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FEBS Letters 341 (1994) 239–243

FEBS 13786

FEBS LETTERS

A porcine gut polypeptide identical to the pancreatic hormone PP (pancreatic polypeptide)

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Received 1 February 1994

Abstract

A peptide hormone has been isolated from porcine intestine. Its primary structure was found to consist of 36 amino acid residues in a sequence identical to that of the porcine pancreatic polypeptide, previously not isolated from intestines or a tissue other than pancreas. The gut polypeptide significantly suppresses glucose-induced insulin secretion in vitro. Using an immunohistochemical technique, we also identified cells in the porcine gastrointestinal tract that were immunoreactive with pancreatic polypeptide. The immunoreactivity disappeared after absorption with the isolated gut polypeptide or synthetic human pancreatic polypeptide.

Key words: Pancreatic hormone; Pancreatic polypeptide; Pig intestine; Radioimmunoassay; Immunohistochemistry

1. Introduction

During studies on the isolation of chicken insulin, Kimmel et al. purified also another peptide from chicken pancreas and named it 'pancreatic polypeptide' (PP) [1]. Later studies showed that an analogous peptide was present in the pancreas of mammals [2]. PP was characterized as a 36-residue peptide with C-terminal tyrosine amide. The accumulated physiological and pharmacological data indicated it to be a pancreatic hormone [3]. Homologous peptides have since been isolated and characterized from human, porcine, canine, bovine [4,5], rat [6], goose [7], alligator [8], turkey [9], ostrich [10], chinchilla [11], frog [12], four species of perissodactyla [13] and recently from five species of the Eurasian crow family [14] and from the European hedgehog [15]. In all studies reported so far, this peptide has been recovered only from the pancreas of these species. Furthermore, PP has been detected by immunofluorescence to occur in specific endocrine cells of the pancreas and gastrointestinal tract of man and rat, and, as judged by radioimmunoassay, at different concentrations in the extracts of these tissues [16–18]. However, we have not found any reports about the distribution of PP cells in porcine gut.

A previous report from our laboratories described the isolation of pancreatic secretory trypsin inhibitor (PSTI) from intestine [19]. Still earlier, the presence in intestine of the two 36-residue polypeptides PYY (Peptide YY) [20] and NPY (Neuropeptide Y) [21] were reported. During the isolation of gut peptides that can influence insulin release, we have now purified the pancreatic polypeptide to high homogeneity and characterized it. The yield is about 3 μg/kg of wet tissue. We also determined the distribution of porcine PP by an immunohistochemical method.

2. Materials and methods

2.1. Materials

Carboxymethyl cellulose CM-23 and diethylaminoethyl cellulose (DEAE) DE-23 were from Whatman (Kent, UK), Sephadex G-25 (fine) from Pharmacia (Uppsala, Sweden), tosyl-L-phenylalanine chloromethyl ketone-treated trypsin from Worthington (Freehold, NJ, USA), and avian pancreatic polypeptide from Peninsula Laboratories Europe (Merseyside, UK).

2.2. Starting material

The starting material was a concentrate of thermostable intestinal peptides prepared from the upper part of pig small intestine and fractionated with ethanol, as described [22–24]. The ethanol precipitate at −20°C [24], 500 g (wet weight), was suspended in 5 l sodium phosphate buffer (0.02 M, pH 6.4) containing 0.5% thiodiglycol. After vigorous stirring for 60 min, the insoluble material was removed by filtration and the clear filtrate was passed through a column (14 × 14 cm) of carboxymethyl cellulose, preequilibrated with the same buffer. Unretained material was precipitated by solubilization of 320 g NaCl/l after adjust-

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SSDI 0014-5793(94)00171-Q

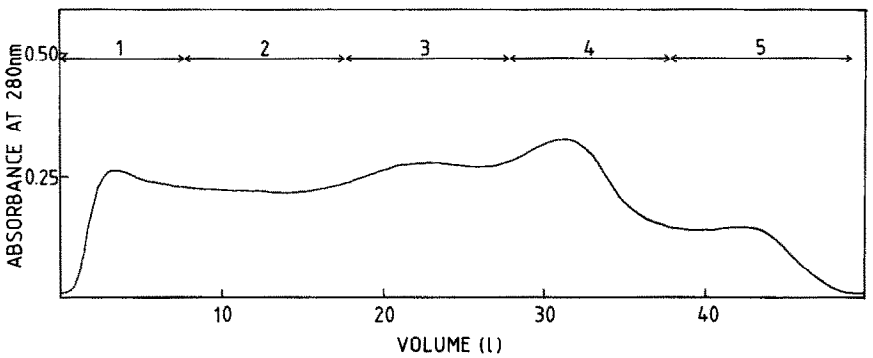

Fig. 1. Exclusion chromatography of the crude material on Sephadex G-25 in 0.2 M acetic acid. Fraction 4 was collected and precipitated (cf. text).

ment to pH 4.0 ± 0.1 with 2 M HCl, and the precipitate was collected by suction filtration for further purification (cf. section 3).

2.3. Bioassay

The effects of peptide fractions on glucose-induced insulin release were monitored using isolated rat pancreatic islets [25]. Tissue cultures were maintained overnight at 37°C under 5% CO_2 and 95% air in medium RPMI 1640 (Flow Laboratories, Irvine, UK) containing 11 mM glucose and 10% (v/v) heat-inactivated calf serum. After culture, insulin release was tested in batches of three islets in 300 μl Krebs–Ringer bicarbonate buffer, supplemented with 2 mg/ml bovine plasma albumin (fraction V, Sigma, St. Louis, MO, USA) and 10 mM HEPES (Sigma), pH 7.4, for 60 min at 37°C during shaking in a water bath. The incubation medium contained either 3.3 or 16.7 mM glucose, with or without peptide fraction. After incubation, samples of the medium were taken for radioimmunoassay of insulin [26].

2.4. Immunohistochemical investigation

Gastrointestinal tract samples were taken from three months old pigs. Tissues from the gastric antropyloric region, upper and lower duodenum, jejunum, ileum, colon, and pancreas were fixed in 10% buffered formalin overnight and routinely processed to paraffin. Sections were incubated with polyclonal antisera against human synthetic PP (A619, Dakopatts, Glostrup, Denmark, diluted 1:1800) and stained [27]. To confirm the specificity of the PP-antiserum, an absorption test was made by incubation overnight at 4–8°C with 0.5, 2 and 10 nmol antigen per ml diluted antiserum. Antigens used were synthetic human pancreatic polypeptide (P-9903, Sigma, St. Louis, MO, USA) and the purified porcine gut polypeptide.

2.5. Structural analysis

Purity of peptide preparations was ascertained by thin-layer chromatography (TLC) on polyamide thin-layer sheets [22] and by capillary zone electrophoresis with a Beckman P/ACE 2000 system, using coated capillaries of fused silica (inner diameter 100 μm, total length 27 cm) and 50 mM phosphate buffer, pH 2.5, as electrolyte.

Molecular masses were determined with a plasma desorption time-of-flight mass spectrometer (Biolon). Samples were dissolved in 0.1% trifluoroacetic acid containing 20% acetonitrile, applied to aluminium foil covered by nitrocellulose and dried in a stream of nitrogen. Data were accumulated for 15 min at 15 kV acceleration voltage. Hydrogen, sodium and nitrous oxide were used as internal calibration standards.

Total compositions were determined with an LKB 4151 Alpha Plus amino acid analyzer after hydrolysis in evacuated tubes at 110°C for 22 h with 6 M HCl containing 0.5% (w/v) phenol. Sequence analysis was carried out with an Applied Biosystems 470A instrument, and phenylthiohydantoin detection was by reverse-phase HPLC [28].

For C-terminal residue determination, 30 μg sample and 30 μg avian pancreatic polypeptide were dissolved in 30 μl 1% NH_4HCO_3, respectively. A trypsin solution (2 μl; 5 mg/ml in 1 mM HCl) was added and the mixtures were incubated at 37°C for 2 h. After lyophilization, half of the digested material was analyzed by HPLC on a Vydac 218TP54 (C_{18}) column (5 μm, 4.6 × 250 mm). Remaining parts were analyzed by TLC as described above and developed with ninhydrin after migration for 4 h.

3. Results and discussion

A 250 g quantity of crude peptide mixture (the precipitate after carboxymethyl cellulose-chromatography, cf. above) was dissolved in 2.5 l 0.2 M acetic acid, mixed with 50 g Hyflo and filtered by suction. The clear filtrate was chromatographed on Sephadex G-25 (35 × 135 cm) in 0.2 M acetic acid, giving five fractions (Fig. 1). Fraction 4 was saturated with NaCl (320 g/l) to produce a peptide precipitate which was collected by suction filtration (yield, about 25 g salt cake). A portion (10 g) was dissolved in 100 ml water, 200 ml 95% ethanol was added, and the solution was adjusted to pH 7.2 with 0.33 M NaOH in 66% ethanol. The suspension was held at −20°C for 48 h, and the precipitate was removed by suction filtration. To the filtrate, an equal volume of ethanol, precooled to −20°C, was added, and a precipitate was obtained after 24 h at −20°C. This precipitate

Table 1
Total composition of porcine gut pancreatic polypeptide

Residue	mol/mol
Asx	3.2 (3)
Thr	2.4 (2)
Ser	–
Glx	5.4 (5)
Pro	4.6 (5)
Gly	1.4 (1)
Ala	4.6 (5)
Cys	–
Val	1.1 (1)
Met	1.6 (2)
Ile	1.0 (1)
Leu	3.1 (3)
Tyr	3.8 (4)
Phe	–
Trp	–
His	–
Lys	–
Arg	4.4 (4)

Values given are molar ratios from acid hydrolysis, without corrections for destruction, incomplete hydrolysis, or impurity, and, within parentheses, from the sum of the sequence analysis.

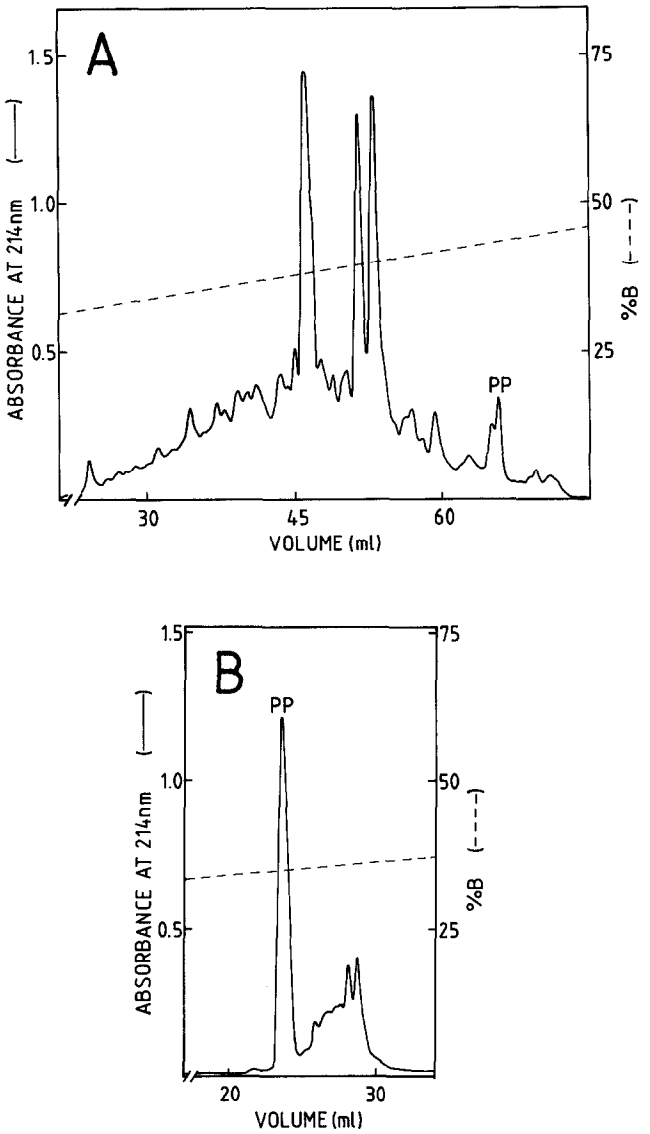

Fig. 2. Reverse-phase HPLC of porcine gut PP. (A) The first HPLC step, from which the fractions were collected and lyophilized for bioassay (cf. text). The peak denoted by PP contained the gut pancreatic polypeptide. (B) Final purification of porcine gut PP. In both A and B, solvent B was acetonitrile with 0.1% trifluoroacetic acid.

was collected by suction filtration (1.5 g wet weight), dissolved in 0.01 M NH_4HCO_3, pH 8, chromatographed on a DEAE-column (3 × 25 cm) equilibrated with the same buffer, and eluted with stepwise increments to 0.05, 0.1 and finally 0.2 M NH_4HCO_3. The fractions eluted with 0.1 M buffer were combined and lyophilized, producing 114 mg dry material. Aliquots (2 mg) were subjected to reverse-phase HPLC on TSK ODS 120-T (10 μm, 7.8 × 300 mm) with a linear gradient (60 min, 1.5 ml/min) of 26–50% acetonitrile in 0.1% trifluoroacetic acid. Eluted components absorbing at 214 nm were collected and analyzed (Fig. 2A). The fraction containing active material was further purified by chromatography on 4.6 × 250 mm Vydac 218TP54 (C_{18}) with a linear gradient (45 min, 1 ml/min) from 30 to 45% acetonitrile in 0.1% trifluoroacetic acid. The active peptide component (Fig. 2B) gave one spot upon TLC, and one symmetrical peak upon capillary zone electrophoresis (Fig. 3). It was found to correspond to porcine gut PP as identified by sequence analysis (below). The yield was about 3 μg/kg pig intestine (wet).

Plasma desorption mass spectrometry (PDMS) with 50 pmol purified gut peptide revealed two prominent peaks at *m/z* 2098.5 and 4194.3, corresponding to the doubly and singly charged species (Fig. 4), resulting in an average mass for this peptide of 4193 ± 4 Da. This mass agrees well with the calculated mass for porcine pancreatic polypeptide, 4196.8 Da [3].

The amino acid sequence of gut PP was determined by Edman degradation. Using 300 pmol, the results were interpretable up to cycle 36, and in agreement with the structure of porcine pancreatic polypeptide [3]. Molecular weight determination and amino acid analysis (Table 1) showed the C-terminal residue to be tyrosine. The precision of PDMS in this mass range is insufficient for determination of whether the tyrosine residue is free or amidated. Therefore, tryptic digestions of purified gut polypeptide and avian pancreatic polypeptide, the latter containing known C-terminal Arg-Tyr-amide, were carried out, followed by HPLC and TLC analyses. HPLC retention time and TLC R_f value, in relation to standard tyrosine and tyrosine amide, clearly showed that the pig gut polypeptide has a C-terminal tyrosine amide, identical to the C-terminal structure of avian PP.

Pancreatic polypeptide-immunoreactive cells (PP-IR) were found in the mucosa of the antropyloric and duodenal regions, and also in some Brunner gland cells. The pancreas contained a large amount of PP-IR cells (Fig. 5), while no PP-IR cells were identified in the jejunum or

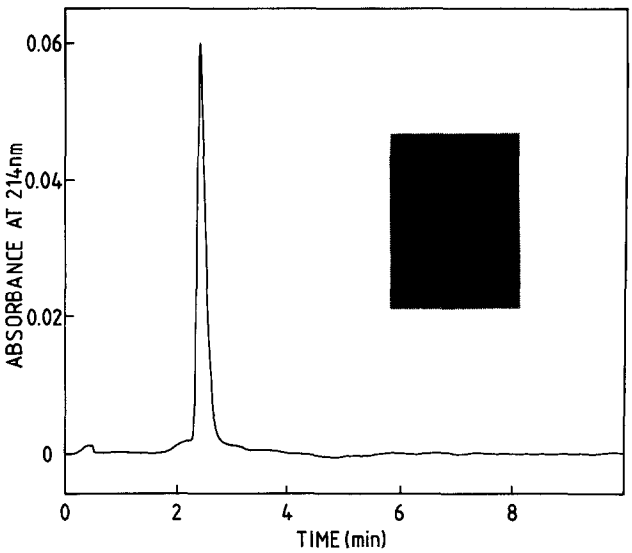

Fig. 3. Capillary zone electrophoresis of gut PP, with inset showing thin-layer chromatography on a silica gel plate. Amount applied to electrophoresis was 10 pmol; 4 nmol was applied to the silica gel plate.

ileum, although frequently in the colonic mucosa. The adsorption test with antigens to both human PP and the porcine gut polypeptide totally abolished the immunoreactivity. In early reports, human PP-cells were mainly found in the pancreas, but a small amount of PP-immunoreactive cells were found throughout the gastrointestinal tract [16]. In adult rats, PP-cells were reported to occur only in the colon and pancreas [17]. In our study, the biochemical and immunohistochemical results support each other and strongly suggest that the small amount of extracted peptide originates from PP-producing cells in the upper part of the intestine.

The secretion of insulin from isolated islets was almost three-fold higher at 16.7 mM than at 3.3 mM glucose (58.9 ± 3.2 vs. 20.5 ± 1.2 μU/islet per h, $n = 8$, $P < 0.001$). Addition of the purified gut polypeptide (100 nM) to 16.7 mM glucose suppressed the insulin release to 31.8 ± 1.8 μU/islet per h ($n = 8$, $P < 0.001$). Thus, the gut peptide inhibited stimulated insulin secretion by 46%. Inhibition of the stimulated insulin secretion has previously been shown in vivo with bovine PP in mice [29] and dogs [30,2], and with rat and human PP in rats [31,32]. For porcine PP, data have appeared conflicting since this peptide suppressed insulin secretion in dogs [33] but not in rats [34]. It is possible, however, that this previously apparent difference was accounted for by the administration of larger, pharmacological, amounts of PP in most studies, showing an effect on the insulin release, while smaller amounts of PP were infused in the second study [34]. In this context, it is of interest that the gut PP, as now shown, exerts a direct insulin-suppressive effect on the isolated pancreatic islet.

In conclusion, we have isolated PP from porcine intestine, demonstrated its primary structure to be identical with that for porcine PP from pancreas, established the PP distribution in pig gut and shown a suppressive effect of PP in vitro on insulin secretion from rat endocrine pancreas. With the establishment of PP production in the gut, the PP actions in the intestinal system will now be available for further investigation to determine the physiological role(s).

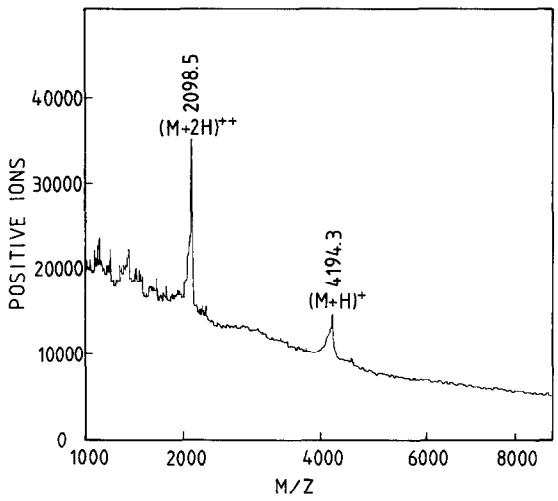

Fig. 4. PDMS spectrum of porcine gut PP after application of 50 pmol to a nitrocellulose-covered aluminized mylar target.

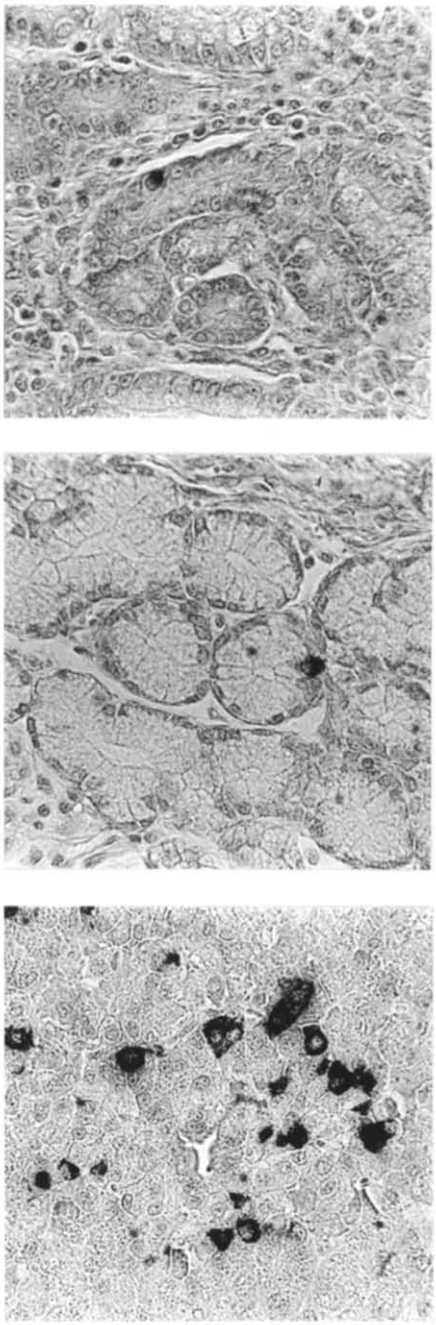

Fig. 5. Photomicrographs of (top) a section from porcine antropyloric region with a single cell (black) immunoreactive to PP antigen; (middle) a PP-immunoreactive cell (black) in the gland of Brunner from a section of porcine duodenum submucosa; and (bottom) a section from porcine pancreas with PP-immunoreactive cells (black) at well-known positions.

Acknowledgements: This work was supported by grants from the Swedish Medical Research Council (projects 12X-102, 13X-1010, 13X-3532, 13X-10832, 19X-00034), the Swedish Cancer Society (project 1806), the Swedish Diabetes Association, the Nordic Insulin Foundation, and the

Magn. Bergvall Foundation. We are indebted to Anita Nylen and Carina Palmberg for excellent assistance.

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