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Post-translational processing of prepro-urotensin II

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The primary structure of a teleost prepro-urotensin II may be deduced from the nucleotide sequence of cloned DNA complementary to carp prepro-urotensin II mRNA but the pathway of post-translational processing of the precursor is unknown. In this study, we have isolated four peptides from an extract of flounder urophysis that are derived from prepro-urotensin II by proteolytic cleavage. The amino acid sequences of the peptides demonstrate that flounder prepro-urotensin II is cleaved at two monobasic processing sites (single arginine residues) to generate peptides with limited homology to carp prepro-urotensin II-(22–41)-, -(42–87)- and -(88–110)-peptides. Cleavage at a tribasic residue processing site generates a urotensin II with the primary structure: Ala-Gly-Thr-Thr-Glu-Cys-Phe-Trp-Lys-Tyr-Cys-Val. Urotensin II-(4–12)-peptide represented a minor component in the extract.

Urotensin II; Post-translational processing; Flounder urophysis

1. INTRODUCTION

The caudal portion of the spinal cord of jawed fishes contains a neurosecretory organ, the urophysis. Extracts of teleost urophyses contain factors with hypotensive, smooth muscle stimulating, osmoregulatory, and steroidogenic activities (reviewed in [1]). These activities may be ascribed, at least in part, to the presence of two peptides in the urophysis: urotensin I and urotensin II. Urotensin II has been isolated in single molecular form from the goby, *Gillichthys mirabilis* [2] and in multiple molecular forms from the white sucker, *Catostomus commersoni* [3] and carp, *Cyprinus carpio* [4]. The peptides comprise 12 amino acid residues and contain the cyclic sequence: -Cys^6-Phe^7-Trp^8-Lys^9-Tyr^{10}-Cys^{11}- that has been conserved between the species. This region is structurally similar to the functionally important central region of somatostatin-14: -Phe^7-Trp^8-Lys^9-Thr^{10}- and urotensin II and somatostatin share some common biological properties [3].

The complete primary structures of two biosynthetic precursors of urotensin II (prepro-urotensin II) may be deduced from the nucleotide sequence of cloned DNAs complementary to mRNAs prepared from the caudal spinal cord region of the carp [5]. The precursors, prepro-urotensin II-α and prepro-urotensin II-γ, are highly homologous and comprise 125 amino acids. Although the pathways of post-translation processing of fish preprosomatostatins have been studied in detail [6,7], the sites of post-translational proteolytic cleavage in prepro-urotensin II are unknown. This study describes the isolation of several peptides derived from prepro-urotensin-II from an extract of the urophysis of the flounder, *Platichytes flesus*. Determination of the primary structures of these peptides has enabled the elucidation of the pathway of post-translational processing of the precursor.

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2. MATERIALS AND METHODS

2.1. *Tissue extraction*

Urophyses from 31 adult flounder were removed following tissue exposure by drilling away the overlying vertebral bone and immediately frozen on solid CO_2. The tissue (35 mg wet weight) was boiled in 0.25% (v/v) acetic acid (7.8 ml) for 3 min [8] and the mixture freeze-dried. The dried material was redissolved in 0.1% trifluoroacetic acid/water and centrifuged (10000 × *g*, 15 min).

2.2. *Purification of the peptides*

The supernatant from the extract was injected onto a semi-preparative (250 × 10 mm) Vydac 218TP510 column (Separations Group) equilibrated with 0.1% trifluoroacetic acid/water at a flow rate of 2 ml/min. The concentration of acetonitrile in the eluting solvent was raised to 48% (v/v) over 90 min using a linear gradient. Absorbance was monitored at 214 nm and 280 nm and peaks were collected manually without using a fraction collector. The peaks designated 1–6 in Fig. 1 were rechromatographed on an analytical (250 × 4.6) Vydac 214TP54 column equilibrated with acetonitrile/water/trifluoroacetic acid (21.0:78.9:0.1) at a flow rate of 1.5 ml/min. The concentration of acetonitrile in the eluting solvent was raised to 48% (v/v) over 60 min using a linear gradient.

2.3. *Characterization of the peptides*

The primary structures of the peptides were determined by automated Edman degradation using an Applied Biosystems model 471A sequenator modified for on-line detection of phenylthiohydan-

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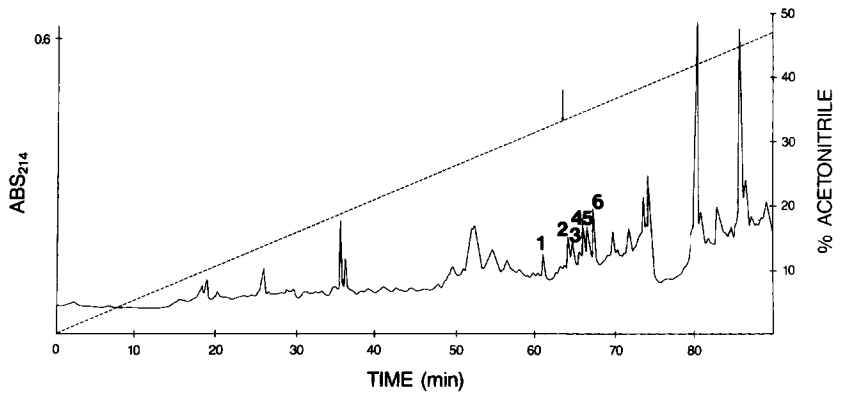

Fig. 1. Reversed-phase HPLC on a semi-preparative Vydac C-18 column of an extract of flounder urophyses. The peaks designated **1–6** were selected for further purification. The arrow shows the retention time of synthetic goby urotensin II and (– – –) the concentration of acetonitrile in the eluting solvent.

toin (PTH) amino acids under gradient elution conditions. The manufacturer's standard operating procedures were used and the detection limit for PTH amino acids was 0.5 pmol. Californium-252 plasma desorption time-of-flight mass spectrometry was carried out using a BIO-ION Nordic BIN-10K instrument as described [9]. The accuracy of the mass determinations was approximately ± 0.1%.

3. RESULTS

3.1. *Purification of peptides derived from pro-urotensin II*

The elution profile on a semi-preparative Vydac C-18 column of the extract of flounder urophyses is shown in Fig. 1. The chromatogram contained six partially resolved peaks (designated **1–6**) whose retention times were similar to that of synthetic goby urotensin II. The peptides in these peaks were purified to apparent homogeneity by rechromatography on a Vydac C-4 column.

3.2. *Structural characterization*

In view of the small amounts of pure material available, approximately 90% of the total quantity of peptides was subjected to automated Edman degradation. The results of the structural analysis are summarized in Table I. Peptide **1** represented flounder urotensin II. Confirmation of the proposed structure, including the presence of a cystine bridge, was provided by mass spectrometry. A strong molecular ion (MH^+) was identified at 1406.7 ± 1.4 amu compared with a calculated MH^+ of 1406 for the proposed sequence. Edman degradation of peptide **3** indicated the probable amino acid sequence: Thr-Glu-X-Phe-Trp-Lys-Tyr-X-

Table I

A comparison of the amino acid sequences of peptides derived from flounder pro-urotensin II with the predicted sequences from the corresponding region of carp pro-urotensin II-α

	10 20
Carp	HPVTDTADMTYSGPDSVEEA
Flounder	--I-ES-E-P-P--A-L--R* (55%)
	30 40
Carp	GGVSPDDFAVSDLNDLLQRAA
Flounder	-VG-L--LSL-EQ-YPP--G-
	50 60
	VVGYSPLLSRENIKVPGQIPKEALR*
	GLR-AT-.....(39%)
	70 80 90
Carp	ELLLEKPYRLIPPSGLWGSRRQFR*K*R*
Flounder	-V----QSL-N-F-RVF-I-K-- (52%)
	100
Carp	GGGADCFWKYCV
Flounder	A-TTE------- (67%)

(-) denotes residue identity and the values in parentheses show % homologies between corresponding regions of the carp and flounder precursors. The R-20, R-66 and R-90 K-91 R-92 residues, denoted by asterisks, represent putative sites of post-translational processing of pro-urotensin II

Val. corresponding to urotensin II-(4–12)-peptide. Mass spectrometry confirmed the proposed sequence and indicated the presence of a cystine bridge in the molecule. A strong molecular ion (MH^+) was detected at 1175.7 ± 1.2 compared with a calculated MH^+ of 1176. A second strong signal was observed at MH^+ 1187.4 ± 1.2 suggesting that peptide **3** may not have been pure.

Peptide **5** was shown to be a 20-amino-acid-residue peptide terminating in an arginyl residue and peptide **6** was a 23-amino-acid residue peptide. Comparison with the predicted amino acid sequence of carp prepro-urotensin II [5] demonstrates that peptide **5** represents flounder pro-urotensin II-(1–20)-peptide and peptide **6** represents flounder pro-urotensin II-(67–89)-peptide. It was not possible to obtain the complete amino acid sequence of peptide **4**. PTH-derivatives were unambiguously identified during cycles (1–26), a threonine residue was detected in trace amount only at cycle 27 and a leucine residue was detected at cycle 28. Comparison with the predicted structure of carp prepro-urotensin II [5] demonstrates that the partial sequence of peptide **4** may represent the NH_2-terminus of flounder pro-urotensin II-(21–66)-peptide.

4. DISCUSSION

A comparison of the amino acid sequences of four peptides derived from flounder prepro-urotensin II with the sequences of peptides from the corresponding region of carp prepro-urotensin II (predicted from the nucleotide sequence of a cDNA) shows that the primary structure of the precursor has not been well conserved between the species. Strong evolutionary pressure appears to have acted to conserve only the sequence at COOH-terminus of the molecule, i.e. the cyclic region of urotensin II. The degree of homology of the three peptides in the NH_2-region of the precursor is only 39–55% (Table I). The data suggest, but do not prove, that the NH_2-flanking peptides in the pro-urotensin II molecule do not have a regulatory role. Similarly, the demonstration that the NH_2-region of the precursor is cleaved into several fragments shows that the prepro-urotensin II gene does not direct the synthesis of a urophysin (urotensin-binding protein) analogous to the neurophysins [10].

It has been proposed that conformation features in the prohormone regulate the specificity of cleavage by the processing enzymes. In the case of processing at the site of multiple basic residues, the cleavage sites are often located inside, or immediately adjacent to, regions with a high probability of β-turn formation [11] or alternatively are associated with Ω loops [12]. Schwartz has identified proximity to proline residues as an important conformational feature regulating cleavage at single arginine residues in some precursor peptides [13] whereas Benoit et al. have claimed that the monobasic site must be in a domain containing an additional basic residue together with leucine and/or alanine residues [14]. Isolation of flounder pro-urotensin II-(1–20)-peptide, which contains a COOH-terminal arginine residue, identifies the site of cleavage of the signal peptide and provides good evidence that the precursor is cleaved a monobasic processing site. The Arg-20 residue, however, is not located in the neighbourhood of a proline residue or in a domain containing a second basic residue. Evidence for the occurrence of a second monobasic cleavage in flounder pro-urotensin II is indirect. Carp prepro-urotensin II contains a single arginine residue at position 87 which corresponds to position 66 in flounder pro-urotensin II. In the carp preprohormone, Arg-87 is located in a region containing an additional basic residue (lysine) and one alanine and four leucine residues. Although it was not possible with the limited amount of pure material available to obtain the amino acid sequence at the COOH-terminus of peptide 4 (the putative pro-urotensin II-(21–66)-peptide), isolation and full structural characterization of pro-urotensin II-(67–89)-peptide suggests that cleavage may have occurred at Arg-66 in the flounder precursor. This study has demonstrated that the Arg-86 Lys-87 potential dibasic residue processing site in flounder pro-urotensin II (corresponding to Arg-107 Arg-108 in the carp preprohormone) is not recognized by the processing enzyme(s). This site is not located in or immediately adjacent to a β-turn.

The pathway of post-translational processing of flounder pro-urotensin II may be compared with the pathway of processing of flounder prosomatostatin II [7]. In the pancreatic islets, prosomatostatin II is cleaved at a dibasic residue processing site in the central region of the molecule and the resulting fragments are cleaved at monobasic (single arginine) sites. The primary structures of the prosomatostatin fragments demonstrated that the COOH-terminal arginine residue was removed following (or concomitant with) cleavage by the monobasic processing enzyme. Determination of the primary structure of flounder pro-urotensin II-(1–20)-peptide has shown that the COOH-terminal arginine residue has not been removed from the fragment. This difference in processing pathways may reflect a difference in concentration of carboxpeptidase E in the urophysis and pancreatic islets.

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