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Purification of Somatostatin from Frog Brain: Coisolation with Retinal Somatostatin-Like Immunoreactivity

Mimi Takami, *Joseph R. Reeve, Jr., †David Hawke, †John E. Shively, ‡Scott Basinger, and Tadataka Yamada

*Department of Internal Medicine, University of Michigan Medical Center, Ann Arbor, Michigan; *CURE, V. A. Wadsworth Medical Center and UCLA School of Medicine, Los Angeles; †Division of Immunology, City of Hope Research Institute, Duarte, California; and ‡Cullen Eye Institute and Program in Neuroscience, Baylor College of Medicine, Houston, Texas, U.S.A.*

Abstract: Somatostatin-like immunoreactivity (SLI) was purified from frog brain and retina, and the structure of the brain peptide was determined. Frog brain (101 g) and retinal (45 g) tissues were extracted with 3% acetic acid, yielding 9.6 and 0.44 nmol of SLI, respectively. SLI was further purified by chromatography on a somatostatin immunoaffinity column followed by sequential application to reverse-phase C-18 HPLC columns. The brain and retinal peptides, purified roughly 100,000-fold with net yields of 7.5 and 2.3%, respectively, appeared identical in the final steps of purification. The amino acid sequence of brain SLI, as determined by a gas-phase automated Edman degradation technique, was as follows: Ala-Gly - (Cys)-Lys - Asn-Phe - Phe-Trp - Lys-Thr - Phe-Thr-Ser- (Cys). Our data indicate that despite structural variations in somatostatins of other lower vertebrates, the amino acid sequence of frog brain and, by deduction, retinal SLI is identical to that of somatostatin tetradecapeptide. These findings support the physiological relevance of studies directed at elucidating the neurotransmitter function of somatostatin using the well-established models of frog brain and retina. **Key Words:** Retinal peptides—Neuropeptides—Somatotropin release-inhibiting factor—Peptide sequence—Peptide purification. **Takami M. et al.** Purification of somatostatin from frog brain: Coisolation with retinal somatostatin-like immunoreactivity. *J. Neurochem.* **45**, 1869–1874 (1985).

Somatostatin, a tetradecapeptide initially isolated from ovine hypothalamus (Brazeau et al., 1973), has been shown by immunochemical techniques to be widely distributed in neural tissues (Hokfelt et al., 1975*a,b*, 1977; Costa et al., 1977). Although evidence points to a role as a neurotransmitter or a modulator of neuronal activity, the function of somatostatin in neurons has yet to be confirmed. The retina provides some specific advantages for study of brain peptides such as somatostatin. Not only is somatostatin-like immunoreactivity (SLI) present in a great variety of vertebrate retinas (Krisch and Leonhardt, 1979; Rorstad et al., 1979; Yamada et al., 1980; Brecha et al., 1981; Marshak and Yamada, 1984), but the anatomy, physiology, and pharmacology of the retina have been studied extensively and each of the major cell types has been characterized (Kaneko, 1979). The retina is easily isolated without contamination from other tissues and can be maintained in organ culture for prolonged periods. The ability to stimulate discrete populations of cells within the retina with light or with pharmacologic reagents permits parallel electrophysiological and biochemical studies. To elucidate the physiological function of somatostatin in the retina, however, it is important first to determine the chemical structure of the peptide from the species being examined because of the possibility of structural heterogeneity, particularly in lower vertebrates (Hobart et al., 1980; Oyama et al., 1980; Magazin et al., 1982).

For the most part, retinal SLI has been identified only immunochemically, with the single exception being a 28-amino-acid somatostatin from bovine retina, which has been purified and its amino acid sequence determined (Marshak et al., 1983). In this study, we sought to determine the structure of SLI from frog retina because of its usefulness as a model

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Address correspondence and reprint requests to Dr. T. Yamada at D2101 Medical Professional Building, University of Michigan Medical Center, Ann Arbor, MI 48109, U.S.A.

Abbreviations used: S14, somatostatin tetradecapeptide; S28, somatostatin octacosapeptide; SLI, somatostatin-like immunoreactivity; TFA, trifluoroacetic acid.

for electrophysiological studies. Unfortunately, the minute amounts of SLI in frog retina made its purification in quantities sufficient for amino acid sequence determination difficult. To circumvent this problem, we purified SLI from frog retina and frog brain in parallel fashion. By elucidating the structure of the latter peptide, we could deduce the structure of the former.

MATERIALS AND METHODS

Tissue extraction

Frogs (*Rana pipiens*) kept on a cycle of 4 p.m.–6 a.m. light/6 a.m.–4 p.m. dark for 30 days were pithed and enucleated under dim red light on the day of dissection. Retinas were removed from the bisected eyecups, separated from pigmented epithelium, and frozen immediately in liquid nitrogen. Whole brains were removed from crania and frozen in a separate container. For extraction, frozen retinal and brain tissues (45 and 101 g, respectively) were placed separately in boiling water (5 ml/g of wet tissue weight) for 15 min and then homogenized in a blender for 5 min. An equal volume of 6% acetic acid was added to the homogenate to make a final concentration of 3% acetic acid (10 ml/g of tissue). After boiling for an additional 15 min, the extract was ultracentrifuged at 10°C for 35 min at 100,000 *g*. The precipitate was discarded, and the supernatant was adjusted to pH 5.0 by addition of ammonium hydroxide.

Chromatography

Small aliquots (2 ml each) of the crude extracts were applied to a Sephadex G50 superfine column (1 × 120 cm) equilibrated with 0.1 *M* ammonium acetate, pH 5.0, and eluted fractions were assayed for SLI. The remainder of the extracts was purified on an affinity column made by linking anti-somatostatin antiserum 1001 to Affi-Gel 10 by previously described methods (Marshak et al., 1983). The brain extracts were applied in batches of 100 ml each, and the entire retinal extract (500 ml) was applied in one batch. The sample flow rate through a 1.5- × 20-cm column containing 25 ml of beads was ~200 ml/h. The column was washed with 3 column volumes of 0.1 *M* ammonium acetate, pH 5.0, and eluted with 60 ml of 2% trifluoroacetic acid (TFA). The void, wash, and TFA eluate fractions from each column were assayed for SLI, and the TFA eluate was pooled, diluted with 5 volumes of water, and applied at a flow rate of 4 ml/min to a reverse-phase Z-Module C-18 HPLC column (10 μm, 3.9 mm, × 30 cm; Waters Associates, Milford, MA, U.S.A.) equilibrated in 0.1% TFA (buffer A). HPLC was performed with a Beckman Scientific (Fullerton, CA, U.S.A.) model 421 HPLC system equipped with model 100A buffer pumps and a model 165 variable wavelength detector. The columns were eluted with a stepwise gradient (0–100%) of a solution containing 50% acetonitrile in 0.1% TFA (buffer B). The eluted fractions containing SLI were pooled, diluted with 3 volumes of buffer A, and rechromatographed on the Z-Module C-18 column one or two additional times as indicated in Table 1. The eluted fractions containing SLI from the final Z-Module column were pooled, diluted with three volumes of buffer A, and chromatographed on a Vydac reverse-phase C-18 column. The peaks of A_{220} corresponding to the peaks of SLI were pooled separately and further analyzed. Recovery at each step of purification was calculated on the basis of specific immunoreactivity per unit A_{220}.

TABLE 1. *Recovery of frog brain and retinal SLI at each step of purification*

Step	SLI (nmol)	Recovery (%)		Purification (fold)
		Step	Cumulative	
Brain				
Extract	9.60	—	100	1
Affinity	6.60/3.30[a]	68.8	68.8	100
HPLC				
Z-Module 1	3.23	97.9	67.3	11,000
Z-Module 2	1.34	41.5	27.9	25,000
Z-Module 3	0.78	58.2	16.2	60,000
Vydac	0.36	46.2	7.5	110,000
Retina				
Extract	0.44	—	100	1
Affinity	0.10	22.7	22.7	200
HPLC				
Z-Module 1	0.06	60.0	13.6	9,000
Z-Module 2	0.02	33.3	4.5	66,000
Vydac	0.01	50.0	2.3	93,000

[a] Only 3.30 of the 6.60 nmol was further purified.

Amino acid sequence analysis

The amino acid sequence of the purified frog brain SLI was determined by automated Edman degradation with a gas-phase sequenator as previously described (Hawke et al., 1985). The phenylthiohydantoin derivatives of amino acids were analyzed by HPLC on an Ultrasphere ODS column (Beckman), using published methods (Hawke et al., 1982). Peaks were integrated and gradient elution was controlled with a Spectra Physics (Santa Clara, CA, U.S.A.) model 4000 integrator system.

Radioimmunoassay

SLI was detected by radioimmunoassay with antibody 1001 using a modification of a previously described technique (Yamada et al., 1980). Antibody 1001 was used at a dilution of 1:100,000, and samples were incubated for 16 h at 4°C in 1 ml of buffer containing 0.05 *M* sodium phosphate (pH 7.0), 0.08 *M* NaCl, 0.01 *M* EDTA, 0.02% sodium azide, 0.25% bovine serum albumin, and 0.1% gelatin. Separation of antibody-bound and free ^{125}I-Tyr^{1}-somatostatin was achieved with 1 ml of assay buffer to which 25 mg/ml activated charcoal and 2.5 mg/ml dextran T-70 (Pharmacia, Piscataway, NJ, U.S.A.) had been added. For the purpose of this study, synthetic somatostatin tetradecapeptide (S14; Peninsula Labs, Belmont, CA, U.S.A.) was used as the standard for radioimmunoassays. Antibody 1001 is specific for the ring structure of S14 and cross-reacts 75% with somatostatin octacosapeptide (S28). Cross-reactivity with peptides altered at the cysteine residues in the 3 and 14 positions was 6% with D-Cys^{3}-S14 and 10% with D-Cys^{14}-S14.

RESULTS

As indicated in Table 1, 9.6 nmol of SLI was extracted from roughly 100 g of brain tissue. The yield of SLI from retinal tissues was ~10-fold less than that from the brain (0.44 nmol from 45 g). Overall recovery of SLI following purification from brain

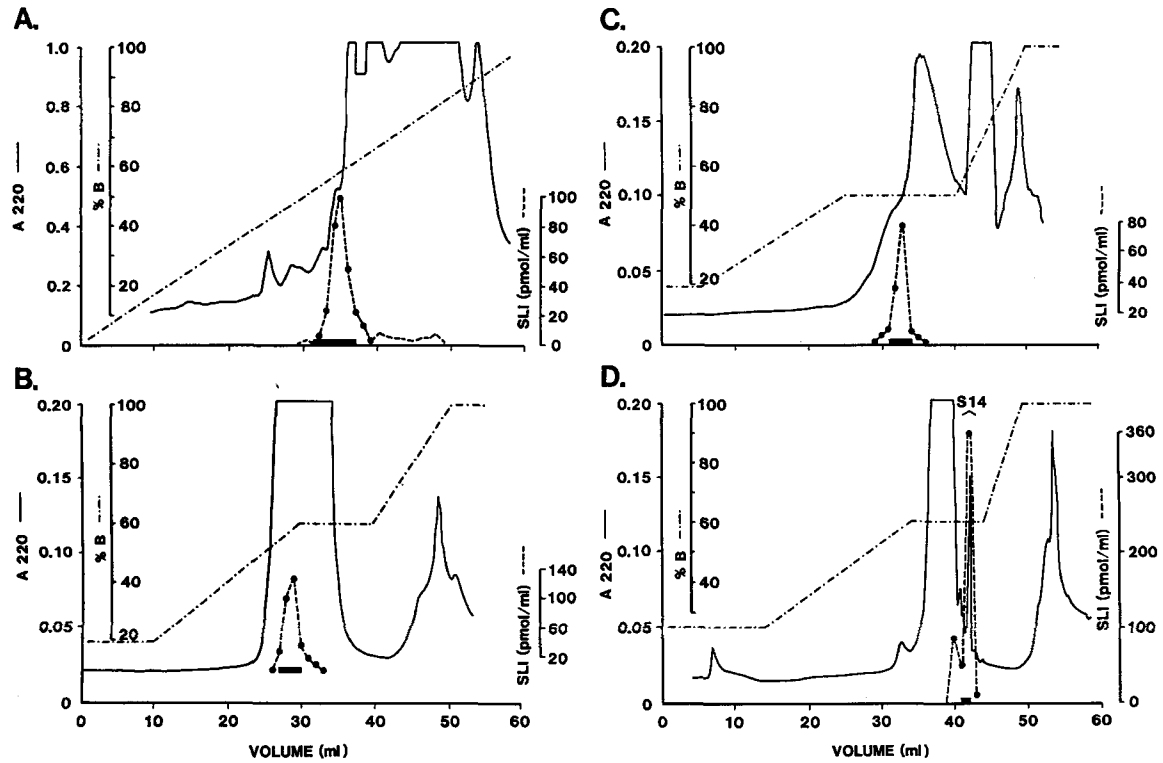

FIG. 1. Purification of frog brain SLI by HPLC. After extraction with 3% acetic acid and purification by affinity chromatography, frog brain SLI was subjected to HPLC on reverse-phase C-18 Z-Module **(A–C)** and Vydac **(D)** columns. The columns were equilibrated in 0.1% TFA and then eluted with a stepwise gradient of 50% acetonitrile in 0.1% TFA (buffer B) as indicated in each panel. A_{220} was monitored, and the SLI in each fraction was quantified by radioimmunoassay. The fractions pooled for further purification (A–C) or amino acid sequencing (D) are indicated by the black bars. The elution position of synthetic S14 is indicated in D.

(7.5%) was better than recovery from retina (2.3%) despite an extra HPLC step. At each step of purification, portions of the SLI peaks were discarded to enhance the purity of the pooled fractions; thus, the actual recovery of SLI was somewhat higher than the values listed in Table 1. The final purification factor was 110,000 for the brain extract and 93,000 for the retinal extract.

For both the brain and the retinal extracts, most of the SLI was contained in one molecular form, because no substantial peak other than the major one could be clearly segregated during the sequential purification procedures (Figs. 1 and 2). Analysis of small aliquots of crude brain and retinal extracts by chromatography on Sephadex G50 revealed that most of the SLI coeluted with S14 (Fig. 3).

At each step of purification, the major SLI peak was associated with multiple peaks of absorbance until the last HPLC step, when a single sharp A_{220} peak corresponding to the SLI peak was clearly segregated (Figs. 1 and 2). The final peak of SLI purified from both brain and retinal extracts coeluted with S14 in the isocratic portion of the elution profile at 60% buffer B.

Approximately 100 pmol of purified frog brain SLI was applied to microsequence analysis, but only 68 pmol of the amino terminal derivative was recovered in the first Edman degradation cycle. Nevertheless, a full sequence was obtained (Table 2) and was found to be identical to that of S14. The absence of derivatives in cycles 3 and 14 is consistent with the presence of half-cystines, which are not detected unless the native disulfide is chemically modified. Furthermore, the high cross-reactivity of the purified peptide with the radioimmunoassay antiserum indicates that the amino acids occupying positions 3 and 14 are likely to be identical to those in S14. Although the quantities of SLI purified from retina were insufficient for sequence analysis, the exact coelution of the purified brain and retinal peptides on the final HPLC column indicates that in all likelihood they have an identical amino acid sequence.

DISCUSSION

Although the structure of somatostatin appears to be well conserved in mammals (Reichlin, 1983), considerable variability exists in the peptide's amino acid sequence in submammalian species

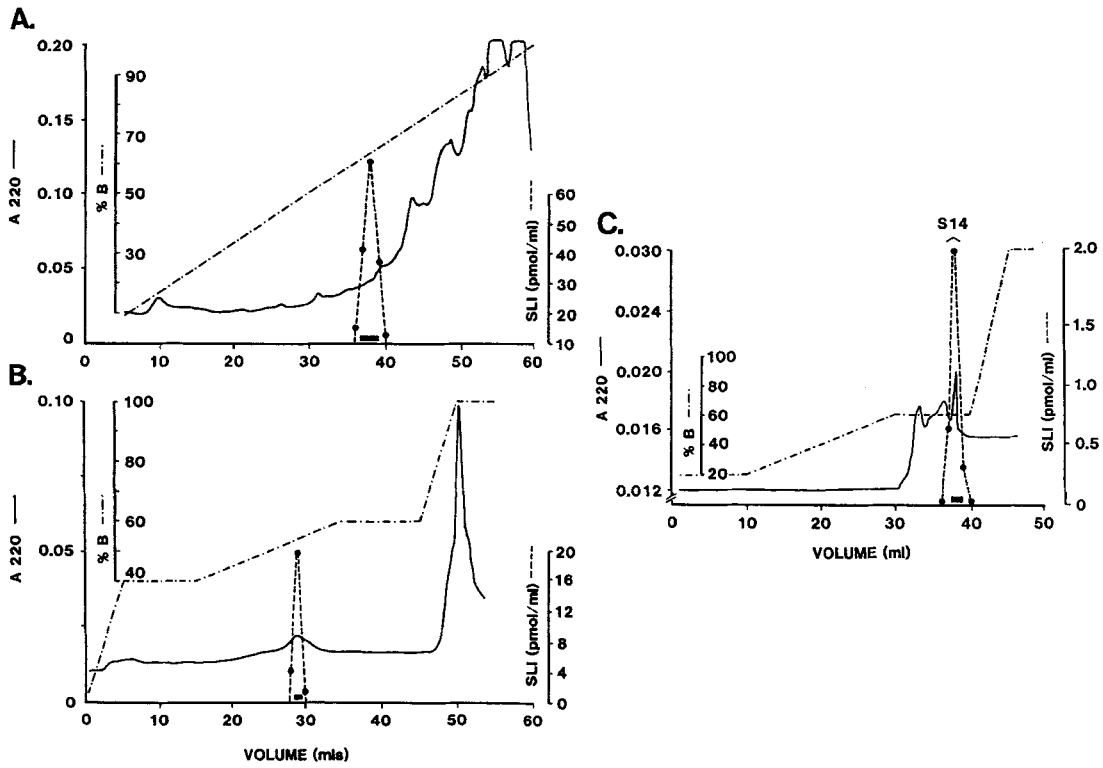

FIG. 2. Purification of frog retinal SLI by sequential HPLC on reverse-phase C-18 Z-Module columns **(A** and **B)** and a C-18 Vydac column **(C).** The columns were equilibrated and calibrated as in Fig. 1.

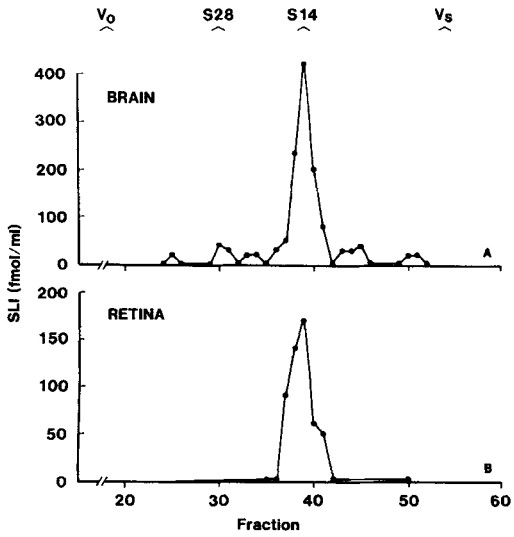

FIG. 3. Gel filtration of frog brain and retinal SLI. Brain **(A)** and retinal **(B)** extracts (3% acetic acid) were applied to a Sephadex G50 superfine column (1 × 120 cm) and eluted with 0.1 *M* ammonium acetate, pH 5.0. Eluted fractions (2.4 ml each) were measured for SLI by radioimmunoassay. The columns were calibrated by elution of bovine serum albumin (V_o), NaCl (V_s), S14, and S28.

(Table 3). Characterization of a 22-amino-acid somatostatin purified from catfish pancreas indicated that these variations could be present even within the biologically active core of S14 (Oyama et al.,

TABLE 2. *Amino acid sequence of purified frog brain SLI by automated Edman degradation*

Edman cycle no.	Amino acid	Quantity (pmol)
1	Ala	68
2	Gly	25
3	(Cys)	ND
4	Lys	60
5	Asn	22
6	Phe	22
7	Phe	22
8	Trp	11
9	Lys	21
10	Thr	[a]
11	Phe	5
12	Thr	[a]
13	Ser	1.5
14	(Cys)	ND

Cysteine is placed in parentheses because its presence was deduced and not actually confirmed (see text). ND, undetectable.

[a] Threonine was assigned on the basis of its dehydro derivative detected at 313 nm.

TABLE 3. *Amino acid sequences of S14 and somatostatin of lower vertebrate species*

Somatostatin	Relative to S14 amino terminus										
	−8	−7	−6	−5	−4	−3	−2	−1	1	2	3
S14									Ala	Gly	Cys
Frog brain									Ala	Gly	(Cys)[a]
Anglerfish I									Ala	Gly	Cys
Anglerfish II									Ala	Gly	Cys
Catfish pancreas	Asp	Asn	Thr	Val	Thr	Ser	Lys	Pro	Leu[b]	Asn[b]	Cys

Somatostatin	Relative to S14 amino terminus										
	4	5	6	7	8	9	10	11	12	13	14
S14	Lys	Asn	Phe	Phe	Trp	Lys	Thr	Phe	Thr	Ser	Cys
Frog brain	Lys	Asn	Phe	Phe	Trp	Lys	Thr	Phe	Thr	Ser	(Cys)[a]
Anglerfish I	Lys	Asn	Phe	Phe	Trp	Lys	Thr	Phe	Thr	Ser	Cys
Anglerfish II	Lys	Asn	Phe	Tyr[b]	Trp	Lys	Gly[b]	Phe	Thr	Ser	Cys
Catfish pancreas	Met[b]	Asn	Tyr[b]	Phe	Trp	Lys	Ser[b]	Arg[b]	Thr	Ala[b]	Cys

Amino acids are numbered from the amino terminus of S14.

[a] Parentheses denote that the cysteine residues in position 3 and 14 of frog brain somatostatin have yet to be confirmed.

[b] Amino acids that vary from those in the sequence of S14.

1980). Subsequent use of recombinant DNA techniques has permitted the elucidation of somatostatin structures from a variety of species. Through such techniques, the amino acid sequence of catfish pancreatic somatostatin was confirmed, although corrections were required (Magazin et al., 1982), and two different somatostatins were identified in anglerfish islets (Hobart et al., 1980). The structure of anglerfish somatostatin I is identical to that of mammalian S14, but that of anglerfish somatostatin II differs by a Tyr-Phe substitution in position 7 and a Gly-Thr substitution in position 10. These differences in the structure of somatostatin may have profound importance in the interpretation of physiological studies with synthetic S14, particularly because lower vertebrates are useful for study of the neural function of somatostatin.

Because the frog retina provides one well-developed model for neurotransmitter studies, we undertook the simultaneous purification of SLI from frog brain and retina. Recent advances in biochemical techniques for purification and structural analysis of minute quantities of peptides have permitted us to purify a peptide roughly 100,000-fold and to determine its structure even when starting with <10 nmol of material. Our studies indicate that frog brain SLI and, by deduction, retinal SLI are identical to S14. These data confirm our previous findings in somatostatin biosynthesis studies using frog retinas (Yamada and Basinger, 1982). Although we were unable to determine the amino acid sequence of retinal SLI directly, our approach using simultaneous purification studies has permitted us to surmise with relative confidence that the sequence is identical to that of brain somatostatin from the same species. Inasmuch as frog retinal SLI, localized and synthesized in situ, appears to be identical to S14, studies to determine the physiology of somatostatin in frog retina will assume functional relevance. We believe that our techniques may be applied to the purification of other neuropeptides in the retina, thus extending the usefulness of the retinal model for study of peptides as neurotransmitters.

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