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Presence of somatostatin-28-(1–12) in hypothalamus and pancreas

(radioimmunoassay/reverse-phase high-performance liquid chromatography/amino acid composition/brain peptide/prosomatostatin)

ROBERT BENOIT, PETER BÖHLEN, NICHOLAS LING, AARON BRISKIN, FRED ESCH, PAUL BRAZEAU, SHAO-YAO YING, AND ROGER GUILLEMIN

Laboratories for Neuroendocrinology, Salk Institute for Biological Studies, 10010 North Torrey Pines Road, La Jolla, California 92037

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ABSTRACT **Acid extracts from rat pancreas and hypothalamus were analyzed for the presence of the antigenic determinant corresponding to the NH_2 terminus of somatostatin-28 (SS28), using an antiserum directed against amino acids 1 to ≤11 of the SS28 molecule. On gel permeation chromatography the majority of the immunoreactive material from each tissue extract eluted in one zone compatible with a peptide of 1250 daltons. Purification of this immunoreactive material by reverse-phase HPLC and cation-exchange chromatography yielded two immunoreactive peptides from each tissue extract. The amino acid compositions of both peptides in pancreas and hypothalamus correspond to the fragment 1–12 of SS28. The more hydrophobic peptide from each tissue coeluted with synthetic SS28-(1–12) on HPLC, while the other one coeluted with synthetic [Met(O)^8]SS28-(1–12). Neither native peptide coeluted with synthetic SS28-(1–12)-amide. We conclude that the prosomatostatin fragment Ser-Ala-Asn-Ser-Asn-Pro-Ala-Met-Ala-Pro-Arg-Glu-OH is present in both rat hypothalamus and rat pancreas.**

Somatostatin was characterized in extracts of mammalian tissues as a tetradecapeptide, somatostatin-14 (SS14) (1–4, *). An NH_2-terminally extended form, somatostatin-28 (SS28), was characterized in extracts of porcine intestine (5) and later in ovine (6, 7) and porcine (8) hypothalami. In these various tissues and species, the same octacosapeptide structure Ser-Ala-Asn-Ser-Asn-Pro-Ala-Met-Ala-Pro-Arg-Glu-Arg-Lys-Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys was found. Results from recent work on murine thyroid (9) and hypothalamus (10) added further evidence for the structural invariability of the octacosapeptide sequence in different mammalian species.

The SS28 molecule contains a pair of basic residues adjacent to the SS14 sequence and now recognized as a potential cleavage site for the posttranslational processing of prohormones (11). We hypothesized that, if SS14 originated from enzymatic cleavage of SS28, then the remaining NH_2 terminus of SS28 could also be present in tissue where SS14 is found. In order to verify this hypothesis, we raised an antiserum directed against the NH_2-terminal region of SS28 and analyzed tissue extracts for SS28-NH_2-terminus-like immunoreactivity (SS28-Nt-LI). The present work describes the isolation and characterization of SS28-(1–12) in both rat hypothalamus and rat pancreas.

MATERIALS AND METHODS

Starting Material. (*i*) For pilot studies, fresh pancreata or hypothalami from adult rats were used (Fig. 2). (*ii*) For peptide isolation, the starting material of hypothalamic origin was a side fraction obtained during the purification of rat hypothalamic SS28 (10). Briefly, hypothalami were extracted with 2 M acetic acid containing pepstatin and phenylmethylsulfonyl fluoride (10 mg/liter each). After centrifugation, the supernatant was defatted with petroleum ether and passed through a Sepharose-4B anti-SS14 immunoaffinity column to remove all material with SS14-like immunoreactivity. Aliquots corresponding to 3000 or 12,500 hypothalami equivalent of the material that did not bind to the affinity column were selected for the present work. The starting material of pancreatic origin was a similarly prepared side fraction of an acid extract from 439 rat pancreata originally used for the characterization of pancreatic SS14 (3).

Synthetic Peptides. SS14, SS28-Tyr, SS28-(1–12), and SS28-(1–12)-NH_2 were synthesized by a solid-phase technique, described previously for SS28 (12). [Met(O)^8]SS28-(1–12) was prepared by oxidizing 10 nmol of synthetic SS28-(1–12) with a solution containing 100 nmol of chloramine-T in 30 μl of 50 mM sodium phosphate (pH 7.4) for 30 sec at room temperature. The resulting [Met(O)^8]SS28-(1–12) was purified by HPLC.

Radioimmunoassay (RIA). Two 3-month-old male New Zealand White rabbits were injected subcutaneously at five dorsolateral sites with 2 ml of a suspension of 6×10^{10} killed *Bordetella pertussis* (Institut Armand-Frappier, Laval, PQ, Canada). Two days later, the emulsion for primary injection was prepared, using 8 mg of synthetic SS28 dissolved in 0.4 ml of sterile NaCl solution (150 mM) in a polypropylene tube kept on ice. Methylated bovine serum albumin (2 mg in 2 ml of distilled water; Sigma) was added to the peptide solution, which was then swirled on a Vortex mixer for 10 sec. Complete Freund's adjuvant containing killed *Mycobacterium butyricum* (2.5 ml, Difco) and 20 mg of killed *Mycobacterium tuberculosis* (Cuti-BCG, Institut Armand-Frappier) were then added. The mixture was emulsified with a Polytron homogenizer (Brinkmann) while being kept in an ice bath. Each rabbit received approximately 100 intradermal injections of this emulsion. Six weeks later the animals received a booster injection, which was repeated four times every 2 weeks. For each boosting, the same preparation as for the primary injection was used except that no mycobacteria were given and one-third of the primary injection dose in terms of peptide and methylated bovine serum albumin was administered. The plasma obtained 12 days after the last boost from rabbit S298 was selected for RIA.

Five micrograms of SS28-Tyr was iodinated with 1.2 mCi (1 Ci = 3.7×10^{10} becquerels) of $Na^{125}I$ (Amersham) by using a modification of the chloramine-T procedure of Greenwood *et al.* (13) (6 μg of chloramine-T, 38-sec reaction time before addition of 19 μg of sodium metabisulfite followed by 80 μl of a

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Abbreviations: SS14, somatostatin-14 (a tetradecapeptide); SS28, somatostatin-28 (an octacosapeptide); SS28-Nt-LI, SS28-NH_2-terminus-like immunoreactivity; Met(O), methionine sulfoxide; RIA, radioimmunoassay.

* Rivier, J., Spiess, J., Rivier, C., Branton, D., Millar, R. & Vale, W., Seventh American Peptide Symposium, Madison, WI, June 14–19, 1981, abstr. S5.

10% human serum albumin solution in 50 mM sodium phosphate, pH 7.4). The tracer was purified at 4°C by cation-exchange chromatography on a CM-32 CM-cellulose column (0.5 × 15 cm; Whatman) at a flow rate of 10 ml/hr by eluting first with 6 ml of 0.02 M ammonium acetate (pH 4.6) and then with 0.9 M ammonium acetate (pH 4.6). The fraction corresponding to the second peak of radioactivity (4×10^8 cpm/ml) was chosen as tracer.

The incubation buffer for RIA was 10 mM sodium phosphate (pH 7.2) containing human serum albumin at 1 mg/ml, 150 mM NaCl, 25 mM EDTA, and 0.2 M sodium acetate. The tracer (10,000 cpm in 50 μl of buffer) was added 8 hr after the first antibody (250 μl). Assay tubes were incubated at 4°C. After 24 hr of incubation, goat anti-rabbit plasma (60 μl) was added, together with 4% normal rabbit plasma (50 μl), for precipitation. Eight hours later the tubes were centrifuged for 30 min at 4°C at 2000 × *g* and the radioactivity of the precipitate was measured in a Micromedic 4/600 gamma counter.

Aliquots of column fractions (from HPLC or CM-cellulose) used for RIA were dried in a Speed-Vac concentrator (Savant) after adding 100 μg of human serum albumin (Pierce).

Peptide Isolation. *Reverse-phase peptide extraction.* The starting material corresponding to the fraction not retarded on the anti-SS14 immunoaffinity column was adjusted to pH 2.5 with acetic acid and then pumped through a column (7.5 × 25 cm) filled with octadecylsilylsilica (ODS, LRP-2, 37- to 53-μm particle size, Whatman) previously equilibrated in 0.2 M acetic acid. Peptides that bound under such conditions were then eluted with a solution of 0.36 M pyridine adjusted to pH 3 with formic acid and containing 60% (vol/vol) 1-propanol, as described (14, 15).

HPLC. A microprocessor-controlled model 332 Altex liquid chromatograph, equipped with an automatic stream-sampling fluorescamine detection system (16), was used in conjunction with C_8- or C_{18}-Ultrasphere reverse-phase semipreparative or analytical columns (5-μm particle size, Altex, Berkeley, CA). The mobile phases used with this system consisted of 0.36 M pyridine formate (pH 3) in a 1-propanol gradient, 0.5% or 0.1% trifluoroacetic acid in an acetonitrile gradient, or 0.5% heptafluorobutyric acid in an acetonitrile gradient. These mobile phases were adaptations of those introduced by Rubinstein *et al.* (17) and Bennett *et al.* (18).

Cation-exchange chromatography. A CM-32 column (7.5 × 0.6 cm, bed volume 2 ml) was coated with 1 mg of human serum albumin (Pierce) washed with 1 M ammonium acetate (pH 6.5), and equilibrated with 0.01 M ammonium acetate to pH 4.4. The sample was loaded in 200 μl of 0.01 M ammonium acetate (pH 4.4). The column was then washed with 250 μl of the same buffer and eluted with a 110-ml linear gradient from 0.01 M ammonium acetate (pH 4.4) to 0.2 M ammonium acetate (pH 6.5), at a flow rate of 6 ml/hr.

Amino Acid Analysis. Peptides (25–50 pmol) were hydrolyzed in sealed evacuated tubes containing 6 M HCl and 2% thioglycollic acid for 24 hr at 110°C (19). To quantitate cysteine, separate peptide aliquots were oxidized with performic acid (20) and then hydrolyzed in 6 M HCl. Amino acid analyses of peptide hydrolysates were performed by using a Liquimat III amino acid analyzer (Kontron, Zürich, Switzerland) equipped with an *o*-phthalaldehyde fluorescence detection system and a proline conversion accessory (21).

RESULTS

At a final dilution of 1:5000, the immune plasma S298 bound 30% of the ^{125}I-labeled SS28-Tyr (SS28-^{125}I-Tyr). In the radioimmunoassay, the lowest detectable amount of SS28 was 35 pg. Specificity studies (Fig. 1) showed that S298 did not read SS14 but crossreacted minimally with SS28-(4–28) (<1:2000). However, it read equimolar amounts of SS28-(1–12), [Met(O)^8]SS28-(1–11), and SS28. Thus we can conclude that the antigenic determinant recognized by the immune plasma is contained in the SS28-(1–11) segment.

Hypothalamic SS28-(1–12). Sephadex G-50 chromatography of fresh rat hypothalamic extract revealed three zones of immunoreactivity (Fig. 2*A*). The first two zones were compatible with peptides having molecular weights corresponding to that of a somatostatin precursor of ca. 12,000 daltons (23) and with SS28. The third and major peak eluted in an area corresponding to a 1200- to 1300-dalton peptide. Initial purification of this third component was carried out with an acid extract of 3000 rat hypothalami by reverse-phase peptide extraction (yield: 52 mg of crude peptide fraction). Peptide isolation was then achieved after several steps on semipreparative HPLC (Fig. 3*A*) in the pyridine formate/1-propanol system (yield 14 μg of SS28-Nt-LI) and analytical HPLC using the trifluoroacetic acid/acetonitrile mobile phase (Fig. 3*B*). Two peaks of immunoreactive material containing 8.1 and 6.1 μg of SS28-Nt-LI were eluted.

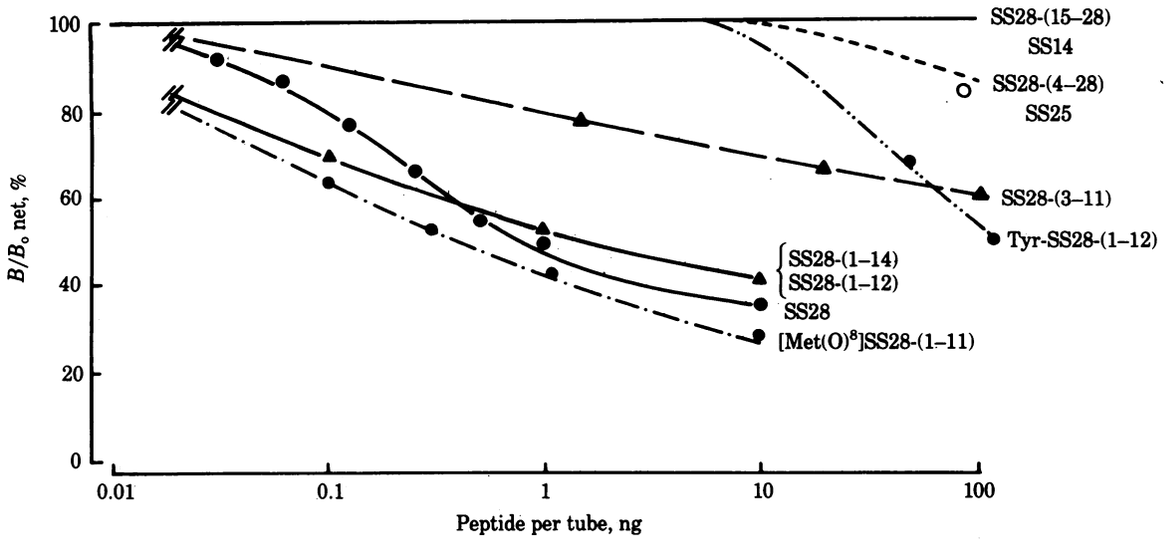

FIG. 1. Crossreactivity studies with S298-11/9/80 immune plasma at a final dilution of 1:5000. The curves represent the displacement of the SS28-^{125}I-Tyr tracer bound to the antibody by SS28 or related peptides. *B*, Tracer bound; B_o, tracer bound in the absence of competitor.

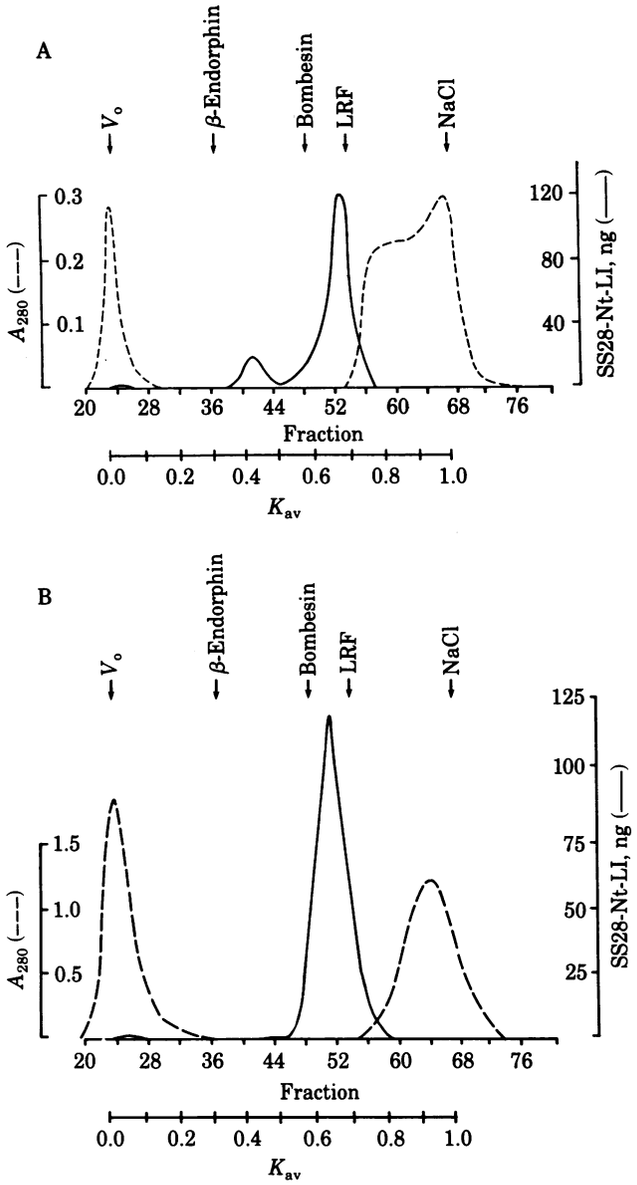

FIG. 2. Gel permeation chromatography (Sephadex G-50 fine) of an acid extract of: (*A*) four rat hypothalami (130 mg wet weight) and (*B*) one rat pancreas (1.3 g wet weight). Fresh tissues had been extracted with a solvent containing 1% (vol/vol) trifluoroacetic acid, 1 M HCl, 1% (wt/vol) NaCl, and 5% (vol/vol) formic acid as described by Bennett *et al.* (22). The tissue extracts were then defatted with petroleum ether and after lyophilization the dry material was dissolved in 4 ml of 30% (vol/vol) acetic acid and loaded onto the Sephadex column (1.6 × 101 cm). Void volume $V_o = V_{albumin} = 76$ ml. $V_{salt} = 195$ ml. Elution with 30% acetic acid; flow rate, 9 ml/hr; fraction size, 3 ml. The column was calibrated with human β-[Leu^5]endorphin (M_r 3400), bombesin (M_r 1618), and luteinizing hormone-releasing factor (LRF; M_r 1181), as indicated by arrows. $K_{av} = (V_x - V_o)/(V_{salt} - V_o)$.

Finally, 1.8 μg of pure peptide was isolated from peak II after two additional partitions in the same system, first using a shallower gradient (14–17% acetonitrile in 75 min, not shown), and then isocratic conditions (Fig. 4). The peptide from peak I of Fig. 3*B* could not be purified to homogeneity due to losses encountered during final isolation steps. More starting material originating from 12,500 hypothalami was subjected to reverse-phase peptide extraction and semipreparative HPLC. At the second HPLC step (conditions as in Fig. 3*B*), 48 μg of SS28-Nt-LI material was recovered in peak I. This peak was rechromatographed twice in the trifluoroacetic acid/acetonitrile system (yield: 5.4 μg of SS28-Nt-LI) and because of cochromatography of impurities, was subjected to cation-exchange chromatography. When the immunoreactive fractions from CM-32 chromatography were pooled and rechromatographed by reverse-phase HPLC in isocratic conditions with 0.5% trifluoroacetic acid/13.8% acetonitrile, 5 μg of pure immunoreactive material was obtained (chromatogram not shown).

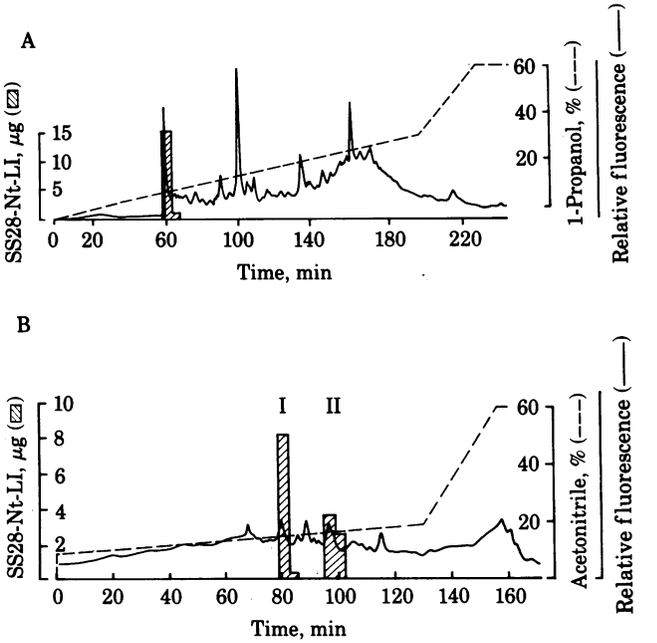

FIG. 3. Reverse-phase HPLC of 52 mg of peptide extract from 3000 rat hypothalami on a C_{18} column (25 × 1 cm) with pyridine formate/1-propanol as the mobile phase. Flow rate, 48 ml/hr; fraction size, 3 ml. (*B*) HPLC purification of the immunoreactive reaction from the HPLC in *A* on a C_8 column (4.6 mm × 25 cm). The mobile phase was 0.5% trifluoroacetic acid with acetonitrile. Flow rate, 0.6 ml/min; fraction size, 2.2 ml.

Amino acid compositions of the two hypothalamic peptides originating from peaks I and II are shown in Table 1. The composition of both peptides was identical in both cases to that of SS28-(1–12). Furthermore, retention times in an isocratic reverse-phase HPLC system using 0.5% heptafluorobutyric acid/22% acetonitrile showed that synthetic SS28-(1–12)-OH co-

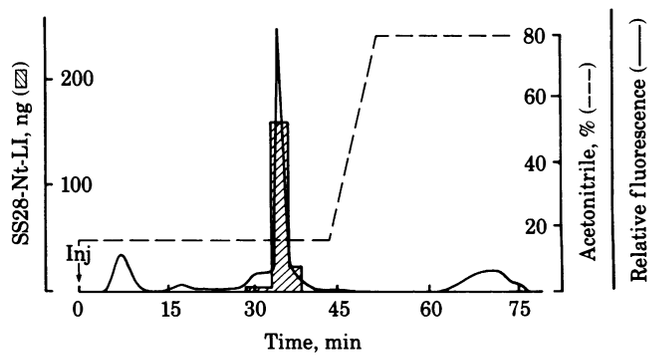

FIG. 4. The SS28-Nt-LI material from peak II of Fig. 3*B* was further purified on a C_8 HPLC column using 0.5% trifluoroacetic acid with an acetonitrile gradient of 14–17% in 75 min (data not shown). The SS28-Nt-LI material eluted from that column was refractionated on the same HPLC system, using 0.5% trifluoroacetic acid under isocratic conditions at 15% (vol/vol) acetonitrile. Flow rate, 0.6 ml/min; fraction size, 2.2 ml. Inj, injection.

Table 1. Amino acid compositions of the two SS28-Nt-LI peptides isolated from rat hypothalamus and pancreas and comparison to SS28-(1–12)

Amino acid residue	Hypothalamus		Pancreas		
	Peptide I (n = 4)	Peptide II (n = 3)	Peptide I (n = 2)	Peptide II (n = 2)	SS28-(1–12)
Asx	1.88 ± 0.19	1.79 ± 0.09	1.96 ± 0.07	1.87 ± 0.17	2
Thr	0	0.05 ± 0.08	0	0	0
Ser	1.82 ± 0.07	1.57 ± 0.06	1.83 ± 0.11	1.63 ± 0.04	2
Glx	1.14 ± 0.08	1.24 ± 0.19	1.24 ± 0.03	1.00 ± 0.03	1
Pro	2.03 ± 0.07	2.23 ± 0.34	2.20	2.19 ± 0.12	2
Gly	0.20 ± 0.08	0.05 ± 0.06	0.23 ± 0.02	0	0
Ala	3.05 ± 0.14	2.88 ± 0.33	2.92 ± 0.05	3.02 ± 0.10	3
Cya	0	0	0	0	0
Val	0	0	0	0	0
Met	0.93 ± 0.13	1.08 ± 0.10	0.92 ± 0.06	1.11 ± 0.04	1
Ile	0	0.04 ± 0.04	0	0	0
Leu	0	0.07 ± 0.06	0	0	0
Tyr	0	0	0	0.05 ± 0.05	0
Phe	0	0	0	0.06 ± 0.05	0
His	0	0.02 ± 0.03	0	0	0
Lys	0	0	0	0	0
Trp	0	0	0	0	0
Arg	1.16 ± 0.15	1.21 ± 0.16	1.01 ± 0.02	1.25 ± 0.22	1

All experimental values are mol/mol of peptide and are corrected for blank contamination. Thr and Ser values are uncorrected for hydrolysis loss. Values are means ± SD from two to four analyses. Cya, cysteic acid.

eluted with the hypothalamic peptide isolated from peak II and synthetic [Met(O)^8]SS28-(1–12)-OH coeluted with the hypothalamic peptide isolated from peak I. Neither natural peptide coeluted with synthetic SS28-(1–12)-NH_2.

Pancreatic SS28-(1–12). Sephadex G-50 chromatography of fresh rat pancreatic extract revealed three zones of immunoreactivity (Fig. 2*B*). The first two zones, accounting for 1.4% of the total SS28-Nt-LI eluted from the gel filtration column, were compatible with peptides of molecular weights corresponding to those of prosomatostatin (ca. 12,000) and SS28. The third peak, accounting for 98.6% of the SS28-Nt-LI, corresponded to a peptide of molecular weight 1200–1400.

The starting material used for purification of this third component consisted of an acid extract from 439 rat pancreata that did not bind to an anti-SS14 immunoaffinity column. This unbound material was then submitted to reverse-phase peptide extraction, Sephadex G-50 fine gel permeation chromatography, and two HPLC steps.

Two peaks of immunoreactive material were obtained, containing 9.7 μg (peak I) and 4.6 μg (peak II) of SS28-Nt-LI (Fig. 5). Both peaks were further purified by CM-cellulose chromatography and various HPLC systems with trifluoroacetic acid/acetonitrile. Five hundred nanograms of pure immunoreactive material from peak I was finally obtained by using 0.5% heptafluorobutyric acid/22% acetonitrile (vol/vol) run isocratically (Fig. 6), and 4 μg of pure material was obtained from peak II with the same heptafluorobutyric acid/acetonitrile system. The two pancreatic peptides from peaks I and II had the same amino acid compositions as the two hypothalamic peptides (Table 1). Both corresponded to the composition of SS28-(1–12). As had been observed for the peptides isolated from the hypothalamus, isocratic HPLC performed with the heptafluorobutyric acid/acetonitrile system showed that pancreatic peptides I and II coeluted with synthetic [Met(O)^8]SS28-(1–12)-OH and synthetic SS28-(1–12)-OH, respectively.

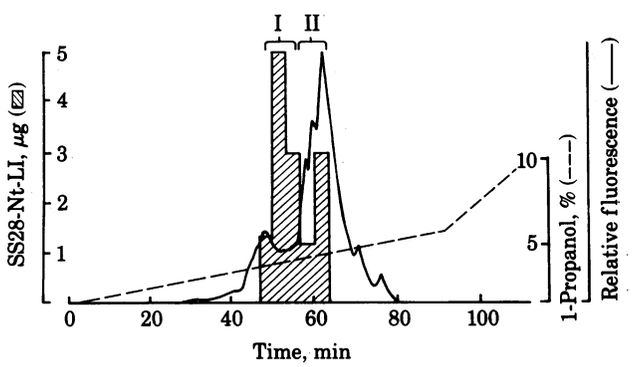

FIG. 5. Reverse-phase HPLC of 15 mg of peptide extract from 439 rat pancreata on a C_{18} column (25 × 1 cm) with pyridine formate/1-propanol as the mobile phase. Flow rate, 36 ml/hr; fraction size, 3 ml.

DISCUSSION

No primary structure was established for these peptides due to the small amount of material available, but on the basis of the

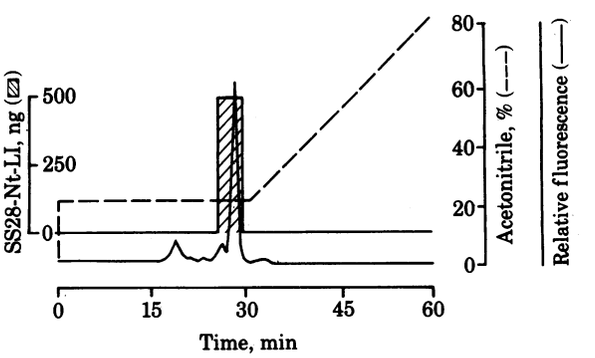

FIG. 6. Final HPLC chromatogram on C_{18} column (4.6 mm × 25 cm), yielding pure pancreatic SS28-Nt-LI peptide. The starting material was obtained from peak I of Fig. 5 after further HPLC and CM-cellulose purification steps. Mobile phase, 0.5% heptafluorobutyric acid/22% (vol/vol) acetonitrile; flow rate, 0.6 ml/min; fraction size, 1.8 ml.

above data we conclude that both rat pancreas and hypothalamus contain the dodecapeptide SS28-(1–12). In both tissue extracts the [Met(O)^8]SS28-(1–12) form was also observed, presumably due to oxidation during the isolation procedure.

Quantitation by RIA in fresh acid extracts after gel filtration indicates that SS28-(1–12) and SS14 are present in approximately equimolar amounts (20–40 pmol per hypothalamus; 140–170 pmol per pancreas). On the other hand, in a rat anaplastic medullary thyroid carcinoma in which SS28 was present but no SS14 could be detected, we were unable to observe SS28-(1–12) (24). These findings are consistent with the hypothesis that SS28 is the immediate biosynthetic precursor of both SS14 and SS28-(1–12).

An important issue remains as to whether SS28-(1–12) is endowed with any physiological role. On the basis of our own bioassays (effect on growth hormone, thyrotropin, and prolactin secretion *in vitro*), no biological function can be attributed to this dodecapeptide at the present time. Demonstrating that SS28-(1–12) is secreted would be interesting, although it could still represent a nonfunctional fragment cosecreted with SS14, a situation similar to the C-peptide for insulin (11). On the other hand, because different natural peptide fragments from the same precursor may play different biological roles [as is the case, for example, with the pro-opiomelanocortin-derived fragments (25)] SS28-(1–12) could possibly have a physiological role of its own. The results reported here add to the growing evidence of posttranslational processing of the precursor proteins of biologically active oligopeptides.

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