Vol. 151, No. 1, 1988 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 29, 1988 Pages 347-354

# PROSOMATOSTATIN PROCESSING IN ANGLERFISH BRAIN, GUT AND PANCREAS

Alain Morel, Paul F.M. Kuks, Julie Bourdais and Paul Cohen

Université Pierre et Marie Curie,
Groupe de Neurobiochimie Cellulaire et Moléculaire, UA 554 au CNRS,
96 boulevard Raspail, 75006 Paris, France

Received January 15, 1988

**Summary : The distribution of somatostatin immunoreactive forms in three tissues of the anglerfish (Lophius piscatorius L.) was analyzed by a combination of gel permeation, High Pressure Liquid Chromatography and amino acid analysis. The data indicate that prosomatostatins I and II are expressed in both neural and gastro-intestinal tissues and that their post-translational processing gives rise to somatostatin-14 I, somatostatin-28 II and to some of its hydroxylysine$^{23}$ -derivative, respectively. It is concluded that, in contrast to the mammals, production of two somatostatins in the Teleostean fish requires two structurally distinct precursors whose processing operates in a fixed pattern rather than in a tissue-specific manner.** 

Brockmann organs, the endocrine pancreas in anglerfish produce two mRNA species encoding for two different somatostatin precursors (1). Prosomatostatin-I (PS-I) generates the tetradecapeptide somatostatin-14 (S-14 I) which is identical to mammalian somatostatin (2). The second precursor (PS-II) gives rise to an octacosapeptide, somatostatin-28II (S-28 II)(3,4) whose COOH-terminus corresponds to the sequence of the [Tyr$^{7}$, Gly$^{10}$]-derivative of S-14 I (3-5).In the anglerfish *Lophius piscatorius L.* no somatostatin-14 II could be detected and it was deduced that post-translational maturation of PS-II terminates at the level of S-28 II formation (3,5). Indeed this finding was rather unexpected since a somatostatin-28 converting activity of rodent origin (6,7) which cleaves mammalian S-28 at the level of the Arg$^{13}$ Lys$^{14}$ doublet, was also able to excise the same dibasic moietie in anglerfish S-28 II and to release, *in vitro*, S-14 II (3). Taken together these facts suggest a physiological role for S-28 II.

Subsequently, two other groups (8,9) showed that in islets of the anglerfish *Lophius americanus Valenciennes*, in addition to S-28 II, a derivative hydroxylated at residue Lys$^{23}$ can be found. It is not clear whether the absence of S-14 II and the presence of hydroxylated S-28 II are typical of pancreatic tissue or the outcome of a fixed pattern of prosomatostatin processing in anglerfish. Likewise, hydroxylation of lysine, which seldom occurs in

0006-291X/88 $1.50

regulatory peptides, might be a peculiarity of *L. americanus* rather than a widespread phenomenon, even in fish metabolism.

In order to analyze the processing of both PS I and PS II, somatostatin-like molecular forms in anglerfish (*L. piscatorius*) brain, gut and pancreas were characterized. These observations are discussed with respect to the situation in mammals and a comparative scheme is proposed.

## MATERIALS AND METHODS.

Brockmann bodies, upper intestine and brain were dissected from live anglerfish (*Lophius piscatorius L.* ) immediately after capture thanks to the kind cooperation of Captain Riou and his crew (the "Gwerch'ez-Benniget", le Conquet, Finistère, France). The animal species and the tissue types were identified with the help of the anatomical descriptions by Baron (10), le Danois (11) and Caruso (12). After dissection, the tissues were immediately frozen on dry ice and kept at -70°C until used. They were extracted by homogenization in 50% v/v acetic acid (3 ml/g of wet tissue). The homogenate was spun at 100,000 x g for 1 hour at 4°C in a Beckman L5-65 ultracentrifuge equipped with a SW 50 rotor. Subsequently, the lipid upper layer and the pellet were discarded and the clear aqueous layer in between was applied onto a Sephadex™ G-50 fine column (90x1.2 cm) equilibrated with 10% v/v acetic acid. The column was eluted at a rate of 6 ml/hour and aliquots of each fraction were assayed for SLI with a radio immmunoassay as previously described (13). Contiguous fractions containing SLI were pooled and evaporated in a Speed Vac™ concentrator. They were redissolved in 100 µl of acetonitrile/water/acetic acid (50/40/10) and injected into an HPLC system which consisted of a 10 RP 8 column (Merck) 4.6x250 mm on a LDC/Milton Roy instrument with a variable wavelength spectrophotometer set at 220 nm. The column was eluted at a rate of 1 ml/min with an acetonitrile/water mixture containing 0.1% v/v of trifluoroacetic acid. It was calibrated with synthetic S-14 I, S-14 II, S-28 II and with [$Hyl^{23}$] S-28-II isolated from anglerfish pancreas (see below). The S-14 pool (see results) was separated by raising the percentage of acetonitrile from 20 to 40% in 20 minutes and collecting fractions of 1 ml. The S-28 pool was resolved with a gradient increasing from 23% to 24% acetonitrile in 30 minutes and 500 µl fractions were collected. Aliquots of each fraction were evaporated with a Speed Vac™ concentrator and assayed for SLI.

Somatostatin-like immunoreactive species in the S-28 pool from pancreas were purified to homogeneity by repeated HPLC steps using the separation method described above. Their amino acid composition was determined according to the method of Chang (14). They were digested with trypsin as described in (15). They were partially sequenced by the manual double coupling method of Chang (16).

## RESULTS

The profile of somatostatin-like immunoreactivity (SLI) recovered from gel permeation shows three peaks in the case of pancreatic tissue and two when intestinal or brain tissue was analyzed (Figures 1A, B and C). These might be attributed to prosomatostatin (11-13 kDa), S-28(3 kDa) and S-14(1.5 kDa) respectively. Table I shows that anglerfish brain and intestine contain far less SLI than pancreas, also that the ratio S-14/S-28 is much greater in these tissues and that only traces of S-28 can be detected in intestine.

The S-28 pools from Brockmann bodies and brain were further separated by HPLC. The S-28 pool from Brockmann bodies contained a sufficient quantity of SLI for chemical characterization. It was resolved into two somatostatin-like immunoreactive species (Figure

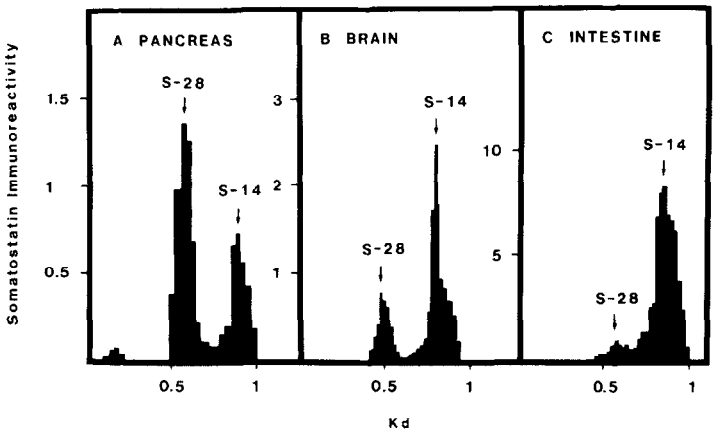


**FIGURE 1 . Molecular sieve filtration of somatostatin immunoreactive forms extracted from *L. Piscatorius* different tissues.**

Acid extracts of Brockmann organ (Panel A), Brain (Panel B) and whole intestine (Panel C) were submitted to gel filtration on a Sephadex G-50 column (90 x 1.2 cm) eluted with 10 % acetic acid at a flow rate of 3 ml/h at room temperature.

Somatostatin immunoreactivity, expressed as µg (Panel A) and ng (Panels B and C) of somatostatin-14 per fraction, was measured in each fraction by RIA.

2), one of which was shown to contain hydroxylysine (Table II), the other one co-eluted with synthetic S-28 II (5). From trypsin digestion experiments it was inferred that, in the former, the site of hydroxylation was $Lys^{23}$ (data not shown). Except for this modified residue, the amino acid composition and partial sequence data were compatible with the sequence of S-28 II (4). The ratio of S-28II to [$Hyl^{23}$] S-28 II was found to represent at most 60/40.

The immunoreactive forms in the S-28 pools from brain were characterized by their co-elution on HPLC with S-28 II and [$Hyl^{23}$]S-28 II purified from anglerfish pancreas.

**TABLE I**

*Levels of somatostatin immunoreactivities in anglerfish tissues.*

| Somatostatin-immunoreactive form | | | |
|---|---|---|---|
| Tissue | Precursor | S-28 | S-14 |
| Pancreas | 400 | 9000 | 5000 |
| Brain | nd | 4 | 12 |
| Mucosa | nd | nd | nd |
| Whole intestine | nd | 2 | 31 |

immunoreactivity expressed as pmole/g of wet tissue.

nd : not detected.

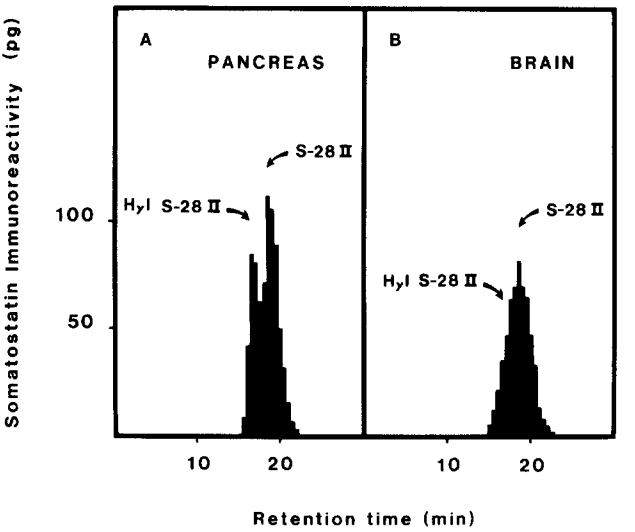


FIGURE 2 . HPLC analysis of somatostatin-28 immunoreactive species.

Somatostatin immunoreactive fractions corresponding to somatostatin-28 from Pancreas (Panel A) and brain (Panel B) were analysed by HPLC on a 10 RP8 column (Merck; 4.6 x 250 mm) eluted with a linear gradient from 23% to 24 % acetonitrile in TFA 0.1 % at a flow rate of 1 ml/min in 30 min.

An aliquot of each fraction was submitted to somatostatin radioimmunoassay. Immunoreactivity is expressed as pg of somatostatin-14 per aliquot.

Arrows indicate the [Hyl$^{23}$]S-28 II and S-28 II retention times.

Compared to the pancreas, this tissue contains very little S-28 (Figure 1). In brain tissue only the non-hydroxylated form of S-28 II could be detected (Figure 2). S-28 I was not detected either in brain, or pancreas, and the intestine contained too little S-28 to allow further characterization.

TABLE II

*Amino acid composition of anglerfish S-28II.*

| Amino Acid | Residues.mol$^{-1}$ | |
|---|---|---|
| | Found | Theoretical |
| Asx | 3.6 | 4 |
| Glx | 1.2 | 1 |
| Ser | 2.6 | 3 |
| Thr | 1.5 | 2 |
| Gly | 1.9 | 2 |
| Ala | 1.1 | 1 |
| Arg | 2.1 | 2 |
| Pro | 2 | 2 |
| Val | 1 | 1 |
| Leu | 1 | 1 |
| Phe | 2.1 | 2 |
| Hyl | 0.4 | 3 |
| Lys | 2.3 | |
| Tyr | 0.5 | 1 |
| Trp | nd | 1 |
| Cys | nd | 2 |

nd : not determined

The immunoreactive species in the S-14 pools from brain and intestine were found to consist exclusively of S-14 I, a situation similar to pancreas. Since the intestinal muscular layer was very tightly attached to the mucosa we were unable to obtain mucosa-free muscle layer. Our results show that intestinal S-14 I must be located in the muscular layer, since no SLI could be detected in the mucosa.

During the analysis of the S-14 pool from pancreas, the S-28 I(1-12) was purified by sequential HPLC separations, and quantified by amino acid composition. It was found to represent at most 30% of the S-14 I found in this tissue.

## DISCUSSION

Our data indicate that anglerfish intestine contains very little SLI. This result was rather unexpected in view of the high levels of SLI in rat intestine (17). It is, on the other hand, in good agreement with results of others (18) who observed very low levels of somatostatin mRNA in anglerfish intestine. However, in contrast to the pattern observed for the pancreas, only one type of mRNA could be detected. The electrophoretic behavior of the *in vitro* translation product of this mRNA was identical to that of the largest preprosomatostatin from pancreas, PS-II. Since we found only S-14 I in the intestinal S-14 pool, there is clearly a discrepancy between our results and those of Goodman et al. (18) which might be explained by tissue-specific differences in gene expression or possibly the existence of a third separate gene responsible for intestinal somatostatin biosynthesis.

The present results demonstrate that about 40% of the S-28 pool in Brockmann bodies from *L. piscatorius* consists of [Hyl$^{23}$]S-28 II, while the majority corresponds to the non-hydroxylated form. This indicates that the occurence of [Hyl$^{23}$]S-28 II is not restricted to *L. americanus*. Anglerfish brain, however, appeared to be devoid of the hydroxylated form. One of the functions of somatostatins in the anglerfish central nervous system is probably the inhibition of growth hormone release as was shown for another teleost species, Sarotherodon mossambicus (19). Therefore, the site of hydroxylation within the S-28 II molecule is of considerable interest since the corresponding lysine residue in mammalian somatostatin-14 appears to be critical for the biological activity of this peptide (20,21). Whether hydroxylation of S-28 II in the pancreas should be regarded as a post-translational modification or possibly as the first reaction of a degradative pathway leading to the inactivation of S-28 II, has not been established.

No evidence for the presence of S-28 I could be obtained from any of the tissues investigated here either by HPLC or immunochemical analysis. Since all peptides from the S-28 pool with SLI have been characterized and their quantity evaluated, it can be inferred that S-28 I remains undetectable.

Mackin and Noe (22) characterized two specific proteolytic activities which are able to release anglerfish somatostatins *in vitro* from their precursors. Indeed, among these no S-28 I releasing activity was found. However, Noe et al. (23) and Andrews and Dixon (24) as well as the present report indicated the presence of S-28 I(1-12) in anglerfish pancreas. This implies that in addition to the activity that releases S-14 I by cleavage at the $Arg^{13}$-$Lys^{14}$ sequence, another activity must be responsible for cleavage at the single arginine residue which separates the S-28 I domain from the remaining of PS-I. The absence of S-28 I could be accounted for by postulating that S-14 I is released prior to S-28 I(1-12). In a number of cases, cleavage at a dibasic sequence was shown to precede cleavage at monobasic sites (e.g. 25,26). On the other hand, the possibility that S-14 I and S-28 I(1-12) are released by a rate-limiting cleavage at the monobasic site followed by a fast dibasic cleavage cannot be excluded. In either case S-28 I would remain undetected.

Overall the present data show that in all tissues investigated both S-28 I and S-14 II are absent. Preliminary results obtained with an antibody to synthetic S-28 II(1-12) (5) indicate that this peptide is also absent in pancreas (A. Morel et al., unpublished results). Therefore, post-translational proteolysis of each of the two prosomatostatins appears to proceed according to a fixed pattern rather than in a tissue-specific fashion. Moreover, the very low level of S-28 species in anglerfish intestine is corroborated by the absence of one type of mRNA (18). These facts raise the interesting possibility of independent control of the levels of somatostatin-14 and -28 by regulation of gene expression or mRNA translation. Obviously, such a mechanism would not operate in organisms possessing only one somatostatin gene. There, the intracellular levels of these peptides are probably regulated via the post-translational processing machinery.

Brockmann bodies have long been considered as an attractive model system for somatostatin biosynthesis in general. It is, however, quite clear now that in anglerfish this biosynthetic pathway is fundamentally different from that in mammals (Figure 3). A number

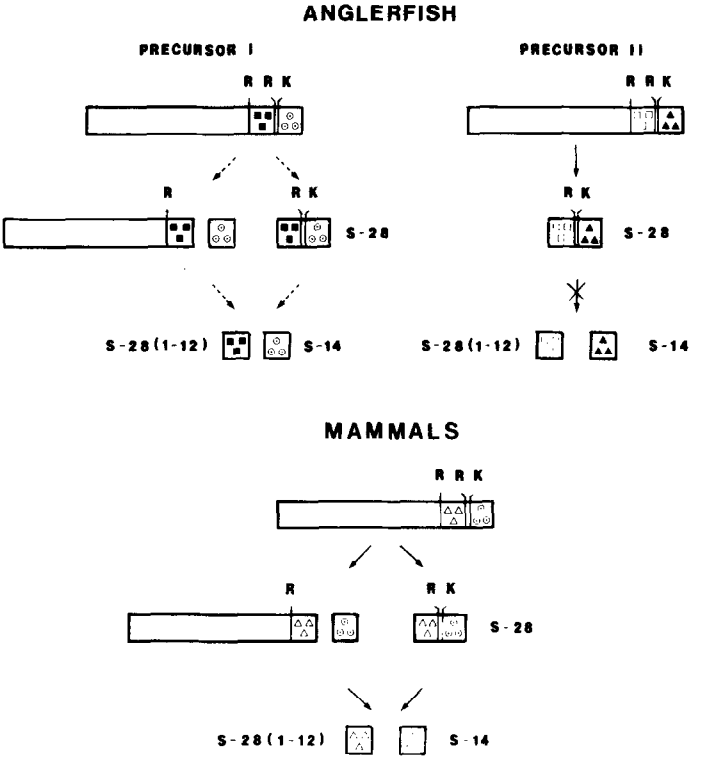


FIGURE 3 . A schematic representation of prosomatostatin processing in anglerfish and mammals.

Dashed arrows represent hypothetical steps of the processing.

of these differences are still poorly understood. Their elucidation might turn out to be very interesting from an evolutionary point of view.

## ACKNOWLEDGEMENTS

We thank C. Rougeot (URIA, Institut Pasteur, Paris) for providing the antibody to somatostatin and Prof. M. Beinfeld for careful reading of the manuscript. This work was supported by funds from the Université Pierre et Marie Curie, the Centre National de la Recherche Scientifique and the Institut National de la Santé et de la Recherche Médicale (CRE : 87-4002).

## REFERENCES

1. Hobart, P., Crawford, R., Shen,, L.P., Pictet, R. and Rutter, W.J. (1980) Nature 288, 137-141.
2. Noe, B.D., Spiess, J., Rivier, J.E. and Vale, W. (1979) Endocrinology 105, 1410-1415.
3. Morel, A., Gluschankof, P., Gomez, S., Fafeur, V. and Cohen, P. (1984) Proc. Natl. Acad. Sci. USA 81, 7003-7006.
4. Morel, A., Chang, J.-Y. and Cohen, P. (1984) FEBS Lett. 175, 21-24.
5. Nicolas, P., Delfour, A., Boussetta, H., Morel, A., Rholam, M. and Cohen, P. (1986) BBRC 140, 565-573.

6. Gluschankof, P., Morel, A., Gomez, S., Nicolas, P., Fahy, C. and Cohen, P. (1984) Proc.Natl. Acad. Sci. USA 81, 6662-6666.
7. Gluschankof, P., Gomez, S., Morel, A. and Cohen, P. (1987) J. Biol. Chem. 262, 9615-9620.
8. Andrews, P.C., Hawke, D., Shively, J.E. and Dixon, J.E. (1984) J. Biol. Chem. 259, 15021-15024.
9. Spiess, J. and Noe, B.D. (1985) Proc. Natl. Acad. Sci. USA 82, 277-281.
10. Baron, H. (1935) Zeit. wiss. Zool. 146, 1-40.
11. Le Danois, Y. (1974) Mem. Mus. Natl. Hist. Natur. (Paris) Ser.A 91, 1-127.
12. Caruso, J.H. (1983) Copeia 1983, 11-30.
13. Morel, A., Nicolas, P. and Cohen, P. (1983) J. Biol. Chem. 258, 8273-8276.
14. Chang, J.-Y., Knecht, R. and Braun, D.G. (1983) Meth. Enzymol. 91, 41-48.
15. Alan, G. (1981) Sequencing of proteins and peptides, p. 55 (North-Holland Pub.).
16. Chang, J.-Y. (1983) Meth. Enz. 91, 455-466.
17. Patel, Y.C., Wheatley, T. and Ning. C. (1981) Endocrinol. 109, 1943-1949.
18. Goodman, R.H., Lund, P.K., Barnett, F.H. and Habener, J.L. (1981) J. Biol. Chem. 256, 1499-1501.
19. Fryer, J.N., Nishioka, R.S. and Bern, H.A. (1979) Gen. Comp. Endocrinol. 39, 244-246.
20. Nutt, R.F., Veber, D.F., Curley, P.E., Saperstein, R., and Hirschmann, R. (1983) Int.J.Pept. Prot. Res. 21, 66-73.
21. Hirst, B.H., Barry, H. and Coy,D.H. (1984) Regul. Pept. 8, 267-271.
22. Mackin, R.B. and Noe, B.D. (1987) J. Biol. Chem. 262, 6453-6456.
23. Noe, B.D., Andrews, P.C., Dixon, J.E. and Spiess, J. (1986) J. Cell Biol. 103, 1205-1211.
24. Andrews, P.C. and Dixon, J.E. (1987) Biochemistry 26, 4853-4861.
25. Camier, M., Barre, N., Morel, A. and Cohen, P. (1986) FEBS Lett. 196, 14-18.
26. Schwartz, T.W. (1987) J. Biol. Chem. 262, 5093-5099.