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The SepOvotropin: A New Ovarian Peptide Regulating Oocyte Transport in *Sepia officinalis*

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In the cuttlefish *Sepia officinalis*, the successive steps of egg laying are controlled by multiple neuropeptides. Recent experiments led us to suppose that there was possible involvement of a second regulation pathway by the release of ovarian regulatory peptides in the genital tract. Using HPLC fractionation and an *in vitro* biological test, a C-terminal amidated peptide modulating the motility of the *Sepia officinalis* oviduct was isolated from an extract of vitellogenic ovarian follicles. The mass of this peptide as determined by MALDI-TOF (1501.8 Da) and analysis by Edman degradation led to the following sequence: Pro-Lys-Asp-Ser-Met-Leu-Leu-Leu-Gln-Val-Pro-Val-Tyr-amide. The peptide mapping performed by LC/MS revealed a distribution restricted to the follicles, the full grown oocytes and the eggs. This new peptide, called SepOvotropin, modulated contractions of the whole genital tract in physiological conditions from a threshold concentration between 10^{-20} and 10^{-19} M, demonstrating for the first time the occurrence of a specific peptidergic control of egg-laying in cephalopods. © 2000 Academic Press

Key Words: **mollusc; cephalopod; *Sepia officinalis;* HPLC; LC/MS; peptide; ovary; oviduct, myotropine; egg-laying.**

If the peptidergic control of reproduction has been fully investigated in gastropods [1, 5] the modality of this regulation in cephalopods is not yet elucidated, whereas the reproductive behavior is clearly stereotyped and described [6]. Nevertheless, recent investigations in *Sepia officinalis* has allowed the identification of peptidic and non peptidic factors involved in the regulation of the first step of egg-laying: the transport of full grown oocytes in the genital tract of the mature females. In addition, a large screening of organs potentially involved in the control of this physiological mechanism, has been performed using an *in vitro* bioassay. The purification by successive steps of HPLC was monitored using a myotropic bioassay based on the recording of the oviduct contractions. This experimental approach led to the isolation and the characterization of neuropeptides related to APGWamide and FMRFamide from optic lobes [7, 8], to the purification of 5-HT from full grown oocytes (smooth oocytes) [9] and the identification of a waterborne pheromonal peptide from eggs [10].

In this paper, the oviduct bioassay was used to investigate the peptide content of vitellogenic follicles putatively involved in the regulation of egg-laying. An original experimental approach was carried out to specially select C-terminal amidated peptides.

MATERIALS AND METHODS

Animals. All cuttlefish were captured in the Western English Channel between January and June by trawling. They were maintained in 1000-liter outflow tanks at the Marine Station of Luc sur Mer (University of Caen, France).

Myotropic bioassay. The myotropic bioassay was performed using different organs. The genital tract including the proximal oviduct containing full grown oocytes, the oviducal gland and the distal oviduct, the isolated distal oviduct, the ovarian stroma, the esophagus, and the arm were dissected from mature females. Each organ was suspended from a displacement transducer (Phymep, Bionic Instruments) connected to a computer controlling the recorder and the DATAC (Dispositif d'Acquisition et de Traitement Automatique de la Contraction). The muscle chamber was perfused at a flow rate of 0.5 ml · min^{-1} with synthetic seawater (Instant Ocean) containing 1 mM glucose and maintained at 15°C. The HPLC fractions were injected in the perfusing flow using a three-way valve in order to avoid mechanical and thermal stress. The flow of the fractions into the muscle chamber was traced by addition of phenol red (1 μM).

Recovery of material from tissues and seawater. For HPLC purification, 200 g of vitellogenic follicles dissected from 10 females were homogenized in 1000 ml of HCl 0.1 N at 100°C and centrifuged 20 min at 30,000*g.* The supernatants were concentrated on C18 Sep-Pak cartridges. For the LC/MS analysis, follicles, full grown oocytes (smooth oocytes) and eggs were extracted as described above. Moreover, the molecules released by 50 full-grown oocytes in 20 ml of

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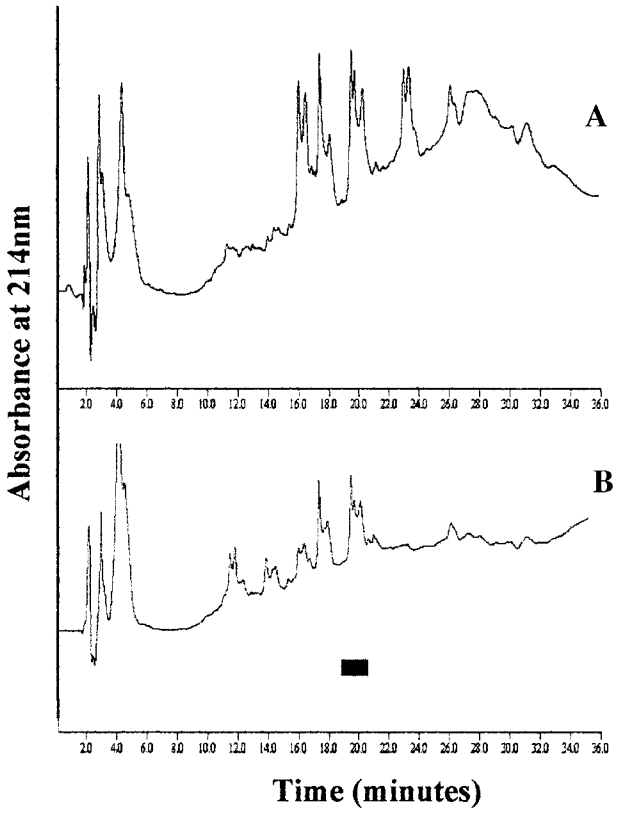

FIG. 1. First purification step of vitellogenic follicles extract (A) without carboxypeptidase A incubation and (B) with carboxypeptidase A incubation, onto a C18 column. The black line indicates myotropic fractions.

seawater or by 1000 just spawned eggs in 1 liter of seawater, during 10 h of incubation, were concentrated on a chromafix C18 column.

Amidated peptides selection. Dry Sep-Pak eluates of vitellogenic follicles were rehydrated in 10 ml of phosphate buffer saline (NaCl 136 mM, KH_2PO_4 14.7 mM, Na_2HPO_4 101 mM, KCl 26.8 mM, pH 7.6, cleaned on a Sep-Pak to avoid organic contamination) and incubated with 10 units of carboxypeptidase A (Sigma, chromatographically purified from bovine pancreas, 200–400 units per mg protein) at 37°C for 60 min. The incubation was stopped in ice by the addition of 2 volumes of cold acetic acid 1 N. After centrifugation, the supernatant was fractionated by HPLC.

High performance liquid chromatography purification. HPLC analysis was performed with a Varian 4050 integrator connected to a Varian 9012 solvent delivery system and a Varian 9050 wavelength UV–VIS detector set at 214 nm. The supernatant obtained after carboxypeptidase incubation was injected onto a C18 column (Macherey-Nagel, 250 × 4.6, 5 μm) with an acetonitrile linear gradient of 2% per minute in TFA 0.1% at the flow of 1 ml/min. Fractions of one minute were collected and tested using the oviduct bioassay. The bioactive fractions eluted at 19 and 20 min were injected onto the column used for the first step with an acetonitrile linear gradient of 1.33% per minute in ammonium acetate 25 mM. The bioactive 29-min fraction was injected onto a nucleosil C4 column (Macherey-Nagel, 250 × 4, 5 μm) with an acetonitrile linear gradient of 0.3% per minute from 24 to 33% of acetonitrile.

MALDI-TOF analysis. The mass spectrometry analysis was performed using a Micromass TOF SPEC-E. The apparent pure peak containing the activity, with a 15.3-min retention time were concentrated and resuspended in 10 μl of 1:1 acetonitrile/01% TFA solution. One microliter of sample was mixed with an equal volume of the MALDI matrix prepared as follows: 10 mg of α-cyano-4-hydroxy cinnamic acid dissolved in 1 ml of a 1:1 acetonitrile/0.1% TFA solution. The sample (1 μl) was spotted into the wells of the MALDI and air-dried in a vacuum chamber prior to submission to multiple shots from the nitrogen laser (337 nm). The peptide of interest was identified in the spectrum by its monoprotonated form $[M + H]^+$.

Sequence analysis. N-terminal sequence analyses were performed using an Applied Biosystems Model 477A protein sequencer, and amino acid phenylthiohydantoin derivatives were identified and quantitated on-line with a Model 120A HPLC system, as recommended by the manufacturer.

Biactivity of SepOvotropin. Aliquots of synthetic SepOvotropin (PKDSMLLLQVPVYamide) (Eurogentec) from 10^{-22} to 10^{-10} M were tested using the oviduct bioassay in order to obtain qualitative and quantitative data regarding their myotropic activity. Moreover, to verify the specificity of SepOvotropin, aliquots from 10^{-14} to 10^{-5} M were tested on different muscles. Each aliquot was resuspended in 100 μl of perfusion liquid and immediately injected as described above.

SepOvotropin mapping by LC/ESI-MS. The Liquid Chromatography/ElectroSpray Ionization Mass Spectrometry (LC/ESI-MS)

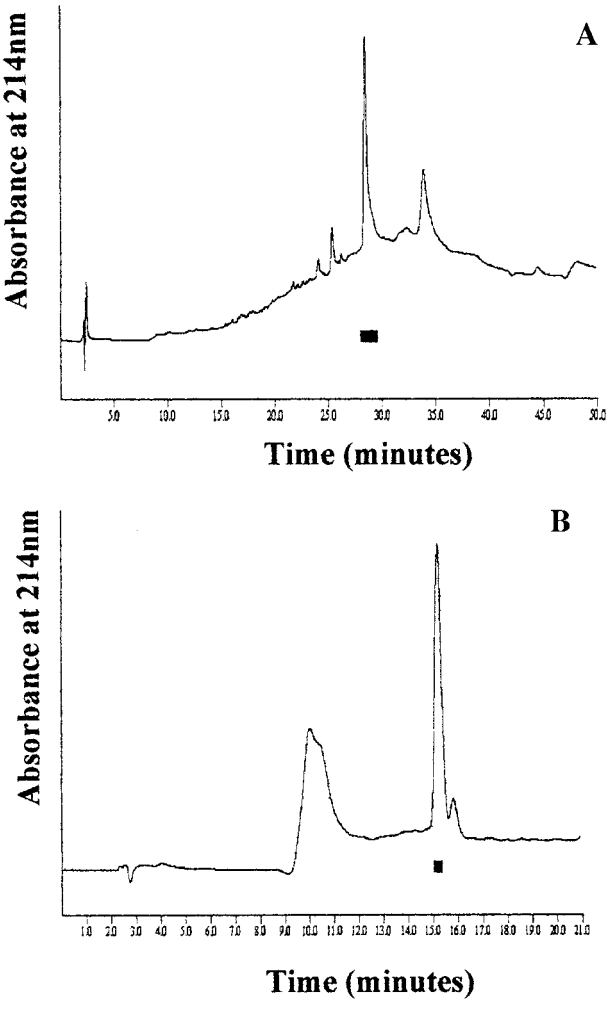

FIG. 2. (A) Second step of purification of myotropic fraction onto a C18 column. (B) Third step of purification of myotropic fraction onto C4 column. The black line indicates myotropic fractions.

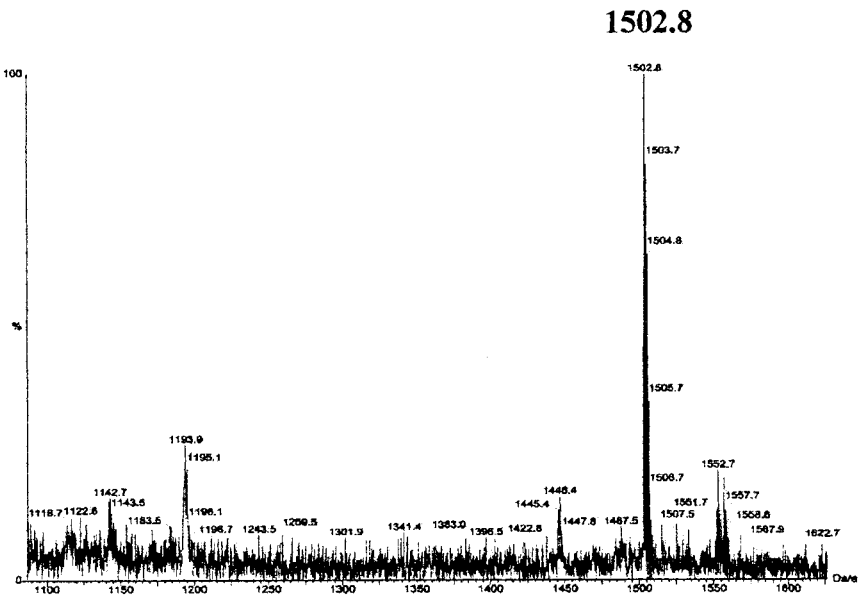

FIG. 3. MALDI-TOF spectrum of the myotropic activity containing peak obtained after three steps of purification.

analysis was performed on an Alliance HPLC (WATERS) connected to the platform LCZ (MICROMASS) which is a single quadrupole analyser. The LC/MS was controlled by the Masslynx NT software. The organic fraction of each extract was injected onto a C18 column (Macherey-Nagel, 250 × 4.6, 5 μm) with an acetonitrile linear gradient of 1.33% per minute in TFA 0.1%, with a flow rate of 1 ml/min. The outlet of the LC column was connected to the ESI interface with Zspray technology, using splitting of 750 μl/min. The MS data were acquired in scan mode considering the positive ion signal. The analysis was performed with a cone voltage of 55 V to obtain the monoprotonated molecule $[M + H]^+$ and the doubly protonated molecule $[M + 2H]^{2+}$.

Threshold of MS sensibility. The threshold of MS detection (Finnigan LCQ^{deca}) was checked with increasing concentrations of synthetic SepOvotropin from 10^{-22} to 10^{-10} M diluted in methanol/water (50:50) (v/v).

RESULTS

HPLC Purification

The C-terminal amidation of bioactive peptides is a protection against carboxypeptidase digestion. Then, the carboxypeptidase digestion of the extract isolated the C-terminal amidated peptides, simplifying the purification by decreasing the number of purification steps. The 1-min HPLC fractions of the carboxypeptidase digested extract (Fig. 1) were tested on the oviduct bioassay. After the first separation, a stimulating activity was then detected in the 19- and 20-min fractions. After two further steps of purification (Fig. 2), the activity was concentrated in an apparently pure peak with a retention time of 15.3 min (Fig. 2B).

MALDI-TOF Analysis and Sequence Determination

The 15.3-min HPLC fraction containing the myotropic activity was submitted to MALDI-TOF analysis. The spectrum revealed a main peak at *m/z* 1502.8 (Fig. 3). The primary sequence of this peptide was determined by Edman degradation: PKDSMLLLQVPVY. The *m/z* measured in MALDI-TOF and the amino acid sequence confirmed the C-terminal amidation of the peptide. Thus, the complete sequence is: PKDSMLLLQVPVY-amide. This new regulatory peptide was called SepOvotropin.

Bioactivity of SepOvotropin

The synthetic peptide was tested on several distal oviducts dissected from females at different stages of sexual maturation. The SepOvotropin had no effect on the oviduct of immature cuttlefish. At 10^{-5} M, it increased the tonus of the oviduct of cuttlefish in vitellogenesis. At 10^{-11} M (Fig. 4B), the synthetic SepOvotropin stimulated the spontaneous contractions of the oviduct of mature cuttlefish as observed during the purification steps (Fig. 4A). At the concentration of 10^{-10} M, the SepOvotropin provoked a cyclic contraction on the mature oviduct which can last for 24 h before recovering a basal activity.

To reproduce the physiological conditions, the SepOvotropin was tested on the whole genital tract of a mature cuttlefish: proximal oviduct containing full grown oocytes, oviducal gland and distal oviduct. From a threshold between 10^{-20} and 10^{-19} M, the SepOvotropin inhibited the contraction of this tract decreasing the amplitude and the tonus of the contractions (Fig. 5).

The specificity of SepOvotropin was verified on multiple muscles as shown in Table 1. The SepOvotropin stimulated esophagus and ovarian stroma only in

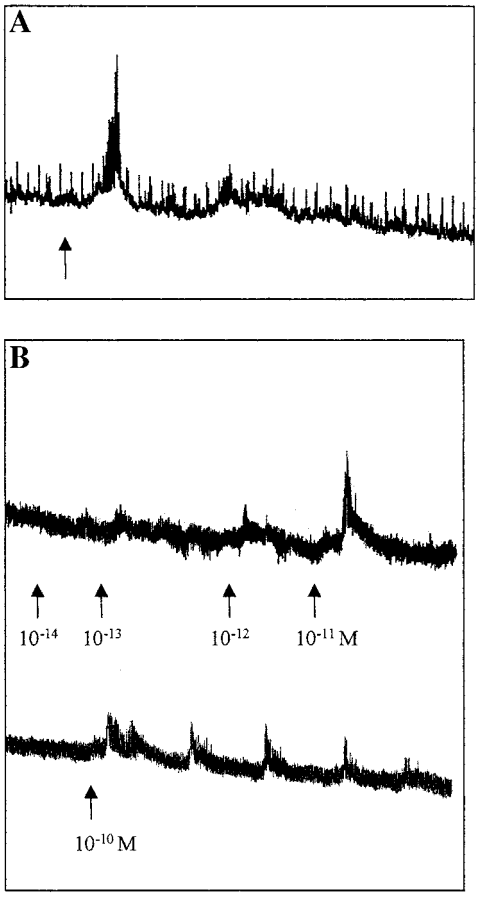

FIG. 4. SepOvotropin-induced contraction of the cuttlefish oviduct: (A) The effect of endogenous SepOvotropin purified, (B) effect of increasing concentrations of synthetic SepOvotropin.

supra-physiological concentrations. Sepovotropin had no effect on the other muscles tested.

SepOvotropin Mapping by LC/ESI-MS

A SepOvotropin mapping was performed by means of LC/ESI-MS. In the Fig. 6A, we can observe the TIC (Total Ion Current) obtained from a follicular extract. The EIC (Extracted Ion Chromatogram) based on the mass of the SepOvotropin double charged ion allowed localisation of a peak at 28.9 min (Fig. 6B), identical to the retention time of synthetic SepOvotropin. The mass spectrum extracted from this peak (Fig. 6C) revealed the occurrence of a predominant double charged ion $[M + 2H]^+$ (*m/z* 753.09) and a single charged ion $[M + H]^+$ (*m/z* 1502.73). The ion mass expected were 752.9 for the double-charged and 1502.8 for the single-charged ion. The occurrence of these two ions at the retention time of the synthetic SepOvotropin on the C18 column confirmed the localization of this peptide in the follicle extract. The LC/ESI-MS analysis performed as described above, revealed the occurrence of SepOvotropin in the full-grown oocytes and in the eggs (Table 2). However the SepOvotropin was not detected in the seawater used for incubation of full grown oocytes or eggs.

Sensibility of MS Detection

The threshold of MS detection for the SepOvotropin was 10^{-10} M. Thus, detecting a concentration of 10^{-20} M would imply a 10^{10}-fold increase of the amount of starting material as well as of the volume of incubation medium, which is, of course impossible.

DISCUSSION

A new ovarian peptide isolated on the basis of its biological activity was characterized in the cuttlefish *Sepia officinalis*. This peptide, called SepOvotropin, did not show any sequence homology with known peptides. Numerous ovarian peptides were already identified in Invertebrates. The SepOvotropin is the first ovarian bioactive peptide characterized in mollusks. In insects, the hexapeptide NPTNLH (Neb TMOF) isolated from vitellogenetic ovaries, exerting folliculostatic and trypsin modulating activity, inhibited ecdysone biosynthesis [10]. The purification and identification of a major ovary component which displayed sequence similarities with a new class of glycine- and proline-rich protein isolated in plants was established in the locust *Schistocerca gregaria* [11]. Moreover the ovary of this insect contained the SGPIP (*Schistocerca gregaria* protease inhibitor protein), a molecule probably involved in insect development [12]. In the echinoderms, DNA clone of the egg peptide speract, which is a chemoattractant increasing spermatozoa motility, was isolated from ovary cDNA library [13].

The multiple targets screened with synthetic SepOvotropin for a wide range of concentrations, clearly demonstrated that the genital tract was the main target in physiological conditions. Even if the SepOvotropin was able to modulate the contractions of the whole genital tract from 10^{-19} M and from 10^{-12} M for isolated oviduct, for organs like esophagus and ovarian stroma, a supra physiological concentration had to be applied to trigger a contractile response. In addition, the tissue specificity of this peptide was associated with a temporal specificity correlated with the sexual stage of the females. Applied on previtellogenic genital

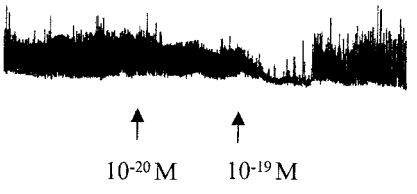

FIG. 5. SepOvotropin-induced contraction of the cuttlefish whole genital tract for 10^{-20} and 10^{-19} M.

TABLE 1

Bioactivity of SepOvotropin

Muscle	Myotropic activity	Threshold concentration
Whole mature genital tract	Inhibition	10^{-19} M
Mature distal oviduct	Stimulation	10^{-12} M
Ovarian stroma	Increase of tonus	10^{-6} M
Esophagus	Stimulation	10^{-6} M
Arm muscular fibers	No activity	—

TABLE 2

LC/ESI-MS Analysis

	Occurrence of SepOvotropin
Follicles	Yes
Full grown oocyte	Yes
Egg	Yes
Incubation seawater with full-grown oocytes	Not detected
Incubation seawater with eggs	Not detected

tract, the SepOvotropin was not able to induce any response demonstrating the absence of receptors. A response was observed at the end of vitellogenesis when the first full grown oocytes were released in the genital coelome.

Among the peptides characterized in invertebrates, the SepOvotropin was the first ovarian peptide modulating the motility of genital tract with a concentration threshold so low. In insect, the threshold of proctolin analogues, which modulated locust oviduct, did not exceed picomolar concentration [14].

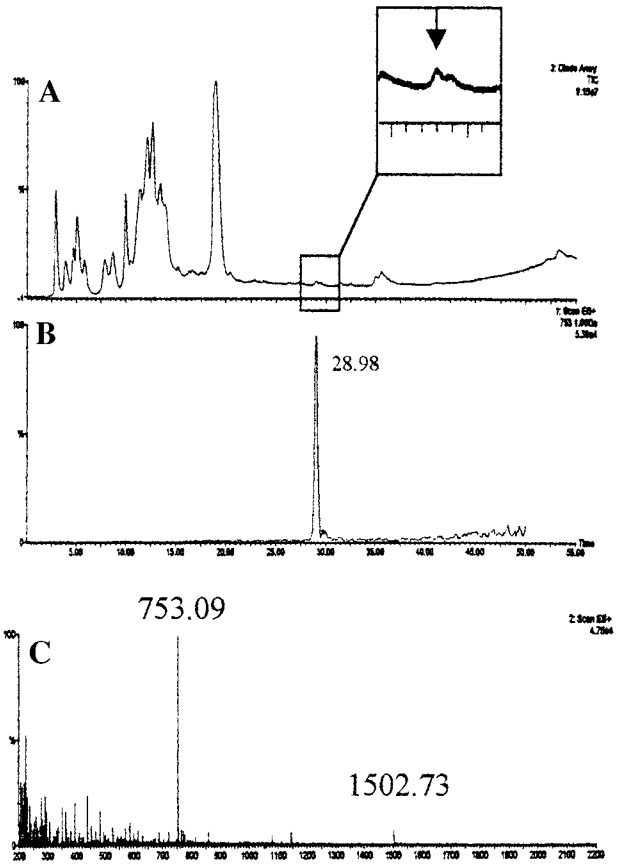

FIG. 6. LC/ESI-MS analysis. (A) Total ion current. (B) Extracted ion chromatogram based on the mass of the SepOvotropin double charged ion. (C) Extracted mass spectrum of the 28.9-min peak. Endogen SepOvotropin was detected at *m/z* 753.09 $[M + 2H]^+$ and *m/z* 1502.73 $[M + H]^+$.

LC/MS analysis used to accurately map the tissues peptide revealed the occurrence of SepOvotropin in the vitellogenic follicles, in the full grown oocytes and in the eggs suggesting a probable expression localized in the follicular cells and/or in the oocyte of the vitellogenic follicles. Associated to this localization, the very low threshold of activity observed for the SepOvotropin suggests a possible pheromonal activity, although the release of this peptide in the external environment had not been established by the LC/MS analysis. Nevertheless, as the SepOvotropin appeared to be able to trigger a response of the whole genital tract from a concentration between 10^{-20} and 10^{-19} M, we cannot rule out the fact that the technique used for the detection of the peptide putatively released from eggs was not sensitive enough, even after a concentration step onto Sep-Pak C18 cartridges. Indeed, even if the LC/MS is a very sensitive approach to detect peptides in a complex mixture, the detection of the SepOvotropin, starting for a concentration of 10^{-20} M, presuppose a concentration step impossible to achieve.

Anyway, the behavior of mature adults during reproduction (1) massive gathering on egg laying areas, (2) mating and fertilization of females, (3) egg laying of multiple females on the same support suggests the existence of chemical messengers released in the environment which are able to induce modifications in the behavior and in the contractile activity of the genital tract. A waterborne pheromonal peptide released in the seawater from egg mass and modulating oviducal contractions has been recently characterized in *Sepia officinalis* [10]. A similar peptide was identified in the marine gastropod *Aplysia californica*. This peptide called attractin was released from egg cordons provoking the gathering of mature adults for reproduction [15]. The SepOvotropin could be one of these chemical messengers involved in the gathering of mature adults and in egg-laying.

In addition to a possible pheromonal activity, the occurrence of SepOvotropin in the full grown oocytes which were transported by the oviduct until to be released in the mantle cavity, suggested a second regulation pathway. The SepOvotropin could be released as a paracrin factor by the full-grown oocytes in the lumen of the genital tract as described for 5-HT in the same

conditions [9] and for the water-borne peptide ILME [10]. This release had not been demonstrated by LC/MS analysis of seawater containing full grown oocytes. Nevertheless, we can suspect that the environment of oocytes in the genital tract: acid pH and mechanical pressure, may play an important role to induce the release of the peptide in the lumen of oviduct.

The effective action of SepOvotropin on egg-laying appeared to be very complex to investigate by *in vitro* experiments, especially this peptide was able to act both as a paracrin factor and a waterborne pheromone.

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