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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-