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Identification of SepCRP analogues in the cuttlefish *Sepia officinalis*: A novel family of ovarian regulatory peptides

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Abstract

In the cuttlefish, *Sepia officinalis*, the ovary appears to be one of the main sources of regulatory peptides involved in the successive steps of egg-laying. Following the identification of the SepCRP-1, which is a peptide extracted from ovary and involved in egg capsule secretion, investigations were focused on the identification of related peptides. Seven related-*Sepia* Capsule Releasing Peptides (R-SepCRPs) were identified by means of mass spectrometry and characterized using MS/MS spectra and/or Edman degradation. Finally, primary structures were verified by the comparison of MS/MS spectra from endogenic and synthetic peptides. This new ovarian peptide family exhibits a conserved SLXKD tag involved in the biological activity. LC–MS/MS screening clearly demonstrates that R-SepCRPs are restricted to the female genital tract. Expressed during vitellogenesis, they are released by vitellogenic follicles and full-grown oocytes (FGO) in the genital coelom. Biological activities suggest that R-SepCRPs would be responsible for the storage of FGO before mating and would take part in the mechanical secretion of egg capsule products, as previously described for SepCRP-1.

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

Since the isolation of GWamide from the cuttlefish *Sepia officinalis* [1], a constant effort has been invested in the characterization of new myotropic peptides modulating the motility of the oviduct. These investigations have led to the identification of numerous factors such as neuromediators, neuropeptides, and ovarian peptides involved in the successive steps of egg-laying. In the cuttlefish, this event is clearly stereotyped. Following an intense vitellogenic period [2], full-grown oocytes (FGO) are released by the ovary and stored into the genital coelom. Ovarian 5-HT synthetized by FGO and able to inhibit oviducal contractions is responsible for the storage of FGO in the genital coelom until mating [3]. After mating, ovarian peptides such as ILME and SepOvotropine [4,5], in association with neuropeptides such as FMRFamide-related-peptides (FaRPs) and APGWamide-related-peptides [1,6], modulate oviduct contractions to ensure the release of oocytes. Then, oocytes receive a first gelatinous envelope secreted by the oviducal gland and a second one released by the nidamental glands [7]. Fertilization, facilitated by ovarian peptides [8], is performed with spermatozoa stored in the copulatory pouch [9]. Recently, our laboratory reported the characterization of the SepCRP (*Sepia* Capsule Releasing Peptide), a myotropic ovarian peptide released by the cuttlefish FGO [10]. This new ovarian peptide, able to inhibit the contractions of the

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whole genital tract and the main nidamental gland (MNG), was suspected to be involved in the storage of FGO in the genital coelom and partially responsible for the mechanical secretion of egg capsule products by the MNG. Characterization of the SepCRP was performed using a mass spectrometry comparative screening associated with a specific bioassay. This approach appeared to be a powerful strategy to identify regulatory peptides from a very small amount of biological material. In this study, using mass spectrometry, we have investigated the putative occurrence of peptides related to SepCRP (R-SepCRPs) in FGO. The first part of this work was aimed at the identification of putative truncated R-SepCRPs. The second part was performed using FGO-conditioned medium and monitored by a myotropic bioassay. Finally, R-SepCRPs were identified by a mass spectrometry screening monitored by sequence libraries focused on the consensus tag. Biological activities were established with synthetic peptides.

Materials and methods

Animals

All the cuttlefish were trapped in the Bay of Seine between January to June. They were maintained in 1000-L outflow tanks at 15 °C ± 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 FGO was homogenized in 6 L HCl 0.1 N at 100 °C and centrifuged 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. In addition, the peptides released from 50 full-grown oocytes or 50 vitellogenic oocytes in 20 ml of synthetic filtered seawater at different pH (5, 5.5, 6, and 6.5) were concentrated on Chromafix C18 cartridges.

MicroLC–ESI–MS/MS analysis

Analysis was performed with a HPLC Surveyor chain connected online 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 300 to 2000 m/z. 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 a 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 electron corporation C18 column (50 × 0.5 mm, 3 µm) with an acetonitrile linear gradient of 3% per minute 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.

Identification of the R-SepCRPs

The whole experimental procedure is summarized in Fig. 1.

Identification of SepCRP-2 and -3

Digestions were performed at 37 °C during 45 s with 100 µg of synthetic SepCRP-1 (EISLDKD). For N-terminal digestion, 4 aminopeptidase units in a sodium citrate buffer (1 M at pH 8.2) were added to the reaction medium, for C-terminal digestion 4 carboxypeptidase units in a PBS buffer at pH 6.7. The reaction was stopped by addition of 2 volumes of 90% methanol/9% H_2O/1% formic acid in ice. Dry samples were analyzed in mass spectrometry and MS/MS spectra obtained from truncated SepCRPs were compared to MS/MS spectra of the FGO extract.

Identification of SepCRP-4

HPLC 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 FGO-conditioned seawater was resuspended in 0.1% formic acid in water and injected into a C18 column (250 × 3 mm, 7 µm) with an acetonitrile linear gradient of 1.33% per minute in 0.1% formic acid at a flow rate of 1 ml/min, during 45 min from 0% to 60% acetonitrile (ACN). One minute HPLC fractions were dried and kept at 4 °C until use.

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 online with a Model 120A HPLC system, as recommended by the manufacturer. The amino acid sequence was checked from MS/MS spectrum with the software Sequest (Thermofinnigan) and MS-Product 1.6.1 (Protein Prospector 3.4.1, University of California).

Identification of SepCRP-5

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. The amino acid sequence was determined from MS/MS spectrum and definitively established by the comparison between synthetic and endogenic peptide MS/MS spectra.

Identification of SepCRP-6, -7, and -8

Fasta libraries were generated with the software Aaseq 5.0 (Zanuttini and Henry, University of Caen). Screening was performed using Sequest software (Thermofinnigan) which is able to compare experimental MS/MS spectra and theoretical MS/MS spectra generated from fasta libraries. The amino acid sequence was determined from MS/MS spectrum with the Sequest software (Thermofinnigan), MS-Tag and MS-Product 1.6.1 (Protein Prospector 3.4.1, University of California). For each peptide, the amino acid sequence was definitely established by the comparison between synthetic and endogenic peptide MS/MS spectra.

Synthetic peptides

Peptides were synthesized using classical Fmoc (*N*-[9-fluorenyl]methoxycarbonyl) solid-phase chemistry using a commercial automatic peptide synthesizer by coupling Fmoc-α-amino acids on preloaded Wang resin. Protected amino acids were coupled by in situ activation with diisopropylcarbodiimide (DIPCDI) and *N*-hydroxybenzotriazole (HOBt). N^{α}-Fmoc deprotection was performed with 20% piperidine in DMF. Side chain deprotection and cleavage of peptides from the solid support were performed by treatment with reagent B (88% trifluoroacetic acid (TFA)/5% phenol/5% water/2% TIS) for 2 h at 20 °C [11]. Peptides were purified by reversed-phase HPLC (RP-HPLC) using a Waters semi-preparative HPLC system on an X Terra 10 µm column (300 × 19 mm). The elution was achieved with a linear gradient of aqueous 0.1% TFA (A) and 0.08% TFA in acetonitrile (B) at a flow rate of 10 ml/min with photodiode array detection at 210–440 nm. The purity of each peptide was controlled by analytical RP-HPLC on the same instrument with a X Terra 5 µm column (250 × 4.6 mm) using a linear gradient of 0.1% TFA in water and aceto-

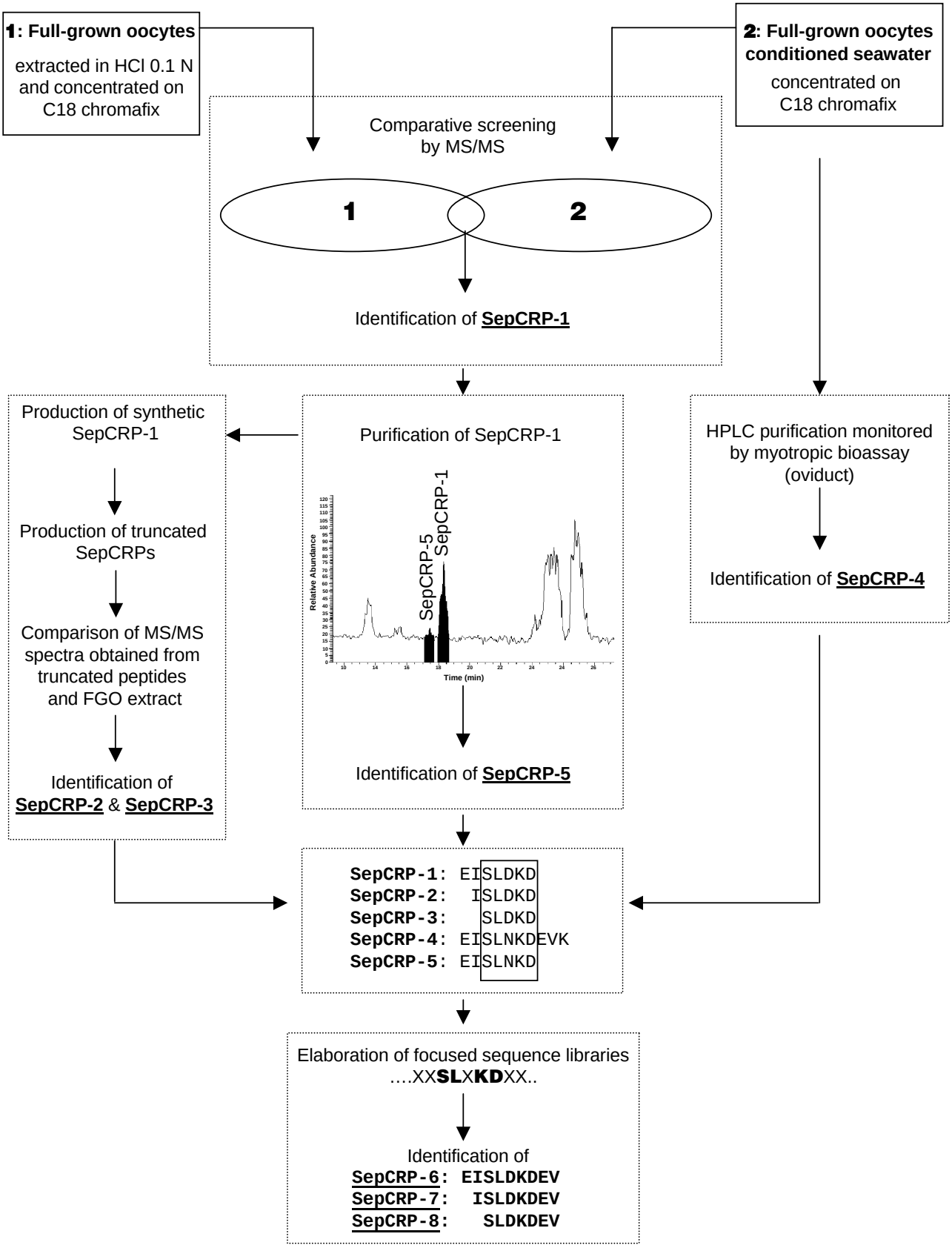

Fig. 1. Experimental procedures performed for the identification and the characterization of R-SepCRPs.

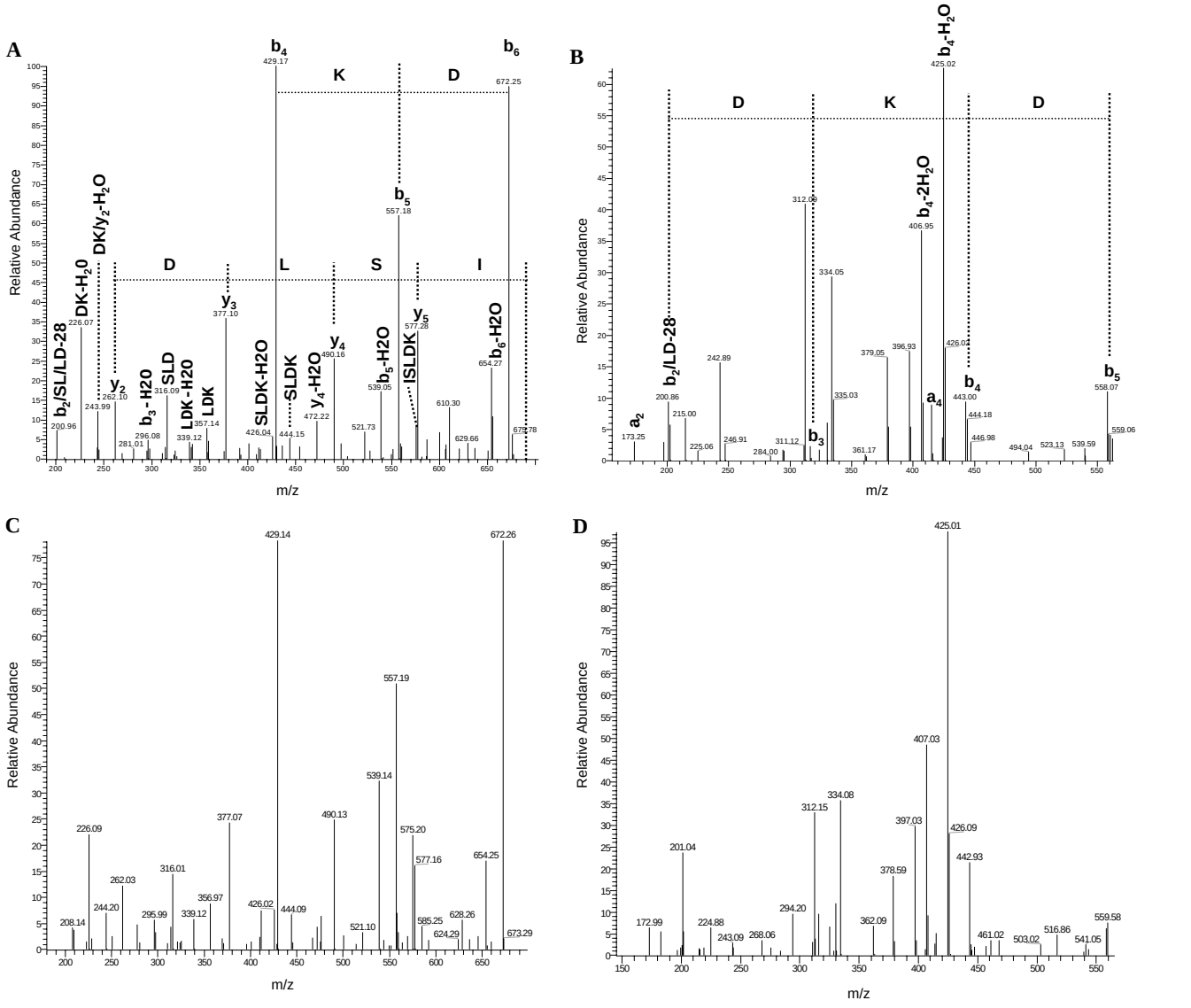

Fig. 2. MS/MS spectra of synthetic SepCRP-2 (A) and SepCRP-3 (B) using the same experimental conditions as for endogenic SepCRP-2 (C), and SepCRP-3 (D).

nitrile containing 0.08% TFA at a flow rate of 1 ml/min. Finally, integrity of each peptide was assessed by LC–ESI–MS/MS analysis.

Biological assay

Several contractile organs were used for the myotropic bioassay. The genital tract (including the proximal oviduct containing FGO, the oviducal gland, and the distal oviduct), the MNG, the esophagus, and the rectum 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 fractions into the muscle chamber was traced by addition of phenol red. Aliquots of synthetic peptide ranging from 10^{-15} to 10^{-6} M were tested on the oviduct and main nidamental glands (MNG) contraction and various somatic muscles to check the specificity of R-SepCRPs.

Results

Identification of SepCRP-2 and -3

Aminopeptidase digestion of synthetic EISLDKD

After the aminopeptidase digestion of the synthetic peptide, 1 μg of the final reaction was injected and analyzed in LC–ESI–MS/MS. The synthetic peptide EISLDKD (SepCRP-1) was completely digested. The *m/z* 690, 577, and 490 corresponding, respectively, to ISLDKD, SLDKD, and LDKD forms were recovered from the sample.

Carboxypeptidase digestion of synthetic EISLDKD

For carboxypeptidase digestion of the synthetic peptide, the *m/z* 704, 576, and 461 corresponding, respectively, to EISLDK, EISLD, and EISL forms were recovered from the sample by LC–ESI–MS/MS analysis.

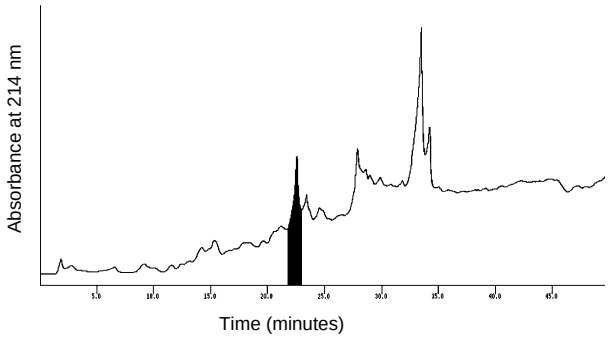

Fig. 3. First step of purification on C18 column of the chromafix C18 eluate of the total FGO conditioned seawater. One myotropic fraction was detected by the myotropic bioassay (shaded part).

FGO sample

The extract of FGO was submitted to LC–ESI–MS/MS analysis. The *m*/*z* 690, 577, 490, 704, 576, and 461 corresponding, respectively, to ISLDKD, SLDKD, LDKD, EISLDK, EISLD, and EISL, previously generated by aminopeptidase and carboxypeptidase digestions, were investigated in the sample. For SepCRPs N-terminal truncated forms, the total ion chromatograms (TIC) revealed two peaks at *m*/*z* 690 and 577. Comparison of MS/MS data obtained from *m*/*z* 690 and 577 from aminopeptidase digestion of synthetic EISLDKD with those of FGO extract revealed the presence of ISLDKD (*m*/*z* 690; SepCRP-2) and SLDKD (*m*/*z* 577; SepCRP-3) in FGO. The comparison of MS/MS data obtained from synthetic and endogenic peptides definitively confirmed the amino acid sequence of SepCRP-2 and -3 (Fig. 2). SepCRPs C-terminal truncated forms were not detected in FGO.

Identification of SepCRP-4

HPLC purification

One single step of purification was performed on HPLC from FGO-conditioned seawater. The 1-min HPLC fractions of the sample were tested on the myotropic bioassay (female whole genital tract). After the first separation step, the activity was concentrated in an apparently pure peak at a retention time of 22.5 min (Fig. 3). This peak was manually collected and concentrated to be submitted to Edman degradation.

Sequence determination

Primary sequence of peptide purified in HPLC was determined by Edman degradation: EISLNKDEVK (SepCRP-4) and checked by the comparison of MS/MS data obtained from synthetic and endogenic peptides (Fig. 4).

Identification of SepCRP-5

During the purification of SepCRP-1 [10], a pure peak eluted at 15.5 min containing the *m*/*z* 818.5 was detected. MS/MS spectrum of this peptide is closely related to SepCRP-1 (EISLDKD) MS/MS spectrum (Fig. 5). Analysis of *m*/*z* 818.5 MS/MS spectrum revealed a loss of 1 Da

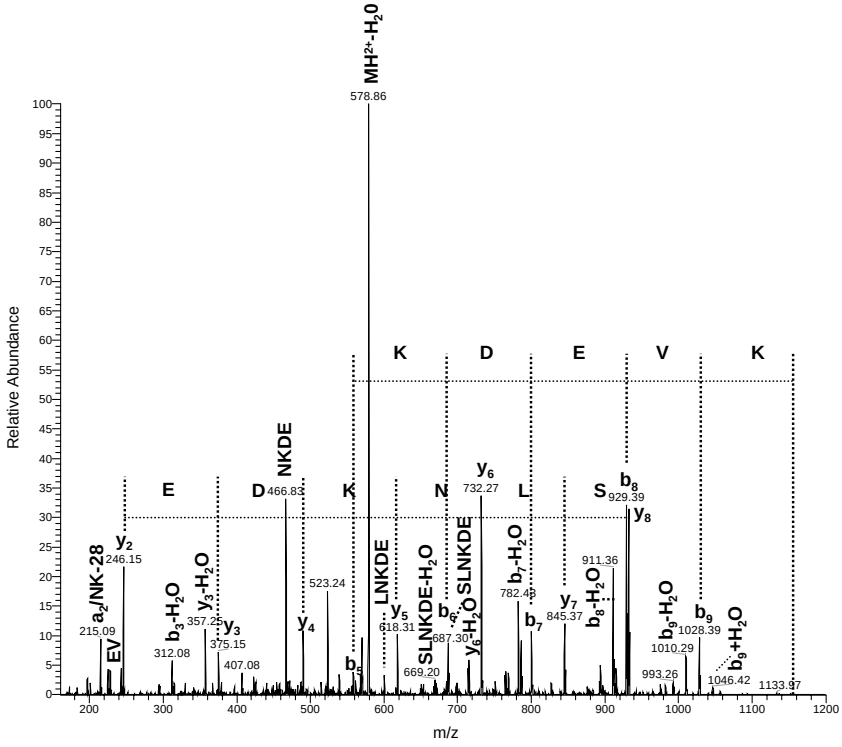

Fig. 4. MS/MS spectrum of endogenic SepCRP-4 (*m*/*z* 1174.6).

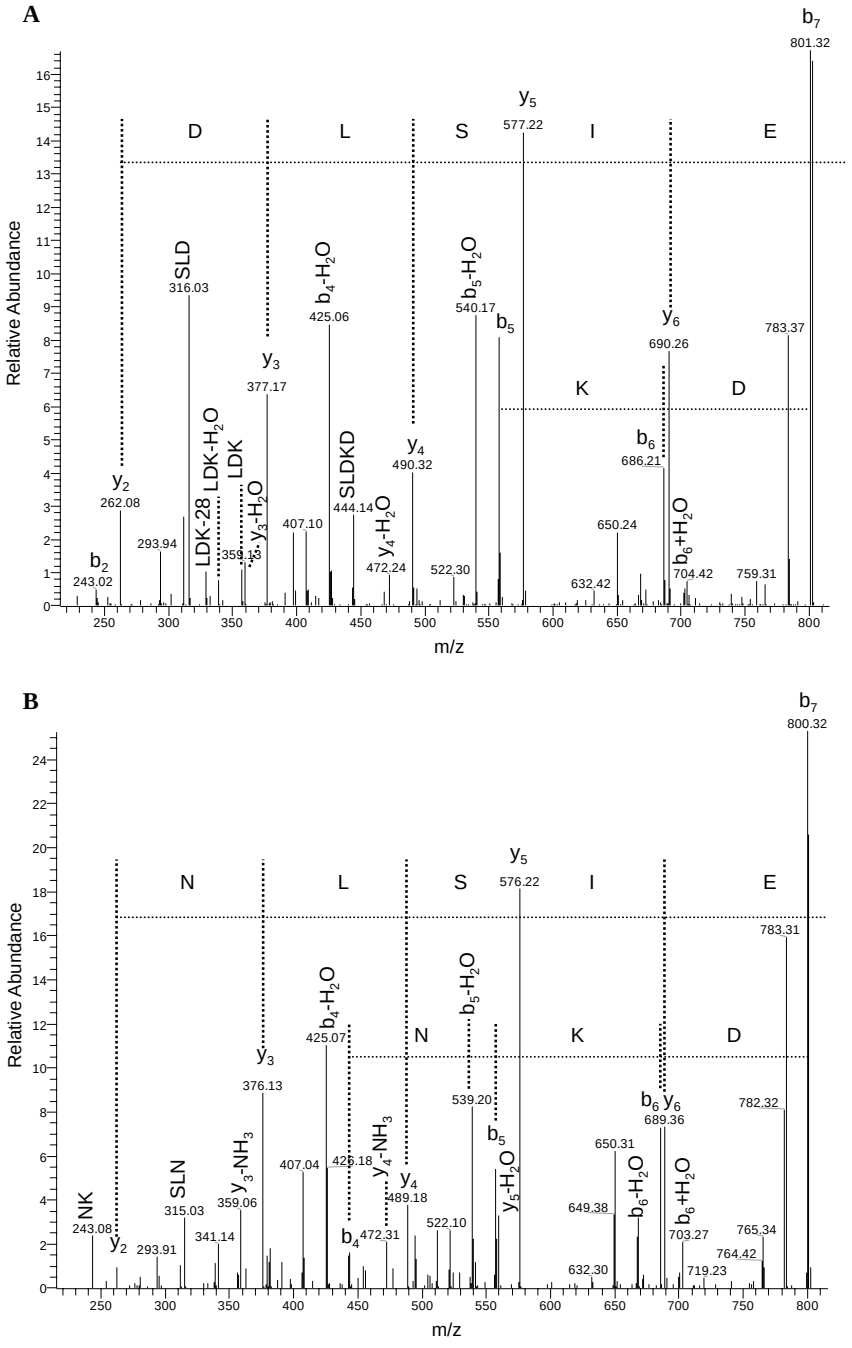

Fig. 5. Comparison of SepCRP-1 (A) and SepCRP-5 (B) MS/MS spectra. MS/MS spectra revealed a loss of 1 Da for y_3, y_4, y_5, and y_6 ions corresponding to the substitution of the internal D (aspartate: 115 Da) by N (asparagine: 114 Da).

for y_3, y_4, y_5, and y_6 ions corresponding to the substitution of an internal D (aspartate: 115 Da) by N (asparagine: 114 Da) leading to the following sequence: EISLNKD. MS/MS data obtained from synthetic EISLNKD confirmed the amino acid sequence and established the occurrence of this new R-SepCRP in FGO.

Identification of SepCRP-6, -7, and -8

Aaseq 5.0 software was used to build a sequence library containing the SLXKD tag and ranging from 500 to 1100 Da. Investigations of R-SepCRPs from FGO, monitored by Sequest software and sequence library [SLXKD; 500–1100 Da], led to the identification of 3 new R-SepCRPs: EISLDKDEV (SepCRP-6), ISLDKDEV (SepCRP-7), and SLDKDEV (SepCRP-8) (Fig. 6). Amino acid sequences of these peptides were checked with MS/MS data obtained from synthetic peptides. Table 1 summarizes all R-SepCRPs characterized and identification methods.

Bioactivity of R-SepCRPs

Increasing concentrations of synthetic peptides were tested on the whole genital tract (proximal oviduct contain-

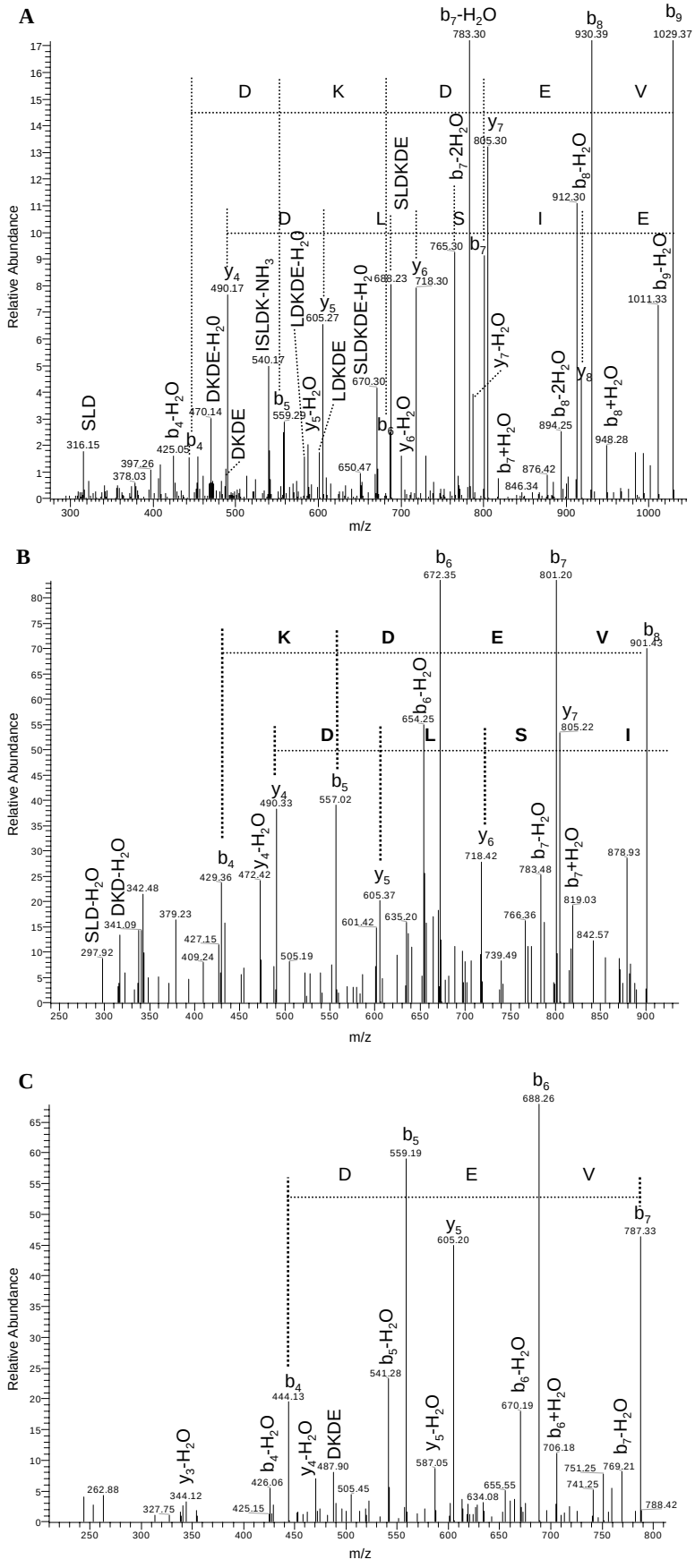

Fig. 6. MS/MS spectra of endogenic SepCRP-6 (A), SepCRP-7 (B), and SepCRP-8 (C).

Table 1
Summary of the eight R-SepCRP primary structures and identification methods

	Sequence	Identification methods
SepCRP-l	EISLDKD	Mass spectrometry comparative screening
SepCRP-2	ISLDKD	Mass spectrometry and aminopeptidase digestion
SepCRP-3	SLDKD	Mass spectrometry and aminopeptidase digestion
SepCRP-4	EISLNKDEVK	HPLC purification monitored by myotropic bioassay (oviduct)
SepCRP-5	EISLNKD	Identification by mass spectrometry
SepCRP-6	EISLDKDEV	Mass spectrometry and specific fasta libraries
SepCRP-7	ISLDKDEV	Mass spectrometry and specific fasta libraries
SepCRP-8	SLDKDEV	Mass spectrometry and specific fasta libraries

Table 2
Bioactivity of R-SepCRPs

Muscles	Threshold concentration				
	SepCRP-1	SepCRP-2	SepCRP-3	SepCRP-4	SepCRP-5
Whole mature genital tract	10^{-18}	10^{-13}	10^{-11}	10^{-9}	10^{-8}
Esophagus	NA	NA	NA	NA	NT
Ovarian stroma	NA	NA	NA	NA	NT
Rectum	NA	NA	NA	NA	NT
Penis	NA	NA	NA	NA	NT
Main nidamental glands	10^{-13}	10^{-9}	10^{-8}	10^{-9}	10^{-13}

The activities of R-SepCRPs appear to be restricted to the female genital tract and the MNG.
NA, no activity; NT, not tested.

ing FGO, oviducal gland, and distal oviduct), MNG, ovarian stroma, esophagus, rectum, and penis of mature cuttlefish. As shown in Table 2, the myotropic activities of R-SepCRPs are restricted to the genital tract and the MNG. SepCRP-2, -3, -4, and -5 modulate the whole genital tract contractions from 10^{-13}, 10^{-11}, 10^{-9}, and 10^{-8} M, and the MNG contractions from 10^{-9}, 10^{-8}, 10^{-9}, and 10^{-13} M (Figs. 7 and 8).

MicroLC–ESI–MS/MS tissue mapping of R-SepCRPs

Tissue mapping was performed in MS/MS mode based on the *m/z* 819 (SepCRP-1), 690 (SepCRP-2), 577 (SepCRP-3), 1174.5 (SepCRP-4), 818 (SepCRP-5), 1047 (SepCRP-6), 918 (SepCRP-7), and 805 (SepCRP-8). The occurrence of peptides was established by the comparison of MS/MS data obtained from the sample and the synthetic peptides. Analysis performed on the different parts of the central nervous system and the hemolymph did not reveal any trace of R-SepCRPs. These peptides appear to be restricted to the vitellogenic follicles, the FGO, and the eggs (Table 3). Moreover, the release of R-SepCRPs by the FGO, and the vitellogenic follicles in various conditions (pH 5, 5.5, 6, and 6.5) was clearly established by the analysis of the conditioned seawater. R-SepCRPs were not recovered in egg-conditioned seawater by MS/MS analysis. This result suggested that the release by eggs seems to be stopped by the internal and external capsules coating the egg. Nevertheless, some traces of R-SepCRPs were detected in the internal capsule, probably trapped during the polymerization of the egg-capsule.

Discussion

The investigation of R-SepCRPs performed in the cuttlefish *S. officinalis* using mass spectrometry screening has led to the identification and the characterization of seven new ovarian regulatory peptides. The addition of a recurrent SLXKD tag (with X = D or N), similar activities, and a tissue mapping restricted to female genital tract clearly demonstrate that these peptides belong to the same family. The multiple organs screened with synthetic peptides revealed that mature female genital tract and MNG are the main targets. The R-SepCRPs, released by vitellogenic follicles and FGO in the lumen of the oviduct, appeared to directly modulate the contractions of oviduct and MNG. In association with SepCRP-1 [10], R-SepCRPs force the storage of the FGO in the genital coelom before mating and induce the mechanical secretion of egg capsule products. Moreover, R-SepCRPs biological activity is restricted to the egg-laying period. The thresholds determined with synthetic R-SepCRPs reveal that SepCRP-1 is more potent than truncated SepCRP-2 and -3 or elongated SepCRP-4. N-terminal elongation of the SLXKD tag appeared to enhance the activity of the peptide, as observed for SepCRP-2 (ISLDKD) and SepCRP-1 (EISLDKD). The substitution of D (aspartic acid) by N (asparagine) reduced the activity of the peptide, as observed for SepCRP-5 and -6. However, R-SepCRPs showed important variations in response thresholds, correlated with the egg-laying stage of the animal. The detection of SepCRP-2, -3, -7, and -8, which are truncated forms, in FGO-conditioned seawater, which is only concentrated (no acidic extraction), demon-

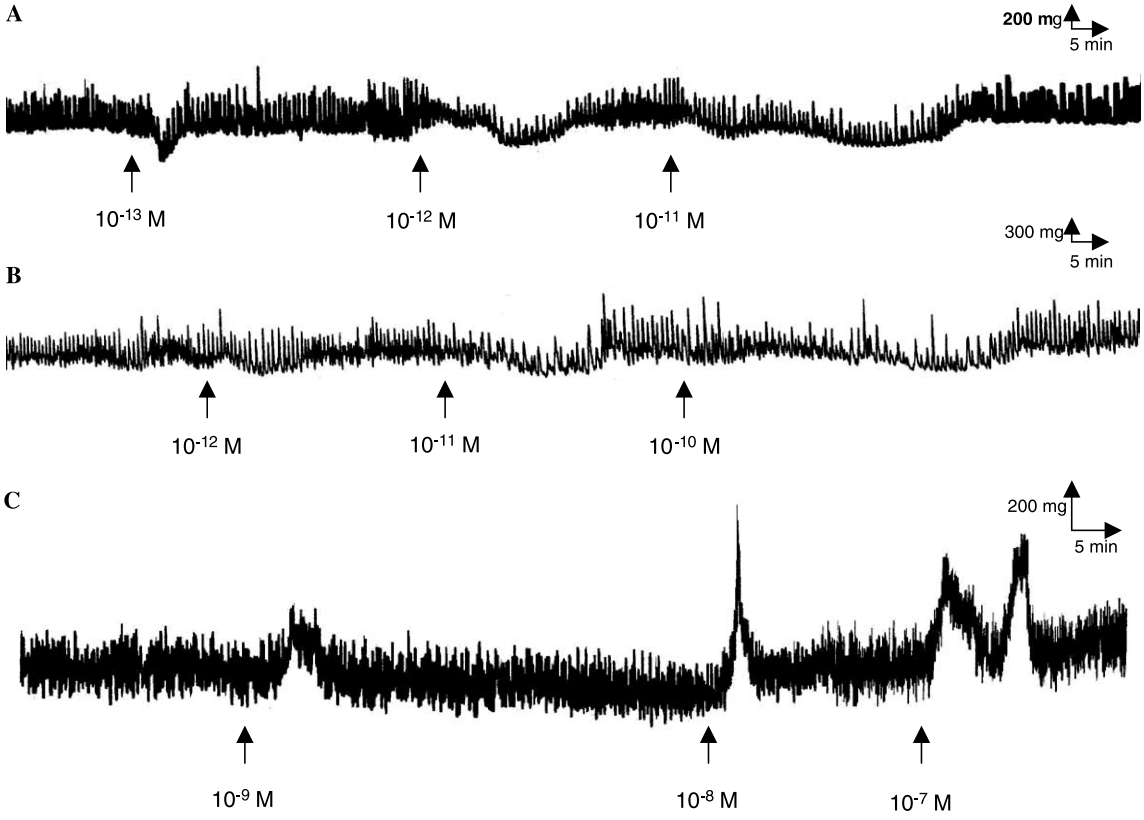

Fig. 7. R-SepCRPs-induced contraction of the cuttlefish whole genital tract for a threshold of 10^{-13} M for SepCRP-2 (A), 10^{-11} M for SepCRP-3 (B), and 10^{-9} M for SepCRP-4 (C).

strates that these forms were not a result of acidic extraction cleavage. Moreover, the investigation of the EISLDKDE, ISLDKDE, and SLDKDE forms which were not recovered in FGO put forward for consideration that SepCRP-2, -3, -7, and -8 are monitored by FGO enzymatic baggage. Despite we do not have any information about R-SepCRPs precursor, we suppose that these peptides results in post-translational modifications, suggesting the occurrence of an original enzymatic baggage in the vitellogenic follicles and the FGO. The recurrent SLXKD tag (X corresponding to D or N) and the biological activity observed for SepCRP-3 suggest that the SLXKD tag is responsible for the activity. If for amidated peptides, the C-terminal extremity is often involved in the activity, for non-amidated families, activity often involves internal tags, such as RLRF/A for BCP (bag-cell peptides) and CDCP (caudodorsal-cell peptides) [12,13] or IEECKTS for attractin [14]. Mass spectrometry analyses clearly demonstrate that R-SepCRPs are expressed during vitellogenesis in vitellogenic follicles and released in genital coelom by vitellogenic follicles and FGO. As the genital coelom pH of cephalopods ranges between 5.2 and 6.4 [15], the detection of R-SepCRPs in FGO-conditioned seawater at pH 5, 5.5, 6, and 6.5 confirms the release of R-SepCRPs in genital coelom under physiological conditions. R-SepCRPs are neither detected in egg-conditioned seawater nor in the external capsule. Thus, internal and external polymerized capsules appear to stop the release of R-SepCRPs into the external medium. This observation demonstrates that R-SepCRPs are only released into the female genital tract close to their targets. Numerous ovarian waterborne peptides have been characterized in marine invertebrates: attractin, emptin, enticin, and seductin in *Aplysia californica* [16–19] and sperm attractive or activating peptides (SAPs) in cnidaria and echinodermata [20–23]. With the identification and characterization of a new regulatory peptidic family, the ovary of the cuttlefish *S. officinalis* appears to be one of the main sources of waterborne regulatory peptides involved in the successive steps of egg-laying. In unmating females, we speculate that a constant concentration of ovarian factors, such as R-SepCRPs and ovarian 5-HT [3,10], is maintained in genital coelom in order to inhibit female genital tract contractions. After mating, peptides involved in oocyte transport such as ovarian peptides, ILME or Sepovotropine [4,5] or neuropeptides, FaRPs or APGWa-RPs [1,6] take over the peptides involved in the storage of FGO. Embedded oocytes released by oviduct in the mantle cavity receive a second envelope secreted by the MNG under control of R-SepCRPs and FaRPs [6]. Finally, the fertilization is facilitated by ovarian SepSAP (*Sepia* Sperm Attracting Peptide) [8]. The occurrence of so many regulatory factors: ovarian peptides, neuropep-

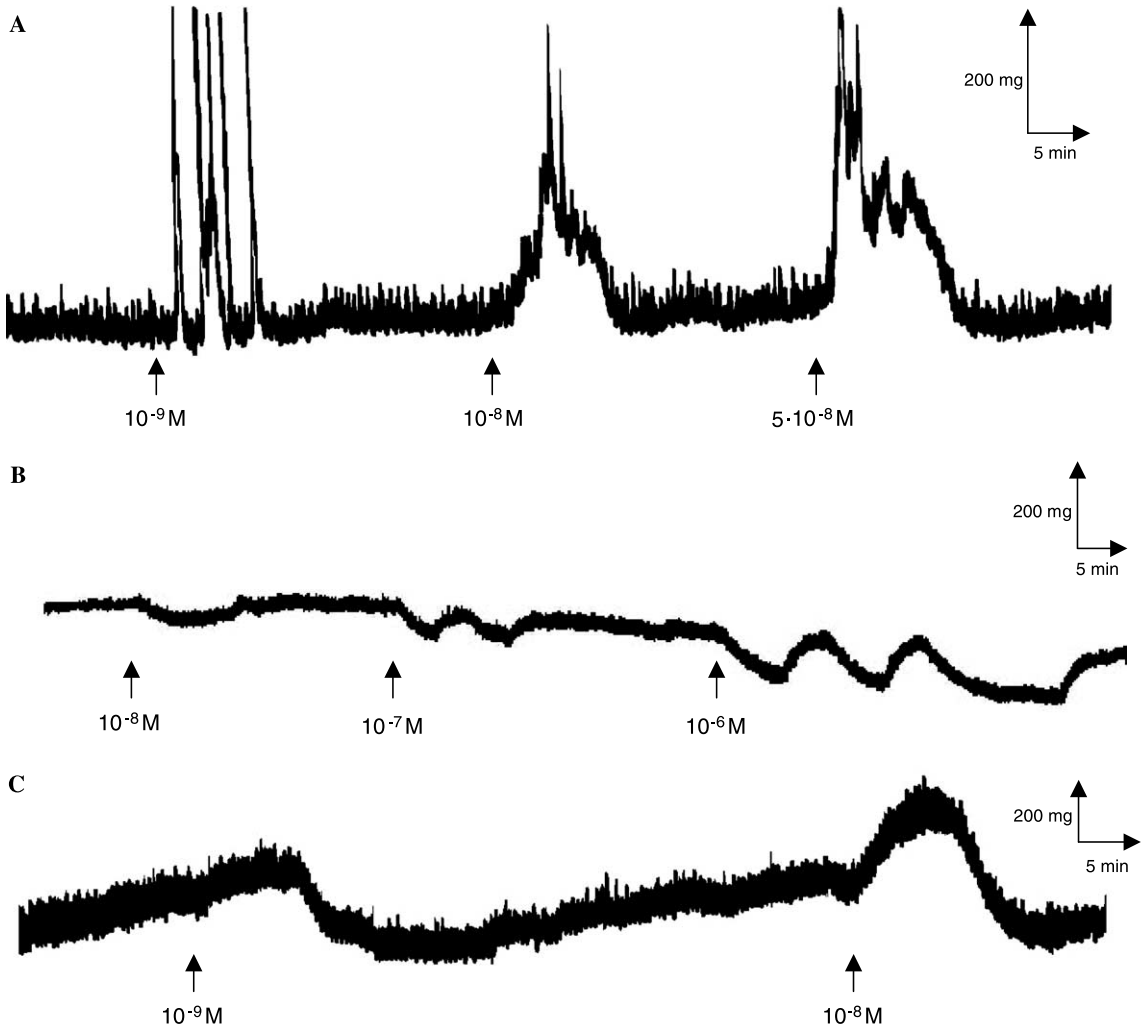

Fig. 8. R-SepCRPs-induced contraction of the cuttlefish main nidamental gland for a threshold of 10^{-9} M for SepCRP-2 (A), 10^{-8} M for SepCRP-3 (B), and 10^{-9} M for SepCRP-4 (C).

Table 3
Tissue mapping of R-SepCRPs performed in microLC–ESI–MS/MS

Occurrence of:	SepCRP-1	SepCRP-2	SepCRP-3	SepCRP-4	SepCRP-5	SepCRP-6	SepCRP-7	SepCRP-8
Previtellogenic follicles	nd	nd	nd	nd	nd	nd	nd	nd
Vitellogenic follicles	d	d	d	d	d	d	d	d
Full-grown oocytes	d	d	d	d	d	d	d	d
Eggs	d	d	d	d	d	d	d	d
Internal capsule	d	nd	d	nd	nd	d	nd	d
External capsule	nd	nd	nd	nd	nd	nd	nd	nd
Vitellogenic follicles-conditioned seawater	d	d	d	d	d	d	d	d
Full-grown oocytes-conditioned seawater	d	d	d	d	d	d	d	d
Egg-conditioned seawater	nd	nd	nd	nd	nd	nd	nd	nd
Central nervous system	nd	nd	nd	nd	nd	nd	nd	nd
Hemolymph	nd	nd	nd	nd	nd	nd	nd	nd

R-SepCRPs are restricted to the vitellogenic follicles, the FGO, and the eggs. nd, not detected; d, detected.

tides, monoamines, and probably neurohormones, demonstrates that egg-laying regulation is a highly complex physiological mechanism. Thus, the association of numerous regulation pathways allows female cuttlefish to achieve an optimal adaptation in response to internal and/or external variations, related to the maturity stage. Indeed, egg-laying involves the ovary, accessory sex glands, and the oviduct which have to be synchronized to succeed in the elaboration and release of embedded oocytes which could be fertilized. Finally, the wide range of peptide combinations

associated with the different steps of egg-laying probably allows for the very fine regulation of this critical phase of the life cycle.

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