SYNTHESIS OF MODIFIED HUMAN C-PEPTIDE AND ITS FRAGMENTS*

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Dedicated to the memory of Dr Karel Bláha.

Syntheses of the modified human C-peptide containing residues suitable for the introduction of the radioactive label (tyrosine) and internal marker for monitoring binding to carrier (norvaline) and five of its fragments are described. The syntheses were performed by solid phase method using either 9-fluorenylmethoxycarbonyl or tert-butyloxycarbonyl protecting groups. The products were purified by gel filtration, ion exchange chromatography and reversed phase HPLC. The reactivity of prepared peptides with antisera was determined and the modified C-peptide was found fully reactive.

The radioimmunoassay (RIA) of C-peptide** (*I*) in the blood plasma, serum or urine of patients gives the reliable information about the function of beta-cells in Langerhans' islets. Insulin is synthesized via the prohormone proinsulin, which differs from insulin by the so-called connecting peptide (C-peptide) corrresponding to positions 33–63 of proinsulin. This is the single-chain, 31-amino acid polypeptide that connects the N-terminal of the A chain with the C-terminal of the B-chain via two dipeptide (Lys-Arg and Arg-Arg) units. The C-peptide is split off by a trypsin-like enzyme, and the insulin is stored in the cytoplasmic granules characteristic of the beta cell. Equimolar amounts of the C-peptide are released together with insulin upon secretion.

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** All optically active amino acids are of the L configuration. Symbols and abbreviations are in accord with recommendations of the IUPAC–IUB Joint Commission of Biochemical Nomenclature[1]. Ape is α-aminopentanoic acid (norvaline), Ahx is α-aminohexanoic acid (norleucine). Numbering of amino acid residues in the C-peptide is based on the numbering of the human proinsulin.

Since insulin is secreted into the portal circulation, all insulin appearing in the peripheral circulation has passed through the liver. The liver takes up about half of the insulin coming through the portal vein. The hepatic insulin extraction is not constant but varies with insulin and glucose concentration. C-Peptide is extracted at the constant rate (about 12%) by the liver and the C-peptide circulates in plasma with a considerably longer half-life (11 min) than insulin (5 min). Thus the C-peptide concentration in peripheral blood represents well endogenous insulin secretion[2-6].

Moreover, only C-peptide determination is possible to estimate the residual secretion of endogenous insulin in diabetics treated with exogenous insulin since insulin of bovine or porcine origin fully cross-reacts in RIA for insulin[7]. The C-peptide is eliminated from blood plasma through the kidney and thus the C-peptide in the urine can be used as the integrated parametr of insulin secretion[8]. On the other side, the plasma C-peptide in diabetics with kidney diseases is false higher and must be corrected.

For the purpose of RIA the chemically synthesized C-peptide is needed, because the availability of natural peptide is extremely limited and human C-peptide cannot be substituted by the animal peptides for their high species-specificity. The syntheses of this peptide were performed (often aimed to the RIA system development) both by the conventional solution method[9-13] and by the solid-phase method[14-16].

X-Glu^{33}-Ala-Glu-Asp-Leu-Gln-Val-Gly^{40}-Gln-Val-Glu-Leu-Gly^{45}-Gly-Gly-
-Pro-Gly-Ala-Gly-Ser-Leu-Gln-Pro-Leu-Ala^{57}-Leu-Glu-Gly-Ser-Leu-Gln^{63}-Y

I, X = H, Y = OH; *II*, X = H-Tyr, Y = Gly-OH;
III, X = H-Tyr, Y = Ape-OH

H-Tyr-Gly^{40}-Gln-Val-Glu-Leu-Gly^{45}-Gly-Gly-Pro-Gly-Ala-Gly-Ser-Leu-Gln-
-Pro-Leu-Ala^{57}-Leu-Glu-Gly-Ser-Leu-Gln^{63}-X-OH

IV, X = Gly; *V*, X = Ape

H-Tyr-Gly^{40}-Gln-Val-Glu-Leu-Gly^{45}-Gly-OH

VI

H-Tyr-Ala^{57}-Leu-Glu-Gly-Ser-Leu-Gln^{63}-Gly-OH

VII

H-Ahx-Gln-Val-Gly^{40}-Gln-Val-Glu-Ile-Gly^{45}-Gly-NH^2

VIII

Madsen et al.[17,18] found that the fragments 40–45 and 57–63 of human proinsulin behave as immunodeterminants. Moreover, the sequence 38–46 was predicted to be the most hydrophilic. We decided to synthesize the peptides containing these sequences to test their usefulness as the immunogens for raising antibodies against human C-peptide. We have synthesized peptides *II–VIII* containing either full (*II, III*) or partial (*IV–VIII*) sequences of the C-peptide, but none of the peptides containing only one of both predicted antigenic determinants exhibit significant response in the RIA test for the C-peptide (Table I). Significant reactivity with antisera was found only for peptides *IV* and *V* containing both of above mentioned sequences, which had almost 30% of the reactivity of the C-peptide. It confirmed the previous findings[17,18] that two not adjacent sites contribute to the binding to antibodies, and that the peptide connecting them is necessary only for their proper space orientation. The use of simpler peptides for immunization was therefore ruled out and we decided to synthesize the full sequence of human C-peptide for this purpose.

TABLE I
Characteristics of prepared peptides

Peptide	Yield[a]	R_F	E^c	Amino acid analysis					Reactivity[d]
Method	Content[b]	S3 S4	E^{picr} E^{Gly}	Asp Ala	Ser Val	Glu Ape	Pro Leu	Gly Tyr	%
II	186	0.11	0.26	0.89	1.66	7.80	2.10	7.43	57.5
Boc	26	0.38	0.34	3.00	1.83	–	5.66	0.84	
III	553	0.12	0.26	1.01	1.83	7.74	2.14	7.20	96
Fmoc	68	0.37	0.34	3.00	1.94	0.98	6.42	0.86	
IV	112	0.11	0.23	–	1.61	5.12	2.18	7.56	29.9
Boc	28	0.40	0.38	2.00	0.92	–	4.87	0.78	
V	180	0.11	0.23	–	1.69	5.03	2.21	6.88	34
Fmoc	72	0.41	0.38	2.00	1.08	0.88	5.12	0.89	
VI	547	0.13	0.11	–	–	2.13	–	2.76	0.1
Boc	66	0.46	0.54	–	1.93	–	1.00	0.92	
VII	153	0.12	0.12	–	0.82	1.86	–	2.08	0.4
Boc	81	0.48	0.53	1.00	–	–	2.20	0.92	
VIII	255	0.13	0.11	–	–	2.79	–	3.12	0.1
Boc	61	0.44	0.54	–	2.00	0.94[e]	0.96[f]	–	

[a] In mg of crude peptide. [b] Relative area of the main peak determined from the HPLC analysis at 220 nm. [c] Electrophoretic mobility in respect to picric acid in buffer pH 5.7 or in respect to Gly in buffer pH 2.4. [d] Reactivity with antisera in % of the human C-peptide response. [e] Ahx. [f] Ile.

We synthesized the modified C-peptide (*III*) bearing both the residue allowing for the easy introduction of the radioactive label and the internal marker allowing for the simple monitoring of the carrier–ligand binding. The first task was easily fulfilled by the inclusion of tyrosine residue into the peptide molecule, the best place being the amino-terminal residue. The second intention was realized by the introduction of non-coded amino acid norvaline (α-aminopentanoic acid) as the carboxy-terminal residue.

For the syntheses of the above mentioned peptides we have used the solid phase methodology[19,20] utilizing either classical Boc/Bzl approach or more advanced Fmoc/Bu[t] approach with the application of acid-labile anchoring of the peptide to the polymer matrix[21]. During the cleavage of the modified peptide *II* from the resin by the HF treatment we have been faced with the significant decomposition of the synthesized peptide. We have therefore tested various scavengers (anisole, thioanisole and ethanedithiol) and evaluated the composition of the crude reaction mixture by reversed-phase HPLC. None of the scavengers tested gave satisfactory results, but the addition of thioanisole was found the most acceptable. The content of the modified C-peptide *II* in the crude reaction mixture was between 18 and 26 percent. As can be seen from the Table I, the Fmoc-synthesis gave us significantly better results. Moreover the final steps of the synthesis are much simpler and the purification of the product is easier.

The modified C-peptide *III* was purified by ion-exchange chromatography, gel filtration and reversed-phase HPLC. The purity was determined by electrophoresis, thin layer chromatography and HPLC. The peptide was characterized by amino-acid analysis, elemental analysis and fast atom bombardment mass spectroscopy. It was found fully reactive with antisera in comparison with the human C-peptide by the RIA determination.

Modified C-peptide *III* was coupled to bovine serum albumine or bovine thyroglobulin using glutardialdehyde[22]. Rabbits and guinea-pigs were immunized by this conjugate. Calibration curves obtained with the best rabbit and guinea-pig antisera are presented in Fig. 1. The guinea-pig antiserum was applicable for routine diagnostics. The evaluation and clinical verification of RIA method for C-peptide determination in human serum will be presented elsewhere.

EXPERIMENTAL

Protected amino acids used in the syntheses were prepared according to the literature[20,23], and were checked prior to the coupling by the ninhydrin test[24], melting point, and thin layer chromatography. Solid phase synthesis was performed on semi-automated synthesizer of our own construction. Completness of the coupling was tested by the ninhydrin test[24], or by the chloranil test[25] (when

proline was penultimative amino acid). Thin-layer chromatography was carried out on silica gel plates (Silufol, Kavalier, Czechoslovakia) in the systems: 1-butanol–acetic acid–water (4:1:1) (S3), and 1-butanol–pyridine–acetic acid–water (15:10:3:6) (S4). Electrophoresis was performed on a Whatman 3MM paper in moist chamber (20 V/cm) for 1 h in 1 M acetic acid (pH 2.4) and a pyridine–acetate buffer (pH 5.7). The compounds were detected by ninhydrin or by the chlorination method[26]. Samples for amino acid analyses were hydrolyzed with 6 M HCl containing 2% phenol at 105°C for 20 h and analyzed on an automatic analyzer, Durrum 500. The high performance liquid chromatography (HPLC) was carried out on an SP-8700 instrument equipped with an SP-8400 detector (Spectra-Physics, Santa Clara, U.S.A.). Fast atom bombardment mass spectra were obtained on a ZAB-EQ spectrometer (VG Analytical Ltd., Manchester) with xenon at 8 kV as the bombarding gas. ^{125}I-C-Peptide was obtained by iodination using Iodogen[27].

The immunoreactivity of synthesized peptides was tested by commercial kit Biodata-Serono using routine procedure.

Synthesis of the Protected Peptide Resin

Boc-*approach*: Merrifield's resin (2 g) cross-linked with 1% divinylbenzene containing 0.32 mmol/g of Boc-Gly (attached by the Gisin's method[28]) was deprotected by the action of 45% trifluoroacetic acid in dichloromethane (2 and 20 min) and Boc-protected amino acids (Gln, Leu, Ser(Bzl), Gly, Glu(OBzl), Ala, Pro, Val, Asp(OBzl), Tyr) were coupled to it by the action of preformed (in dichloromethane) symmetrical anhydrides (3 equivalents) in dimethylformamide[29]. The following sequence was used in each step: *1*) 3 × 1.5 min dichloromethane wash, *2*) 4 × 1.5 min 10% diisopropylethylamine in dichloromethane, *3*) 2 × 1.5 min dichloromethane wash, *4*)

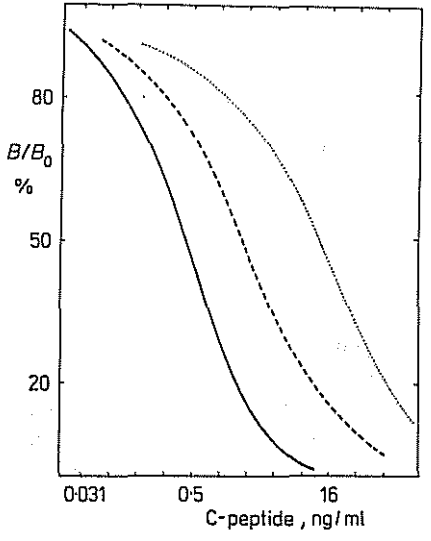

FIG. 1

Calibration curves obtained with the best rabbit (····) and guinea-pig (———) antiserum using ^{125}I-peptide *III*, and calibration curve obtained with commercial kit Biodata (– – –)

2 × 1 min dimethylformamide, *5*) coupling 30 min – 15 h according to the ninhydrin test (if incomplete after 15 h, the coupling was repeated with the fresh symmetrical anhydride), *6*) 2 × 1 min dimethylformamide, *7*) 3 × 1.5 min dichloromethane, *8*) 45% trifluoroacetic acid and 2% anisole in dichloromethane – 2 and 20 min.

After the coupling of glycine in position 40 and alanine in position 57 the sample of the peptide resin (c. 0.3 g) was taken out, acylated by Boc-Tyr, and peptide was cleaved from the resin (see Table I). Peptide resin containing the full sequence of protected modified C-peptide was thoroughly washed and dried in vacuo. The peptides *VI* and *VIII* were synthesized similarly, the latter being prepared on benzhydrylamine resin.

Fmoc-*approach*: To the benzhydrylamine resin (1% of divinylbenzene, substitution 0.4 mmol of NH_2 per 1 g, 1.5 g) the 3-(4'-(N^{α}-fluorenylmethyloxycarbonyl-2-aminopentanoyloxymethyl)-phenoxy)propionyl residue was coupled by the active ester method[21], and the protecting group was removed by the treatment with 50% piperidine in dimethylformamide. Fmoc protected amino acids (Gln, Leu, Ser(Bu^t), Gly, Glu(OBu^t), Ala, Pro, Val, Asp(OBu^t) – 3 molar excess) were coupled to this resin via preformed (1 h at room temperature) active esters with the N-hydroxybenzotriazole (with the exception of Fmoc-Gln, which was coupled as symmetrical anhydride). The following sequence was used in each step: *1*) 2 × 1.5 min dichloromethane wash, *2*) 2 × 1.5 min dimethylformamide, *3*) 50% piperidine in dimethylformamide 2 and 20 min, *4*) 2 × 1.5 min dimethylformamide, *5*) 2 × 5 min dioxane–water (1:1), *6*) 3 × 1.5 min dimethylformamide, *7*) coupling 30 min – 2 h (only in two cases – Gln in the 11th step, and Gly in the 20th step, the chloranil or ninhydrin test was positive after 2 h and coupling was repeated) *8*) 5 × 1.5 min dimethylformamide, *9*) 5 × 1.5 min 2-propanol, *10*) 3 × 1.5 min dichloromethane. As the last amino acid Boc-Tyr was coupled to the resin and the resin was washed and dried in vacuo. (After the coupling of glycine in position 40, the sample of the resin was taken out, acylated by Boc-Tyr, washed and dried.)

Preparation of the Free Peptides

HF-*cleavage*: The procedure is exemplified on the case of the modified C-peptide *II*. Appropriate peptide-resin prepared by the Boc-approach (1 g) was mixed with 2 ml of thioanisole in the reaction vessel of HF cleavage line and approximatelly 20 ml of liquid HF were distilled in it. After 1 h at 0°C the mixture was evaporated and the residue was washed with ethyl acetate (3 × 60 ml) and peptide was extracted by 30% acetic acid (3 × 60 ml). After lyophilization the half of the product was dissolved in degassed water with the addition of ammonia (to pH 7) the solution was applied onto the column of DEAE-Sephadex (18 × 2.2 cm). Elution was performed with gradient composed of 0.01 M $AcONH_4$ (350 ml) and 1 M $AcONH_4$ pH 7.2 (350 ml). The major peak was pooled and lyophilized. This product (142 mg) contained according to the HPLC analysis 78% of the peptide *II*, and was used without further purification for the immunization purposes. Part of the lyophilizate (4.4 mg) was dissolved in 0.1 M $AcONH_4$ pH 7.2 and injected in two portions onto a column (Separon SIC18 150 x 4 mm) equilibrated with 0.1 M $AcONH_4$ pH 7.2. Elution was performed isocratically with 50% methanol. The major peak was collected, methanol evaporated and the residue was lyophilized. The product was dissolved in 3 ml of 3 M acetic acid and applied onto the column of Biogel P-4 (100 × 1 cm); elution was performed with the same solvent. After the lyophilization 1.8 mg of the product pure according to analytical HPLC (retention time 8.2 min, gradient 50 to 100% MeOH in 0.1 M $AcONH_4$, pH 7.5, 2%/min, flow rate 1 ml/min) was obtained. Other analytical data are given in Table I. For $C_{143}H_{239}N_{37}O_{51}.3CH_3COOH.10H_2O$ (3 643) calculated: 49.13% C, 7.22% H, 14.23% N; found: 49.53% C, 7.21% H, 14.54% N. FAB MS (*m/z*): 3 284 $(M+H)^+$.

TFA-*cleavage*: The peptide resin (1g) (prepared by the Fmoc-approach) was treated with 50% of trifluoroacetic acid in dichlormethane containing 2% of anisole for 2 h at room temperature. The resin was filtered off, washed by the same solvent mixture and the filtrate was diluted by dry ether, the precipitate was collected and dried in vacuo (498 mg). The resin was further washed by 30% acetic acid and the residue was lyophilized (55 mg). The part of the product (250 mg) was dissolved in water with the addition of ammonia (to pH 7) and applied onto the column of DEAE-Sephadex. Purification was performed as in HF-cleavage.

Immunization

Modified C-peptide *III* was coupled to bovine serum albumine using glutardialdehyde[22]. The number of attached molecules was determined by amino acid analysis utilizing norvaline as the internal marker. Seven molecules of C-peptide were found to be coupled to one molecule of bovine serum albumine. To enhance the immune response, the conjugate C-peptide–bovine thyroglobulin was used for the last three injections. The thyroglobulin conjugate was prepared by the same manner and tested after addition of tracer amount of ^{125}I-peptide *III* into reaction mixture. About 30 molecules of compound *III* were bound to one molecule of thyroglobulin.

Twenty two rabbits and forty guinea-pigs were immunized for 4 — 12 months in 3 weeks intervals. Conjugate (0.25 mg) in complete Freund's adjuvant was injected subcutaneously for one dose. Titre and affinity were tested after 2 months every 6 weeks.

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