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Isolation and Characterization of [Pro^2]Somatostatin-14 and Melanotropins from Russian Sturgeon, *Acipenser gueldenstaedti* Brandt

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A new form of somatostatin (SRIH), along with melanotropins (MSHs), was isolated from pituitaries of the Russian sturgeon *Acipenser gueldenstaedti* Brandt by gel filtration, ion exchange, and reversed-phase HPLC following acid–acetone extraction. The sturgeon SRIH consists of 14 amino acid residues and differs from mammalian SRIH-14 by the substitution Pro for Gly at position 2. Synthetic [Pro^2]SRIH-14 was as potent as mammalian SRIH-14 in inhibiting release of growth hormone into medium from the organ-cultured pituitary of rainbow trout. Sturgeon α-MSH has the same amino acid sequence as those found in mammals. Sturgeon β-MSH is composed of 17 amino acid residues, and its amino acid sequence is identical to the N-terminal 15 residues of salmon β-MSH I and to the C-terminal 2 residues of mammalian β-MSH. © 1995 Academic Press, Inc.

Somatostatin (SRIH) was originally isolated from ovine hypothalamus as a factor that inhibits the secretion of growth hormone (GH) in the pituitary (Brazeau *et al.*, 1973). This peptide hormone was subsequently identified in a number of tissues where it inhibits the release of bioactive substances such as insulin and glucagon in the pancreas (Koerker *et al.*, 1974) and gastrin and cholecystokinin in the gastrointestinal tract (Polak *et al.*, 1975; Bloom *et al.*, 1975; Konturek *et al.*, 1976).

In mammals, two major forms of SRIH are produced by tissue-dependent processing of a single precursor protein (Patzelt *et al.*, 1980). One consists of 14 amino acids, SRIH-14, and the other has an N-terminal extension, SRIH-25 or -28 (Bohlen *et al.*, 1980). However, teleost fishes studied to date are associated with two genes encoding distinct pro-SRIH, I and II (anglerfish: Goodman *et al.*, 1980, Andrews *et al.*, 1987; catfish: Andrews and Dixon, 1981; salmon: Plisetskaya *et al.*, 1986; daddy sculpin and flounder: Conlon *et al.*, 1987; eel: Conlon *et al.*, 1988). Pro-SRIH-I contains SRIH-14 at its C-terminus. There is remarkable conservation of the amino acid sequence of this SRIH among different vertebrate species. Only three different molecular forms of SRIH-14 have been found in lower vertebrates; [Ser^{12}]SRIH-14 from the pancreas of sea lamprey (Andrews *et al.*, 1988) in addition to mammalian SRIH-14 from the brain (Sower *et al.*, 1994), [Ser^5]SRIH-14 from ratfish islets (Conlon, 1990), and [Pro^2, Met^{13}]SRIH-14 as a minor component from frog brains (Vaudry *et al.*, 1992). On the other hand, pro-SRIH-II contains putative [Tyr^7,Gly^{10}]SRIH-14 at the C-terminus, but actually it is processed to larger peptides (Morel *et al.*, 1984).

Sturgeons are one of the four extant representatives of chondrosteans (Order Chondrostei) which lie on the line of evolution that leads to, in turn, holosteans (Order Holostei) and teleosts (Order Teleostei). In spite of a close resemblance with teleost fishes, sturgeons exhibit dramatic similarities with amphibians; the sturgeon has a hypothalamic–hypophysial portal system which is comparable to that of tetrapods but missing in teleosts (Polenov and Garlov, 1971). Due to this taxonomic position, the modern representative of the primitive bony fish is an intriguing species that can provide a

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better understanding of the molecular evolution of hormones in the hypophysial–pituitary system. Indeed, amino acid sequences of GH (Yasuda *et al.*, 1992) and prolactin (Noso *et al.*, 1993) of the Russian sturgeon indicate its closer relationship to tetrapods than to teleosts.

During the isolation and characterization of proopio-melanocortin-related peptide from the Russian sturgeon, we found a new molecular form of SRIH. The present paper describes the chemical and biological characterization of this SRIH and melanotropins (MSHs).

MATERIALS AND METHODS

Materials

The pituitary glands were collected from prespawning female and male Russian sturgeons *Acipenser gueldenstaedti* Brandt during the period of anadromous migration in the delta of the Volga River in April 1992. The weight of the fish ranged from 12 to 15 kg (110–130 cm in length) in males and from 18 to 26 kg (120–150 cm in length) in females. Pituitaries were frozen immediately in liquid nitrogen and transferred to the Laboratory of Molecular Endocrinology. Immature rainbow trout (average body weight 90 g) were obtained from Iwate Prefectural Fisheries Experimental Station. Synthetic SRIH-14 was purchased from Sigma (St. Louis, MO). [Pro^2]SRIH-14 was custom-made at Peptide Institute, Inc. (Osaka, Japan). Chum salmon GH and its antiserum were prepared as described (Kawauchi *et al.*, 1986).

Purification

Frozen pituitary glands (5 g; mixed sexes) were homogenized in 100 ml of acid–acetone (35% HCl:acetone = 1:28) at 4° for 1 hr, and the residue was extracted with 100 ml of 80% acetone at 4° for 1 hr. Both extracts were combined (ca. 200 ml) and poured into 3 liters of prechilled acetone at 4°. The resulting precipitate was collected by centrifugation (10,000*g*, 20 min, 4°) and subjected to gel filtration on a Sephadex G-25 column (2.7 × 70 cm) in 1 *M* acetic acid. The smaller peptide fraction was contaminated with salt and therefore applied to SEP–PAK C-18 cartridges for desalting. Peptides were recovered by elution with 60% acetonitrile in 0.1% trifluoroacetic acid (TFA) and, after lyophilization, subjected to ion-exchange HPLC on a CM-2SW column (0.46 × 25 cm, particle size 10 μm). Linear gradient elution was performed using 10 m*M*–1 *M* ammonium formate buffer (pH 6.5) containing 10% acetonitrile. All fractions were further purified by reversed-phase (rp)HPLC on an ODS-120T column (0.46 × 25 cm, particle size 5 μm) using a linear gradient of 10–50% acetonitrile in 0.1% TFA. Peptides were monitored by absorption at 220 nm.

Sequence Analysis

Peptides were hydrolyzed in 6 *M* HCl containing 0.6% phenol at 110° for 18–24 hr. Amino acid composition was determined by use of an amino acid analyzer (HITACHI L-8500). Amino acid sequences were determined with an

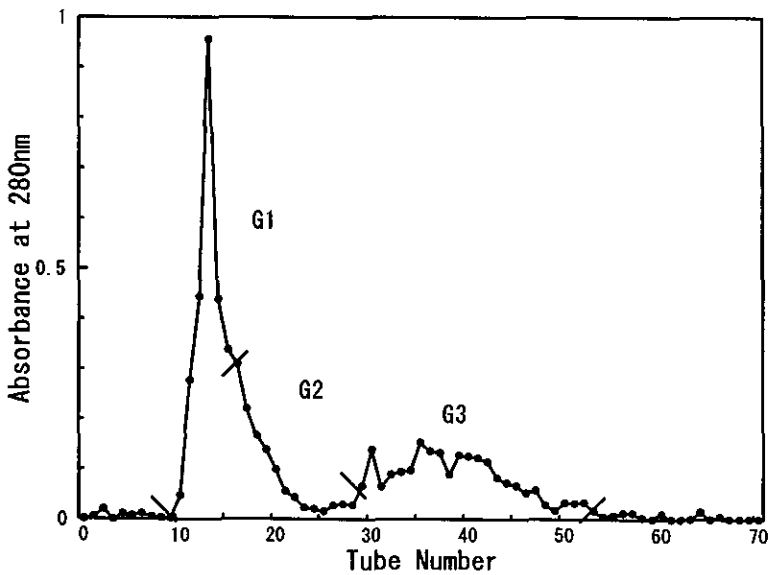

FIG. 1. Gel filtration of acid–acetone extract of Russian sturgeon pituitaries on Sephadex G-25 column (2.46 × 70 cm) with 1 *M* acetic acid at a flow rate of 5 ml/30 min/tube.

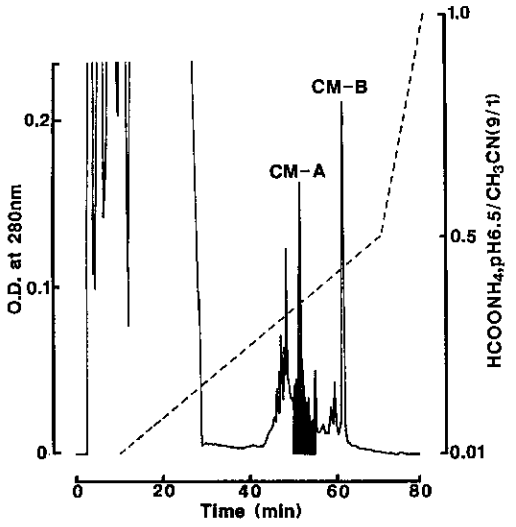

FIG. 2. Ion-exchange HPLC of G3 in Fig. 1 on a TSK-Gel CM-2SW column (46 × 250 mm, particle size 10 μm) with a linear gradient of ammonium formate in 10% acetonitrile at a flow rate of 1.0 ml/min.

automated gas-phase sequencer (Shimadzu PSQ-1) equipped with an on-line phenylthiohydantoin analyzer (Shimadzu C-R4A).

Incubation of Pituitaries

Pituitaries were taken from rainbow trout by decapitation and cultured by the method of Yada *et al.* (1991). In brief, pituitaries were placed individually in wells on a 96-well multiplate. Each well contained 200 μl Eagle's minimum essential medium (pH 7.1–7.4) comprising 6.8 g NaCl, 0.4 g KCl, 0.2 g $CaCl_2$, 0.1 g $MgSO_4$, 0.125 g NaH_2PO_4, 2.2 g $NaHCO_3$, and 1 g glucose/liter supplemented with kanamycin (60 μg/ml). Pituitaries were incubated at 15° under an atmosphere of 95% O_2 and 5% CO_2 for 24 hr and subsequently in the medium containing SRIH for 24 hr. Five pituitaries were cultured for each dose.

Estimation of GH

GH concentration in the culture medium of rainbow trout pituitary was determined by competitive enzyme-linked immunosorbent assay (ELISA) for sGH (Nishii, unpublished). Standard sGH and diluted culture medium were incubated with the antiserum in a siliconized glass tube at 4° overnight. After the incubation, an aliquot of the solution was transferred to a 96-well ELISA plate (Corning) coated with sGH. Each well was treated subsequently with Vectastain ABC–AP kit (Vector Lab.) and *p*-nitrophenylphosphate (Sigma). Absorbance at 405 nm was monitored by a microplate reader (Model 450, Bio-Rad). Statistic differences were assessed by Duncan's new multiple range test.

RESULTS

Identification of SRIH and MSHs

The gel filtration profile of the acid–acetone extract from 5 g of pituitaries is shown in Fig. 1. The most retarded fraction, G3, was desalted

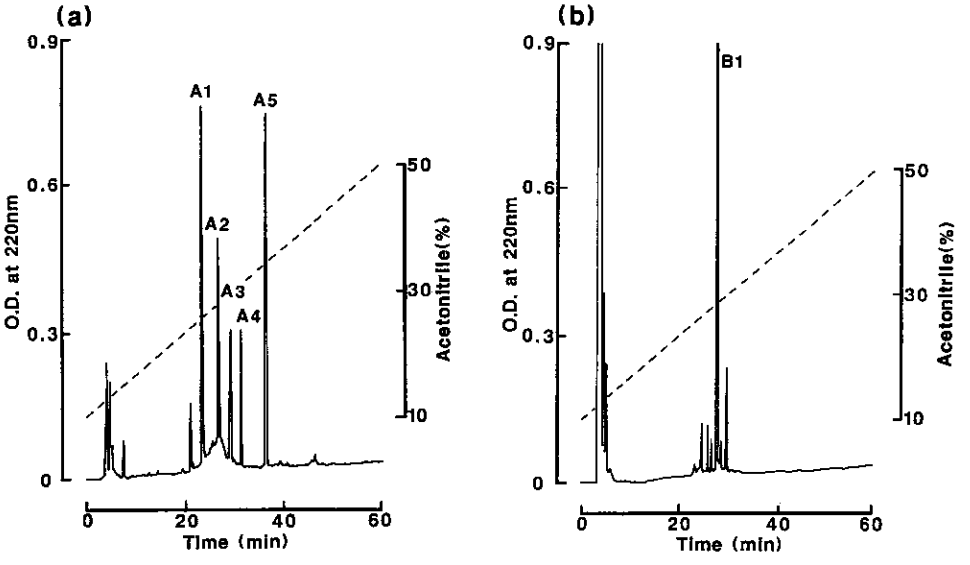

FIG. 3. Reversed-phase HPLC of CM-A (a) and CM-B (b) on TSK-Gel ODS-120T column (46 × 250 mm, particle size 5 μm) with linear gradient of acetonitrile in 0.1% TFA at a flow rate of 1.0 ml/min.

TABLE 1
YIELDS OF PHENYLTHIOHYDANTOIN AMINO ACIDS FROM SOMATOSTATIN AND MELANOTROPINS (pmol)

Cycle	β-MSH	ACTH(1-13)	SRIH-14
1	Asp (296)	Ser (64)	Ala (188)
2	Gly (331)	Tyr (193)	Pro (73)
3	Ser (89)	Ser (95)	——
4	Tyr (356)	Met (173)	Lys (84)
5	Lys (506)	Glu (210)	Asn (74)
6	Met (380)	His (87)	Phe (64)
7	Asn (364)	Phe (147)	Phe (82)
8	His (156)	Arg (121)	Trp (18)
9	Phe (241)	Trp (37)	Lys (34)
10	Arg (293)	Gly (52)	Thr (8)
11	Trp (207)	Lys (37)	Phe (26)
12	Ser (25)	Pro (3)	Thr (7)
13	Gly (114)	Val (1)	Ser (4)
14	Pro (90)		——
15	Pro (118)		
16	Lys (143)		
17	Asp (4)		

with SEP-PAK C-18 cartridges and subsequently fractionated by ion-exchange HPLC on a CM-2SW column (Fig. 2). RpHPLC of CM-A resulted in the separation of five major peptides (A1–A5) (Fig. 3a), while CM-B contained only one major peptide, B1 (Fig. 3b).

A1 consisted of 17 amino acids with the following sequence: H–Asp–Gly–Ser–Tyr–Lys–Met–Asn–His–Phe–Arg–Trp–Ser–Gly–Pro–Pro–Lys–Asp–OH. The yield of phenylthiohydantoin amino acid is listed in Table 1. On the basis of sequence similarity, the peptide was categorized as β-MSH (Fig. 4).

Both B1 and A2 consisted of 13 amino acid residues with a sequence identical to that of mammalian ACTH (1–13) (Table 1). Both A3 and A4 were similar in amino acid composition to B1 and A2, although no data were obtained in the sequence analysis of the intact peptides. These results suggest that A3 and A4 are blocked at the N-terminus.

Peptide A5 consisted of 14 amino acid residues with the following sequence: H–Ala–Pro–X–Lys–Asn–Phe–Phe–Trp–Lys–Thr–Phe–Thr–Ser–X–OH. The yield of phenylthiohydantoin amino acids is listed in Table 1. This amino acid sequence is similar to that of mammalian SRIH-14 if X is read as Cys (Fig. 5). Since the total yield of A5 was estimated to be only 7 μg on the basis of amino acid analysis, the structure was confirmed using synthetic peptide. Synthetic [Pro^2]SRIH-14 was eluted with the same retention time as the natural peptide in rpHPLC.

Inhibition of GH Release

The synthetic peptide inhibited GH release from cultured rainbow trout pituitaries in a dose-dependent manner. The potency of [Pro^2]SRIH-14 appeared to be equivalent to that of mammalian SRIH-14 (Fig. 6).

DISCUSSION

In the present study, a new molecular form of SRIH was isolated and characterized from sturgeon pituitaries. It consists of 14 amino acid residues and differs from mammalian SRIH-14 by the substitution Pro for Gly at position 2. This is the fourth natural variant of SRIH-14. Although the absence of mammalian SRIH-14 was not conclusive, the peptide is, at least, a major form of sturgeon SRIH.

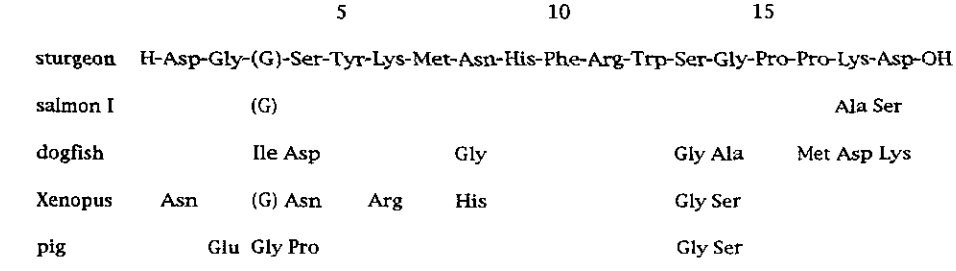

FIG. 4. Comparison of the amino acid sequence of sturgeon melanotropins with those of other vertebrates. Data are taken from Eberle (1988). (G) indicates a gap.

	5								10
sturgeon	H-Ala-Pro-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys-OH								
ratfish	Gly	Ser							
lamprey *	Gly							Ser	
lamprey**	Gly								
frog								Met	
sheep	Gly								

FIG. 5. Comparison of the amino acid sequence of sturgeon somatostatin with that of somatostatin-14 of other vertebrates. Data are taken from frog (Vaudry *et al.*, 1992), ratfish (Conlon, 1990), lamprey* (Andrews *et al.*, 1988), lamprey** (Sower *et al.*, 1994), and sheep (Brazeau *et al.*, 1973).

Since trout pituitaries continuously release a large amount of GH in serum-free culture, somatostatic activity of the sturgeon SRIH was evaluated using this system (Yada *et al.*, 1991; Yada and Hirano, 1992). Synthetic [Pro^2]SRIH-14 was as potent as the mammalian SRIH-14 in inhibiting release of GH *in vitro*.

The primary structure of SRIH-14 has been strongly conserved during evolution of vertebrates, although four positions have been found to be substituted: Pro for Gly at position 2 in the sturgeon in the present study and in the frog (Vaudry *et al.*, 1992), Ser for Asn at position 5 in the ratfish (Conlon, 1990), Ser for Thr at position 12 in the lamprey (Andrews *et al.*, 1988), and Met for Ser at position 13 in the frog (Vaudry *et al.*, 1992). Obviously, these substitutions do not affect biological activity. Studies on structure–activity relationships with synthetic analogues demonstrated that only the central residues Phe–Trp–Lys–Thr between positions 7 and 10 in the cyclic conformation for the tetradecapeptide are required for biological activity. The disulfide bond between positions 3 and 14 is necessary for activity as a conformational constraint (Rivier *et al.*, 1975; Veber *et al.*, 1981).

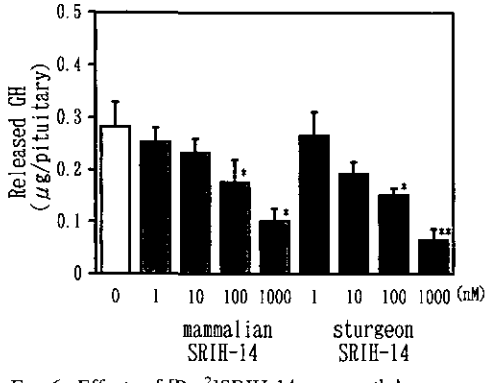

FIG. 6. Effects of [Pro^2]SRIH-14 on growth hormone release from cultured pituitaries of rainbow trout for 24 hr. Data are presented as means ± SEM ($n = 5$). *Significantly different from control at $P < 0.05$. **Significantly different from the control at $P < 0.01$.

Sturgeon β-MSH was composed of 17 amino acid residues, the N-terminal 15 and the C-terminal 2 residues of which are identical to those of salmon β-MSH I and amphibian and mammalian β-MSHs, respectively (Fig. 4). This structural feature appears to be symbolic of the taxonomic position of sturgeon. Phylogenetically, α-MSH is an ancient molecule, and there is remarkable conservation of the amino acid sequence. Keller *et al.* (1994) identified chromatographically multiple forms of α-MSH from the pituitaries of the white sturgeon, in which N-acetylated forms of α-MSH represented over 90% of the total α-MSH, and suggested that sturgeon α-MSH has the same primary sequence as mammalian α-MSH. The present study confirmed that Russian sturgeon α-MSH also has the same amino acid sequence as its teleost and tetrapod counterparts, although ACTH(1–13) is a major form in this extract.

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