) *Horm. Metab. Res.* **6**, (Suppl. 5) 40 - 44.
[12] Markussen, J., Jørgensen, K. H. & Heding, L. G. (1970) *Horm. Metab. Res.* **2**, 53 - 55.
[13] Kaneko, T., Oka, H., Munemura, M., Oda, T., Yamashita, K., Suzuki, S., Yanaihara, N., Hashimoto, T. & Yanaihara, C. (1974) *Endocrinol. Jpn.* **21**, 141 - 145.
[14] Mathur, K. B., Klostermeyer, H. & Zahn, H. (1966) *this J.* **346**, 60 - 68.
[15] Naithani, V. K. (1973) *this J.* **354**, 659 - 672.
[16] Hunter, W. M. & Greenwood, F. C. (1962) *Nature (London)* **194**, 495 - 496.
[17] Markussen, J. & Vølund. Å. (1975) *Int. J. Pept. Protein Res.* 7, 47 - 56.
[18] Greenfield, N. & Fasman, G. D. (1969) *Biochemistry* **8**, 4108 - 4116.
[19] Chen, Y.-H., Yang, J. T. & Martinez, H. M. (1972) *Biochemistry* **11**, 4120 - 4131.
[20] Arx, E. von & Neher, R. (1963) *J. Chromatogr.* **12**, 329 - 341.

Note added in proof: For information on human C-peptide radioimmunoassay kit, please, write to Novo Research Institute, Novo Alle, DK-2880 Bagsvaerd, Denmark.