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Isolation and characterization of somatostatin from pigeon pancreas

(*S*-carboxymethylation/high-pressure liquid chromatography/spinning cup sequencer/Polybrene)

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ABSTRACT **Most of the somatostatin-like activity from pigeon pancreas was found to correspond to a small species with an apparent molecular weight of 1500–2500. This species was isolated under conditions minimizing intermolecular interactions and protease activities. The isolated product was characterized by two somatostatin radioimmunoassays, a bioassay, endgroup determination, and amino acid analysis. The structure of the isolated compound was determined to be H-Ala-Gly-cyclo-(Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys)-OH. Additionally, small amounts of des-Ala[1]-somatostatin, a possible degradation product of pancreatic somatostatin, and a large somatostatin-like species with an apparent molecular weight of 11,000–12,500 were detected. It is concluded that the main somatostatin-like polypeptide isolated from pigeon pancreas is identical to the mammalian hypothalamic tetradecapeptide somatostatin.**

In 1973, the amino acid sequence of somatostatin (SS) from ovine hypothalamus was reported (1, 2). The sequence for porcine hypothalamic SS was shown to be identical (3). Biologic and immunologic SS-like activities (SSLA) have also been detected in extrahypothalamic parts of the brain (4, 5), pancreas (6, 7), and gastrointestinal tract (7). Although these activities had been observed by bioassay and by immunologic techniques with several antisera directed against different portions of the SS molecule (8), the chemical identities of extrahypothalamic SS-like substances had not been established. In two earlier reports (9, 10) we described the initial purification of SS-like substances from pigeon pancreas, which was chosen as a source of extrahypothalamic SS-like species because of its high content of SSLA (8). We report here details of the isolation and characterization of SS from pigeon pancreas.

METHODS AND MATERIALS

Purification. Male pigeons were obtained from Carpenter Squab Ranch (Ventura, CA). For one experiment, 5–20 pigeons were decapitated. The pancreata were removed and submitted to either one of the following procedures:

(*A*) The pancreata were frozen in liquid nitrogen within 1 min after removal from the animal. The frozen material was then pulverized in liquid nitrogen. The powder was almost totally dissolved in 8 M guanidine hydrochloride/2 M ammonium acetate, pH 2.5, at room temperature and centrifuged for 20 min at 48,000 × *g*. The supernatant was defatted with an equal volume of diethyl ether/hexane (2:1, vol/vol) and submitted to column chromatography (Bio-Gel P-100, Bio-Rad), which was performed in the presence of 6 M guanidine hydrochloride/1 M ammonium acetate, pH 2.5. The chromatographic elution pattern was characterized by the following molecular weight markers: bovine serum gamma globulin, bovine ribonuclease A, cytochrome *c* (horse heart), lima bean trypsin inhibitor, bovine pancreatic trypsin inhibitor, porcine insulin, porcine ^{125}I-labeled glucagon (New England Nuclear), [^{125}I-Tyr^{1}]SS, bacitracin, and tyrosine. All elution volumes were calculated as partition coefficients K_D, referring to the internal volume of the gel. $K_D = (V_e - V_o)/(V_s - V_o)$; V_e, elution volume of sample; V_o, void volume; V_s, elution volume of small solutes (molecular weight <200). Fractions containing SSLA were desalted on Bio-Gel P-2 in the presence of 3 M acetic acid and further purified by DEAE-Sephadex ion-exchange chromatography (A25, Pharmacia) in 0.1 M ammonium acetate, pH 7.0. Final purification was achieved by high-pressure liquid chromatography (HPLC).

The apparatus used for HPLC consisted of Waters Associates models 204 liquid chromatograph, UK6 injector, two 6000A pumps, a 660 programmer, and a 450 variable wavelength detector, a Minigrator from Spectra Physics, and a model 455 chart recorder from Linear Instruments Corporation.

HPLC was performed as reverse-phase chromatography on μ-Bondapak C_{18} columns (0.39 × 30 cm or 0.70 × 30 cm, Waters Associates) and μ-Bondapak CN columns (0.39 × 30 cm, Waters Associates). μ-Bondapak C_{18} and μ-Bondapak CN contain octadecylsilanyl and cyanopropylsilanyl groups, respectively, covalently coupled to silica particles. The columns were eluted by a mixture containing 24–26% (vol/vol) acetonitrile and 76–74% (vol/vol) TEAP or TEAF buffer. These buffers consisted of 0.08 M phosphoric acid (TEAP) or 0.25 M formic acid (TEAF) adjusted to pH 2.5 or pH 3.0, respectively, by triethylamine. Because TEAP buffer often gives higher resolution than TEAF buffer (11), most samples were first applied to μ-Bondapak C_{18} columns, eluted by TEAP buffer and acetonitrile, and then desalted by reverse-phase HPLC using μ-Bondapak CN columns and TEAF buffer.

Ten to 20 μg of SS equivalents, as determined by SS radioimmunoassay, were usually loaded on a μ-Bondapak C_{18} (0.70 × 30 cm) column. The protein load was less than 40 mg.

(*B*) After removal from the animal, the pancreata were suspended in hot (100°C) 2 M acetic acid containing, per ml, 200 μg of soya bean trypsin inhibitor, 30 μg of bacitracin, and 10 μg of pepstatin A. After 10 min, the suspension was cooled to *ca.* 2°C, homogenized with a Polytron homogenizer (Kinematica), and centrifuged as in procedure *A*. Ice-cold acetone was added to the supernatant to a final concentration of 75% (vol/vol). After centrifugation at 28,000 × *g* for 30 min, the supernatant was decanted, freed of acetone by a stream of nitrogen gas, and dried by lyophilization. The residue was incubated in 1–2 ml of 8 M guanidine hydrochloride/2 M ammoniun acetate, pH 2.5, at 100°C for 5 min, defatted with an equal volume of diethyl ether/hexane (2:1), and submitted to reverse-phase HPLC, performed as in procedure *A*.

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Abbreviations: SS, somatostatin; SSLA, somatostatin-like activity; Cm-Cys, *S*-carboxymethylcysteine; Cm-SS, carboxymethyl-SS; HPLC, high-pressure liquid chromatography; TEAP, triethylammonium phosphate; TEAF, triethylammonium formate; >PhNCS, phenylthiohydantoin.

S-Carboxymethylation. Tetradecapeptide SS and pancreatic SS-like polypeptide were carboxymethylated at pH 6.0 according to Burgus *et al.* (2) or at pH 8.7 according to Crestfield *et al.* (12). The procedure recommended by Crestfield *et al.* (12) for S-carboxymethylation of proteins was modified by reducing peptides with 50 mM dithiothreitol rather than 119 mM thioglycol. The carboxymethylation was stopped after 3 min with acetic acid (final concentration 10% vol/vol), and the products were purified by reverse-phase HPLC on a μ-Bondapak C_{18} column (0.39 × 30 cm) with the TEAF buffer and acetonitrile as described above.

Amino Acid Analysis. Peptide (1 to 4 μg) was hydrolyzed for 24 or 48 hr at 110°C with 4 M methanesulfonic acid/0.2% tryptamine according to Moore (13). Analysis was performed with a Beckman 121 MB amino acid analyzer equipped with a model 126 data system. Samples containing 180–500 pmol per amino acid were applied to a 0.28 × 20 cm column (Beckman AA-10 resin), using a standard sodium citrate program (Beckman) with ninhydrin detection (single column system).

Endgroup Determination. Peptide (0.5 to 1 μg) was dansylated in 5 mM dansyl chloride, 25 mM $NaHCO_3$, pH 9.2, at 37°C and hydrolyzed by 6 M HCl for 4 hr at 105°C (14, 15). Dansylated amino acids were chromatographed on 5 × 5 cm polyamide sheets (16).

Protein Determination. Protein concentrations were usually determined according to Lowry *et al.* (17). After several purification steps, amino acid analysis was used.

Edman Degradation. Sequence analysis of the carboxymethylated product after final purification was performed with a Beckman 890C sequencer by using a modified program of Hunkapiller and Hood (18) with the following changes: a single cleavage, cyclohexane in place of benzene, and no automated conversion step. Four milligrams of Polybrene [poly(*N,N,N',N'*-tetramethyl-*N*-trimethylenehexamethylene diammonium dibromide)] as recommended by Tarr *et al.* (19) was introduced into the cup as an aqueous solution and dried. The sample (*ca.* 21 μg) in 0.48 ml of 3 M acetic acid was then added, mixed with the Polybrene film, and dried. By treatment with aqueous HCl, the 2-anilino-5-thiazolinones of the amino acids were converted into the phenylthiohydantoin (>PhNCS) amino acids, which were identified by gas chromatography (20) and reverse-phase HPLC (21). The HPLC procedure (21) was modified, the main change being the column resin (a Lichrosorb Rp 8 mixture: 40% 5 μ; 60% 10 μ, Merck, Darmstadt). All >PhNCS amino acids could be resolved and quantitated except Met>PhNCS and Val>PhNCS. The detection limit was found to be about 60 pmol. Usually, 1–2 nmol of >PhNCS amino acid was applied. Details about packing of the columns, elution program, and resolution will be described elsewhere (J. A. Rodkey and C. D. Bennett).

Radioimmunoassays. SSLA was determined by radioimmunoassays employing the centrally directed SS antibody S201 (22) or the NH_2-terminally directed SS antibody S39 (8). [^{125}I-Tyr^{1}]SS and [^{125}I-Tyr^{11}]SS were prepared as described (8).

SS Bioassay. The biologic activity of purified pancreatic fractions was tested by their ability to inhibit growth hormone secretion by primary rat anterior pituitary cell cultures used 4 days after plating (23).

RESULTS

Each of the isolation procedures was successfully applied to the isolation of SS from pigeon pancreas. In procedure *A*, three to five pancreata were homogenized in liquid nitrogen and extracted by 8 M guanidine hydrochloride at pH 2.5. About 20–30 μg of SS equivalents as determined in the SS radioimmunoassay S201, was found per pigeon pancreas. The guanidine hydrochloride extract was defatted and filtered through Bio-Gel P-100 in the presence of 6 M guanidine hydrochloride. The bulk of SSLA was eluted as a small species referred to as pancreatic SS with the SSLA elution maximum at a partition coefficient K_D of 0.78. The apparent molecular weight of pancreatic SS was estimated to be in the range of 1500–2500. Only about 2% of the gel-filtered SSLA was eluted significantly earlier than pancreatic SS.

Table 1. Typical purification of pancreatic SS from five pigeon pancreata after extraction by 8 M guanidine hydrochloride (procedure *A*)

Fraction	SSLA,* μg SS	Protein, μg	Specific activity, μg SS/μg protein	Yield, %
Crude extract before centrifugation	95	743,000†	0.00013	100
Crude extract after centrifugation	101	721,000†	0.00014	107
Defatting	94	559,000†	0.00017	99
Gel filtration (Bio-Gel P-100)	53			56
Ion-exchange chromatography (DEAE-Sephadex)	36			38
Reverse-phase HPLC	15.2	15.7‡	0.97	16

* SS radioimmunoassay S201 was used (see ref. 22).
† Protein was determined according to Lowry *et al.* (17).
‡ Protein was determined by amino acid analysis.

Pancreatic SS was further purified by ion-exchange chromatography (DEAE-Sephadex) and reverse-phase HPLC. The purification was monitored by SS radioimmunoassay (antiserum S201) and protein determination. A 7500-fold purification and a specific activity of 0.97 μg of SS per μg of protein were finally achieved (Table 1). The overall yield was determined to be 15–20% (Table 1). Because guanidine hydrochloride is not lyophilizable, the volume of the initial extract limited this procedure to only a few pancreata per extraction.

For the preparation of larger amounts of pancreatic SS, another purification procedure was developed (procedure *B*). In this procedure, SSLA was extracted from 10–20 pigeons by hot 2 M acetic acid, purified by acetone precipitation, defatted, and then submitted to reverse-phase HPLC. Procedure *B* gave the same overall yield as procedure *A* (Table 2) and required only 2 days for isolation.

Table 2. Typical purification of pancreatic SS from 20 pigeon pancreata after extraction by 2 M acetic acid (procedure *B*)

Fraction	SSLA,* μg SS	Protein, μg	Specific activity, μg SS/μg protein	Yield, %
Crude extract after centrifugation	301	878,000†	0.00034	100
Acetone precipitation	141	64,090†	0.0022	47
Reverse-phase HPLC	55	59.1‡	0.93	18

* SSLA was measured by SS radioimmunoassay S39 (8, 22).
† Protein was determined according to Lowry *et al.* (17).
‡ Protein was determined by amino acid analysis.

An aliquot of the acetic acid extract after acetone treatment and defatting was filtered through Bio-Gel P-100 in the presence of 6 M guanidine hydrochloride. More than 99% of the gel-filtered SSLA appeared as small species (Fig. 1).

Additionally, a large SS-like species was found which represented 0.3 to 0.5% of the gel filtered SLA and eluted with K_D of 0.22 in the experiment presented here (Fig. 1). In other experiments, the K_D varied between 0.17 and 0.22, corresponding to an apparent molecular weight range of 11,000–12,500. This large SS-like species was also stable in 8 M urea. However, it could be almost totally (95%) dissociated into a small SS-like species, coeluting with tetradecapeptide SS in gel filtration, when pretreated with 8 M urea and 5% (wt/vol) thioglycol (10).

Pancreatic SS purified by procedure *A* (guanidine hydrochloride extraction) was eluted from μ-Bondapak columns in reverse-phase HPLC with the same retention time as hypothalamic tetradecapeptide SS (Fig. 2*A*). Under the same chromatographic conditions, closely related compounds such as [Gly^{15}]SS and tetradecapeptide SS were resolved.

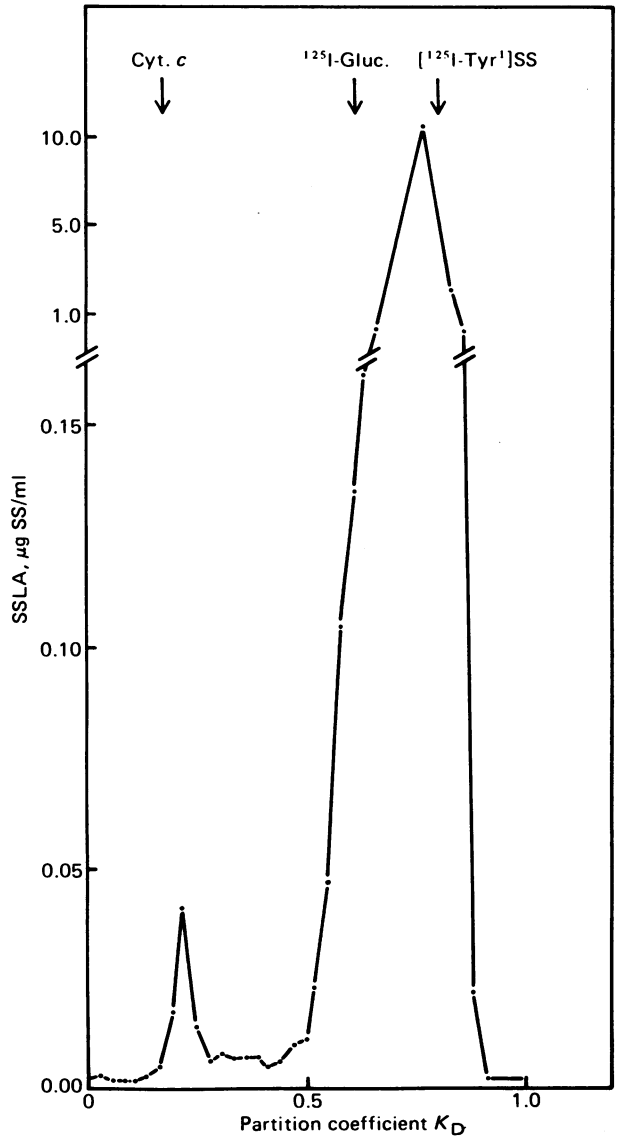

FIG. 1. Gel filtration of an extract of nine pigeon pancreata on a Bio-Gel P-100 column (2.2 × 58 cm). The gel was eluted by 6 M guanidine hydrochloride/1 M ammonium acetate, pH 2.5, at room temperature. Arrows show the K_D values of the markers cytochrome *c*, ^{125}I-labeled-glucagon, and [^{125}I-Tyr^1]SS (internal standard). SSLA was measured by radioimmunoassay S201.

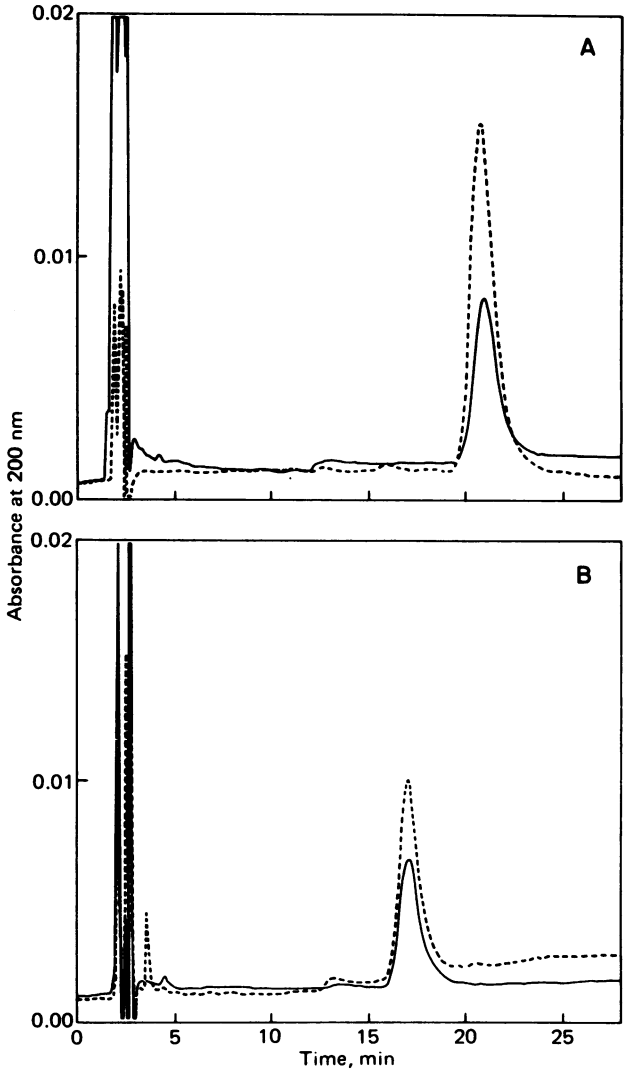

FIG. 2. Reverse-phase HPLC of synthetic hypothalamic and isolated pancreatic SS on μ-Bondapak C_{18} (0.39 × 30 cm). The column was isocratically eluted by 75.4% (vol/vol) TEAP buffer, 24.6% (vol/vol) acetonitrile [1.5 ml/min, 900 pounds/inch^2 (6.2 MPa), room temperature]. (*A*) One microgram of synthetic hypothalamic SS (---) and 0.5 μg of pancreatic SS (——) were applied in separate runs. (*B*) Nine-tenths microgram of synthetic Cm-SS (---) and 0.3 μg of pancreatic Cm-SS (——) were applied in separate runs. Carboxymethylation was performed at pH 8.7.

Pancreatic SS (from procedure *A*) was hydrolyzed for 24 and 48 hr by 4 M methanesulfonic acid/0.2% tryptamine (13) and analyzed for amino acids. The amino acid concentrations, as determined in the amino acid analyzer, were normalized to alanine and extrapolated to starting time of hydrolysis. The concentrations of almost all amino acids closely approximated integer values after normalization (Table 3), indicating that the hydrolyzed fraction probably contained only one compound. The amino acid composition of pancreatic SS did not deviate from the composition of hypothalamic SS. Only the concentrations of phenylalanine and cysteine deviated by 3% and 5%, respectively (Table 3).

Pancreatic SS purified by procedure *B* behaved as did the product isolated by procedure *A* in reverse-phase HPLC and amino acid analysis. It therefore was concluded that both purification procedures yielded the same product.

Table 3. Amino acid composition of pancreatic SS

Amino acid	mol amino acid/mol Ala: Hypothalamic SS*	Pancreatic SS†	Pancreatic Cm-SS†
Asx	1	1.0	1.1
Thr	2	2.0	2.0
Ser	1	1.0	0.9
Glx	0	0.0	0.0
Pro	0	0.0	0.0
Gly	1	1.0	1.0
Ala	1	1.0	1.0
Cys	2	1.9	0.0
Cm-Cys	0	0.0	2.0
Val	0	0.0	0.0
Ile	0	0.0	0.0
Leu	0	0.0	0.0
Tyr	0	0.0	0.0
Phe	3	3.1	3.1
Lys	2	2.0	2.0
His	0	0.0	0.0
Trp	1	1.0	1.0

* See ref. 2.

† Samples were hydrolyzed for 24 and 48 hr at 110°C with 4 M methanesulfonic acid/0.2% tryptamine. Amino acid concentrations (mol/mol Ala) were extrapolated to starting time of hydrolysis. Three analyses were performed for each hydrolysis time. Standard deviations were between ±0.02 and ±0.04 mol/mol Ala.

‡ Carboxymethylated SS samples were hydrolyzed for 24 hr at 110°C with 4 M methanesulfonic acid/0.2% tryptamine. Three analyses were performed. Standard deviations were between ±0.02 and ±0.04 mol/mol Ala.

For further characterization, pancreatic and synthetic hypothalamic SS were reduced under alkaline conditions and carboxymethylated with iodoacetate at pH 8.7 or 6.0. The reaction could easily be followed by reverse-phase HPLC, because hypothalamic and pancreatic SS and their reduction and carboxymethylation products were resolved on μ-Bondapak C_{18} columns, eluted by TEAP or TEAF buffers.

At pH 8.7, hypothalamic and pancreatic SS were almost quantitatively (>95%) carboxymethylated to products that were eluted from μ-Bondapak C_{18} columns with the same retention time but earlier than tetradecapeptide SS (Fig. 2*B*).

When carboxymethylated pancreatic SS was hydrolyzed by the methanesulfonic acid method and analyzed for amino acids, 2.0 mol of Cm-cysteine per mol of alanine was found (Table 3). No cystine or cysteine was detectable. The other amino acid concentrations deviated after normalization to alanine by maximally 10% from the amino acid composition of hypothalamic SS (Table 3). Similar results were found for the carboxymethylation product of synthetic hypothalamic SS. These results suggest that both cysteine residues of pancreatic SS were carboxymethylated at pH 8.7 and that no other amino acid was affected.

Carboxymethylation at pH 6.0 resulted in products that again were eluted with the same retention time but later than tetradecapeptide SS in reverse-phase HPLC under the conditions described above. The yield was 60–70%. These carboxymethylation products contained 1 mol of Cm-cysteine per mol of alanine, according to amino acid analysis, performed after hydrolysis with the methanesulfonic acid method (13). Probably only one cysteine residue of hypothalamic or pancreatic SS had reacted under these carboxymethylation conditions.

Endgroup determination of purified pancreatic SS was performed with the dansyl technique. As for hypothalamic SS, alanine was found as the only NH_2 terminus.

When the immunoreactivity of pancreatic SS was determined in radioimmunoassays using the NH_2-terminally directed SS antibody S39 or the centrally directed SS antibody S201, an S201-to-S39 activity ratio of 0.98 ± 0.12 (SD) was found. In contrast to hypothalamic and pancreatic SS, few SS analogs have shown the same immunologic activity versus both antibodies (22).

Purified pancreatic SS inhibited growth hormone secretion from cultured rat anterior pituitary cells (Fig. 3). The biologic potency of the pancreatic SS referred to synthetic hypothalamic SS was calculated to be 114% with the 95% fiducial limits of 66% and 192% (Harvard University Bioassay Computer Program). According to these results, there is no significant difference between the biologic potencies of hypothalamic and pancreatic SS.

Carboxymethylated pancreatic SS (21 μg, purified by procedure *B*) that was at least 95% pure on the basis of HPLC and amino acid analysis, was submitted to Edman degradation in an automated spinning cup sequencer. In the presence of 4 mg of Polybrene, an average repetitive yield of 93% was found (Table 4). On the basis of the sequence analysis (Table 4), pancreatic SS was a polypeptide with the same primary structure as hypothalamic SS: H-Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys-OH.

Whether pancreatic SS contained a disulfide bridge, like hypothalamic SS (2), could not be determined from the sequence analysis data alone (Table 4). But because isolated pancreatic SS behaved like synthetic hypothalamic cyclic SS in HPLC (Fig. 2*A*) under conditions that resolve linear and cyclic SS, it was concluded that primary and secondary structure of the isolated pancreatic and hypothalamic somatostatin were the same, suggesting the structure H-Ala-Gly-cyclo-(Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys)-OH for the isolated polypeptide. The sequence analysis data showed, besides the structure of tetradecapeptide SS, the structure of des-Ala^1-SS as a contaminant representing about 4% of SS.

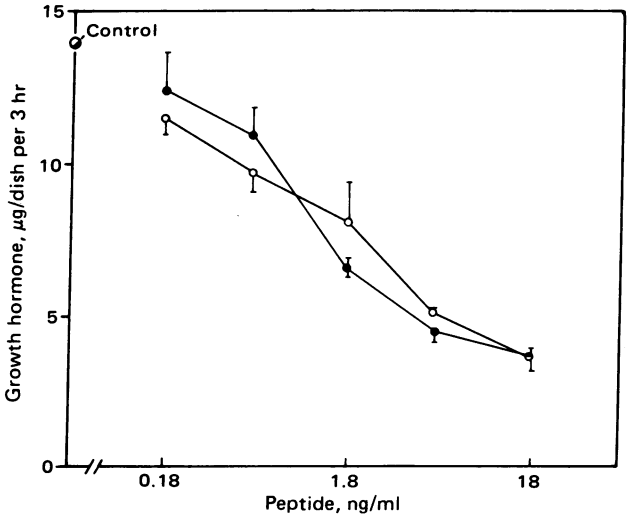

FIG. 3. Inhibition of growth hormone release from cultured anterior pituitary cells (rat) by synthetic hypothalamic (○) and pancreatic (●) SS. During the test period, primary anterior pituitary cell cultures (4 days after plating; 5.12×10^5 cells per dish) were incubated at 37°C for 3 hr in 1.5 ml of Hepes-modified Dulbecco's modified Eagle's medium containing 2% fetal calf serum and 0.4 mM 3-isobutyl-1-methylxanthine to stimulate release of growth hormone. Additions of pancreatic SS are noted as SS equivalents determined by SS radioimmunoassay. Each point on the dose–response curves represents three dishes. Deviations are given as SEM.

Table 4. Sequence analysis of carboxymethylated pancreatic SS

Cycle	>PhNCS residue	Yield,* nmol
1	Ala	11.0
2	Gly	8.8
3	Cm-Cys	5.2
4	Lys	7.2
5	Asn	9.2
6	Phe	7.7
7	Phe	7.6
8	Trp	5.1
9	Lys	5.9
10	Thr	6.1†
11	Phe	5.9
12	Thr	5.2†
13	Ser	4.6‡
14	Cm-Cys	2.3

* The >PhNCS amino acids were quantitated by HPLC.
† Thr>PhNCS was determined and quantitated as dehydro-Thr>PhNCS by gas chromatography.
‡ Ser>PhNCS was quantitated by gas chromatography.

DISCUSSION

The experimental data presented here demonstrate that most of the pancreatic SSLA is due to a small species with an apparent molecular weight in the range of 1500–2500. This finding is in agreement with a report of Dupont and Alvarado-Urbina (24), who found that a large SS-like species from rat pancreas could be converted into a small species coeluting with tetradecapeptide SS in gel filtration, by treatment with urea independent of the presence of the reducing agent thioglycol.

In contrast to Dupont and Alvarado-Urbina (24) and in agreement with earlier observations in our laboratory (9), we found a large SS-like species in pancreatic extracts that was stable in 8 M guanidine hydrochloride or 8 M urea but could be almost totally dissociated into a small species of SS size when pretreated with urea and thioglycol. These results, which will be discussed in more detail elsewhere (J. Spiess and W. Vale), indicate that most of the SS component of the large pancreatic SS-like species is not part of a single chain polypeptide.

Our main concern during the purification of pancreatic SS-like species was the high proteolytic activity of pancreas, which could cause partial digestion of the peptides to be isolated. We have approached this problem by using specific and nonspecific methods of protease inhibition. Soya bean trypsin inhibitor, bacitracin, and pepstatin A were used as specific inhibitors. Especially, application of guanidine hydrochloride was considered to be an efficient nonspecific method to inactivate proteases (25). From the observation that the same SS-like species was isolated by two different purification procedures, it is suggested that the isolated compound is not a digestion product formed during purification.

The biologic significance of the observation that one of the isolated fractions of pancreatic SS contained a small quantity of des-Ala^1-SS is not clearly understood. Whether this tridecapeptide occurs naturally or is only generated during extraction and isolation has not been determined. Because SS analogs with modifications (including deletions) of the first two amino acids have high potency (26), des-Ala^1-SS may be of physiologic significance.

Characterization of pancreatic SS has revealed that hypothalamic and pancreatic SS do not differ in biologic potency, immunologic activity in two different radioimmunoassays, NH_2 terminus, amino acid composition, HPLC before and after two chemical modifications, and primary and secondary structure. These results demonstrate that the main SS-like polypeptide isolated from pigeon pancreas is identical to ovine and porcine hypothalamic SS. Thus, the identical tetradecapeptide sequence is found not only outside the central nervous system, but in different vertebrate orders as well. This observation supports the concept that somatostatin has regulatory roles in various organs and species.

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