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Oxidation/reduction explains heterogeneity of pancreatic somatostatin

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Somatostatin 14 (SS 14) has been isolated from pancreatic extracts, but open gel filtration immunoreactive SS often elutes in two peaks. We isolated both peaks, but upon sequence analysis only authentic SS 14 could be identified. By further gel filtration experiments it turned out that both synthetic and extractable SS appeared homogeneous at neutral pH 7.5, but showed an additional, earlier peak in acetic acid. After addition of mercaptoethanol, all of the SS eluted at this earlier position regardless of the pH. We conclude that partial reduction/oxidation of SS explains the heterogeneity.

Pancreatic somatostatin; Prosomatostatin processing; DesAla^1-somatostatin; Pancreatic islet

1. INTRODUCTION

The somatostatin gene [1] is expressed in numerous cells of the body [2], including the D-cells of the pancreatic islets [3]. Prosomatostatin (proSS), the primary translation product is, however, subjected to differential processing in the different tissues in which it is produced. In the endocrine cells of the small intestinal mucosa, the C-terminal part of proSS is cleaved to give rise to somatostatin 28 (corresponding to proSS 65–92), whereas in the pancreas this sequence of proSS gives rise to somatostatin 14 (corresponding to proSS 79–92) and the 1–12 fragment of somatostatin 28 (corresponding to proSS 65–76) [4–6].

In many studies pancreatic somatostatin, however, shows some heterogeneity. The heterogeneity appears by size fractionation (gel filtration) of pancreatic extracts, whereby somatostatin-like immunoreactivity (SLI) typically elutes in a double-peaked pattern around the position of synthetic somatostatin [6–10]. The two peaks have been interpreted to indicate the presence of two immunoreactive molecular forms with a slight difference in size. In the circulation, somatostatin 14 is very rapidly degraded to desAla^1-somatostatin 14 [11–13] and these two molecular forms, when mixed together, elute by gel filtration in a similar double-peaked pattern. It was therefore speculated [6,7,10] that the molecular heterogeneity in pancreatic extracts could be due to the presence of approximately equal amounts of somatostatin 14 and desAla^1-somatostatin.

The present study was undertaken to elucidate the chemical structures responsible for the heterogeneity.

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2. MATERIALS AND METHODS

Pancreatic tissue was obtained from anaesthetized pigs and immediately frozen in dry-ice. The frozen tissue was crushed and homogenized in ice-cold acid-ethanol and further processed according to method II in (10). In short, the acid-ethanol extract was mixed with 5 vols of ice-cold diethyl ether. The aqueous phase, containing the extracted proteins and peptides, was collected at −50°C and reconstituted in distilled water containing in addition 2 mol/l urea (analytical grade). This method was chosen because among several tested it gave the highest yield of somatostatin [10]. After removal of a small sample for chromatographic analysis the extract was applied to a 3 × 15 cm glass column packed with Techoprep C-18, 40–63 μm (HPLC Technology, Macclesfield, Cheshire, UK) and eluted with a gradient of 20–80% ethanol in water containing, in addition, 0.1% trifluoroacetic acid (Merck, Darmstadt, Germany). All fractions containing somatostatin-like immunoreactivity were pooled and subjected to gel filtration on a 50 × 1000 mm borosilicate glass column (K 50/1000, Pharmacia Fine Chemicals Uppsala, Sweden) packed with Sephadex G50 SF, which was equilibrated and eluted at 4°C with 0.5 M acetic acid at a flow rate of 60 ml/h. Further chromatographic analysis was performed on 16 × 1000 mm columns (K 16/100, Pharmacia) packed with Sephadex G50 F (Pharmacia) and eluted at a flow rate of 20 ml/h at 4°C, using as eluants either 0.5 mol/l acetic acid as above or the radioimmunoassay buffer: 0.05 mol/l sodium phosphate, pH 7.5, containing in addition 0.1% human serum albumin (Reinst, Trocken, Behrringwerke, Marburg/Lahn, Germany), 0.1 mol/l NaCl, and 0.6 mol/l thiomersal. ^{125}I-labelled albumin and ^{22}NaCl in trace amounts were added to all samples applied to gel filtration as internal markers. Elution positions are referred to by the coefficient of distribution, $K_d = (V_e - V_o)/(V_i - V_o)$, where V_e is the elution volume of the substance in question, V_o the elution volume of ^{125}I-albumin and V_i that of ^{22}Na.

HPLC analysis was performed using a 4 × 250 mm column of Nucleosil C_{18} 300, 10 μm (Macherey-Nagel, Duren, Germany) eluted with mixtures of distilled water containing 0.1% of trifluoroacetic acid (Pierce Chemical Co., Rockford, IL, USA) (phase A) and acetonitrile (Grade S, Rathburn Chemicals Ltd, Walkerburn, UK), 80% (v/v) in distilled water containing 0.1% TFA (phase B).

Sequence analysis was performed using an automatic protein sequencer (Model 475A, Applied Biosystems) equipped with on-line HPLC detection of the PTH-derivatives. To improve the separation of Trp from the sequencer byproduct, *N,N'*-diphenylurea, a

modification of the gradient (RUN 470-L) recommended by Applied Biosystems was employed. Cysteine residues were not derivatized, so they were not detected.

Radioimmunoassay for somatostatin-like immunoreactivity was performed as described in detail in [14] using antiserum 1758, raised against cyclic somatostatin 14. This antiserum is directed against the 4–11 sequence of somatostatin 14 and reads equally well linear and cyclic somatostatin as well as extended, modified and shorter forms of somatostatin that contain the 4–11 sequence [4,10,14]. Synthetic, cyclic somatostatin (Peninsula Europe, Merseyside, St. Helens, UK), was used for standards and monoiodinated ^{125}I-Tyr^{1}-somatostatin (a generous gift from the Novo Research Institute, Bagsværd, Denmark) was used as tracer. Fractions eluted from the columns were assayed directly when the eluant was assay buffer or else after evaporation in a vacuum centrifuge and reconstitution in assay buffer.

3. RESULTS

Fig. 1A shows the elution profile of immunoreactive somatostatin in the extract prepared from porcine pancreas, a sample of which was applied to a Sephadex G50 F column and eluted with acetic acid. The SLI appeared as a broad double peak with K_d values around 1.0. The extract was concentrated by chromatography on Techoprep C_{18} (Fig. 1B), from which the SLI eluted as a narrow peak. Upon rechromatography on Sephadex G50 (acetic acid) the double peak profile was retained (not shown). The immunoreactive fractions were pooled, and half of the material was applied to the HPLC column and eluted with a linear gradient of acetonitrile (0–100% B in 105 min). The immunoreactive material, which eluted as a single homogenous peak, was pooled and rechromatographed twice. From the last run a single, immunoreactive peak eluted which also appeared homogenous by UV absorption (Fig. 1C). The recovery of the immunoreactive material applied to HPLC analysis was 90%. The material was subjected to sequence analysis. It was essentially pure, and the sequence corresponded to that of somatostatin 14: Ala-Gly-(Cys)-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-(Cys). The cysteine residues were not identified, but 'holes' in the sequence fitted with Cys in position 3 and 14.

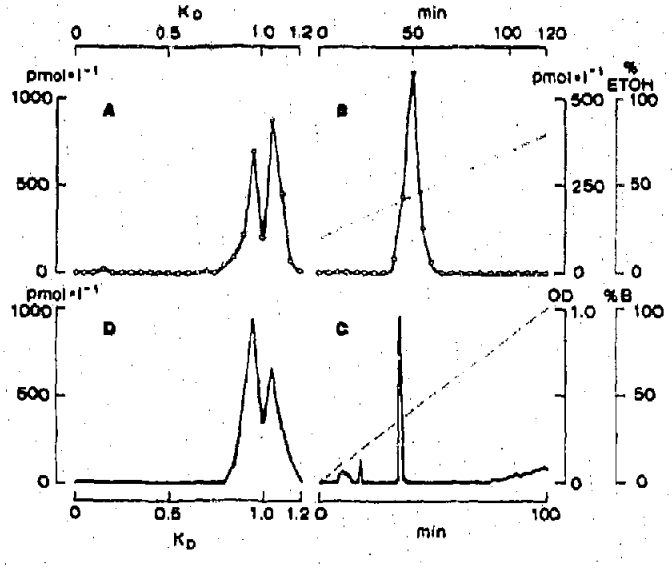

Fig. 1. Purification of porcine pancreatic somatostatin. Panel A shows the gel filtration profile of SLI in an acid-ethanol extract of pancreas applied directly to a Sephadex G50 column eluted with acetic acid. The effluent SLI concentration is plotted against coefficient of distribution, K_d. Panel B shows the SLI eluted from a Techoprep column with a gradient of ethanol as indicated by the dotted line. Effluent SLI concentration is plotted against retention time. Panel C shows the optical density, monitored continuously at 226 nm, of the final HPLC run of a pancreatic extract. The HPLC column was eluted with a gradient of acetonitrile as indicated by the dotted line. All of the SLI coeluted with the homogenous peak with a retention time of 36 min (not shown). Panel D shows the SLI elution profile of synthetic cyclic somatostatin, dissolved in acetic acid and subjected to gel filtration in acetic acid.

Fig. 1D shows the elution profile of synthetic cyclic somatostatin, dissolved in acetic acid and subjected to gel filtration in acetic acid on a K 16/100 Sephadex G50 column. The elution pattern was very similar to that of extractable pancreatic SLI (Fig. 1A).

The second half of the material was used for gel filtration analysis on both acetic acid and assay buffer columns (Fig. 2A). Again, in acetic acid the SLI eluted in a broad double peak with one peak at K_d 0.9–0.95 and another around 1.05. In assay buffer (Fig. 2B) (pH 7.5) an apparently homogenous peak eluted at K_d 1.05. To another sample of the pool mercaptoethanol was

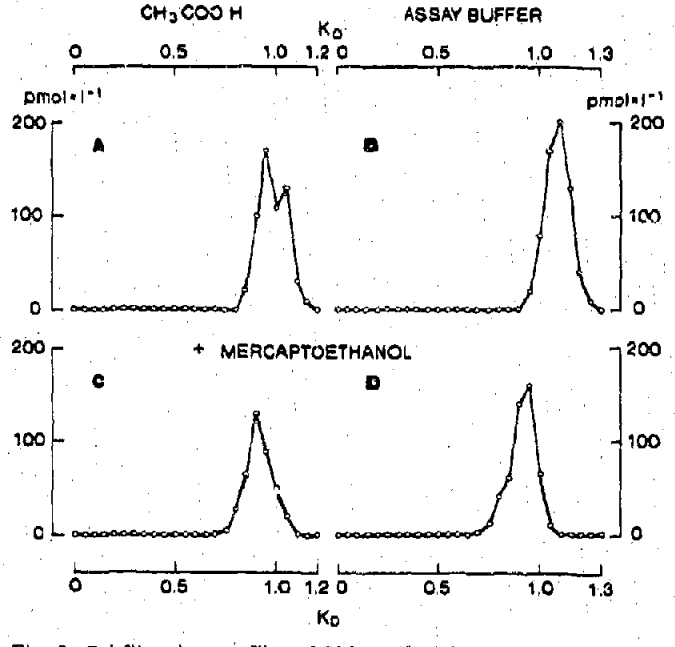

Fig. 2. Gel filtration profiles of SLI purified from pancreas extracts and subjected to gel filtration analysis in acetic acid (panels A and C) and assay buffer at pH 7.5 (panels B and D), without (top panels) or with addition of 2% mercaptoethanol to the applied sample (panel C) or to both the sample and the elution medium (panel D). Effluent concentrations of SLI are plotted against the coefficients of distribution, K_d.

added to a concentration of 2% (v/v), and the material was chromatographed on both the acetic acid column and on the assay buffer column. Mercaptoethanol was added to the assay buffer to a concentration of 2%. As shown in Fig. 2C and D, the mercaptoethanol treatment changed the elution pattern of SLI. The elution position of SLI on the assay buffer column now had a K_d of 0.90, and on the acetic acid column the heterogeneity disappeared and all of the SLI eluted as a homogenous peak at K_d 0.90.

4. DISCUSSION

In order to explain the apparent heterogeneity of immunoreactive somatostatin in pancreatic extracts our first approach was to isolate the responsible peptides by high pressure liquid chromatography. According to the data reported by McMartin and Purdon and by Shoelson et al. [11,12] somatostatin 14 and desAla^1-somatostatin 14 would be readily separable by HPLC. However, in spite of the fact that the starting material clearly contained moieties with different gel filtration elution positions and the fact that neglible amounts were lost during the HPLC purifications, the SLI always eluted as a single and eventually pure peptide, with the sequence of somatostatin 14.

We then considered the possibility that varying degrees of reduction or oxidation of the somatostatin molecule could explain the heterogeneity. It was found that the same material which upon gel filtration in acetic acid showed a double peak, eluted as a single peak when run at a neutral pH in assay buffer. However, the elution position at K_d 1.05, i.e.with an elution volume exceeding the volume of the total mobile phase of the column as determined with ^{22}NaCl, clearly indicated that an interaction between somatostatin and the column matrix had taken place, causing the delayed elution. This interaction might apply equally to both of the peaks whereby the heterogeneity would be obscured. Another explanation could be that somatostatin spontaneously cyclizises at pH 7.4 and that the homogenous appearance was due to a predominant presence of cyclic somatostatin. The experiments with the same column and the same buffer but with addition of the reducing agent, mercaptoethanol, showed that this explanation could be true. Thus, all of the immunoreactivity now eluted at K_d 0.9 as a slender, apparently homogenous peak. Similar results were obtained with the acetic acid column. After mercaptoethanol addition the double peak pattern disappeared and all of the SLI eluted at K_d 0.9. In further support of this interpretation, we found that synthetic cyclic somatostatin, dissolved in acetic acid, also eluted in a double-peaked manner upon gel filtration in acetic acid, suggesting that even the synthetic material was partly converted to reduced, linear somatostatin in acetic acid.

We conclude from these results that the heterogeneity was not, as previously believed [6,10], due to the presence of molecular forms with an amino acid composition different from that of somatostatin 14. Rather, it was due to the formation of a mixture of oxidized and reduced somatostatin in acetic acid. By reduction, the molecule acquires a linear conformation, whereby its hydrodynamic volume increases, causing it to elute earlier upon gel filtration. Apparently a mixture of reduced and oxidized molecules occur in acetic acid and this explains the appearance of SLI at different elution positions.

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