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Identification and tissue mapping of APGWamide-related peptides in *Sepia officinalis* using LC-ESI-MS/MS

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Abstract

This paper demonstrates for the first time the occurrence of tetrapeptides related to APGWamide in the mollusk cephalopod *Sepia officinalis*. LC-ESI-MS/MS analysis allowed the identification of the APGWamide-related peptides predicted by the two genes cloned previously in *Lymnaea stagnalis* and in *Mytilus edulis*, as well as the dipeptide GWamide released from the processing of the tetrapeptides by a dipeptidyl aminopeptidase (DPAP). TPGWamide and GWamide appeared to be exclusively located in the CNS, and the APGWamide in both the CNS and the nerve endings. The RPGWamide and the KPGWamide were not detected by LC-ESI-MS/MS suggesting they could be totally processed into GWamide. The in vitro processing of the tetrapeptides into GWamide by optic lobe extract revealed a differential processing for each, with APGWamide (44.7%) > RPGWamide (24.3%) > KPGWamide (19.3%) > TPGWamide (11.7%). The tissue mapping results, together with the processing efficiency data suggest that the GWamide is mainly produced from the *M. edulis* gene products RPGWamide and KPGWamide.

Keywords: LC-ESI-MS/MS; APGWamide; Neuropeptide; Cephalopods; Cuttlefish; *Sepia officinalis*; Oviduct; Egg laying; DPAP

1. Introduction

APGWamide-related peptides are expressed by two different genes which have been cloned in *Lymnaea stagnalis* [12] and *Mytilus edulis* [3]. The full processing of the gastropod gene led to the synthesis of 10 copies of APGWamide. The expression of the bivalve gene led to the synthesis of five copies of RPGWamide, one of KPGWamide, and one of TPGWamide. The recent demonstration of the occurrence of the two gene products in both gastropods and bivalves suggests that these genes are both present in these mollusk classes [6]. The absence of homologous domains in the *Lymnaea* precursor and the *Mytilus* precursor eliminates the hypothesis of an alternative splicing of a single gene. This suggests the likelihood of two genes, probably resulting from duplication of an ancestral gene before the divergence between gastropods and bivalves. The comparative biological activities of these peptides on the *Mytilus* muscle contraction appeared to be very similar [6]. In *L. stagnalis*, the APGWamide was responsible for the relaxation of the penis retractor muscle [7]. Moreover, in association with the *Lymnaea* neuropeptide Y (LNPY), the pedal peptide and the conopressin which are co-expressed in the right cerebral ganglion [2], it controlled muscles involved in the ejaculation of semen during copulation [14]. In the cuttlefish *Sepia officinalis*, a dipeptidyl aminopeptidase (DPAP) located in the central nervous system (CNS) has been shown to process APGWamide tetrapeptides into GWamide, a dipeptide which inhibited the contraction of the distal oviduct [5]. Recently, Electrospray ionization mass spectrometry (ESI-MS) analysis of prepurified HPLC fractions demonstrated the occurrence of the GWamide in *Lymnaea* and *Mytilus* as well as a particular tissue mapping of APGWamide-related peptides [6]. Thus, TPGWamide and GWamide appeared to be located in the CNS, KPGWamide and RPGWamide in the nerve endings (NE), and APGWamide both in CNS and NE [6]. This differential distribution suggested that this neuropeptide family could target many organs and thus exert a wide variety of biological activities.

In this paper we investigate the occurrence and the mapping of tetrapeptides related to APGWamide in *S. officinalis* by analysis using LC-ESI-MS/MS of samples from the CNS and NE.

We used in vitro processing of each APGWamide-related peptides as described in [5] to elucidate the occurrence of GWamide and we checked the ability of each APGWamide tetrapeptide to be processed into GWamide by the DPAP.

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2. Materials and methods

2.1. Animals

All the cuttlefish were trapped in the Bay of Seine between January and June. They were maintained in 1000 l outflow tanks at the Marine Station of Luc sur Mer (University of Caen, France).

2.2. Extraction of tissues for LC-ESI-MS/MS analysis

Two animal equivalents of each organ studied corresponding approximately to 500 mg of sus-oesophagal mass, 400 mg of sub-oesophagal mass, 1 g of optic lobe, 100 mg of oviduct gland NE, and 100 mg of veina cava neurohemal area were crushed to obtain powder in nitrogen liquid before being extracted in 10 ml of cold methanol:water:acetic acid (90:9:1, v/v) and centrifuged 20 min at 30,000 × *g* at 4 °C. The supernatants were evaporated in a Rotavap and concentrated using Chromafix C18.

Fifty microliter of hemolymph were extracted in an equal volume of cold methanol:water:acetic acid (90:9:1, v/v) and concentrated as described above.

2.3. LC-ESI-MS/MS analysis

Peptide identifications were performed on the LC/MSD Trap (Agilent Technologies) comprising a high performance liquid chromatography connected online to an electrospray ion source. The ions were focused into an ion-trap, capable of MS and MS/MS analyses.

2.3.1. Liquid chromatography procedure

The extracts were resuspended in 100 μl 0.1% acetic acid/0.01 M ammonium acetate in water, and 5 μl were injected onto a C8 Zorbax RX column (2.1 mm × 150 mm) with an acetonitrile linear gradient of 0.72% per minute from 5 to 30% at a flow rate of 0.3 ml/min. HPLC column was rinsed between each injection to avoid contamination of the natural products with the synthetic peptides.

2.3.2. Peptide identification

The MS data were acquired in positive ion mode by selected ion monitoring (SIM) for the following *m*/*z* values: 261.1 (GWamide), 429.0 (APGWamide), 459.1 (TPGWamide), 486.2 (KPGWamide), and 514.2 (RPGWamide). The peptides were identified at the retention times observed for synthetic peptides by monitoring their monoprotonated forms $[M + H]^+$, in association with MS/MS spectra of the mass selected $[M + H]^+$ ions which were compared to those of the corresponding synthetic peptides.

The mass spectra were acquired during 50 ms from *m*/*z* 100–600. The capillary exit of the electrospray ion source was set at 70 V and the octapole at 3 V.

2.4. In vitro processing of APGWamide-related peptides

Optic lobes kept at −80 °C were homogenized in an ice-cold Potter apparatus in saline phosphate buffer (1 g of tissue for 10 ml of PBS; KH_2PO_4 2.35 mM, K_2HPO_4 3.11 mM, NaCl 154 mM, pH 6.5), and then centrifuged for 2 min at +4 °C. The supernatants were pooled and stored at −80 °C until incubation. Aliquots of 100 μl of extract were incubated for 1 min at 35 °C with 1 μg of each synthetic peptide. The incubation was stopped by addition of 500 μl of ice-cold methanol:water:acetic acid (90:9:1). After a 5 min centrifugation at 12,000 × *g*, 500 μl of the supernatant were collected, evaporated in a SpeedVac concentrator and kept at −20 °C until HPLC fractionation. For each experiment ($n = 8$), a control corresponding exactly to the experiment but without incubation was performed to eliminate variations induced by the endogenous concentration of the studied peptides.

2.5. HPLC analysis

HPLC analysis was performed using the Varian software LC-STAR, with a Varian 9012 solvent delivery system and a Varian 9050 UV-VIS detector set at 214 nm. The dry samples were resuspended in 250 μl of buffer A (0.1% TFA in water), injected into a 100 μl injection loop and eluted by a 60 min linear gradient of 1% of acetonitrile per minute on a Nucleosil C18 column (250 mm × 4 mm, 7 μm). The peptide concentrations were calculated by comparison of the peak area obtained at 214 nm with a reference peak corresponding to 0.1 μg of synthetic peptide, eluted using the same HPLC conditions.

3. Results

3.1. LC-ESI-MS/MS

The results obtained for the standards, used for peptide identification, are summarized in Fig. 1. The total ion current (TIC) chromatogram is shown in Fig. 1(A). The extracted ion chromatograms (EIC) can be superposed due to the purity of the synthetic peptides. The MS and MS/MS spectra of each synthetic peptide, shown in Fig. 1(A)–(F), can be used as standard spectra for the identification of APGWamide-related peptides in the tissues of *S. officinalis*.

Fig. 2 shows the MS data obtained for the optic lobes (OL) extract. In Fig. 2(A), the TIC chromatogram illustrates the complexity of the sample analyzed. The occurrence of three APGWamide-related peptides in the OL is clearly demonstrated in Fig. 2(B, GWamide), (C, APGWamide), and (D, (TPGWamide). The mass and MS/MS spectra extracted for each detected APGWamide-related peptide were compared with the standard data obtained for the synthetic peptides under the same analysis conditions. The MS/MS spectra

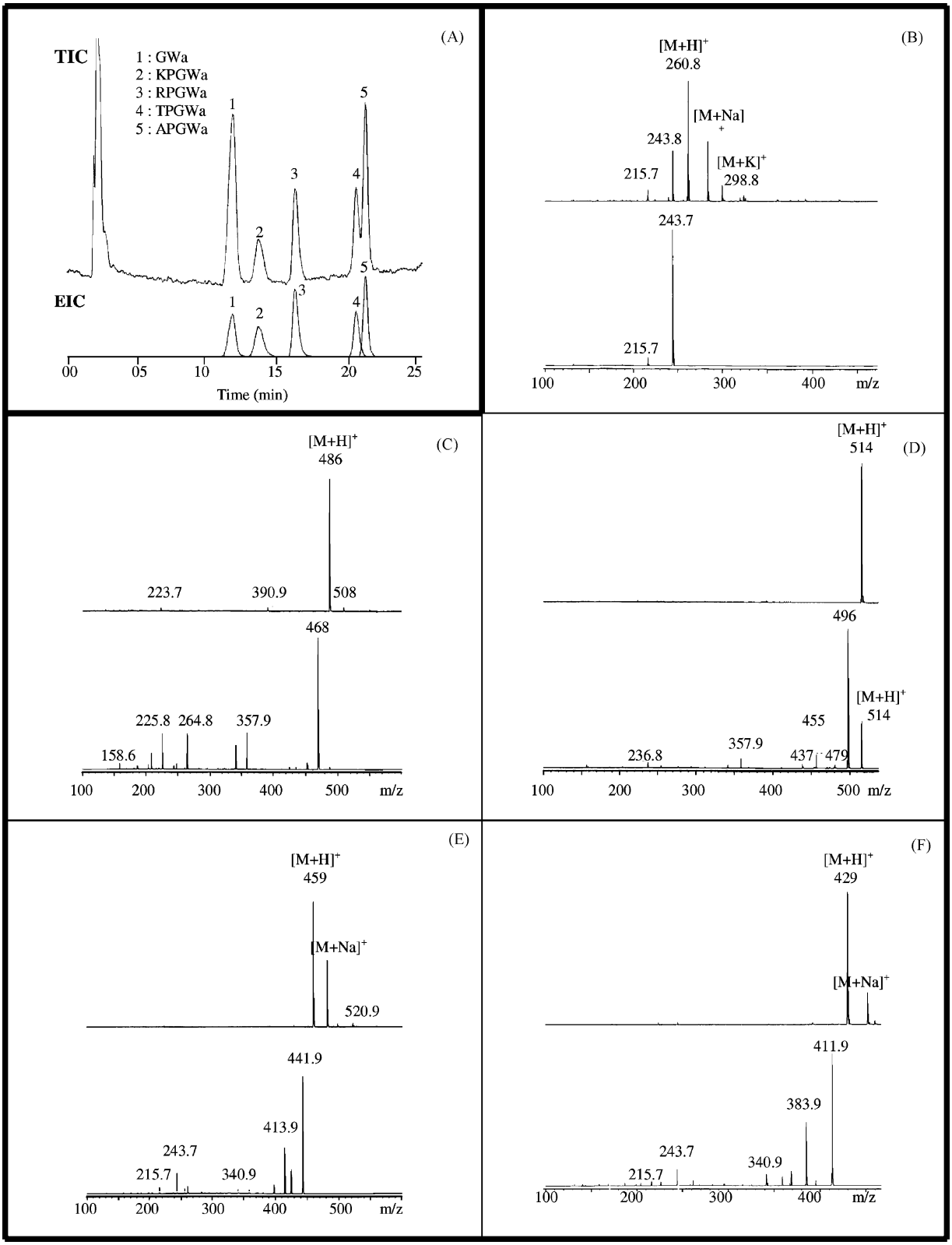

Fig. 1. LC-ESI-MS/MS of the synthetic peptides. The fractionation was performed on a Zorbax C8 column. (A) Total ion chromatogram and EIC of the *m/z* values corresponding to the synthetic peptides. (B) Mass spectrum and MS/MS spectrum of the $[M+H]^+$ ion at *m/z* 261 for the peptide GWamide. (C) Mass spectrum and MS/MS spectrum of the $[M+H]^+$ ion at *m/z* 486 for the peptide KPGWamide. (D) Mass spectrum and MS/MS spectrum of the $[M+H]^+$ ion at *m/z* 514 for the peptide RPGWamide. (E) Mass spectrum and MS/MS spectrum of the $[M+H]^+$ ion at *m/z* 459 for the peptide TPGWamide. (F) Mass spectrum and MS/MS spectrum of the $[M+H]^+$ ion at *m/z* 429 for the peptide APGWamide.

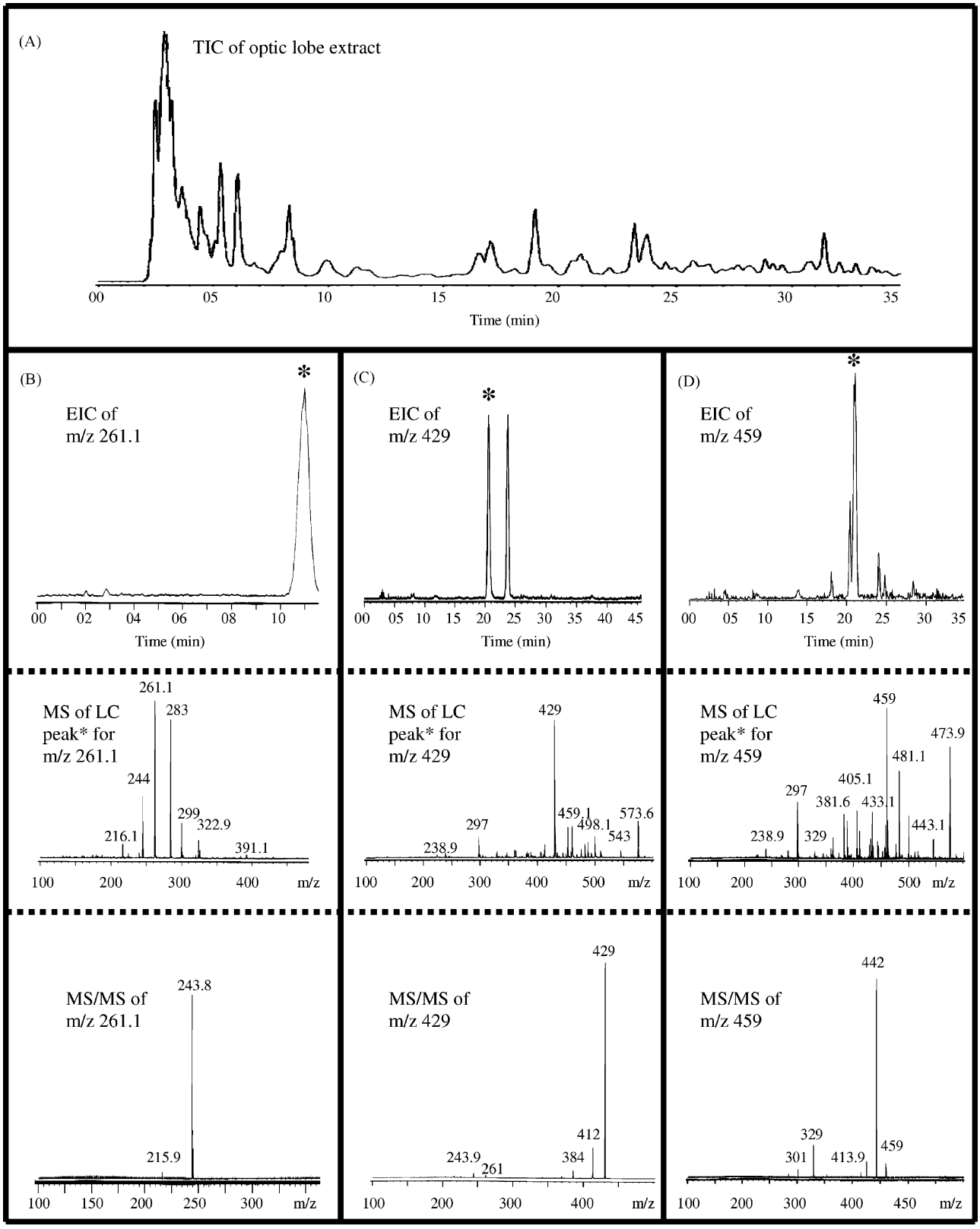

Fig. 2. Detection of APGWamide-related peptides in *S. officinalis* tissues. (A) Total ion chromatogram obtained from the LC-ESI-MS/MS analysis of the optic lobe extract performed using the same experimental conditions as for the synthetic peptides (Fig. 1). For each *m/z* value corresponding to APGWamide-related peptides, the EIC, the MS and the MS/MS spectra were acquired. (B) GWamide. (C) APGWamide. (D) TPGWamide.

Table 1
Peptide fragment ions obtained from MS/MS analysis

m/z	Identified peptidic fragments from MS/MS
441.9	Loss of C-terminal NH_2 (17 Da) from $[M + H]^+$ TPGWamide
413.9	Loss of CO (28 Da) for *m/z* 441.9
411.9	Loss of C-terminal NH_2 (17 Da) from $[M + H]^+$ APGWamide
383.9	Loss of CO (28 Da) for *m/z* 411.9
357.9	$[M + H]^+$ for PGW
340.9	Loss of C-terminal NH_2 (17 Da) from $[M + H]^+$ PGW
260.8	$[M + H]^+$ for GWamide
243.7	Loss of C-terminal NH_2 (17 Da) from $[M + H]^+$ Gwamide
215.7	Loss of CO (28 Da) for *m/z* 243.7
158.6	Immonium ion of W

contained, for APGWamide, TPGWamide, and GWamide a peak corresponding to the *m/z* value of the monoprotonated form less 17 Da (*m/z* 244 for GWamide; *m/z* 412 for APGWamide; *m/z* 442 for TPGWamide). These fragments are clearly C-terminal desamidated ions. The identified peptide fragment ions observed in the MS/MS analysis are listed in Table 1.

The results of the peptide mapping performed for the different tissues, and summarized in Table 2, showed, as observed in gastropods and bivalves, that TPGWamide and GWamide were exclusively located in the CNS, and APGWamide in both CNS and NE. RPGWamide and KPGWamide, which were detected in peripheral organs of gastropods and bivalves, were not detected in any tissue of *S. officinalis*. In the neurohemal area, as well as in the hemolymph, no APGWamide-related peptides were detected.

3.2. Comparative in vitro processing of APGWamide-related peptides into GWamide in the optic lobe

The 1 min incubations of each of the four synthetic APGWamide tetrapeptides with optic lobe extract allowed observation of a neo-synthesis of GWamide (Fig. 3). When the four tetrapeptides were thus processed by the optic lobe DPAP, their processing percentages were statistically different. APGWamide was the best substrate with 44.7% processed, compared to only 11.7% for TPGWamide, and 24.3 and 19.3% for RPGWamide and KPGWamide respectively (Table 3). For one tetrapeptide molecule processed, 0.83 molecule of GWamide was produced. The difference of 17% was probably associated with the digestion of the tetrapeptides by other endogenous peptidases released in the optic lobe extract.

Table 2
Tissue mapping of APGWamide-related peptides

	APGWamide	RPGWamide	KPGWamide	TPGWamide	GWamide
Sus-oesophagal mass (CNS)	d	nd	nd	d	d
Sub-oesphagal mass (CNS)	d	nd	nd	d	d
Optic lobes (CNS)	d	nd	nd	d	d
Nerve endings of oviductal gland	d	nd	nd	nd	nd
Veina cava neurohemal area	nd	nd	nd	nd	nd
Hemolymph	nd	nd	nd	nd	nd

The abbreviations d: detected and nd: not detected.

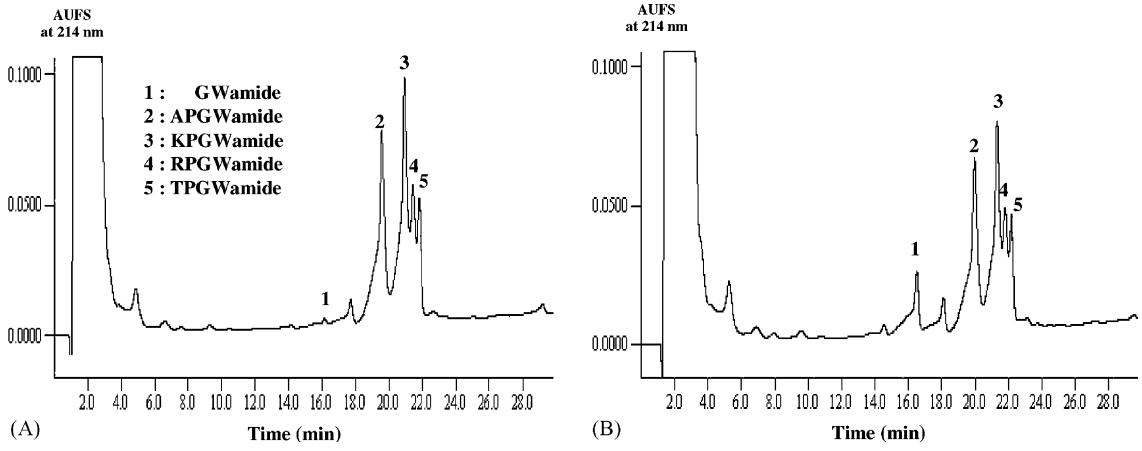

Fig. 3. Dipeptidyl aminopeptidase activity in the optic lobes: processing of APGWamide-related tetrapeptides. (A) A/K/R/TPGWamide + optic lobe extract, no incubation. (B) A/K/R/TPGWamide + optic lobe extract, 1 min incubation at 35 °C. The samples ($n = 8$) were fractionated after incubation by reverse phase chromatography using a Nucleosil C18 column and UV detection at 214 nm.

Table 3
In vitro processing of APGWamide-related peptides into GWamide

	Weight (μg)	Molecules	Processing (%)
GWamide neo-synthetized	0.39	8.89×10^{14}	
APGWamide processed	0.34	4.80×10^{14}	44.7
KPGWamide processed	0.17	2.07×10^{14}	19.3
RPGWamide processed	0.19	2.61×10^{14}	24.3
TPGWamide processed	0.10	1.26×10^{14}	11.7
APGWamide-related peptides processed		10.74×10^{14}	
Processing output		0.83	

4. Discussion

The recent isolation and characterization of the dipeptide GWamide in the mollusk cephalopod *S. officinalis*, as well as the demonstration of its synthesis from the processing of APGWamide by a DPAP, suggested the occurrence of APGWamide-related tetrapeptides in this mollusk [5]. MALDI-TOF analysis performed in a previous study did not allow the detection of any of these tetrapeptides in the CNS of *Sepia* [5]. In the present paper, by the use of LC-ESI-MS/MS, two tetrapeptides related to APGWamide, together with the cephalopod dipeptide, were detected in different parts of the CNS and in the NE which innervate the distal oviduct. APGWamide appeared to be located both in CNS and in NE, whereas TPGWamide and GWamide were located only in the CNS.

The occurrence of APGWamide and TPGWamide confirmed the expression of the two genes as described for *L. stagnalis* and *M. edulis* [6]. The differential distribution of APGWamide-related peptides showed that these neuropeptides were probably able to target various central and peripheral tissues. Moreover, the hypothesis of blood transport can be discarded in view of the results obtained from the veina cava neurohemal area and the hemolymph, where the APGWamide-related peptides were not detected.

The experiments on in vitro digestion by optic lobe extract showed that the four tetrapeptides can be processed into GWamide but not with the same efficiency as reflected by their calculated processing percentages for 1 min incubations: APGWamide (44.7%) $\ggg$ RPGWamide (24.3%) $>$ KPGWamide (19.3%) $\gg$ TPGWamide (11.7%).

The aminopeptidase responsible for the post-translational maturation of APGWamide-related peptides is a DPAP able to cleave the peptide bonds involving the cyclic amino acid proline located in the N-terminal penultimate position. The activity of these particular aminopeptidases is influenced by the nature of the N-terminal amino acid. Indeed, the hydrophobic and basic amino acids are preferred [8]. Therefore, the processing percentage observed for each APGWamide-related peptides can be directly related to the nature of the N-terminal amino acid. Alanine, an hydrophobic amino acid, and the basic amino acids arginine and lysine, are responsible for the high level of processing of APGWamide, RPGWamide, and KPGWamide, whereas threonine, a neutral and hydrophilic amino acid, provides some protection for the TPGWamide.

The values of processing percentage obtained from the HPLC analysis (Table 3) suggested that DPAP digestion occurred only for the *Mytilus* gene products, RPGWamide, KPGWamide and TPGWamide. The best DPAP substrates, RPGWamide and KPGWamide, were not detected by MS/MS as if they were totally processed into GWamide (though perhaps at trace levels for the RPGWamide in the sub-oesophagal mass).

Similar post-translational maturations by DPAP have been reported for peptides such as melittin, a lytic peptide of honeybee venom [4], the antibacterial proline-rich peptides isolated from honeybee hemolymph [1], the skin peptides of Amphibians such as caerulein [11], xenopsin [13] and leviticide [10]. In addition to allow the maturation of regulatory propeptides, the DPAP could be involved in the degradation pathways. Degradation mechanisms have been described for Substance P peptide, which can be subjected to the sequential liberation of two N-terminal dipeptides without loss of its biological activity at the tachykinin receptors [9]. If the truncated substance P conserved its biological properties, the release of the N-terminal X-Pro makes possible its degradation by classical aminopeptidases. Thus, we can suspect that the DPAP activity observed in the *S. officinalis* nervous system is the first step of the inactivation pathway of APGWamide-related peptides, as described previously for Substance P. The cleavage of the N-terminal X-Pro led to a biologically active dipeptide GWamide, one of the more active APGWamide-related peptides, which can be further hydrolyzed by most aminopeptidases whereas the C-terminal amidation enables a carboxypeptidase cleavage.

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References

[1] Casteels P, Ampe C, Jacobs F, Vaeck M, Tempst P. Apidaecins: antibacterial peptides from honeybees. EMBO J 1989;8: 2387–91.
[2] De Lange RPJ, Van Golen FA, Van Minnen J. Diversity in cell specific co-expression of four neuropeptide genes involved in control of male copulation behaviour in *Lymnaea stagnalis*. Neuroscience 1997;78:289–99.
[3] Favrel P, Mathieu M. Molecular cloning of a cDNA encoding the precursor of Ala-Pro-Gly-Trp amide-related neuropeptides from the bivalve mollusc *Mytilus edulis*. Neurosci Lett 1996;205:210–4.
[4] Habermann E. Bee and wasp venoms (Review). Science 1972;177:314–22.
[5] Henry J, Favrel P, Boucaud-Camou E. Isolation and identification of a novel Ala-Pro-Gly-Trp-amide-related peptide inhibiting the motility of the mature oviduct in the cuttlefish, *Sepia officinalis*. Peptides 1997;18:1469–74.
[6] Henry J, Zatylny C, Favrel P. HPLC and electrospray ionization mass spectrometry as tools for the identification of APGWamide-related peptides in gastropod and bivalve mollusks: comparative activities on *Mytilus* muscles. Brain Res 2000;17:162–70.
[7] Li KW, Smit AB, Geraerts WPM. Structural and functional characterization of neuropeptides involved in the control of male mating behavior of *Lymnaea stagnalis*. Peptides 1992;13:633–8.
[8] Mentlein R. Dipeptidyl-peptidase IV (CD26)—role in the inactivation of regulatory peptides (Review). Regul Peptides 1999;30:9–24.
[9] Mussap CJ, Geraghty DP, Burcher E. Tachykinin receptors: a radioligand binding perspective (Review). J Neurochem 1993;60:1987–2009.
[10] Poulter L, Terry AS, William DH, Giovanini MG, Moore CH, Gibson BW. Levitide, a neurohormone-like peptide from the skin of *Xenopus laevis*. Peptide and peptide precursor cDNA sequences. J Biol Chem 1988;263:3279–83.
[11] Richter K, Egger R, Kreil G. Sequence of preprocaerulein cDNAs cloned from skin of *Xenopus laevis*. A small family of precursors containing one, three, or four copies of the final product. J Biol Chem 1986;261:3676–80.
[12] Smit AB, Jiménez CR, Dirks RW, Croll RP, Geraerts WPM. Characterization of a cDNA clone encoding multiple copies of the neuropeptide APGWamide in the mollusk *Lymnaea stagnalis*. J Neurosci 1992;12:1709–15.
[13] Sures I, Crippa M. Xenopsin: the neurotensin-like octapeptide from *Xenopus*skin at the carboxyl terminus of its precursor. Proc Natl Acad Sci USA 1984;81:380–4.
[14] Van Golen FA, Li KW, De Lange RPJ, Van Kesteren RE, Van Der Schors RC, Geraerts WPM. Co-localized neuropeptides conopressin and ALA-PRO-GLY-TRP-NH_2 have antagonistic effects on the vas deferens of *Lymnaea*. Neuroscience 1995;69:1275–87.