Glicentin is present in the pig pancreas

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Received 16 October 1984

Specimens from porcine pancreas and ileal mucosa were extracted in acid/ethanol, subjected to gel permeation chromatography, ion-exchange chromatography, enzymatic peptide degradation, reverse-phase HPLC, and analysed for glucagon-like and glicentin-like immunoreactivity by region-specific radioimmunoassays. Results obtained with all methods were consistent with the hypothesis that glicentin is present in the pig pancreas in small amounts.

Glicentin *Proglucagon* *Gastrointestinal hormone* *Prohormone*

1. INTRODUCTION

Glicentin is a 69-amino-acid protein found in pig intestinal mucosa. It contains the entire sequence of glucagon (residue 33–61) and is closely homologous to that of the 180-amino-acid glucagon precursor of hamster and man [1,2]. These preproglucagons contain 5 functional regions: a signal or pre-peptide, a 30-amino-acid NH_2-terminal peptide related to GRPP (glicentin-related pancreatic peptide of pigs) [3], the glucagon sequence itself, and two carboxy-terminal glucagon-like peptides (GLP-1 and GLP-2). We here report the isolation of apparently intact glicentin from the pig pancreas, thus providing evidence that this gut peptide is formed during the processing of pancreatic glucagon precursor as well.

2. MATERIALS AND METHODS

Porcine ileal mucosa and pancreatic tissues were extracted by an acid/ethanol method (method II in [4]). Tissues, obtained from anesthetized animals and frozen, immediately after excision, between blocks of dry ice, were homogenized in acid/ethanol at −20°C and centrifuged. Diethyl ether, precooled to −20°C, was then added to the supernatant and the aqueous proteinaceous phase was isolated and redissolved in distilled water + 8 M urea. Gel filtrations were performed on 50 × 1000 mm columns (K 50/100) packed with Sephadex G-50 superfine (both from Pharmacia), equilibrated and eluted at 4°C with 0.5 M acetic acid at a flow rate of approx. 60 ml/h. Sample size never exceeded 2% of bed volume. Elution positions are referred to by their coefficient of distribution $K_d = (V_e - V_o)/V_i$, where V_e is the elution volume of the substance in question, V_o is the exclusion volume of the gel, and V_i the available inner volume, determined as the difference between the elution volumes of ^{125}I-labelled human albumin and ^{22}NaCl (both from The Radiochemical Centre, Amersham) added to all samples for internal calibration. In the enzymatic degradation experiment (see below) gel filtrations were performed on 16 × 1000 mm columns (K 16/100, Pharmacia) of Sephadex G-50 fine grade, equilibrated and eluted at 4°C with 0.125 M NH_4HCO_3, adjusted to pH 9.0 and supplemented with human serum albumin (1 g/l), NaCl (0.1 M) and thiomersal (0.6 mM), at a constant flow of approx. 20 ml/h. Glicentin-containing fractions (as determined by radioimmunoassay, see below) were

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00145793/85/$3.30

freeze-dried, redissolved in 50 mM sodium phosphate buffer, pH 5.2, and subjected to ion-exchange chromatography. Ion-exchange experiments were performed on 9 × 150 mm columns of CM-Sepharose-CL-6B (Pharmacia), equilibrated and eluted at 4°C with the same buffer at pH 5.2 and a linear gradient reaching 0.5 M NaCl. Glicentin-containing fractions were pooled, freeze-dried, reconstituted in distilled water and applied to Sep-Pak C-18 cartridges (Water Associates, Milford, MA). The peptide was eluted at 4°C with a linear gradient of 0.1% trifluoroacetic acid (TFA) (Pierce, Rockford, IL) in water, reaching 90% ethanol. Relevant fractions were collected, freeze-dried, redissolved in 0.1% TFA–2 M CH_3COOH and subjected to HPLC. Reverse-phase HPLC was performed on 8 × 250 mm Nucleosil® 10 μ C-18 columns, employing LKB (Bromma, Sweden) equipment (2150 HPLC pump, 2152 HPLC controller, 2040-203 low-pressure mixer valve). Elution phases were: (A) 0.1% TFA in double-distilled deionised water; (B) 0.05% TFA in 60% ethanol (Merck, Darmstadt, art.no.11727). The flow rate was 1.5 ml/min and eluted fractions, collected at 1-min intervals, were blown dry under N_2 and redissolved in assay buffer. Radioimmunoassays: (i) assays for the glucagon sequence 6–15 were made with antiserum 4304, glucagon standards and ^{125}I-labelled glucagon as in [5]; (ii) assays for the glucagon sequence 19–29 were made with antiserum 4305, glucagon standards and ^{125}I-labelled glucagon as in [5,6]; (iii) assays for the glicentin sequence 61–90 [7] were made with antiserum 4804 [8], glicentin standards and ^{125}I-labelled glicentin as in [9]; (iv) assays for the glicentin sequence 15–30 [7] were made with antiserum R64, glicentin standards and ^{125}I-labelled glicentin [9]. Incubation and separation procedures have been described [10]. Antiserum 4304 binds labelled and unlabelled glucagon and glicentin with identical affinity [6], whereas antiserum 4305 does not bind intact glicentin. Purified porcine glicentin and glucagon standards, as well as monoiodinated tracers for both peptides, were kindly provided by Drs A.J. Moody and U.D. Larsen, The Novo Research Institute, Bagsværd, Denmark. Enzymic degradations of both intestinal glicentin and of pancreatic K_d 0.25 GLI peak were performed at 22°C for 30 min with bovine trypsin (TPCK-treated) (Worthington, Freehold, NJ) as in [5], followed by inhibition by the addition of soya-bean trypsin inhibitor (T 9003, Sigma, St. Louis, MO). A further degradation was performed as above with carboxypeptidase B (phenylmethane-sulphonyl fluoride-treated) (Merck, art.no.2300). The incubations were terminated by the addition of aprotinin (5000 KIU at 0°C) and the mixture was immediately applied to the column.

3. RESULTS

The binding sites of the antisera for the glucagon and the glicentin sequences are shown in fig.1. Fig.2 (the whole right panel) is representative of the gel filtration profiles of the extracts of porcine pancreas (n = 6) as compared to the profiles of purified intestinal glicentin (from Novo); identical profiles were obtained with the peak fractions isolated from ileal mucosa. The assay for the N-terminal sequence of glicentin (residues 15–30) with antiserum R 64 shows a well defined peak at K_d 0.25 (range 0.24–0.26) (fig.2F); the assay against the midpart of glicentin (residues 39–48) with antiserum 4304 (fig.2B) showed recognition of immunoreactivity at K_d 0.25, which was also the case for antiserum 4804, directed against the C-terminal sequence of glicentin (residues 61–69) (fig.2H). Parallel results were obtained with the

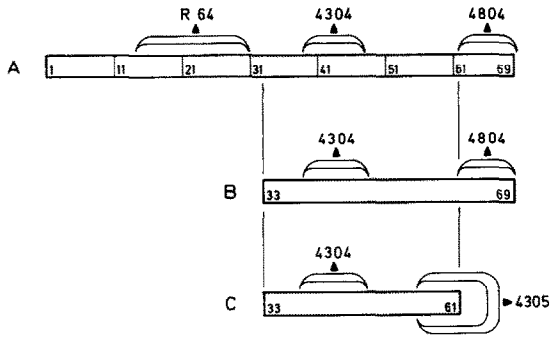

Fig.1. Schematic representation of the binding regions of the assay systems employed, as illustrated against the sequence of the glicentin molecule. A represents the entire glicentin molecule; B represents the 33–69 sequence of glicentin, and C the 33–61 sequence, which is identical with glucagon (stippled area). Note that the assay for the C-terminal glucagon sequence (with antiserum 4305) require unextended terminals of the glucagon sequence for binding.

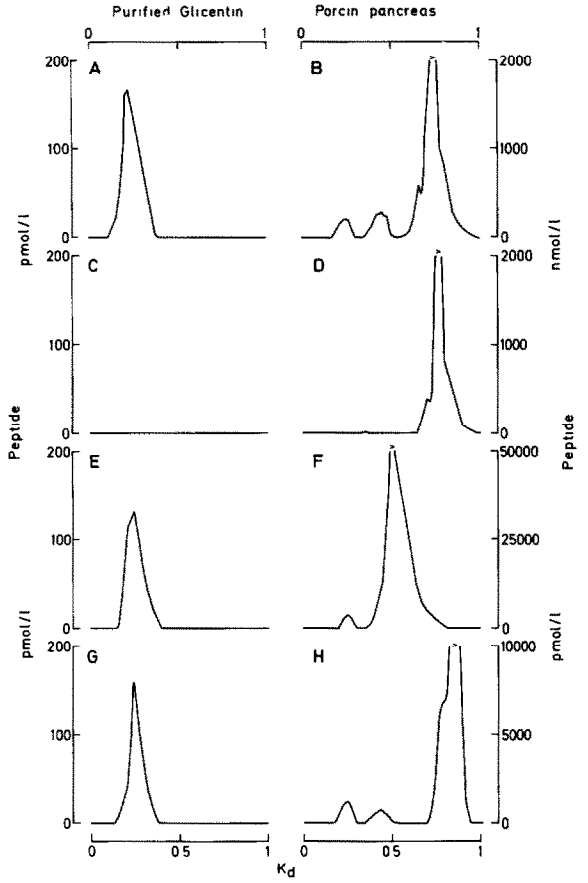

Fig.2. Gel filtration profiles of extracts of porcine pancreas (B, D, F, H) and of purified glicentin (A, C, E, G) as measured by 6–15 immunoreactive (A, B) and 19–29 immunoreactive (C, D) antisera to glucagon; 15–30 immunoreactive (E, F) and 61–69 immunoreactive (G, H) antisera to glicentin, and plotted against the coefficient of distribution, K_d.

glicentin standards (fig.2A,C,E,G). Fractions corresponding to the K_d 0.25 immunoreactivity from the gel filtration of several pancreas specimens were pooled, freeze-dried, and subjected to ion-exchange chromatography. Radioimmunoassays of the eluted fractions elicited a single immunoreactive peak, at the same ionic strength, with both antiserum R64 and with antiserum 4804 (fig.3). Fig.4 shows the results of limited tryptic digestion (10 μg/ml, 30 min, peptide/enzyme ratio approx. 1) followed by carboxypeptidase B treatment of both pancreatic K_d 0.25 immunoreactivity and of intestinal glicentin. Glicentin is no longer homogeneous: a new component is generated, active in the glucagon 19–29 assay (antiserum 4305) at K_d 0.85; in addition, a compound with exclusively glucagon 6–15 immunoreactivity appears at K_d 0.78. Finally, glucagon 19–29 immunoreac-

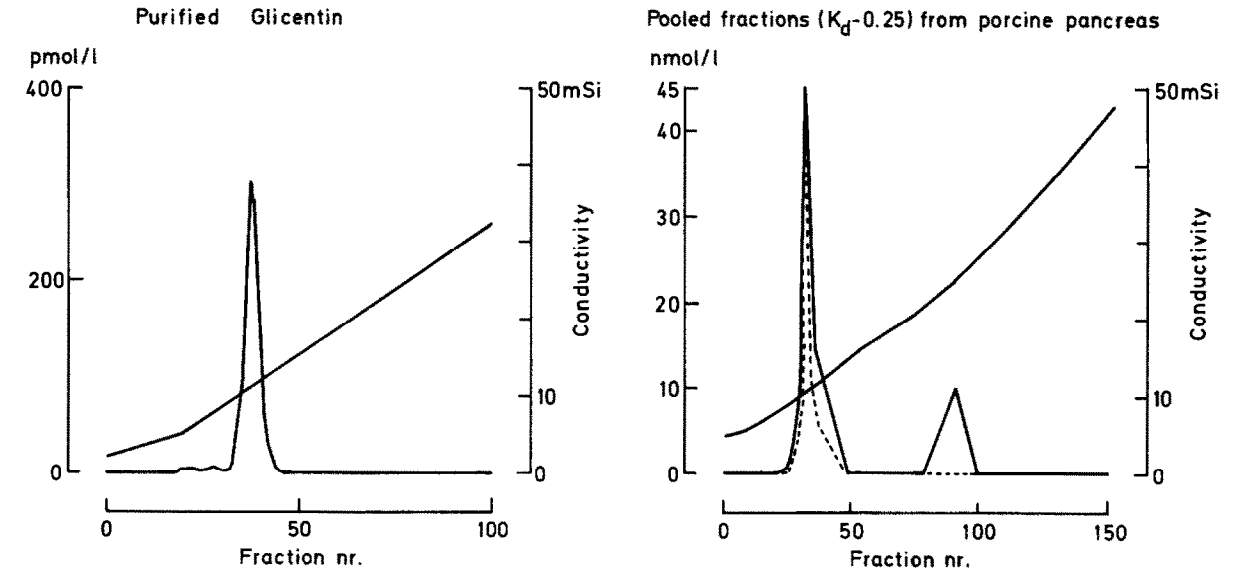

Fig.3. Ion-exchange chromatographic elution profiles of purified glicentin (left panel) and of pooled fractions from extracts of porcine pancreas (K_d 0.25, right panel), as measured by 15–30 immunoreactive (——) and 61–69 immunoreactive (– – –) antisera to glicentin. The NaCl gradient, determined by measurement of conductivity, is also shown.

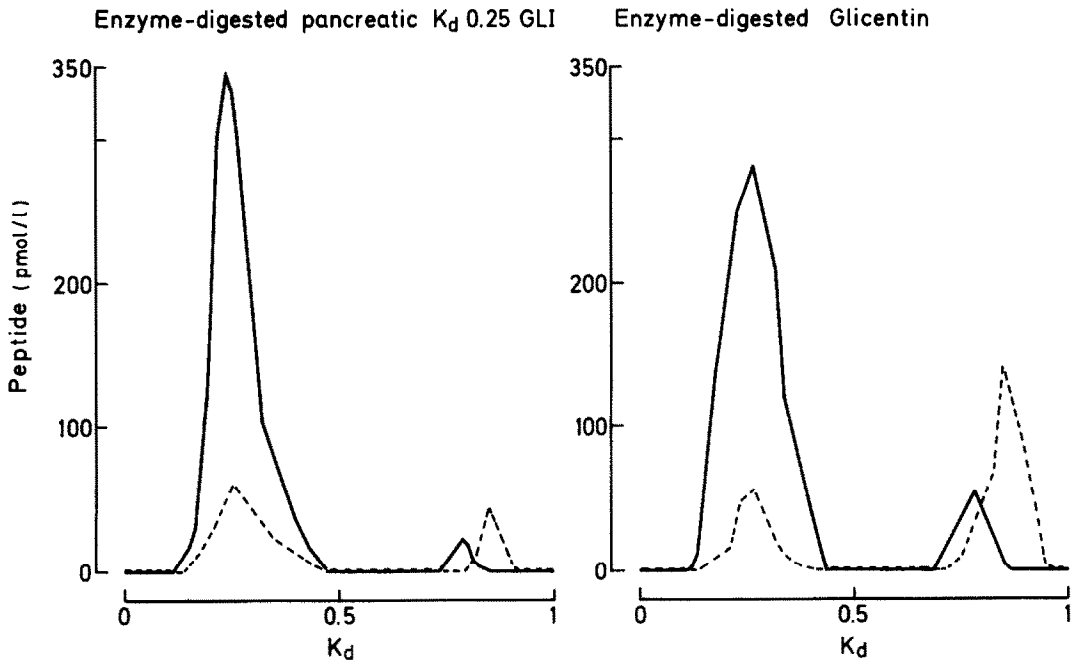

Fig.4. Gel filtration profiles of trypsin + carboxypeptidase B-digested K_d 0.25 GLI (left panel) and of intestinal glicentin (right panel) as measured by 6–15 immunoreactive (——) and 19–29 immunoreactive (– – –) antisera to glucagon and plotted *vs* the coefficient of distribution, K_d.

tivity appears at K_d 0.25. Furthermore, the pancreatic K_d 0.25 immunoreactive peak was redissolved in 0.1% TFA–2 M CH_3COOH and subjected to HPLC. As shown in fig.5, the peptide eluted at the expected retention time of purified glicentin, as was the case for intestinal mucosa K_d 0.25 GLI peak when subjected to the same chromatographic procedure (fig.5).

4. DISCUSSION

Glicentin, first isolated and purified from pig intestinal mucosa [11] and recognized as the major form of enteroglucagon in the pig [6,10,12], has the entire glucagon sequence at positions 33–61 [5,13], and N-terminally, at positions 1–30, and is composed of the glicentin-related pancreatic polypeptide (GRPP), a peptide which is secreted from the porcine pancreas synchronously and in equimolar amounts with glucagon [9]. Small amounts of a peptide with immunological and chromatographic characteristics of glicentin were found [9], and it was hypothesized that glicentin is part of a common precursor molecule giving rise to both pancreatic and non-pancreatic glucagons [9–11,13]. Pancreatic glucagon precursors as from anglerfish pancreas [15–17] show the following organisation: a signal or prepeptide, the N-terminal GRPP, glucagon and one C-terminal glucagon-like peptide. Hamster pancreatic pre-proglucagon shows a similar structure, but for the presence of two C-terminal glucagon-like peptides (GLP-1 and GLP-2) [1], and is almost identical to the human pre-proglucagon [2]. This study was undertaken to elucidate further the processing of proglucagon in pig pancreas. Glicentin found in pig ileal mucosa has been well characterized by immunological and chromatographic studies [5,6,9–13]. We have here shown (fig.2) that the peptide isolated from pig pancreas has the same chromatographic characteristics of glicentin standards, thus pointing to its identity with the intestinal mucosa peak I GLI [6]. The enzymatic digestion of our K_d 0.25 pancreatic peak gave rise to a degradation fragment at K_d 0.78 bearing the immunodeterminant for the glucagon sequence

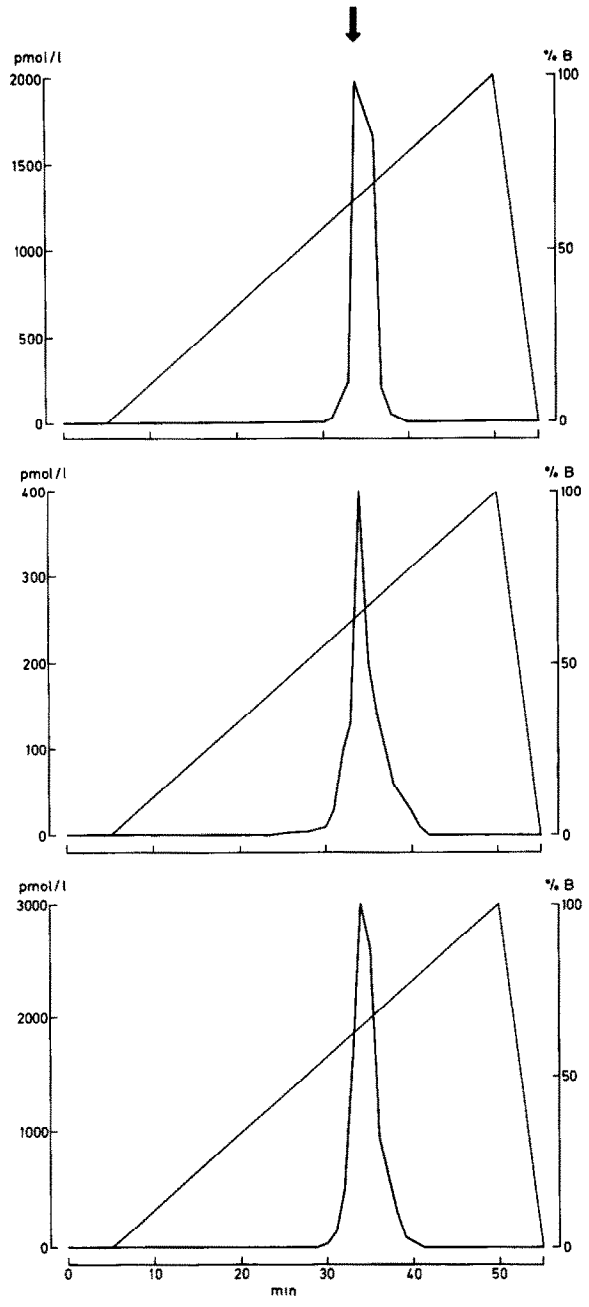

Fig.5. Reverse-phase HPLC profile of purified glicentin (upper panel), pig pancreatic K_d 0.25 GLI (middle panel) and of pig ileal mucosa K_d 0.25 GLI (lower panel), as measured by the 6–15 immunoreactive antiserum to glucagon (——). The UV detection at 226 nm of glicentin standard is indicated by an arrow. (The figure is representative of two identical experiments for each peptide.)

6–15, common for any glucagon-like immunoreactivity, and to a fragment at K_d 0.85 bearing the immunodeterminant for the unextended glucagon sequence 19–29, specific for pancreatic glucagon (fig.4); according to previous experience with the same column system [5], these elution positions correspond to the elution positions of fragments of glucagon corresponding to the 1–17 and 19–29 sequences, respectively. Such was the case for both our pancreatic K_d 0.25 peptide and for the purified glicentin standard, suggesting that the pancreatic K_d 0.25 peptide contains sequences corresponding to the glucagon sequences 1–17(–18) and 18(19)–29. Furthermore, the results of the radioimmunoassays of the fractions eluted from the ion-exchanger (fig.3) and the identity of the HPLC profile with that of purified glicentin (fig.5), are of considerable support in favour of the identity of the pancreatic K_d 0.25 peptide here described with glicentin.

ACKNOWLEDGEMENTS

This investigation was supported by grants from The Danish Medical Research Council, Novo Fond, and P. Carl Petersen Fond.

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