Isolation of ovine pancreatic icosapeptide: a peptide product containing one cysteine residue

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1. INTRODUCTION

The dominating endocrine cell type of the duodenal pancreas is the pancreatic polypeptide (PP) cell [1]. The initially identified product of this cell, PP, is a 36 amino acid, carboxyamidated peptide [2,3] which is part of a family of biologically active peptides also including the homologous peptides PYY [4] and NPY [5]. PP itself seems to be a hormone involved in the regulation of zymogen secretion from the exocrine pancreas and in the regulation of biliary tract motility [6,7]. PP is synthesized as the NH_2-terminal part of a precursor of M_r 8000–10000 [8]. The COOH-terminal part of this precursor gives rise to an icosapeptide which initially is excised from the precursor as an intermediary form bearing a COOH-terminal extension of 5 amino acids [9,10]. The following lines of evidence indicate that the pancreatic icosapeptide is a second product of the PP precursor. Firstly, peptide mapping of biosynthetically labeled peptides shows that the icosapeptide and the PP precursor have tryptic fragments in common [8]. Secondly, during pulse-chase experiments the icosapeptide and PP follow a parallel rate of appearance [8]. Thirdly, antibodies raised against the icosapeptide can specifically precipitate the PP precursor [9]. And finally, immunohistochemistry shows that icosapeptide immunoreactivity is exclusively localized to PP storing cells [11]. The pancreatic icosapeptide and its biogenesis were originally studied in the dog. We describe here the isolation and characterisation of the homologous peptide from sheep pancreas. The sheep was chosen because the ovine pancreas is especially rich in PP.

2. METHODS AND RESULTS

Pancreas were excised from sheep at a local abattoir within 30 min of death. The tissue was cut into small pieces and homogenized with a Braun blender in ice-cold acidified ethanol; final concentration of ethanol was 68%, acidified with 0.1 M HCl. The homogenate was incubated for 18 h at 4°C and then centrifuged at 11000 × *g* for 20 min at 4°C. The supernatant was neutralized with aqueous ammonia and centrifuged at 4°C at 3000 × *g*. The new supernatant was then supplemented with 2 vols absolute ethanol and 4 vols diethyl ether (Merck, no.921). The peptide precipitate which formed through incubation overnight at 4°C was dried over nitrogen and dissolved in 3 M acetic acid and gel filtered at 4°C on a 5 × 180 cm Sephadex G-50 superfine column using 1 M acetic acid as eluant. Aliquots from fractions corresponding to peptides of M_r ~10000 and down to fractions close to the salt volume of the column

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were analysed by polyacrylamide slab gel electrophoresis using 15% acrylamide at pH 8.7 [12]. In this way the elution position of insulin, glucagon and PP could be identified (fig.1). The sheep pancreatic icosapeptide was not readily identifiable. In extracts from dog pancreas the icosapeptide elutes as a conspicuous protein peak just after the insulin and PP peaks; however, in the sheep pancreas no real absorption peak at 280 nm was observed (fig.1). Furthermore, the canine icosapeptide has an electrophoretic mobility slightly slower than that of insulin in the present gel system and no peptide with this mobility was observed in the sheep extract. However, a more anionic peptide eluting in the correct position on the G-50 column was identified and it was decided to purify this anionic peptide (fig.1). Fractions corresponding to the anionic peptide were pooled from the gel filtration column, evaporated and reconstituted in 3 M acetic acid and the peptides separated on a C_{18} reverse-phase column (10 × 240 nm; VYDAC C_{18}, 5 μm particles with 300 Å pores) eluted with a gradient of acetonitrile (Merck, no.30) in trifluoroacetic acid (Pierce, sequanal grade) [0.1% (w/v) in water]. Aliquots from the different peptide peaks were analysed by polyacrylamide gel electrophoresis and amino acid analysis. Peptides comigrating with the anionic peptide were identified in two different peaks (fig.2). If care was taken initially to use fresh diethyl ether and to minimize air contact of the ether–ethanol mixture during preparation, the majority of the anionic peptides eluted as the more hydrophobic peptide species on the reverse-phase column (fig.3A); whereas deliberate aeration of

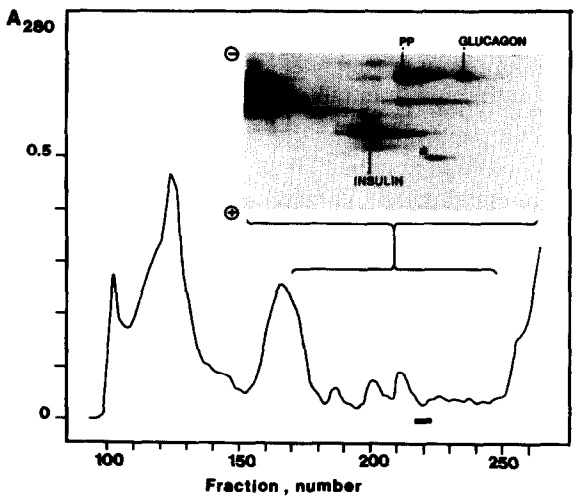

Fig.1. Gel filtration profile of proteins extracted from sheep pancreas. The column (5 × 180 cm) was packed with Sephadex G-50 superfine and eluted at 4°C with 1 M acetic acid at a flow rate of 80 ml/h; fractions of 13 ml each were collected. The inset shows polyacrylamide slab gel electrophoresis of aliquots from every fourth fraction from the indicated area of the column profile. The positions taken by insulin, PP, and glucagon are marked. The anionic peptide marked by an asterisk was further purified by reverse-phase liquid chromatography of fractions pooled corresponding to the solid bar.

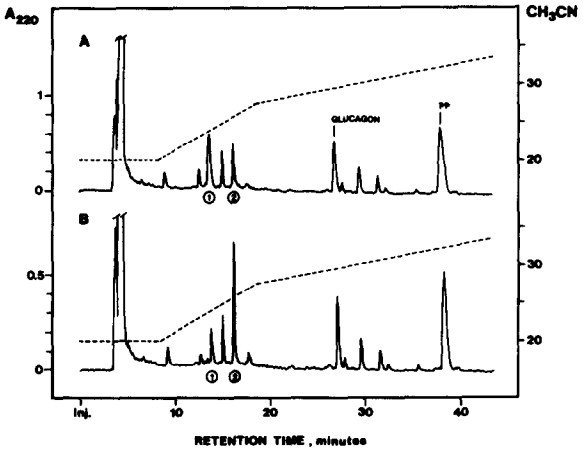

Fig.2. Elution profile of peptides on reverse-phase liquid chromatography of two aliquots from the gel filtration pool shown in fig.1. In this experiment the mixture of ether–ethanol used to precipitate the peptides was deliberately aerated by stirring for 30 min. The column (8 × 240 mm) was packed with 10 μm VYDAC C_{18} beads with 300 Å pores, and eluted with trifluoroacetic acid in water (0.1%, w/v) and a gradient of acetonitrile as indicated by the dotted line. (A) Elution profile of peptides in an aliquot of the pool of peptides which was injected shortly after they had been reconstituted in 1 M acetic acid after evaporation of the gel filtration eluant. (B) Elution profile of peptides in an aliquot of the same reconstituted peptides after incubation overnight in 1 M acetic acid at 25°C. Analytical gel electrophoresis indicated that the anionic peptide shown in the inset of fig.1 eluted in two peaks: '1' and '2'. Note that the ratio between the areas of corresponding peptide peaks in the two different chromatographs is similar for the majority of peptides, however peptide '1' decreased and peptide '2' increased to the same extent during the incubation.

the mixture, e.g., by stirring for 30 min in open air would cause the majority of the anionic peptides to elute as the less hydrophobic peptide species (see fig.2A). Storage of the peptide mixture in 1 M acetic acid at room temperature overnight did not change the elution pattern of the majority of peptides from the gel filtration pool, however, among the anionic peptides discussed above, the amount of the less hydrophobic peptide species decreased and that of the more hydrophobic one increased

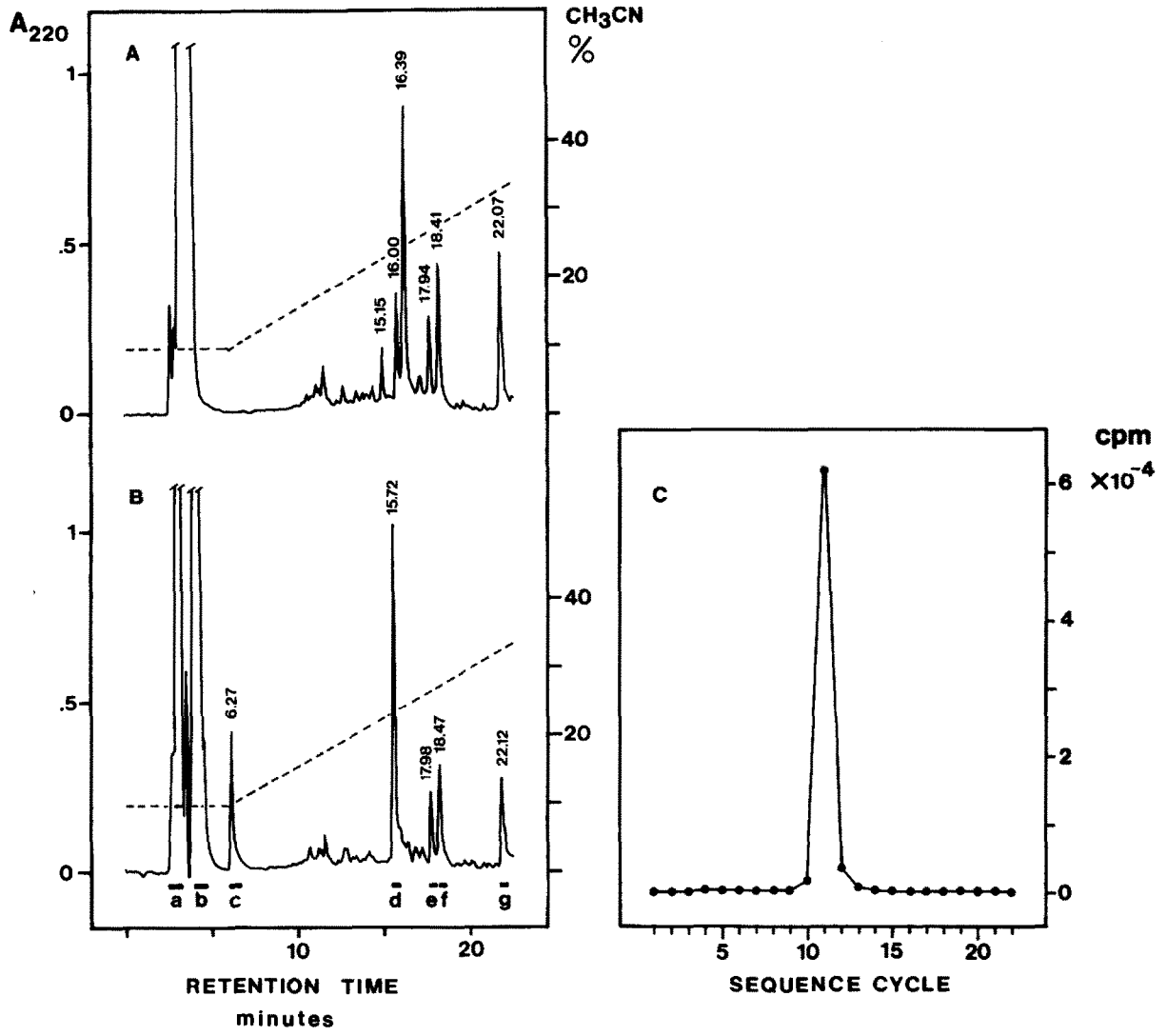

Fig.3. Purification of [^{14}C]amidocarboxymethylated icosapeptide and the occurrence of radioactivity during Edman degradation of the alkylated peptide. Peptides were characterized on a spherisorb C_{18} reverse-phase column (8 × 240 mm), eluted with trifluoroacetic acid (0.1%, w/v) and a gradient of acetonitrile, indicated by the dotted line. (A) Unreacted peptide pool from a gel filtration purification corresponding to that shown in fig.1. In this case care was taken to avoid contact with air of the ether–ethanol mixture. (B) Peptides from the same pool which have been reacted with iodo[^{14}C]acetamide as described in the text. Peaks a, b and d were radioactive after the amidocarboxymethylation. Note that peaks e–g do not change position whereas the peaks eluting in A at 15.15 and 16.39 min disappeared after the alkylation. (C) The occurrence of radioactivity in consecutive cycles of Edman degradation of the [^{14}C]amidocarboxymethylated peptide, peak d from B. One tenth of the phenylthiohydantoin derivatives were counted.

(fig.2A,B). When the less hydrophobic peptide (peak 1) was rechromatographed after similar storage more than half of the peptide eluted as peak 2 (not shown). The most hydrophobic of these peptides (peak 2, fig.2) was shown to contain 20 amino acids and to be homologous to the previously isolated canine pancreatic icosapeptide; the term sheep pancreatic icosapeptide will from this point on be used to designate this peptide. Amino acid analysis of the sheep pancreatic icosapeptide indicated that it contained a single cysteine residue; this was confirmed and the residue localized to position 11 by Edman degradation of peptide reacted with iodo[^{14}C]acetamide (fig.3C). The alkylation was performed at room temperature in the dark on 5 nmol peptide in 0.1 M Tris buffer (pH 7.5), after reduction with a 5-fold excess of dithreothiosol for 30 min; iodo-[1-^{14}C]acetamide (New England Nuclear, NEC-221) was used for the amidocarboxymethylation. The alkylated peptide was purified by liquid chromatography on a spherisorb C_{18}-column eluted with 0.1% (w/v) trifluoroacetic acid in water and a gradient of acetonitrile, and the peptide was subjected to sequence analysis on a Beckman 890C spinning-cup sequencer (generously placed at our disposal by Dr S. Magnusson, Department of Molecular Biology, University of Aarhus). The total amino acid sequence of the sheep pancreatic icosapeptide was determined on 60 nmol peptide also on the Beckman 890C sequencer, except for the COOH-terminal arginine residue which was put in sequence by degradation of 4 nmol peptide with carboxypeptidase Y (a generous gift from Dr J. Johansen, Carlbiotech, Copenhagen) performed at 37°C with a molar enzyme:substrate ratio of 1:50 in 0.1 M *N*-ethylmorpholine brought to pH 7.0 with acetic acid and using norleucine as internal standard. Amino acyl phenylthiohydantoin derivatives from the Edman degradation were identified on a spherisorb S5 ODS2 column and an ethanol gradient system [13]. The sequence of the sheep icosapeptide is shown in fig.4 together with the homologous human and dog peptides.

As discussed above two peptides had the same apparent electrophoretic mobility, but different elution characteristics on reverse-phase liquid chromatography (fig.2). The amino acid compositions of these peptides were identical except that

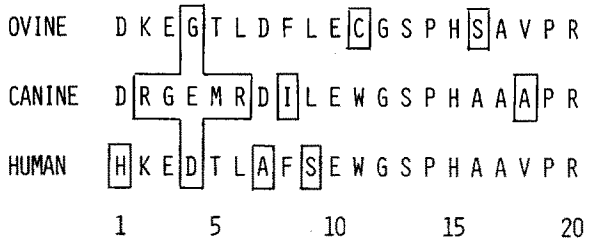

Fig.4. Amino acid sequence of the ovine, canine, and human pancreatic icosapeptides. Amino acid residues 1–19 of the ovine peptide were placed in sequence by Edman degradation and the COOH-terminal arginine residue was identified through degradation with carboxypeptidase Y as described in the text. The sequence of the canine and human peptides has been published [9,11]. Amino acid residues which differ among the 3 species are indicated by boxes. The icosapeptide is excised from the precursor as an intermediate form with a short COOH-terminal extension.

the less hydrophobic peptide (peak 1, fig.2) had an extra residue of Glu, Cys, and Gly. However, this peptide had an identical amino acid sequence to the sheep pancreatic icosapeptide as determined by both Edman degradation and carboxypeptidase Y digestion, thus the 3 extra amino acid residues could not be localized in the larger peptide (see below).

3. DISCUSSION

In dogs the pancreatic icosapeptide has been shown to be a stable peptide product of the COOH-terminal part of the PP precursor [8–11]. In this paper the amino acid sequence of the ovine pancreatic icosapeptide is presented (fig.4). Only 11 of the 20 amino acid residues were identical between the sheep and the dog pancreatic icosapeptide (fig.4). When the sequences of pancreatic icosapeptides from 3 different species are compared it is apparent that the NH_2-terminal half of the molecule is poorly preserved whereas the COOH-terminal part of the molecule is fairly well conserved (fig.4). Significantly, the COOH-terminal sequence, Pro_{19}–Arg_{20}, which forms the cleavage site in the biosynthetic intermediate form is found in all 3 species [9,10].

One interesting characteristic of the sheep pancreatic icosapeptide in contrast to the canine and

human counterparts is that it contains a single cysteine residue, and thus a free, reactive thiol group. It cannot be excluded that the cysteine residue in the sheep icosapeptide reacts with another cysteine residue somewhere in the precursor molecule forming a disulfide bridge. Another possibility would be that the icosapeptide formed dimers also held together by a disulfide bridge. If that were the case, the free icosapeptide observed in the present study would then have been formed by reduction during the isolation of the peptide. We find this very unlikely, since there is very little room for another cysteine-containing peptide in the common precursor for the icosapeptide and the pancreatic polypeptide which is only marginally larger than the combined intermediate forms of these two peptides [8–10]. Furthermore, although the amount of icosapeptide recovered here is somewhat smaller than that of the co-synthesized pancreatic polypeptide, the amount is of a magnitude such that it does not appear likely to be an extraction artefact. However, the final answer to this question must await clarification of the structure of the primary translation product for the pancreatic icosapeptide in the sheep.

On the other hand, the presence of the variant of the sheep pancreatic icosapeptide which could be separated on liquid chromatography (fig.2,3) might very well be an extraction artefact due to reaction of the free thiol group of the icosapeptide. At least, both peptides could be transformed into the amidocarboxymethylated icosapeptide by reaction with iodoacetamide (fig.3). The least hydrophobic of these icosapeptide variants seems to be a peptide composed of glutathione, connected to the icosapeptide through a disulfide bridge. The extra amino acids which were not recovered during the different sequence analyses of this peptide are those of glutathione which is found in millimolar concentrations within cells, although the phenylisothiocyanide probably would react with the α-amino function of the glutathione molecule. The following reactions, cyclization and cleavage, would not occur since the first peptide bond involves the γ- and not the α-carboxyl group of the glutamic acid residue. Thus, no phenylthiohydantoin derivative would be formed from the glutathione chain. Further, it is likely that the carboxypeptidase would not remove the terminal glycine residue from glutathione, at least not readily enough for the amino acid to be noticed during degradation of the main peptide chain.

We are currently raising antibodies against the sheep icosapeptide to study this cysteine-containing peptide in more detail, especially to try to answer the question whether the icosapeptide before and after secretion is found mainly as a monomer, dimer or combined with another peptide or glutathione. Large proteins occasionally have cysteine residues with freely exposed thiol functions, e.g., in thiol proteases. However, although regulatory peptides frequently encounter cysteine residues, to our knowledge these have only been reported to occur when they are part of intra- or interchain disulfide bridges, except in the case where they are found in the signal or 'pre' peptide, which after the translocation event rapidly is cleaved off and degraded [14].

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