Heterogeneity of somatostatin like immunoreactivity (SLI) in extracts of porcine, canine and human pancreas

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Abstract. Four different extraction procedure representative of methods commonly employed in the isolation of somatostatin like immunoreactivity (SLI) were tested for their ability to extract large MW forms of SLI from porcine, canine and human pancreas. The yield of SLI and recovery of added somatostatin was much higher with methods involving traditional acid/ethanol extraction (methods I and II) than with methods involving boiling of tissues in water or 2 M CH3COOH (methods III and IV). Porcine and canine pancreases extracted by methods III and IV (but not methods I and II) revealed remarkable molecular heterogeneity upon gel filtration, but immuno-affinity-chromatography eliminated the largest forms. A component of approximately 3000 daltons was immunosorbable and resisted refiltration in 8 M urea. No large forms were detectable in human pancreases. The SLI peaks eluting at the position of synthetic somatostatin could be resolved into two components, one of which was lacking C-terminal immunoreactivity. It is concluded that the lack of extraction as well as the species investigated and the specificity of the antisera employed will influence significantly the results of studies of the tissue forms of somatostatin.

Molecular heterogeneity was demonstrated in the first attempts of characterizing SLI from rat pancreas and stomach (Arimura et al. 1975b) and porcine hypothalamus (Schally et al. 1976). Continued efforts by a number of groups to demonstrate SLI heterogeneity have resulted in discrepancies concerning the number of identifiable forms, their structural integrity, and their possible physiological roles (Arimura et al. 1975b; DuPont & Alvarado-Urbina 1976; Vale et al. 1976; McIntosh et al. 1978a,b; Patel & Reichlin 1978; Conlon et al. 1978; Noe et al. 1978; Spiess & Vale 1978; Zyznar et al. 1979; Pradayrol et al. 1978a,b, 1980). The wide variety of experimental animals and tissues employed coupled with differences in extraction and isolation procedures utilized from group to group certainly contributes to the confusion surrounding this issue.

In this study we have compared the effect of a variety of commonly used extraction and isolation procedures on the chromatographical pattern of pancreatic SLI from man, pig, and dog.

Materials and Methods

Extraction

Whole pancreases were obtained from 20–30 kg pigs of Danish landrace and 20–40 kg mongrel dogs and placed on dry ice immediately after surgical removal. Human pancreatic tissue was obtained from kidney donors during transplantation operations and frozen immediately after excision on dry ice.

Extraction method I. (Holst 1977; Best et al. 1939). Crushed frozen tissue was added to 5 vols acid-ethanol pre-cooled to −20°C (635 ml ethanol: 15 ml 37% HCl: 150 ml deionized water) homogenized in a Waring blender and centrifuged after 4 h at 4°C. The supernatant was brought to pH 8 with aqueous ammonia. Five vols of a 5:3 diethyl ether/absolute ethanol solution, pre-cooled to −20°C was added to the extract and the mixture allowed to stand overnight at 4°C. The resulting precipitate was saved and re-constituted in 0.01 M HCl.
Table 1.
Region specificity of somatostatin-antisera 213 and 433. Immunological potency of synthetic analogue as compared with cyclic somatostatin*.

<table>
<thead>
<tr>
<th></th>
<th>Dihydro-somatostatin</th>
<th>Tyr1-somatostatin</th>
<th>Tyr11-somatostatin</th>
<th>DesAla1-somatostatin</th>
<th>DesAla1-Gly2 somatostatin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antiserum 213</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>75%</td>
</tr>
<tr>
<td>Antiserum 433</td>
<td>100%</td>
<td>100%</td>
<td>&lt;1%</td>
<td>100%</td>
<td>100%</td>
</tr>
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* The amount of cyclic somatostatin needed to cause a 50% reduction of the binding of 10 fmol of \([^{125}I]\)Tyr1-somatostatin to antibody expressed as per cent of the amount of synthetic analogue needed to cause the same effect.

Extraction method II. As above, but instead of neutralizing, 5 vols of pure, pre-cooled diethyl ether was added to the acidified extract. The denser, proteinaceous aqueous phase was separated out by placing the flask with the solution into a -50°C methanol bath and pouring off the unfrozen ether phase. The protein material was re-dissolved in distilled water.

Extraction method III. (Holst 1977; Mutt 1959). Crushed tissue was added to 5 vols of boiling water and allowed to boil for 20 min, then immediately placed in an ice bath to cool, then 1 mol/l acetic acid was added to a concentration of 1 mol/l, the extract was homogenized in a Waring type blender for 1 min and centrifuged at 5000 g for 30 min.

Extraction method IV. (Conlon et al. 1978; modified after Schally et al. (1971)). Crushed tissue was heated at 90°C for 5 min in 2 m CH3COOH, then cooled in an ice bath and homogenized in a Waring type blender for 1 min. The crude extract was centrifuged at 4000 g for 15 min and the supernatant decanted and brought to pH 7.5 with 10 N NaOH. This solution was subsequently centrifuged at 4000 g for 15 min, the supernatant representing the final extract.

Radioimmunoassay
Two antisera, coded R213 and R433, respectively, were raised in rabbits against synthetic cyclic somatostatin covalently bound to bovine serum albumin using ethyl carbodiimide (Rehfeld et al. 1972). Binding specificity was determined with somatostatin analogues with various deletions or substitutions (Table 1). The results suggest that R213 binds to the sequence between residue 3 and 10, whereas the binding region of R433 involves the sequence around position 11.

The assays were performed using synthetic cyclic somatostatin (Beckman Bioproducts, Geneva, Switzerland) as standard, and \([^{125}I]\)Tyr1-somatostatin labelled according to Arimura et al. (1975a) with slight modifications (a generous gift from U. D. Larsen, the Novo Research Institute, Bagsvaerd, Denmark). Detection limit of the assay was 1 pmol/l. The inter-assay coefficient of variation was better than 6% in the working range. Bound and free somatostatin were separated with plasma coated charcoal (Stadil & Rehfeld 1973). The antisera employed do not cross-react with any other known pancreatic peptides.

Immunofinity chromatography
Immunopurification of SLI from crude pancreatic extracts was carried out on a 3 ml column of gamma globulin from antisera R213 immobilized on CNBr activated Sepharose 4B (Pharmacia, Uppsała, Sweden) (Holst 1975) according to the manufacturer's instructions. Prior to a run the column was equilibrated with phosphate buffer containing 1% HSA for ½ h. The sample, adjusted to pH 7.5 was then applied, followed by re-application of phosphate buffer. Non-specifically bound materials were removed by irrigation with 1 m NaCl brought to pH 10.4 with aqueous ammonia. Specifically bound SLI was eluted with 2-3 bed volumes of 1 m formic acid. Column runs were monitored by addition of small amounts of \([^{125}I]\)somatostatin to the extracts. Radioactive fractions were pooled and lyophilized for subsequent radioimmunoassay. In some instances immunopurification efficiency was monitored by somatostatin and glucagon radioimmunoassay on all eluted fractions after lyophilization (Fig. 1).

Gel filtration
All gel filtrations of crude extracts were carried out on K 50/100 (Pharmacia) 50 x 1000 mm, 1600 ml columns containing Sephadex G-50 Superfine equilibrated with 0.5 m CH3COOH. Re-filtrations were performed on smaller (K 16/100, 16 x 100 mm) columns containing the same gel and elution buffer. Constant flow rates (0.8 and 0.2 ml/min, respectively) were maintained by means of peristaltic pumps, and fractions collected by means of an automatic fraction collector. The void volume \((V_0)\) was determined by the elution position of albumin, and the available inner volume of the gel bed \((V_i)\) by the position
Elution pattern for a representative immunoaffinity chromatography run performed on a section of human pancreas extracted by method IV. The solid lines (---) represent extracted glucagon-like immunoreactivity; dashed lines (----) extracted somatostatin-like immunoreactivity; dotted lines (· · ·) counts/5 min attributable to $[^{125}I]$somatostatin added to the sample prior to application to the affinity column.

**Fig. 1.**

Table 2.
Yield and recovery of somatostatin upon extraction of porcine pancreas.

<table>
<thead>
<tr>
<th>Extraction</th>
<th>Content of SLI pmol/g pancreas (wet weight)</th>
<th>Content of SLI after addition of 2000 pmol/g</th>
<th>Recovery of added somatostatin %</th>
<th>Recovery of added $[^{125}I]$Tyr$^1$-somatostatin %</th>
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</thead>
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<tr>
<td>Extraction II</td>
<td>702 ± 51</td>
<td>2516 ± 157</td>
<td>92 ± 7.7</td>
<td>65 ± 8.4</td>
</tr>
<tr>
<td>Extraction III</td>
<td>176 ± 13</td>
<td>366 ± 26</td>
<td>9.5 ± 1.3</td>
<td>86 ± 0.5</td>
</tr>
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Frozen porcine pancreas was crushed finely, mixed vigorously and divided into 24 portions. Each portion was then extracted with either method II or III. Synthetic somatostatin (2000 pmol/g, wet weight) was added to the extraction fluid (water or acid ethanol) in 8 cases, and appr. 40 000 CPM $[^{125}I]$Tyr$^1$-somatostatin was added to the extraction fluid in another 8 cases. The final extracts were then either assayed for their radioactivity or content of radioimmunoassayable somatostatin. Results are presented as mean ± SEM ($n = 4$).
of Na$^{22}$Cl added to the sample prior to gel filtration. The coefficient of distribution, $K_d$, was calculated for all peaks of SLI using the formula $K_d = (V_e - V_0)/V_i$, where $V_e$ is the elution volume of the SLI peak in question. The elution positions of $[^{125}\text{I}]$labelled somatostatin as well as unlabelled synthetic cyclic somatostatin were also determined. Recovery of synthetic somatostatin applied to the columns varied between 61–91% ($n = 8$). Protein determinations were performed according to Lowry et al. (1951).

**Fig. 2.**
Somatostatin-like immunoreactivity extracted from equal sections of porcine pancreas by methods II (upper panel), III (mid panel), and IV (lower panel), and gel filtered on G-50 superfine 0.5 M CH$_3$COOH. Somatostatin was measured with antisera R213 (——) and R433 (———). Panels A: extract submitted directly to gel filtration. Panels B: extract submitted to immunoaffinity chromatography and subsequently gel filtered. This figure is representative of 4 experiments. 'S' denotes the elution position of synthetic cyclic somatostatin. (The amount of extract applied to the columns varied).
Results

Extractions

In Table 2 are presented the yields of somatostatin when porcine pancreas was extracted with either an acid-ethanol method (method II) or a boiling method (method III). The acid-ethanol method was almost 5 times as efficient as the boiling method. The superiority of the acid ethanol method is also apparent from the recovery studies (Table 2) which show that the recovery of synthetic somatostatin with this method was almost 10 times higher than that obtained with the boiling method. Table 2 also shows that recovery studies based on tracing of the radioactivity of labelled peptides may not be reliable; in this study, the recovery of [125I]Tyr1-somatostatin determined by γ-counting was best with the boiling method. Experiments on a single human pancreas indicated also that methods I and II gave higher yields of SL1 (86–225 pmol/g wet tissue vs 54–77 pmol/g with methods III and IV). For porcine pancreas the yield of somatostatin with method III ranged from 159 to 205 pmol/g (n = 8) and with method IV from 18 to 47 pmol/g inspite of identical yields of total protein (8–9.1 mg/g) (n = 4).

Immunoadfinity chromatography

Fig. 1 characterizes a representative affinity chromatography run as performed on a section of human pancreas extracted by method IV and illustrates the effectiveness of immunoabsorption. The average recovery of [125I]somatostatin added as a marker to the respective samples was 73.5 ± 5.6% (SEM, n = 9). The binding capacity of the 213 immunoabsorbent, determined from batchwise incubation of the immunosorbent with trace amounts of [125I]somatostatin and excess unlabelled somatostatin, exceeded 30 nmol.

Gel filtration

A. Porcine. Fig. 2 depicts a representative experiment in which equal sections of the same porcine pancreas were extracted by methods II, III, and IV, respectively; a total of 4 pancreases was studied this way. In all cases, the extracts were divided in half. One half was placed directly on a 1.6 litre Sephadex G-50 Superfine column, 0.5 m CH₃COOH while the other was subjected to immunoaffinity chromatography prior to gel filtration on the same column. Thus, each individual figure shows the gel filtration pattern of immunoabsorbed vs untreated material (B vs A) extracted by one of the three methods.
For all samples, a large peak of SLI is seen eluting with $K_d$ of between 0.85–1.1. Synthetic cyclic somatostatin has a $K_d$ of 0.97 on the columns utilized for these experiments.

Samples extracted by method II had very little SLI in fractions other than those close to the elution position of synthetic somatostatin. This was true for portions of the same extracts regardless of whether they were applied directly to the Sephadex column or immunoabsorbed first (Fig. 2, upper panel).

In addition to a SLI peak with $K_d$ of 0.95, extraction by methods III and IV resulted in three large MW SLI peaks (Fig. 2, lower 2 panels). After immunoabsorption of portions of the same extracts, however, the two largest peaks disappeared and only SLI at $K_d$ 0.60 and 0.95 remained (Fig. 2 lower 2 panels, part B).

The fractions comprising the SLI peaks labelled A, B, C, D, and E in Fig. 2 were pooled respectively, incubated with 8 M urea and an aliquot of each removed and applied to smaller (K 16/100) Sephadex G-50 Superfine, 0.5 m CH$_3$COOH columns.

Re-filtration of the 'true somatostatin' peaks B and D which were not immunoabsorbed prior to gel filtration resulted in two peaks. The first, with $K_d$ of 0.95–1.05 was detected equally by both antisera. The second, with $K_d$ 1–1.12 was measured only by R213 (Figs. 3A and 3C). Re-filtration of peak E, immunoabsorbed 'true somatostatin' resulted in a single peak of SLI, measurable with both antisera with an elution coefficient close to that of synthetic somatostatin (Fig. 3B).

**Fig. 4.**
Re-filtration of peak A from Fig. 2 (mid panel). After incubation in 8 M urea for 1 h, somatostatin-like immunoreactivity was measured by R213 (-----) as well as R433 (------).

**Fig. 5.**
Representative gel filtration pattern for equal sections of the same human pancreas extracted by methods I (panel A), II (panel B), and III (panel C). Somatostatin-like immunoreactivity was determined with antisera R213 (-----) and R433 (------). Abscissa: numbers of eluted fractions.
Re-filtration of peak A (Fig. 4) resulted in two peaks, with a substantial portion of the SLI originally at $K_d$ 0.62 migrating to the elution position of synthetic somatostatin. Re-filtration of peak C, the 0.60 $K_d$ peak obtained for method IV, resulted in a similar pattern (not pictured).

B. Human. Representative gel filtration patterns obtained for equal sections of the same human pancreases extracted by methods I–II are depicted in Fig. 5.

For all extractions performed on human pancreases ($n = 2$) the predominant peak of SLI eluted in the same position as synthetic somatostatin. Only in the case of method III is there a small amount of large SLI measurable by both antisera eluting with a $K_d$ of 0.37.

C. Canine. Fig. 6 depicts the gel filtration pattern of equal sections of a canine pancreas extracted by methods II and III. In both cases, the predominant peak of SLI is seen eluting with a $K_d$ of approximately 0.95. As with porcine and human pancreases, no large forms are present in the elution profile for extraction method II. Extraction by method III resulted in three peaks of SLI in addition to the 0.95 peak. The largest, representing approximately 20% of the eluted SLI, had a $K_d$ of 0.65. Two smaller peaks eluted with a $K_d$ of 0.43 and the void volume, respectively.

Fig. 6.
Representative gel filtration patterns for equal sections of the same canine pancreas extracted by methods II (panel A) and III (panel B). Somatostatin-like immunoreactivity was measured with antisera R213 (-----) and R433(-----).
Abscissa: numbers of eluted fractions.
Discussion

In this study we were unable to demonstrate large forms of SLI in acid-ethanol pancreatic extracts. The rather low percentage ethanol used (61% v/v) was chosen to ensure efficient extraction of molecules as large as e.g. intestinal glucagon-like peptides with molecular weight above 10 000 daltons (Holst 1977). The acid pH should facilitate tissue disruption and dissociation of SLI non-covalently bound to structures which precipitate in acid/ethanol; also degrading enzymes should be efficiently inactivated. The possibility, that degrading enzymes were responsible for the lack of large forms, seems excluded by the results with methods I and II immediately followed by affinity chromatography, in which immunoreactive moieties were separated from the remaining extract within a few minutes and at 4°C. The ability of methods III and IV in extracting large SLI cannot be due to acidity, and therefore probably resides in the use of heating for tissue disruption and enzyme inactivation. However, heat inactivation of proteins may be a slowly reversible process with a risk of subsequent reactivation of enzymes, and boiling at an acid pH could promote acid hydrolysis of peptides. The latter methods would therefore be anticipated to generate more artifacts than the acid/ethanol procedures. As discussed below, part of the large MW SLI identified by gel filtration of method III and IV extracts was lost after immunoaffinity chromatography of the extracts, and part of the Kd 0.60 SLI which remained after affinity chromatography dissociated into low molecular weight SLI by re-chromatography. It cannot, therefore, be excluded that the gel filtration pattern of SLI extracted by acid/ethanol is closer to the true distribution in vivo than that obtained with the heat inactivation methods. The results of the recovery studies support this theory.

In all species surveyed in this study, the predominant form of SLI seen in gel filtration profiles corresponded in elution position to synthetic cyclic somatostatin. In porcine and canine pancreases extracted by methods III or IV, however, this peak shows some heterogeneity, pointing to the presence in the extracts of a somatostatin-related peptide, from which the C-terminal immunodeterminant (including residue 11) is missing.

In porcine pancreases, extraction by methods III and IV resulted in 3 large peaks of SLI. For both methods, the 2 largest peaks disappeared after affinity chromatography, implying that these peaks either represented co-extracted substances, which interfered non-specifically in the radioimmunoassays or were comprised of somatostatin non-covalently bound to a larger protein which was dissociated under the conditions of affinity chromatography. The last large peak, which eluted with Kd 0.58–0.68 for both extraction methods was present in roughly equal concentrations irrespective of whether it was immunoabsorbed or untreated.

The results obtained for extraction method III on canine pancreases are in good agreement with those obtained by Conlon et al. (1978) for the same tissue. SLI extracted from porcine pancreases, on the other hand, seems to differ in several respects. The number of SLI peaks obtained on extraction by either method III or IV was 4 (this investigation) as compared to 3 (Conlon et al. 1978).

The two largest peaks disappeared when exposed to affinity chromatography (this investigation) while a large peak of approximately 12 000 daltons was isolated utilizing affinity chromatography and shown to be resistant to treatment with 8 M urea or 6 M guanidine HCl in canine extracts (Conlon et al. 1978). In both studies, a peak of SLI is seen eluting in the MW range 3–4500 daltons. Treatment with 8 M urea revealed that a substantial portion of this SLI was dissociable (this investigation) vs the peak was left essentially intact (Conlon et al. 1978). The results of the present study are more in agreement with those of Spiess et al. (1979) for pigeon pancreas, DuPont & Alvarado-Urbina (1976) for rat pancreas and Chavyvialle et al. (1978) for human pancreases. In these cases, the majority of large SLI was found to be dissociable. However, some porcine pancreatic SLI remained at Kd 0.60 (Fig. 4) after affinity chromatography as well as urea treatment and may therefore represent a true biosynthetic precursor; such precursors were recently shown to exist in anglerfish islets (Noe et al. 1979a,b). Also a 28 amino acid peptide containing the full somatostatin sequence was recently isolated from upper intestinal wall by Pradayrol et al. (1978a,b, 1980); the relationship of this peptide to the pancreatic SLI eluting at Kd 0.60 remains to be established, but it co-elutes on these columns with synthetic somatostatin 1–28 (Holst et al., unpublished).
Acknowledgment

The authors wish to thank Ms. Merete Hagerup and Ms. Grethe Thaarup for their expert technical assistance. This study was supported by grants from the Danish Medical Research Council.

References


Received on December 23rd, 1980.