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Egg capsule secretion in invertebrates: a new ovarian regulatory peptide identified by mass spectrometry comparative screening in *Sepia officinalis*

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Abstract

Mass spectrometry comparative screening was used to identify ovarian regulatory peptides involved in the successive steps of egg-laying in the cuttlefish *Sepia officinalis*. The peptide content of full-grown oocytes (FGO) was compared with that of oocyte-conditioned medium, which resulted in the detection of peptides that were present in both samples. These peptides, which are suspected of being released by the oocyte in the genital tract, were submitted to a structural analysis. This strategy led to the characterization of a new ovarian regulatory peptide (EISLDKD) able to inhibit the contractions of the whole female genital tract and of the main nidamental glands (MNG). As EISLDKD appeared to be the first regulatory peptide directly involved, at physiological concentrations, in the secretion of the egg capsule by the main nidamental glands, it was named SepCRP for *Sepia* Capsule Releasing Peptide. Mass spectrometry analysis clearly demonstrated that SepCRP was expressed during vitellogenesis by the ovarian follicles and released by the FGO in the lumen of the female genital tract. In association with the ovarian 5-HT, SepCRP would be responsible for the storage of FGO to avoid the spawning of unfertilized oocytes before mating. Released by the distal oviduct in the mantle cavity, SepCRP probably in association with a cocktail of ovarian regulatory factors targets the MNG to regulate the egg capsule secretion. Thus, the ovary appeared to be one of the main sources of regulatory peptides involved in the successive steps of egg-laying in the cephalopod mollusk *S. officinalis*.

Keywords: Egg capsule secretion; Egg-laying; Ovarian regulatory peptide; Myotropin; *Sepia officinalis*; Cephalopod; Invertebrate; Mass spectrometry

In the English channel cuttlefish, *Sepia officinalis*, the reproductive season takes place in the second and last year of the life cycle, between February and June. During this period, the successive steps of egg-laying are clearly stereotyped. Following previtellogenesis which is characterized by an intense phase of cell multiplication, the follicular syncytium synthesizes the yolk proteins leading to the growth of the oocyte. The first step of egg-laying is the release by the ovary of full-grown oocytes (FGO) in the genital coelom [1]. This period is characterized by the release of ovarian 5-HT synthesized in the FGO and responsible for the inhibition of oviducal contractions [2]. The ovarian 5-HT inhibits oocyte transport of the unmated mature females and leads to the accumulation of FGO in the genital coelom. The mating is probably one of the main events which trigger the egg-laying. The oocyte transport performed by the oviduct contractions is also modulated by neuropeptides such as FMRFamide related Peptides (FarPs) and APGWamide related Peptides [3,4] in association with ovarian peptides such as ILME and SepOvotropin [5,6]. During their transport, and before they are released into the mantle cavity by the contractions of the distal oviduct, the oocytes receive a first gelatinous envelope secreted by the oviducal gland. A second egg encapsulation is performed by the secretions of the nidamental

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glands (MNG) [7]. The embedded oocytes are forced into the funnel tube and fertilized by spermatozoa stored in the copulatory pouch [8]. Finally, the eggs are attached to the substrate until hatching. After the first egg-laying, the genital coelom and the main nidamental glands are usually empty. Some females are then able to reconstitute the egg capsule products as well as produce a new batch of oocytes which allows for the occurrence of multiple spawning [9].

The aim of this study was to characterize a peptidic factor released by the oocytes and involved in at least one step of egg-laying. The identification of the peptide was based on a comparative screening performed in microLC–ESI-MS/MS on full-grown oocytes and oocyte-conditioned seawater. The peptides that were detected in the two samples were then suspected to be expressed by the oocytes and released to target organ tissues involved in the successive steps of egg-laying.

Similar factors have been identified in marine invertebrates. In sea urchins, sperm attracting or activating peptides (SAPs) are released by the oocytes to facilitate fertilization [10–17].

In *S. officinalis*, we have recently characterized two ovarian regulatory peptides involved in oocyte transport and sperm attraction which are released by the oocytes and/or the eggs [5,17]. The approach used, which consisted of associating a mass spectrometry screening with a specific myotropic bioassay, appeared to be a powerful strategy to identify regulatory peptides controlling egg-laying and egg capsule secretion.

Materials and methods

Animals. All the cuttlefish were trapped in the Baie de Seine between January and June. They were maintained in 1000-liter outflow tanks at 15 ± 1 °C at the Marine Station of Luc sur Mer (University of Caen, France) under a natural photoperiod.

Recovery of material from tissues and seawater. For HPLC purification and microLC–ESI-MS/MS analysis, 600 g of FGO was homogenized in 10 volumes of HCl 0.1 N at 100 °C and centrifuged for 30 min at 35,000*g* at 4 °C. The supernatants were concentrated on Chromafix C18 cartridges. For the microLC–ESI-MS/MS analysis, previtellogenic follicles, vitellogenic follicles, and eggs were extracted as described above. Moreover, the molecules released from 50 full-grown oocytes in 20 ml of synthetic filtered seawater were concentrated after various incubation times on Chromafix C18 cartridges.

MicroLC–ESI-MS/MS analysis. Analyses were performed with a HPLC Surveyor chain connected on-line to an orthogonal electrospray source (Deca XP MS-n Thermofinnigan) operated in the positive electrospray ionization mode (ESI+). The ions were focused into an ion trap, capable of MS and MS/MS analyses. The mass spectra were acquired during 35 ms from m/z 300 to 2000. The capillary exit of the electrospray ion source was set at 70 V, the octapole at 3 V, and the capillary temperature at 200 °C. A counter flow of nitrogen was used as nebulizing gas. Xcalibur data system was used to acquire the data, which were further processed with Turbo Sequest data system. The organic fraction of each extract was resuspended in 10 µl of 0.1% formic acid in water and injected onto a C18 Thermo Hypersil column (0.5×50 mm, 3 µm) with an acetonitrile linear gradient of 3%/min in formic acid 0.1%, from 2 to 60%. A split ratio of 30:1 was used to perfuse the column at a flow rate of 10 µl/min. HPLC column was rinsed with 90% acetonitrile in 0.1% formic acid between each injection. The MS data were acquired in scan mode considering the positive ion signal.

High performance liquid chromatography purification. The FGO extract was resuspended in 100 µl of 0.1% formic acid in water and injected into a MN 250/4 nucleosil 100-7 C18 column with an acetonitrile linear gradient of 0.36%/min in 0.1% formic acid at a flow rate of 1 ml/min, during 25 min. A split ratio of 100:1 was used to perfuse the electrospray source at a flow rate of 10 µl/min and 1-min fractions were collected.

Biological assay. The myotropic bioassay was performed with several contractile organs. The genital tract (including the proximal oviduct containing FGO, the oviducal gland, and the distal oviduct), the ovarian stroma, the main nidamental gland, and the esophagus were dissected from mature females sacrificed before and after egg-laying. 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 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 fractions into the muscle chamber was traced by addition of phenol red.

Amino acid sequencing. N-terminal sequence analyses were performed using an Applied Biosystems Model 477 A protein sequencer, and amino acid phenylthiohydantoin derivatives were identified and quantified on-line with a Model 120A HPLC system, as recommended by the manufacturer. The amino acid sequence was checked from MS/MS spectrum with the softwares Turbosequest (Thermofinnigan) and MS-Product 1.6.1 (Protein Prospector 3.4.1, University of California).

Bioactivity of EISLDKD. The whole genital tract was perfused with 10 ml FGO conditioned seawater. Moreover, aliquots of a synthetic peptide (Millegen) ranging from 10^{-19} to 10^{-7} M were tested using the oviduct bioassay in order to obtain qualitative and quantitative data about its myotropic activity. To check the specificity of EISLDKD, aliquots from 10^{-15} to 10^{-5} M were tested on different muscles. Each aliquot was resuspended in 100 µl perfusion liquid and immediately injected as described above.

EISLDKD kinetic release from FGO. FGO were displayed in samples of five oocytes in 15 ml of Chromafix C18 filtered seawater. Volumes of 10 ml of seawater were collected by step of 10 min during

Table 1
Counts of m/z 316, 577, and 801 from MS/MS spectra performed from samples of increasing concentrations of synthetic SepCRP

Synthetic peptide (pg)	Counts of m/z 316	Counts of m/z 577	Counts of m/z 801
1	1.2	1.4	7.4
10	1.6	3.2	17.7
100	28.8	41.2	195
1000	204	302	1340
	$y = 1.3205x + 23.235$ $r^2 = 0.9982$	$y = 0.2016x + 2.90$ $r^2 = 0.9983$	$y = 0.2987x + 3.9544$ $r^2 = 0.9988$

1 h and by step of 1 h during 4 h before being concentrated on Chromafix C18 cartridges and analyzed in microLC–ESI-MS/MS as described above.

Dosage of EISLDKD. The quantities of peptide were estimated in MS/MS mode from samples collected from 10 to 300 min and selecting the m/z 819 as the product ion. The relative abundance of the fragment ions SLD-H_2O (m/z 316), SLDK (m/z 577), and EISLDKD-H_2O (m/z 801) was used to establish a relation between the concentration of synthetic peptide and the ion counts obtained from 1-min acquisition time (Table 1).

Results

Mass spectrometry comparative screening

The samples of FGO and full-grown oocytes conditioned seawater were submitted to LC/MS analysis. The total ion chromatograms (TIC) revealed several common peaks. We focused on the peak eluted between 15.5 and 18.9% of acetonitrile corresponding to the single

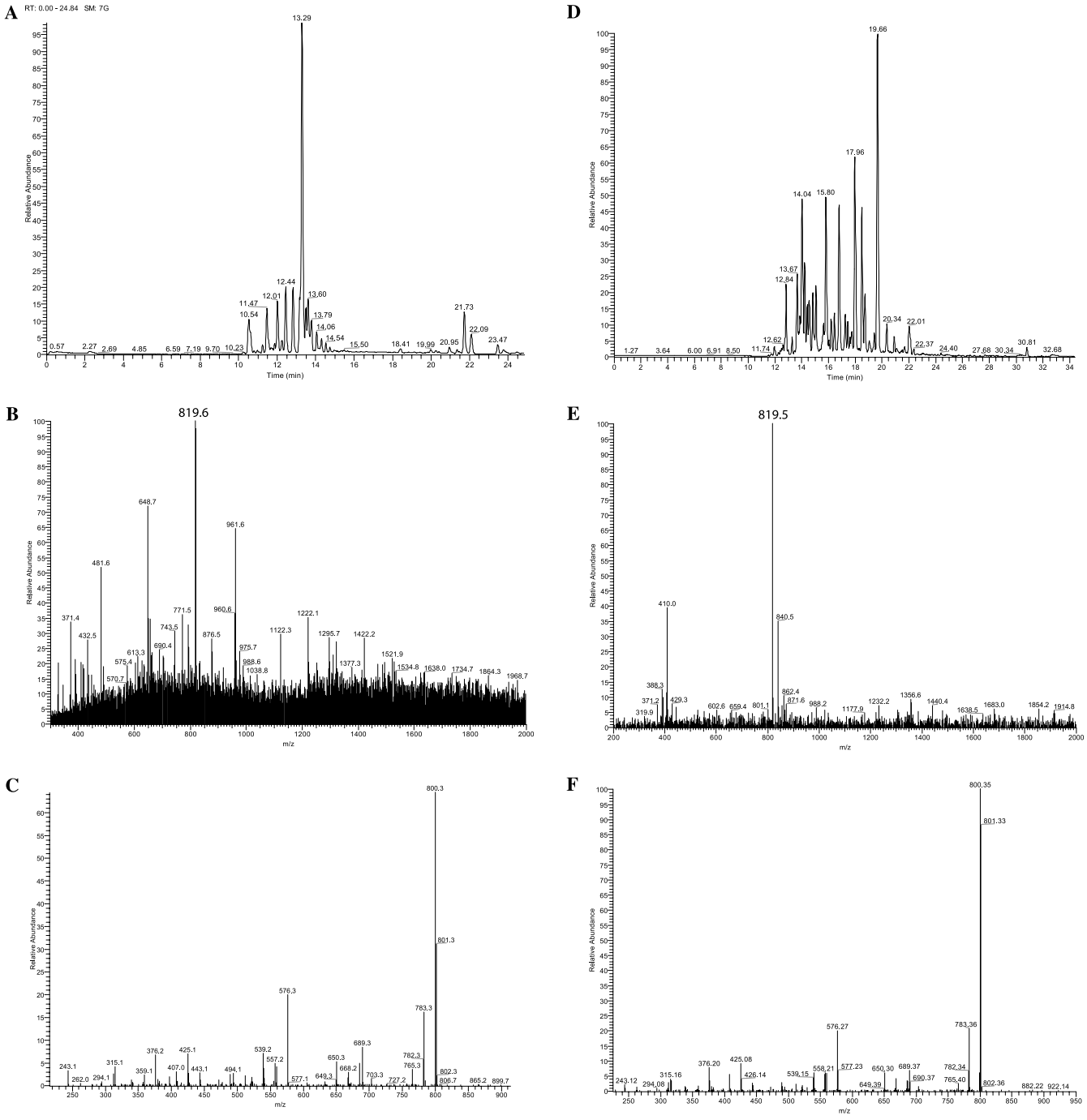

Fig. 1. Ionic chromatograms showing the mass spectrometry comparative screening between full-grown oocytes conditioned seawater (A) and full-grown oocyte extract (D). MS spectra of the two samples for 12.84-min retention time (B,E). MS/MS spectra of the m/z 819 from full-grown oocytes conditioned seawater (C) and full-grown oocyte extract (F).

charged ion at m/z 819 ± 0.5. The similarity of MS/MS spectrum obtained from the two samples (Fig. 1) clearly demonstrated that the two single charged ions at m/z 819 corresponded to the same peptide.

HPLC purification

The purification was performed in microLC–ESI-MS/MS on the basis of the MS data. The 1-min HPLC fractions were tested on the in vitro bioassay. The activity was concentrated in the apparently pure peak for a retention time of 16.34 min (Fig. 2) containing the single charged ion at m/z 819 ± 0.5 detected by the comparative screening described above. This peak eluted in the fractions 16 and 17 was concentrated in order to be submitted to Edman degradation.

Sequence determination

The primary sequence of this peptide was determined by Edman degradation: EISLDKD. The confrontation of MS/MS data obtained from synthetic and endogenic peptide confirmed the amino acid sequence (Fig. 3) and the absence of C-terminally amidation. The apparent molecular weight (818.5 ± 0.1 Da) was very close to the calculated molecular weight (818.41 Da).

Bioactivity of EISLDKD

Increasing concentrations of synthetic peptide were tested on the whole genital tract of a mature female cuttlefish (proximal oviduct containing vitellogenic oocytes associated to oviducal gland and distal oviduct). The results summarized in Table 2 showed that the activity was closely related to the maturity stage. Indeed, the inhibition of the whole genital tract was observed from 10^{-10} M before egg-laying against only 10^{-18} M after egg-laying. For the main nidamental glands responsible for the secretion of the egg capsule, the thresholds of activity decreased after egg-laying (10^{-13} M against $10^{-8}/10^{-7}$ M before egg-laying) (Figs. 4 and 5). The basal activity was recovered after a time of 5–20 min depending on the peptide concentration applied. As shown in Table 2, the activity of SepCRP was restricted to the genital tract and the main nidamental glands. In addition, a similar bioactivity was recovered by the perfusion of the whole genital tract by the FGO conditioned seawater, as yet described in [5].

MicroLC–ESI-MS/MS tissue mapping of SepCRP

The mapping was performed in MS/MS mode based on the m/z 819. As shown in Table 3, SepCRP was strictly localized in the genital apparatus: the vitellogenic follicles, the FGO, the eggs, and the internal egg-capsule (secreted by the oviduct gland). Moreover, the occurrence of a release by the FGO was clearly established. The analysis performed on the different parts of the central nervous system and the hemolymph did not reveal any trace of this peptide.

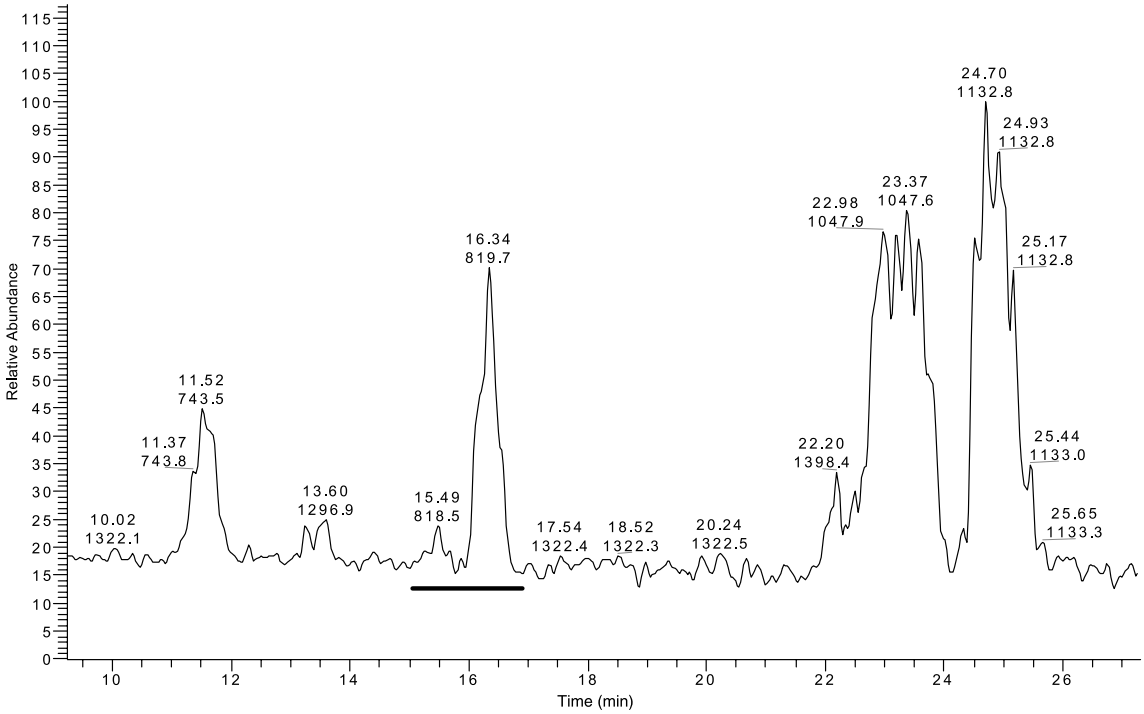

Fig. 2. Ionic chromatogram of the first purification step of full-grown oocytes onto a C18 column. The black line indicates myotropic fraction.

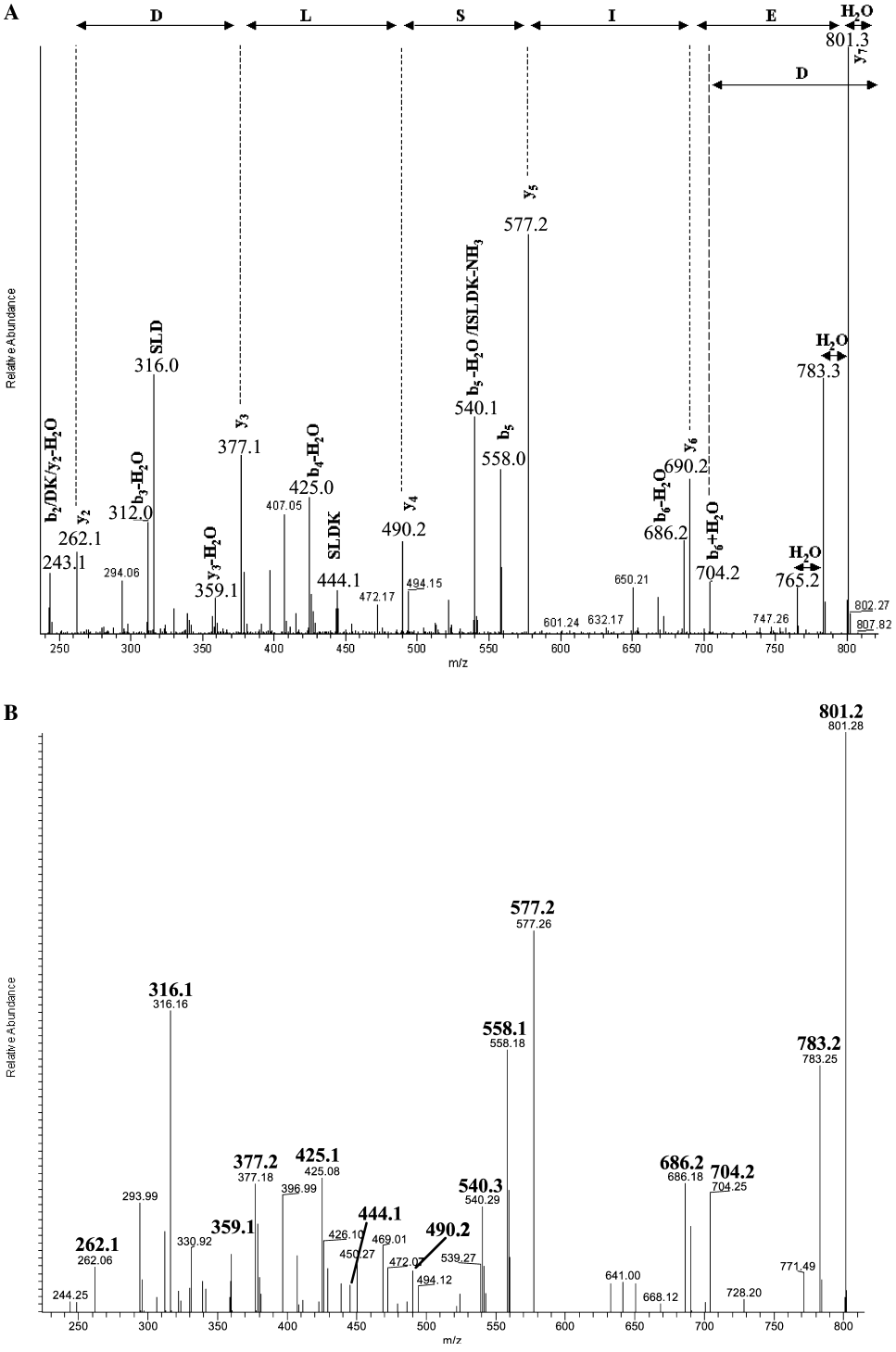

Fig. 3. MS/MS spectra of synthetic SepCRP (A) and endogenic SepCRP (B).

SepCRP kinetic release from full-grown oocytes

The level of SepCRP released was measured in MS/MS mode. The concentration of the m/z 819 was directly correlated to the incubation time. The accumulation of SepCRP in the incubation medium corresponded to a calculated release of 0.16 pmol/FGO/min.

Discussion

A new ovarian regulatory peptide named SepCRP was characterized in the cuttlefish *S. officinalis* on the criteria of its ability to be stored (and maybe expressed) and then released in the external medium by the full-grown oocytes. The investigations have been performed using mass

Table 2
Bioactivity of synthetic SepCRP

Muscle	Myotropic activity	Threshold concentration (M)
Whole mature genital tract		
Before the first egg-laying	Inhibition	10^{-18}
After egg-laying	Inhibition	10^{-10}
Before the following egg-laying	Inhibition	10^{-15}
Esophagus	No activity	—
Ovarian stroma	No activity	—
Rectum	No activity	—
Main nidamental glands		
Before egg-laying	Inhibition	10^{-8}
After egg-laying	Inhibition	10^{-13}

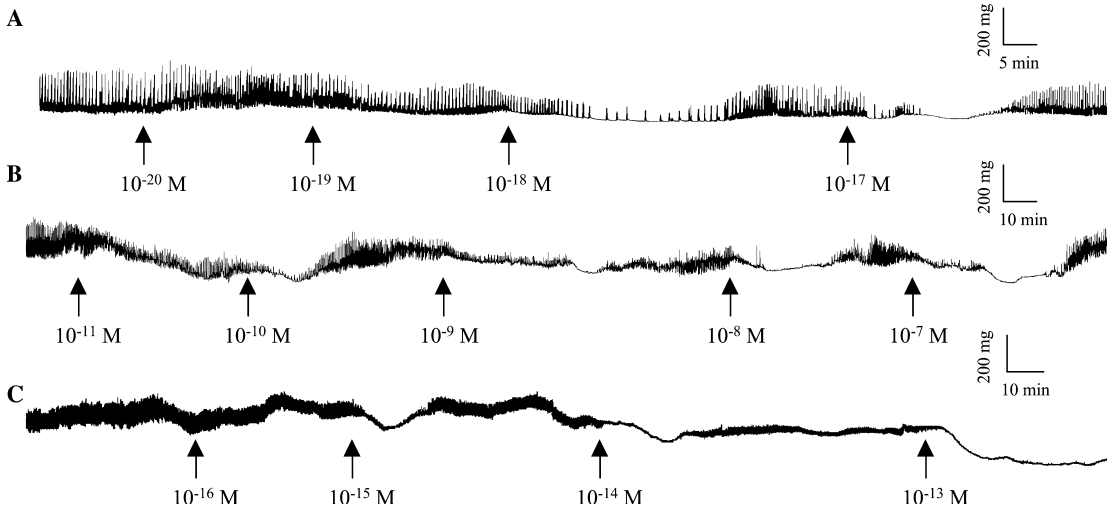

Fig. 4. SepCRP-induced contraction of the cuttlefish whole genital tract for a threshold of (A) 10^{-18} M before the first egg-laying, (B) 10^{-10} M after the first egg-laying, and (C) 10^{-15} M before the following egg-laying.

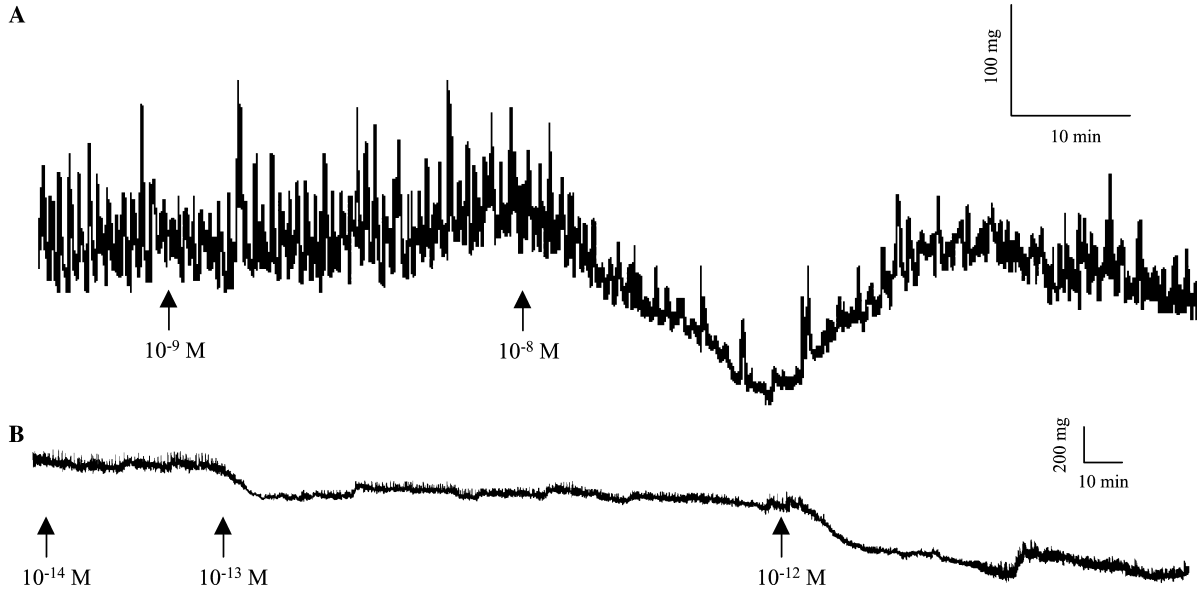

Fig. 5. SepCRP-induced contraction of the cuttlefish main nidamental gland for a threshold of (A) 10^{-8} M before egg-laying and (B) 10^{-13} M after egg-laying.

Table 3
Tissue mapping in microLC-ESI-MS/MS

	Occurrence of EISLDKD
Previtellogenic follicles	Yes
Vitellogenic follicles	Yes
Full-grown oocytes	Yes
Eggs	Yes
Internal capsule	Yes
External capsule	Yes
Vitellogenic follicles conditioned seawater	Yes
Full-grown oocytes conditioned seawater	Yes
Eggs conditioned seawater	Yes
Central nervous system	No
Hemolymph	No

spectrometry comparative screening. This approach appeared to be a powerful means of identification of regulatory factors involved in seasonal and/or local regulations. From a very low level of biological material, a peptide can be identified on the basis of its localization and isolated following the corresponding single and/or multicharged ions in microLC–ESI-MS/MS. The biological activity of the new peptide was then elucidated by testing the effect of increasing concentrations of a synthetic peptide on multiple in vitro bioassays involving the different parts of the genital apparatus. These investigations led to the characterization of a totally new heptapeptide expressed in the vitellogenic ovarian follicles as previously described for ILME and SepOvotropin [5,6]. Released by the FGO in the lumen of the oviduct, SepCRP directly targeted the oviduct and inhibited contractions. Similar to what was observed with the ovarian 5-HT [2], the first action of this peptide could be to force the storage of FGO in the genital coelom of unmating mature females in order to avoid the release of unfertilized eggs. The second action could be related to the second target organ identified in this paper: the main MNG. Concurrent with the arrest of FGO transport, the mechanical secretion of egg capsule products by the MNG would be inhibited by the release of SepCRP in the lumen of the oviduct. The distal oviduct opening in the mantle cavity close to the MNG opening would be the route followed by SepCRP to reach the second target and induce the mechanical release of the egg capsule at a concentration as low as 10^{-13} M.

In addition, in vitro experiments have clearly demonstrated important variations in response thresholds. These variations appeared to be directly correlated with the egg-laying stage of the animal. Before egg-laying, the inhibition threshold of the oviduct contractions was very low (10^{-18} M) probably to avoid the release of unfertilized eggs even if the genital coelom was not yet full. After mating, when the egg-laying has started, the increase of the threshold (10^{-13} M) allows for the maintenance of FGO transport by the genital tract and the continuation of the egg-laying.

For the MNG, we demonstrated in a previous study the occurrence of FMRFamide related peptides (FaRPs) at the level of the nerve endings surrounding the secretory tissue and their ability to exert a strong stimulation of MNG contractions at physiological concentrations [4]. In association with SepCRP released in the mantle cavity by the FGO at the level of the distal oviduct, the FaRPs could induce the secretion of egg capsule products. Alternating stimulations (FaRPs) and inhibitions (SepCRP) of the muscular fibers surrounding excretory ducts could induce the mechanical transport followed by the secretion of egg capsule products in the mantle cavity necessary for the embedding of the FGO. Indeed, during egg-laying and just before the fertilization, the MNG orifices were in close proximity to the FGO. When maintained during 3 or 4 min (egg-laying frequency) against the orifices of the MNG, each FGO has the potential to release approximately 131 pg/min of SepCRP (0.16 pmol/FGO/min). This release rate was sufficient to maintain a local peptide concentration capable of inducing a response from the MNG (SepCRP threshold: 10^{-13} M = 0.08 pg/ml) despite the stream and dilution caused by the gills in the mantle cavity. We suspect that this local regulation was one of the steps allowing the synchronization between genital coelom content, ovary maturation and egg capsule synthesis, and secretion.

In this context, the ovary of *S. officinalis*, as source of regulatory peptides, appears to be one of the main organs involved in the control of successive reproductive steps such as oocyte transport [2,5,6], egg capsule secretion, oocyte fertilization [17], and adults gathering in the egg-laying area. Moreover, in association with the CNS, the ovary is directly involved in the stimulation of the MNG secretion synthesis [18]. We therefore suspect the occurrence of an ovarian synchronization of growth and maturation of the accessory sex glands (ASG) during gametogenesis. As mass spectrometry analysis clearly demonstrates, SepOvotropin, ILME, and SepCRP are expressed during vitellogenesis, however, we cannot rule out the hypothesis that they could target ASG to regulate the synthesis level.

The regulatory peptides expressed in the ovary or in the ASG, also called sex-peptides, have been characterized in many invertebrates. In the marine snail *Aplysia californica*, a peptide called attractin released by the egg cordons is responsible for the gathering of adults [19,20]. In the insect *Drosophila melanogaster*, the sex-peptides induce oviposition and ovulation [21–24], and in *Neobelliera bullata* the hexapeptide Neb TMOF inhibits ecdysone biosynthesis [25]. Thus, the expression and the release of regulatory peptides by the ovary appears to be a widespread strategy in invertebrates. In *S. officinalis*, even though ovarian peptides did not seem able to trigger egg-laying, they appear to play an important role in this mechanism. Observations performed

in aquaria (unpublished results) demonstrate that mating is one of the *stimuli* (visual, mechanical or chemical?) capable of inducing the egg-laying behavior leading to oocyte transport, egg capsule secretion, fertilization, and constitution of the egg mass.

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