Journal of Peptide Science
J. Peptide Sci. **6**: 413–419 (2000)

Primary Structure of Fox (*Vulpes vulpes*) Proinsulin Based on Sequence Studies of Pancreatic Peptides and cDNA

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Received 16 March 2000
Accepted 3 April 2000

Abstract: Insulin and C-peptide were extracted and purified from fox (*Vulpes vulpes*) pancreas using gel filtration, ion-exchange chromatography and HPLC. Chromatographic data for the insulin, as well as for its oxidized and carboxymethylated chains proved it to be identical to that of polar fox (*Alopex lagopus*) and dog. The sequence analysis of a peptide which was assumed to be the corresponding C-peptide revealed that it comprises 23 amino acid residues and is identical to the C-peptide fragment isolated from dog pancreas; it differs from polar fox C-peptide by a single substitution (Asp → Glu). mRNA was isolated from pancreatic tissue and cDNA was obtained by reverse transcription. A polymerase chain reaction was performed using gene-specific primers to obtain a DNA fragment corresponding to part of fox proinsulin. DNA sequencing revealed 100% identity to dog proinsulin at the protein level, although some amino acids were encoded by different codons. The total sequence of proinsulin was deduced from these results. Copyright © 2000 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: C-peptide; cDNA; fox; proinsulin; PCR; sequence studies; *Vulpes vulpes*

INTRODUCTION

The amount of proinsulin in the pancreas is relatively small, and this presents some difficulty in determining the amino acid sequence of this prohormone. Only a few proinsulins have been isolated and sequenced: bovine [1,2], porcine [3,4], human [5] and rat [6].

Since the conversion of proinsulin yields insulin and C-peptide in equimolar amounts, isolation of these peptides from the pancreas, where they are stored in substantially higher amounts than proinsulin itself, proved to be a more convenient route to establishing the structure of proinsulin. The primary structure of insulin is known for more than 25 mammalian species. C-peptide sequences have been reported for pig [7], ox [2,7–9], man [10,11], rat [12,13], monkey [14], sheep [14], dog [14], horse [13], rabbit [15], guinea pig [16,17], chinchilla [18], bison [19,20], polar fox [19], zebra [21], Przewalski's horse [21], rhinoceros [21] and tapir [21]. Since the sequences of the respective insulins were known, this allowed the proinsulin molecules to be reconstructed. In all cases, two pairs of basic amino acids Arg-Arg and Lys-Arg at *N*-terminal and *C*-terminal end of the C-peptide respectively, have been placed by analogy to the already known sequences of proinsulin.

C-peptides have been isolated from two species of the family *Canidae*, namely dog and polar fox. In both cases the C-peptides appeared to be shorter (23 amino acid residues) than those isolated from other species (e.g. 31 amino acid residues for man). The nucleotide sequence of dog proinsulin revealed however that the isolated peptide is a fragment generated from a larger native C-peptide [22]. Nucleotide sequences corresponding to proinsulin,

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have also been reported for man [23,24], monkey [25], lamb [26], rat [27,28], ox [29] and guinea pig [30].

In this report, the isolation and identification of insulin and C-peptide from a new *Canidae* species, namely fox (*Vulpes vulpes*), is described. Also presented are the results of sequencing a cDNA encoding a fragment of proinsulin containing the C-peptide sequence and flanking basic amino acid pairs.

MATERIALS AND METHODS

Extraction of Fox Pancreas

The extraction of fox pancreas was performed using the procedure described for the isolation of insulin and C-peptide of polar fox [19], with some modifications. Frozen pancreatic tissue (about 130 g from 15 animals) was extracted with a solution comprising 93 ml of ethanol, 107 ml of H_2O and 21.5 ml of conc. HCl. After centrifugation, the supernatant was adjusted to pH 8 with ammonia. The precipitate formed was removed and the supernatant was adjusted to pH 5.3 with 6 *M* HCl and 9.2 ml of 2 *M* ammonium acetate buffer, followed by the addition of 700 ml of ethanol, 150 ml of diethyl ether and one drop of 30% aqueous NaCl. The mixture was left overnight at 4°C. The precipitate which formed (510 mg) was collected by filtration.

Isolation of Insulin and C-peptide

The dried precipitate (510 mg) was dissolved in 3 *M* acetic acid and chromatographed on a Sephadex G-50 column (84 × 5.6 cm). Fractions of 20 ml were collected. Lyophilization of fractions 56–69 gave 45.0 mg of material containing insulin and C-peptide (Figure 1). Fractions 70–92 (98 mg) were saved for further experiments. The material obtained from fractions 56–69 was subjected to ion exchange chromatography on a carboxymethylcellulose CM-23 column (49 × 2 cm) using 0.01 *M* citrate buffer in 7 *M* urea. After the C-peptide had been eluted, 2 *M* NaCl solution was used to elute the insulin. Fractions 10–15 and 45–49 (Figure 2), containing C-peptide and insulin, respectively, were combined and desalted on a Sephadex G-10 column. The yield was 5.1 mg of crude C-peptide (Figure 3(a)) and 22.5 mg of crude insulin. C-peptide and insulin were purified using HPLC with Waters equipment on a Vydac C_{18} column (250 × 8 mm, 5 μm). The

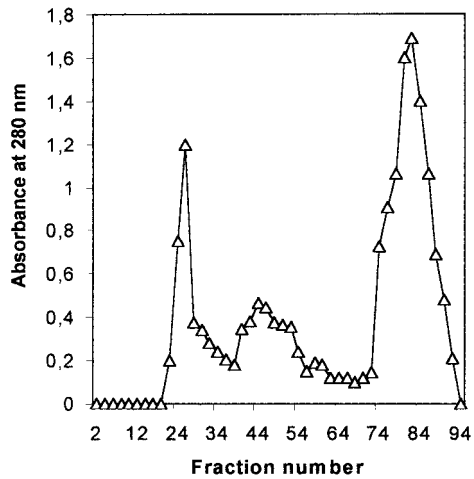

Figure 1 Gel filtration in 3 *M* acetic acid of fox pancreatic extract on a Sephadex G-50 column (84 × 5.6 cm). Fractions 59–69 were combined and used for further purification.

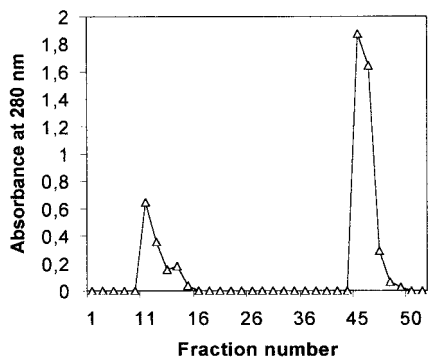

Figure 2 Ion exchange chromatography of the insulin and C-peptide containing fractions from gel filtration. Column 50 × 2 cm; buffer: 0.01 *M* sodium acetate, pH 5, comprising 7 *M* urea. Linear gradient in sodium chloride has been introduced after fraction 20. Fractions 10–15 and 45–49 were combined to receive C-peptide and insulin, respectively.

solvent system was: (A) 0.1% TFA in water and (B) 0.1% TFA in water: CH_3CN (1:4). Elution was effected using a linear gradient of 20–50% B in 60 min. Homogeneous peptides were obtained: insulin (retention time (RT) 30 min) and C-peptide (RT 27.3 min) as determined by RP-HPLC on a Nucleosil 10 C_{18} column (250 × 4 mm, 4 μm) in a gradient of 20–60% B in 40 min. The material containing low molecular weight compounds (Figure 1, fractions 70–92) was desalted on a G-10 column and lyophilized. Yield: 68 mg.

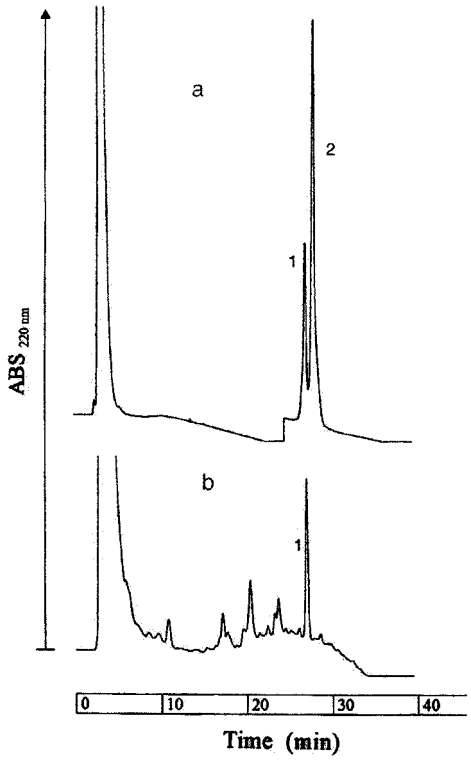

Figure 3 Analytical HPLC of crude C-peptide containing fraction (b), and mixture of purified synthetic peptides: 23-peptide (first peak at 27.3 min) and 31-peptide (second peak at 28.3 min) (a).

Identification of Peptides

Samples of fox insulin (0.1 mg) and pig insulin (0.1 mg) were treated with performic acid (3.6 μl of formic acid plus 8 μl of 30% H_2O_2) for 20 min. The same RT in HPLC was observed for oxidized A chains (29.03 min) and for oxidized B chains (33.96 min). Samples of insulins (0.25 mg each) in 100 μl of buffer (0.4 *M* Tris, 6 *M* guanidine · HCl, 2 m*M* EDTA, pH 8.1) were reduced with dithioerithritol (0.5 mg) at 37°C for 2 h and carboxymethylated with iodoacetate at 37°C (2 h). The carboxymethylated insulin chains were separated using HPLC under the conditions used for the purification of insulin. The RTs of the carboxymethylated A and B chains were 29.03 and 33.96 min, respectively. These compounds co-eluted with standards obtained in an identical manner from porcine insulin. The observation of a molecular ion of 3515 $(M + 1)^+$ confirmed that the carboxymethylated B-chain of fox is identical to that of pig.

Sequence Determination

Edman degradation of peptides was performed with an Applied Biosystem model 494 Procise (in pulsed liquid mode). The sequences of peptides are presented in Table 1.

Synthesis of Peptides

C-peptide (31 amino acid residues, structure deduced from nucleotide sequence, see Table 1) and (9–31) C-peptide (sequence determined by Edman degradation, see Table 1). The synthesis was accomplished by the solid-phase method using 0.6 mmol Boc-L-Gln-Pam-resin (1.07 g, 0.56 meq/g). The function was α-amino protected with the Boc group, with the side chain of Glu, Asp and Arg being protected by Bzl, cHx and Tos, respectively. Three molar excess of symmetrical anhydride, preformed with the use of *N*-*t*-butyl-*N*′-ethylcarbodiimide, was applied for acylation. Glutamine was introduced using the *p*-nitrophenyl ester (DMF, 16 h). The synthesis was carried out according to the standard procedure, as described earlier for the synthesis of bovine C-peptides [20]. After introducing Asp (position 9 in the 31-peptide) the peptide–resin was split with one part being saved for the preparation of the 9–31 sequence, and the second for the synthesis of the 31-peptide identical to the C-peptide of dog. The peptide–resin conjugates were treated with liquid HF and peptides were purified using a Waters System consisting of two Waters 501 pumps, an Automated Gradient

Table 1 Alignment of *Canidae* Species C-peptides in Frame of the 31 Amino Acid Residues of Human C-peptide

Man[a]	**E A E D L Q V G Q V E L G G G P G A G S L Q P L A L E G S L Q**
Fox[b]	**E V E D L Q D V E L A G A P G E G G L Q P L A L E G A L Q**
Fox[c]	*E V E D L Q V R D V E L A G A P G E G G L Q P L A L E G A L Q*
Polar fox[d]	**D V D L A G A P G E G G L Q P L A L E G A L Q**
Dog[e]	**D V E L A G A P G E G G L Q P L A L E G A L Q**
Dog[f]	*E V E D L Q V R D V E L A G A P G E G G L Q P L A L E G A L Q*

The sequences shown *in italic* are deduced from nucleotide sequences, the isolated peptides are shown in bold. [a] [10]; [b,c] this paper; [d] [19]; [e] [14]; [f] [22].

Controler, a Waters 486 Tunable Absorbance Detector and Vydac 250 × 8 mm column. The column was eluted with a solvent system consisting of solvent A (0.1% aqueous TFA) and solvent B (80% MeCN in A), with a linear gradient of 20–40% of B in solvent A and then isocratically. Peptides were detected at 220 nm. The flow rate was 3 ml/min. The purity of peptide fractions was determined using the same system equipped with an analytical column (Nucleosil 10, C_{18}, 250 × 4 mm) with a gradient of 20–50% B in 30 min and flow rate of 1 ml/min. Pure 23-peptide was eluted at 27.3 and 31-peptide at 28.2 min. Liquid secondary ionization mass spectrometry (LSIMS) performed on an AMD-604 Intectra mass spectrometer gave a molecular ion 2205.0 $(M + H)^+$ for the 23-peptide and 3176.5 $(M + H)^+$ for the 31-peptide.

(1–8) C-peptide (sequence deduced from nucleotide sequence, see Table 1). The synthesis of *N*-terminal fragments of the C-peptide was accomplished as described above using BocArg(Tos)-Merrifield resin. The crude product was purified by HPLC using a Vydac Column 250 × 8 mm (gradient 5–45% B in 80 min). Analytical run (Nucleosil 10, C_{18}, 250 × 4 mm) gave a RT of 25.94 min. The structure of the peptide was confirmed by LSIMS: $(M + H)^+ - 986.9$.

Comparison of synthetic and natural peptides. Samples of 31-peptide, 23-peptide and 8-peptide were compared with crude material containing acidic peptides (fractions 9–15, Figure 2). The main product was 23-peptide (RT = 27.3 min, identical to that of the synthetic peptide, confirmed by co-elution). Neither 31-peptide nor 8-peptide was found in this material. 8-Peptide was also not found in the salt peak (fractions 70–93, Figure 1); instead, the main product, with RT of 20.9 min, was found to be a 6-peptide, EVEDLQ.

Cloning by Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Approximately 1 g of pancreas was crushed under liquid nitrogen. Trizol (GIBCO-BRL, Life Technologies, Rockville, USA) was added to extract total RNA. Chloroform was added to cause phase separation and the water phase containing total RNA was extracted once with phenol/chloroform and once with chloroform. RNA was precipitated with 0.3 *M* NaOAc and 3 vol. ethanol, and dissolved in 100 μl of H_2O. Reverse transcription of mRNA into cDNA was performed using SuperScript II reverse transcriptase (GIBCO-BRL Life Technologies, Rockville, USA) on 2 μg of total RNA.

Gene-specific primers, corresponding to the highly conserved amino acid sequences of insulin, were constructed as follows: upstream primer 5′-CAYCTBTGYGGMTCHMAYYT-3′ encoding the amino acid sequence HLCGS(H/N)L located in the *N*-terminus of the insulin B-chain; downstream primer 5′-AGTTRCAGTAGYTCTSYAG-3′ encoding the amino acid sequence L(Q/E)(N/S)YCN located in the *C*-terminus of the insulin A-chain (Figure 4).

PCR with 100 pmol of these insulin-specific DNA primers was performed using EXPAND HighFidelity PCR system from Boehringer Mannheim. After 50 PCR cycles, an insulin-specific DNA product of

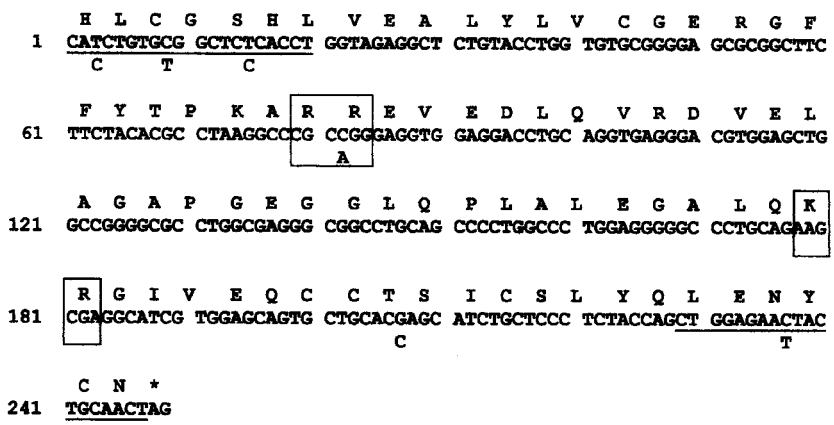

Figure 4 Partial RT-PCR sequence of fox (*V. vulpes*) proinsulin cDNA. The dibasic cleavage sites flanking the C-peptide are shown in boxes. The encoded amino acid sequence is shown above the DNA sequence in one letter code. Underlined sequences denote the position of the primers used for RT-PCR. The nucleotides differing in the dog proinsulin gene [22] are shown below the fox DNA sequence.

approximately 250 base pairs, was evident after agarose gel electrophoresis. This PCR product was cloned using a TOPO TA cloning kit from Stratagene and subjected to DNA sequencing using a Thermo Sequenase radiolabelled terminator cycle sequencing kit (Amersham Farmacia Biotech, Uppsala, Sweden; vector primers M13 forward and M13 reverse). The resulting DNA sequence was shown to correspond to a partial cDNA sequence of proinsulin (Figure 4).

RESULTS AND DISCUSSION

Material containing C-peptide and insulin was obtained from fresh fox pancreas using a standard procedure [10] which includes extraction in acid–ethanol and removal of high molecular weight proteins by precipitation with ether. The resulting material was then filtered in 3 *M* acetic acid on a Sephadex G-50 column (Figure 1). C-peptide and insulin which co-eluted in the third peak were separated by ion-exchange chromatography on a carboxymethylcellulose column (Figure 2). The insulin, after purification by HPLC, was compared with pig insulin (identical to that of polar fox and dog). It co-eluted with pig insulin when submitted to HPLC. Also carboxymethylated and oxidized A and B chains of fox insulin co-eluted with those of pig insulin. Carboxymethylated B chain gave molecular ion of 3515, which confirmed that the fox B chain is identical to that of pig and several other mammalian species. Crude C-peptide gave only one major peak at 27.3 min (Figure 3(a)). After purification to homogeneity this product was sequenced. The sequence was: Asp-Val-Glu-Leu-Ala-Gly-Ala-Pro-Gly-Glu-Gly-Gly-Leu-Gln-Pro-Leu-Ala-Leu-Glu-Gly-Ala-Leu-Gln. This structure was consistent with the mass spectrum $(M+H)^+$, 2205. This peptide appeared to be eight amino acid residues shorter than most mammalian C-peptides, but the same length as peptides isolated so far from other carnivorous species: dog and polar fox. Since examination of the dog gene revealed that the isolated peptide is a fragment of the 31-peptide which is formed by cleavage at the Arg-Asp site, we suspect that this is also the case for the fox.

To draw final conclusions regarding the total structure of fox proinsulin, we decided to study the nucleotide sequence of proinsulin. Using a pair of degenerate primers, corresponding to highly conserved regions in the *N*-terminal of the B-chain and the *C*-terminal of the A-chain, it was possible to isolate a DNA fragment encoding an amino acid sequence corresponding to a major part of proinsulin including the connecting peptide and flanking basic amino acid pairs (Figure 4). This result showed that the fox C-peptide was actually 31 amino acid residues long, as in most mammalian species. Comparing the DNA sequence of fox to that of dog [22] reveals that they encode identical amino acid sequences with only two silent mutations being present within the region unaffected by primer sequences (Figure 4).

To support the conclusions we had drawn from sequence studies of 23-peptide, we synthesized this peptide by the solid phase method. The synthetic peptide was confirmed to be identical with the isolated peptide as judging by HPLC. It was found that the 23-peptide is the main component of the acidic fractions (Figure 3). We also synthesized the whole C-peptide based on the sequence deduced from nucleotide studies. Comparison of the synthetic peptide with the natural material (Figure 3) revealed that this compound was not present. This prompted us to look for the missing *N*-terminal fragment of C-peptide in fractions containing low molecular weight substances (Figure 1, tubes 70–94). We isolated the main component, which appeared to be a 6-peptide (Glu-Val-Glu-Asp-Leu-Gln) corresponding to the *N*-terminal sequence of the predicted C-peptide. Using the *N*-terminal 8-peptide as a standard, we were able to demonstrate that this peptide was not present in this material. It was clear from these results that fox C-peptide indeed contains 31 amino acid residues, as predicted from the corresponding nucleotide sequence, and that the peptides we were able to isolate were the products of processing by proteolytic enzymes. Since the C-peptides of many different species are sufficiently stable to be isolated from the pancreas it seems likely that Arg in position 8 is the primary site of cleavage of fox C-peptide as well as of the previously studied polar fox and dog C-peptides. Dog, polar fox and fox are the only *Canidae* species examined so far. A trypsin-like enzyme, or a subtilisin-related enzyme, which was found to cleave not only at paired basic residues [31] but also at a single basic residue [32], may be involved in degradation of fox C-peptide or proinsulin. Since not *N*-terminal 8-peptide but a *N*-terminal 6-peptide was found in the natural material, it is likely that cleavage at the Arg residue is followed by chymotrypsyn-like degradation at the Gln residue.

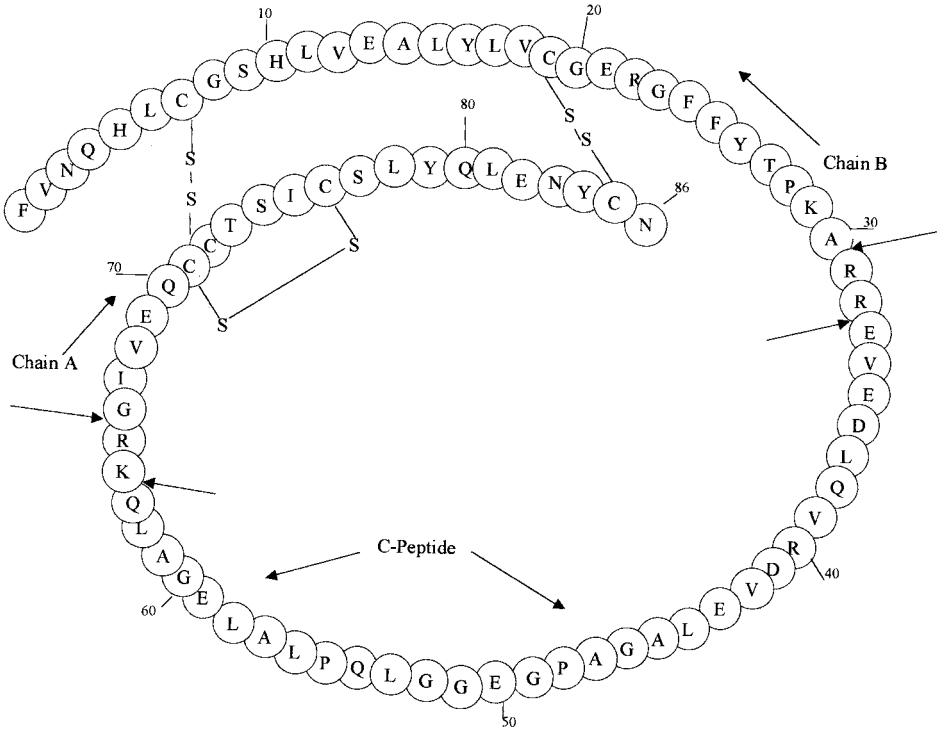

Figure 5 The amino acid sequence of fox (*V. vulpes)* proinsulin.

CONCLUSIONS

The results of isolating C-peptide fragments from dog [14], polar fox [19] and fox (this paper), together with studies of nucleotide sequences of dog [22] and fox (this paper) indicate that the C-peptides of these three closely related species, contain 31 amino acid residues like most other species, but differ from them in that they contain an Arg residue in position 8. Due to rapid hydrolysis at the Arg residue, a *C*-terminal 23-peptide is isolated from the pituitary instead of a 31-peptide.

Fox C-peptide differs from that of polar fox by one amino acid residue (Asp → Glu in position 11) and is identical to that of dog. Consequently, the structure of fox (*V. vulpes)* proinsulin structure (Figure 5), based on integrated studies including sequence determination of pancreatic peptides and cDNA, is identical to that of dog, concluded from DNA sequence. However, some amino acids are coded by different codons.

Acknowledgements

This work was supported by the University of Warsaw. We acknowledge the excellent technical assistance of Elisabeth Veyhe Andersen.

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