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Ovarian jelly-peptides (OJPs), a new family of regulatory peptides identified in the cephalopod *Sepia officinalis*

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ABSTRACT

In marine invertebrates, numerous water-borne peptides involved in reproductive behavior have been characterized. In this study, we focused on three ovarian water-borne peptides, released by full-grown oocytes (FGO) in the genital coelom and in the lumen of the oviduct in the cuttlefish *Sepia officinalis*. The first one (DQVKIVL), was characterized by the monitoring of HPLC purified fraction using a myotropic bioassay. Subsequently, a peptidomic approach consisting of a mass spectrometry comparative screening performed between the peptide content of FGO with that of FGO-conditioned medium, led to the identification of two additional water-borne peptides. The second peptide identified (DEVKIVL) was characterized by MS/MS and the primary structure of the third one (DEVKIVLD) was elucidated by a combination of Edman degradation, acid hydrolysis and MS/MS analysis. Sequence homology, tissue mapping and bioactivity demonstrate that these peptides belong to the same family. DQVKIVL-related-peptides strictly localized in the female genital tract modulate the whole female genital tract and the main nidamental gland contractions. Furthermore, these peptides form a jelly, when resuspended in water. This particular property could play an important role in the kinetics of peptide diffusion in the external medium. Thus, these regulatory peptides were named ovarian jelly-peptides (OJPs).

1. Introduction

Since the introduction of the term "pheromone" by Karlson and Luscher [15], a number of water-borne peptides and proteins which play an important role in the coordination of the reproductive behavior have been discovered in aquatic organisms. In protozoans, peptide pheromones are responsible for mating interactions [17]. In amphibians, sodefrin and silefrin, two sex-pheromone peptides characterized in *Cynops pyrrhogaster* and *C. ensicauda*, respectively [16,28] attracted conspecific females. In the polychaete worm *Nereis*, the peptide nereithione which modulates male nuptial behavior and induces sperm release, was isolated from the coelomic fluid of sexually mature females [21,35]. In cnidaria and echinodermata, more than 80 sperm attracting and activating peptides (SAPs), are released by

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the eggs in seawater to facilitate fertilization by increasing chances of gamete collision [10,18,23,26]. In molluscs, water-borne peptides and proteins have also been identified from gastropods and cephalopods. In *Aplysia*, at least four water-borne peptides, attractin, temptin, enticin and seductin, are released by the egg cordons to induce aggregation of mature adults and egg-laying [6,7,19,20]. In the cuttlefish *Sepia officinalis*, numerous water-borne peptides and one neuromediator able to modulate the successive steps of egg-laying, are released by oocytes in the genital coelom as well as in the lumen of the oviduct [31–33]. The peptidomic or peptide display technique which consists of the comparison between the peptide content of different samples, is a powerful means of identification of putative regulatory peptides. In the cuttlefish *S. officinalis*, this experimental strategy led to the characterization of *Sepia* capsule releasing peptide (SepCRP), a water-borne peptide released by the full-grown oocytes (FGO) in the genital coelom, and involved in the successive steps of egg-laying [2]. After the release of FGO in the genital coelom (ovulation) [9], the ovarian 5-HT and the SepCRP released by the FGO inhibit the contractions of the oviduct until mating [2,31]. After mating, the oocytes are transported by the oviduct contractions controlled by neuropeptides [11,12] and ovarian peptides [32,33]. Then, FGO receive a first gelatinous envelope secreted by the oviduct gland and a second one secreted by the nidamental glands [14]. Finally, oocytes are fertilized by spermatozoa stored in the copulatory pouch and fertilization is facilitated by ovarian peptides [3,34]. In this context, we focused on water-borne peptides expressed in the ovary and targeting oviduct and accessory sex glands. By means of mass spectrometry screening and HPLC purification, we identified three novel ovarian peptides putatively involved in the regulation of egg-laying. Primary sequence, tissue mapping, activity and chemical properties demonstrate that these peptides belong to a new single family.

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 15 °C ± 1 °C at the Marine Station of Luc sur Mer (University of Caen, France) under a natural photoperiod.

2.2. Recovery of material from tissues and seawater

For HPLC purification and microLC-ESI-MS/MS analysis, 600 g of FGO were homogenized in 6 L of 0.1N HCl 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. Moreover, the molecules released from 50 FGO or 50 vitellogenic oocytes in 20 mL of synthetic filtered seawater were concentrated after various incubation times on Chromafix C18 cartridges to be analyzed in microLC-ESI-MS/MS.

2.3. 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 the 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 (50 mm × 0.5 mm, 3 μm) with an acetonitrile (ACN) linear gradient of 3%/min in 0.1% formic acid, from 2 to 60%. A split ratio of 30:1 was used to perfuse the column at a flow rate of 10 μL/min. The HPLC column was rinsed with 90% ACN in 0.1% formic acid between each injection. The MS data was acquired in scan mode considering the positive ion signal.

2.4. LC-ESI-MS purification

The FGO extract was resuspended in 100 μL of 0.1% formic acid in water and injected into Nucleosil C18 column (250 mm × 4 mm, 7 μm) with an ACN 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. The second step was performed in UV-HPLC. The bioactive fraction eluted at 22.20 min was injected onto a Nucleosil C18 column (250 mm × 4 mm, 5 μm) with an ACN linear gradient of 1.33%/min in 10 mM ammonium acetate at a flow rate of 1 mL/min.

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

2.6. Edman degradation

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 120 A HPLC system, as recommended by the manufacturer. The amino acid sequence was checked from MS/MS spectrum with the softwares

Sequest (Thermofinnigan) and MS-Product (Protein Prospector 3.4.1, University of California).

2.7. 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 and *N*-hydroxybenzotriazole. N^{α}-Fmoc deprotection was performed with 20% piperidine in DMF. Side chain deprotection and cleavage of peptides from the solid support was performed by treatment with reagent B (88% trifluoroacetic acid (TFA)/5% phenol/5% water/2% TIS) for 2 h at 20 °C [22]. Peptides were purified by reversed-phase HPLC (RP-HPLC) using a Waters semi-preparative HPLC system on an X Terra 10 μm column (300 mm × 19 mm). The elution was achieved with a linear gradient of aqueous 0.1% TFA (A) and 0.08% TFA in ACN (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 mm × 4.6 mm) using a linear gradient of 0.1% TFA in water and ACN 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.

2.8. Biological assay

The myotropic bioassay was performed with the female genital tract (including the proximal oviduct containing FGO, the oviducal gland and the distal oviduct), the main nidamental glands, the esophagus, the rectum and the penis which were dissected from mature females and males. Each organ was suspended from a displacement transducer (Phymep, Bionic Instruments) connected to a computer controlling the recorder and the Dispositif d'Acquisition et de Traitement Automatique de la Contraction (DATAC). 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. Aliquots of synthetic peptide ranging from 10^{-15} to 10^{-6} M were tested on the oviduct and the main nidamental gland contractions and various somatic muscles to check the specificity of the peptides.

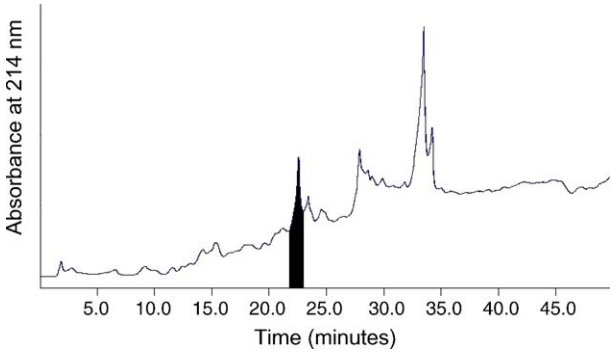

Fig. 1 – First step of purification on C18 column of the chromafix C18 eluate of the total FGO-conditioned seawater. One myotropic fraction (22–23 min) was detected by the cuttlefish oviduct myotropic bioassay (shaded).

3. Results

3.1. Identification of DQVKIVL

3.1.1. HPLC purification

One single step of purification was performed in UV-HPLC from FGO-conditioned seawater. The activity was concentrated in an apparently pure peak at a retention time of 22.5 min (Fig. 1). This peak, manually collected, was concentrated to be submitted to Edman degradation.

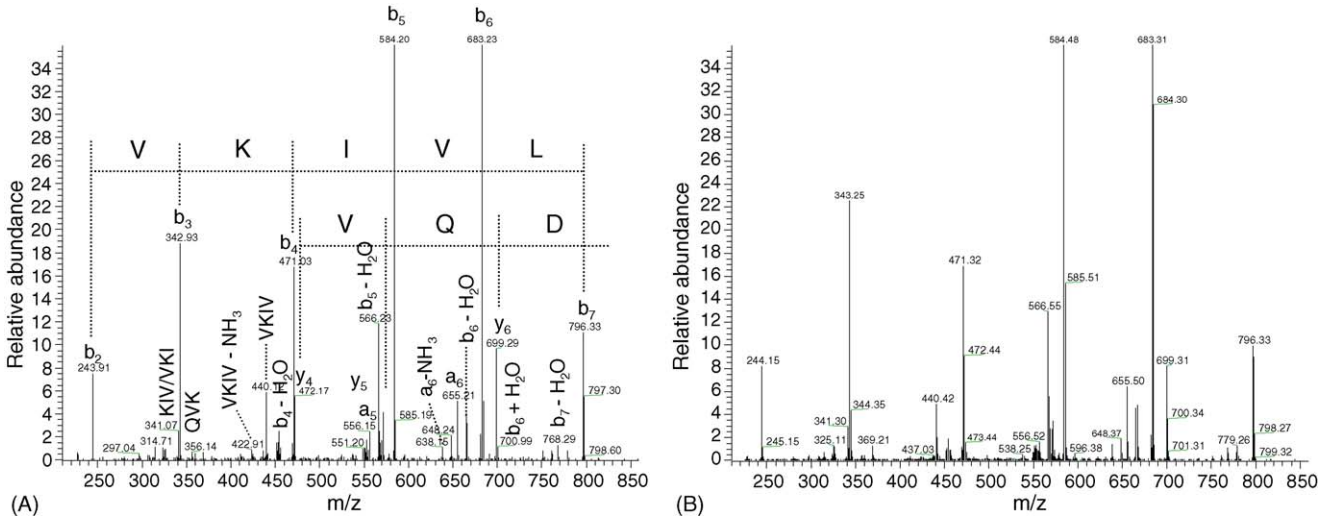

Fig. 2 – MS/MS spectra of endogenous DQVKIVL from FGO-conditioned seawater (A) using the same experimental conditions as for synthetic DQVKIVL (B).

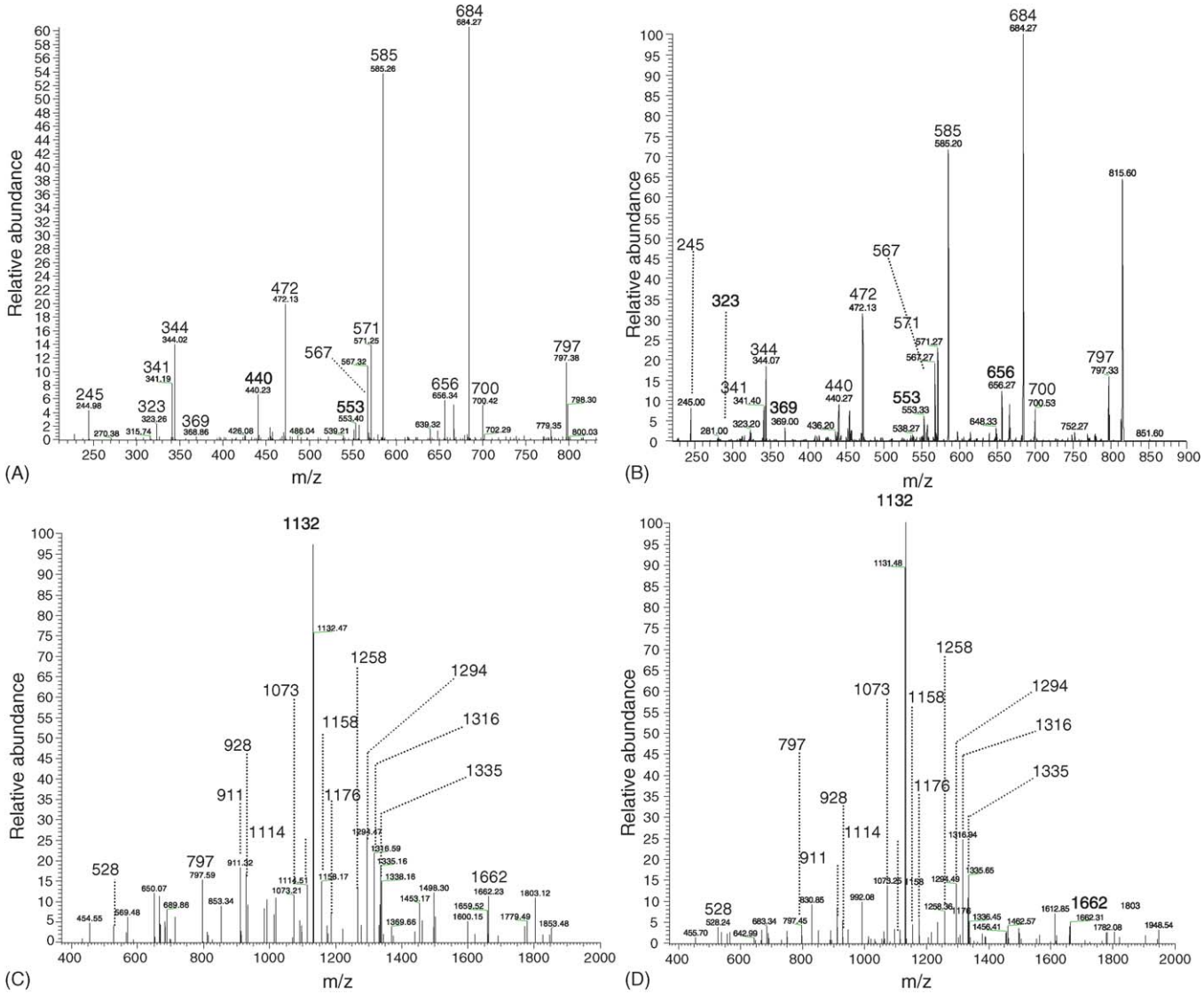

Fig. 3 – MS/MS spectra of the *m/z* 815 from FGO (A) and FGO-conditioned seawater (B). MS/MS spectra of the *m/z* 1397 from FGO (C) and FGO-conditioned seawater (D). The similarity of MS/MS spectra from FGO and FGO-conditioned seawater demonstrated that these peptides occurred in the two samples.

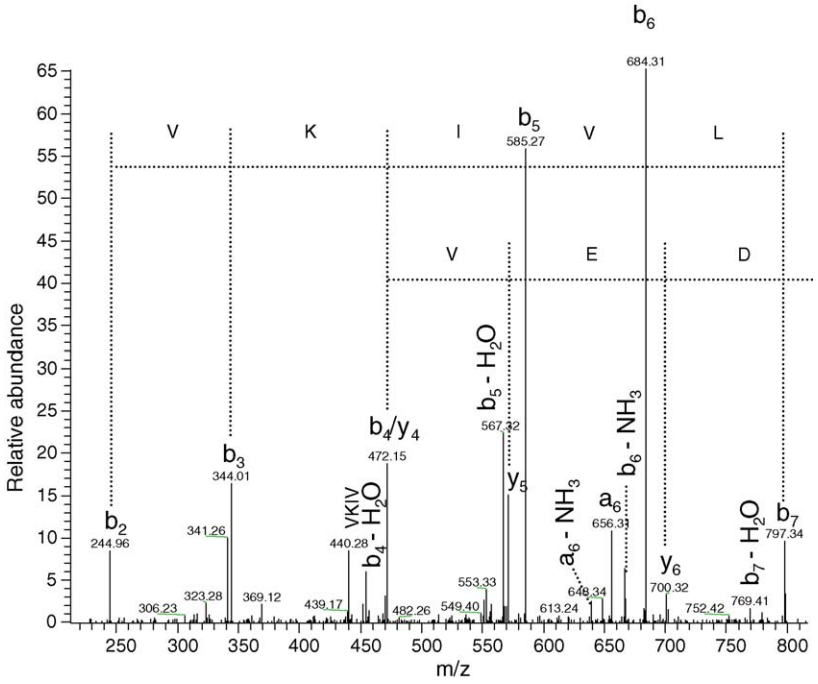

Fig. 4 – MS/MS spectra of the FGO single charged ion at *m/z* 815.5. MS/MS ion analysis led to the sequence DEVKIVL.

3.1.2. *Sequence determination*

The primary sequence of this peptide, DQVKIVL, was determined by Edman degradation and definitively checked by the comparison of MS/MS data obtain from DQVKIVL synthetic and endogenous peptide (Fig. 2).

3.2. *Identification of DEVKIVL and DEVKIVLD*

3.2.1. *Mass spectrometry comparative screening*

The samples of FGO and FGO-conditioned seawater were submitted to microLC-ESI-MS/MS analysis. The total ion chromatograms revealed several common peaks. We focused on the two peaks eluted between 27 and 28.5% and between 25 and 26.3% of ACN corresponding, respectively, to the *m/z* 815.5 and 1397.5. The similarity of retention time, MS and MS/MS spectra in the two samples demonstrated that peptides of *m/z* 815 and 1397 occurred in the two samples (Fig. 3).

3.2.2. *Identification of DEVKIVL*

3.2.2.1. *Sequence determination*. The primary sequence of the single charged ion at *m/z* 815.5 was determined from MS/MS spectrum. Identification of internal fragments, y, b and a ions led to the sequence DEVKIVL (Fig. 4). The combination of MS/MS data obtained from synthetic and endogenous peptides definitively confirmed the amino acid sequence and the absence of C-terminal amidation.

3.2.3. *Identification of DEVKIVLD*

3.2.3.1. *HPLC purification*. First step of purification was performed from FGO in microLC-ESI-MS/MS and 1 min LC–MS fractions were tested on the cuttlefish oviduct myotropic bioassay. After the first purification step, the activity was concentrated in the peak at 22.20 min retention time (Fig. 5A) containing the *m/z* 1397.5 detected by the comparative screening described above. After a second purification step in UV-HPLC (Fig. 5B), the *m/z* 1397.5 was concentrated in a pure peak eluted at 21.63 min (Fig. 5C). As the MS/MS spectrum did not allow to elucidate the primary sequence, the 21.63 min peak was concentrated to be submitted to Edman degradation.

3.2.3.2. *Sequence determination*. Primary sequence of this peptide was partially elucidated by Edman degradation: X-EVKIVL and acid hydrolysis: Glu (1), Val (2), Lys (1), Ile (1) Leu (1) and Asp (2). According to this amino-acid composition, the *m/z* of the peptide should be 930.5.

3.2.3.3. *microLC-ESI-MS analysis*. The zoomscan performed in microLC-ESI-MS, showed a ΔM of 0.5 between monoisotopic ions, demonstrating that the *m/z* 1397 was a dicharged ion (Fig. 6). From the putative *m/z* of 930.5, we deduced that the *m/z* 1397 corresponded to dicharged $3M2H^+$ of a peptide with a molecular weight of 929.5. Thus, the monocharged ion at *m/z* 930.5 was detected from FGO sample. According to the Edman degradation, acid hydrolysis and MS data, we proposed the following putative sequences: DDEVKIVL or DEVKIVLD. Finally, the comparison of MS/MS data issued from synthetic peptides (DDEVKIVL and DEVKIVLD) and endogenous *m/z* 930.5 peptide led to the single sequence: DEVKIVLD (Fig. 7). The occurrence of the *m/z* 1397.5 ($3M2H^+$) during ESI-MS analysis of synthetic peptide definitively established the sequence (Fig. 8). As the retention times of MH^+ and $3M2H^+$ were not similar, we concluded that the formation of the $3M2H^+$ was performed in the mobile phase before ESI-MS analysis. These data have probably to be associated to the property of these peptides to gel in water.

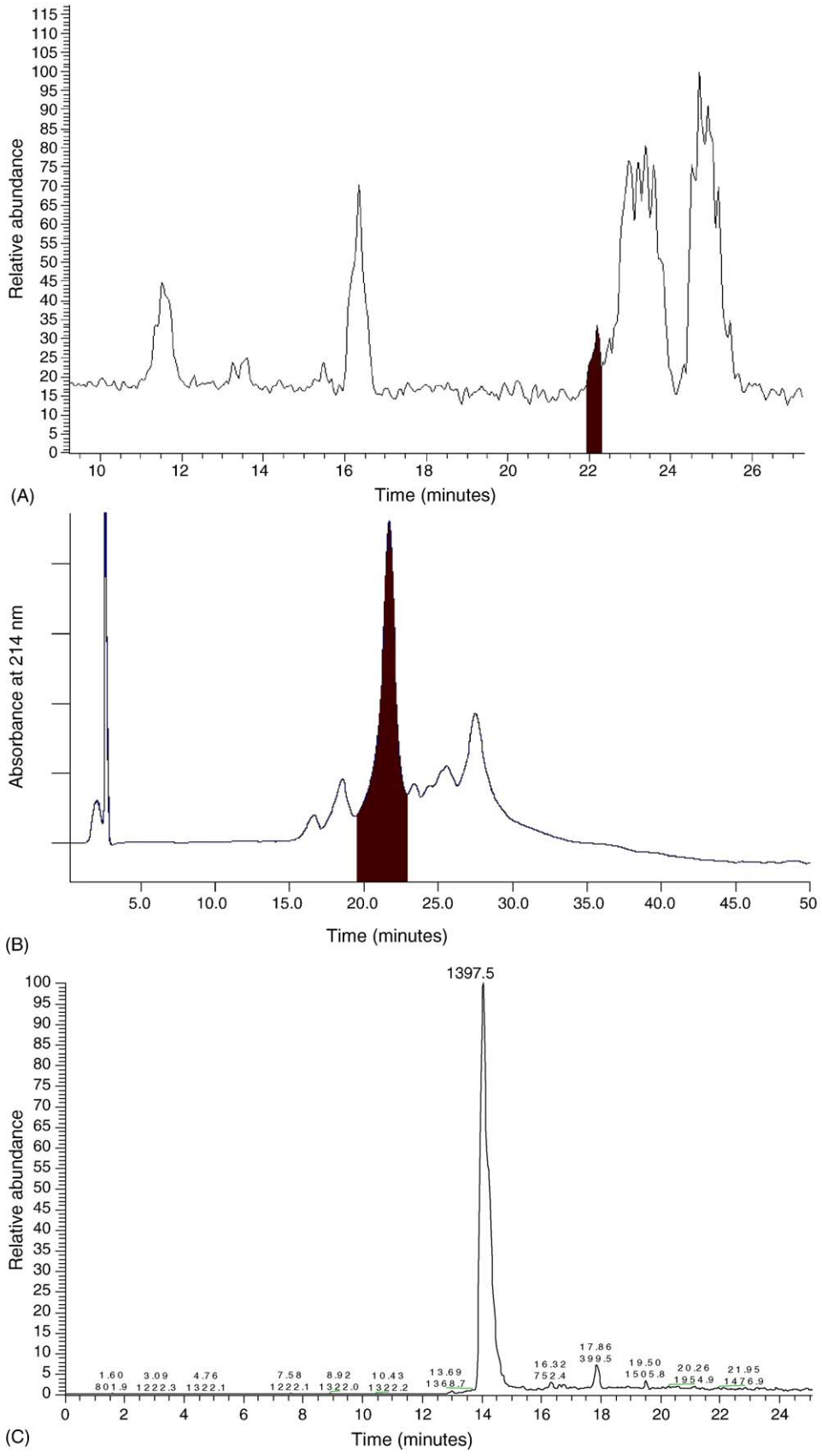

Fig. 5 – Ionic chromatogram of the first purification step of FGO in RP-HPLC with mass spectrometry detection. The myotropic fraction is indicated in black (A). Second step of purification of myotropic fraction in RP-HPLC with UV-detection. The 21.63 min fraction containing the dicharged ion at *m/z* 1397 is indicated in black (B). Total ionic chromatogram of the 21.63 HPLC fraction containing *m/z* 1397 obtained after two purification steps (C).

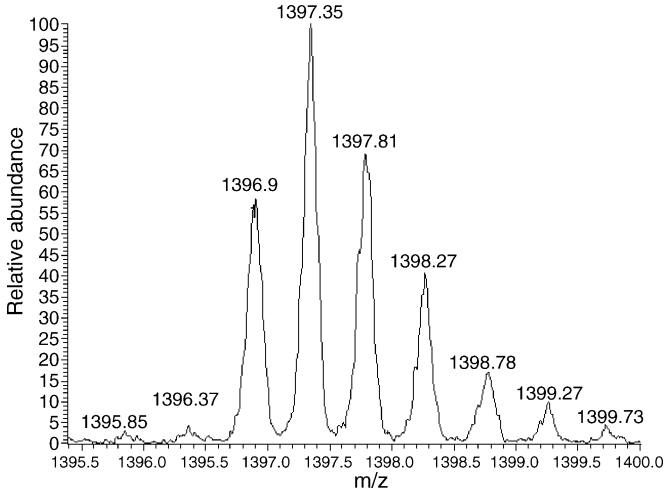

Fig. 6 – Zoomscan analysis of the *m/z* 1397.5. ΔM of 0.5 Da between monoisotopic peaks revealed that *m/z* 1397 is a dicharged ion.

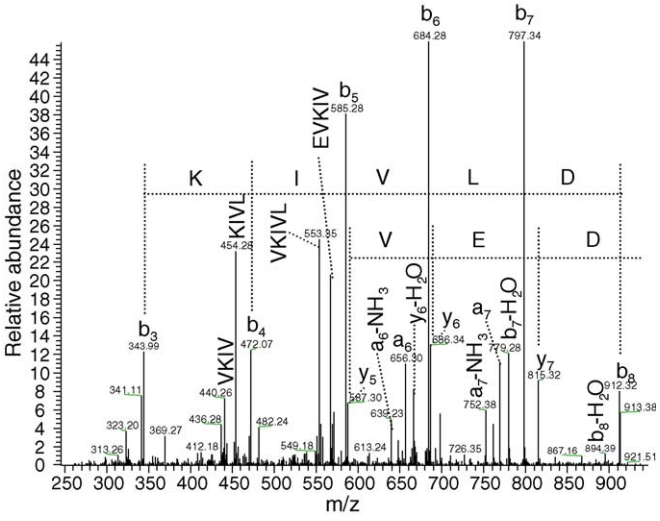

Fig. 7 – MS/MS spectra of endogenous DEVKIVLD from FGO.

3.3. *Bioactivity of synthetic peptides*

Increasing concentrations of synthetic peptides were tested on the female whole genital tract (proximal oviduct containing FGO, oviducal gland and distal oviduct), the main nidamental glands and ovarian stroma of mature females, the esophagus, rectum and penis. As shown in Table 1, the activities of ovarian jelly peptides (OJPs) are restricted to the genital tract and the main nidamental glands. DEVKIVL and DQVKIVL modulate the whole genital tract from 10^{-13} to 10^{-11} M and the main nidamental glands from 10^{-13} to 10^{-12} M (Figs. 9 and 10).

3.4. *MicroLC-ESI-MS/MS tissue mapping*

The mapping was performed in MS/MS mode based on the *m/z* corresponding to the peptides previously characterized (814, 815 and 930, respectively). As shown in Table 2, these peptides are strictly localized in the genital apparatus: the vitellogenic follicles, the FGO, the eggs and the internal egg-capsule (secreted by the oviduct gland). Although these peptides are released by FGO and vitellogenic follicles in the conditioned seawater, they are not detected in egg-conditioned seawater. The release by eggs could be stopped by the internal and/or external capsules. Some traces of OJPs were detected in the internal capsule, probably trapped during the polymerization of the egg-capsule. The analyses performed on the different parts of the central nervous system and the hemolymph did not reveal any trace of these peptides.

4. Discussion

Few ovarian peptide families have been characterized in invertebrates. In cnidaria and echinodermata, the SAPs

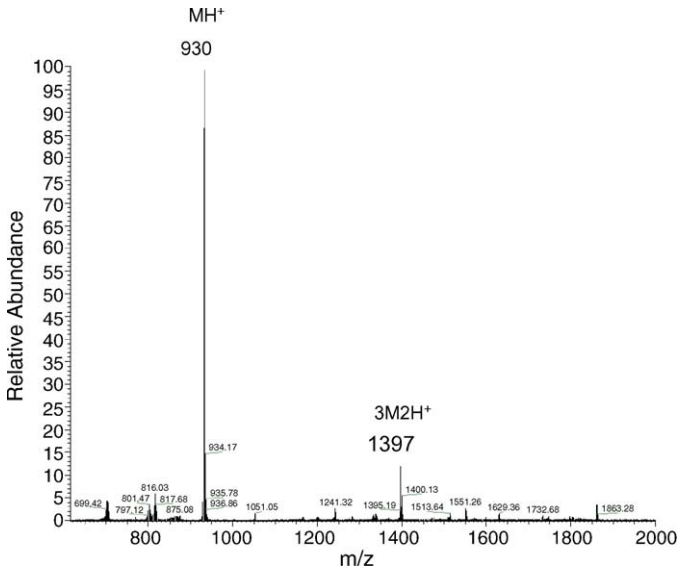

Fig. 8 – ESI-MS analysis of synthetic DEVKIVLD (*m/z* 930) revealed the formation of $3M2H^+$ (*m/z* 1397.5).

Table 1 – Bioactivity of OJPs

Organs bioassayed	Threshold concentration	
	DEVKIVL	DQVKIVL
Whole mature genital tract	10^{-13}	10^{-11}
Esophagus	NA	NA
Ovarian stroma	NA	NT
Rectum	NA	NA
Penis	NA	NA
Main nidamental glands	10^{-13}	10^{-12}

NA: no activity; NT: not tested.

Table 2 – Tissue mapping of OJPs using microLC-ESI-MS/MS detection

	DEVKIVL	DQVKIVL	DEVKIVLD
Previtellogenic follicles	nd	nd	nd
Vitellogenic follicles	d	d	d
Full-grown oocytes	d	d	d
Eggs	d	d	d
Internal capsule	d	d	d
External capsule	nd	nd	nd
Vitellogenic follicles-conditioned seawater	d	d	d
Full-grown oocytes-conditioned seawater	d	d	d
Egg-conditioned seawater	nd	nd	nd
Central nervous system	nd	nd	nd
Hemolymph	nd	nd	nd

nd: not detected; d: detected.

(Sperm Activating or Attracting Peptides) which are short peptides, large glutamine-rich peptides, circular or linear, have been classified by Suzuki into five groups: SAP-I [26]; SAP-II [24,27]; SAP-III [25]; SAP-IV [30] and SAP-V [29] in relation to their amino acid sequence and structure (for review, see [23]). In insects, trypsin modulating oostatic factors (TMOF) control egg development [4,5] and inhibits ecdysone biosynthesis [13]. In mollusks, attractin is released from egg cordons and induces the aggregation of adults [8,19,20]. In invertebrates, ovarian regulatory peptides are involved in the main egg-laying steps: oocyte transport, egg capsule secretion, fertilization and gathering of mature individuals for reproduction. In this context, we focused on the identification of regulatory peptides expressed in the ovary and targeting the oviduct and accessory sex glands of the cephalopod *S. officinalis*. After the identification of ILME, SepOvotropin and SepCRP [2,32,33], we investigated the occurrence of new regulatory water-borne peptides using mass spectrometry. UV-HPLC purification and mass spectrometry screening led to the identification of three related peptides: DQVKIVL, DEVKIVL and DEVKIVLD. Although DEVKIVL and DEVKIVLD are also present in FGO-conditioned seawater, only one peak of myotropic activity (which was identified as the peptide DQVKIVL) was detected after UV-HPLC purification. We can speculate that the kinetics of OJPs release are not similar for the three peptides. Sequence homologies, biological activities and tissue mapping, clearly demonstrate that these peptides belong to a single peptide family. Although DEVKIVL appeared as a breakdown product of DEVKIVLD, its detection in FGO-conditioned seawater, which is only concentrated on C18 chromafix without any acidic extraction, leads to the conclusion that this peptide does not result from acidic cleavage. Moreover, the investigation of putative breakdown products of DEVKIVL (DEVKIV, DEVKI, ...) which were not recovered from FGO neither from FGO-conditioned seawater put forward for consideration that DEVKIVL is dued to the product of a post-translational processing performed in vitellogenic follicles and FGO. A similar processing was recently reported from SepCRP related peptides in *S. officinalis* [1]. Biological activities established from synthetic peptides showed that OJPs modulate the whole genital tract and the main nidamental gland contractions of mature females. OJPs probably force the storage of the FGO in the genital coelom before mating and are involved in the

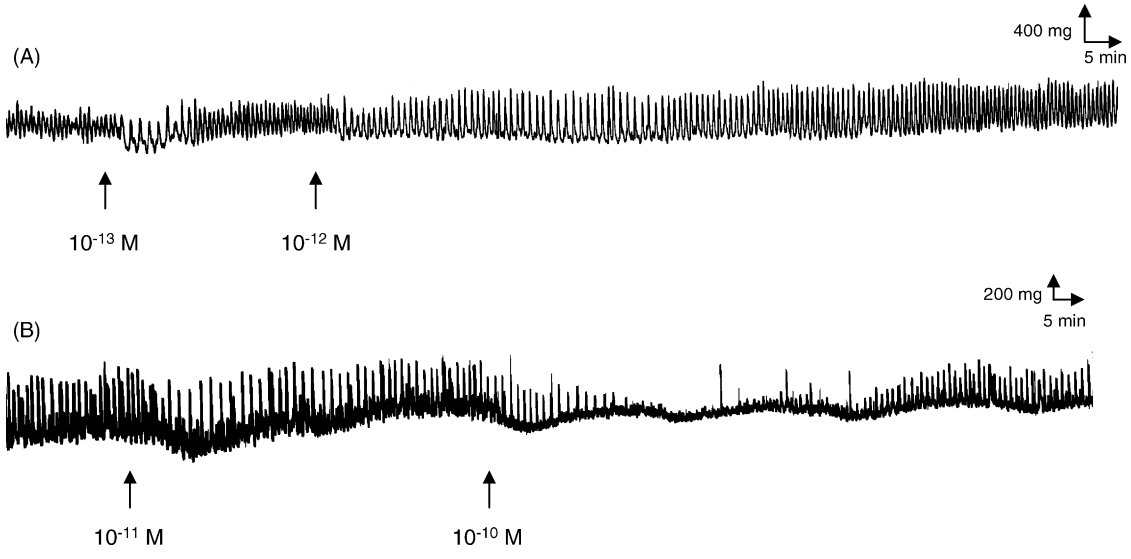

Fig. 9 – Contractions of the cuttlefish whole genital tract in response to threshold concentrations of (A) DEVKIVL (10^{-13} M) and (B) DQVKIVL (10^{-11} M).

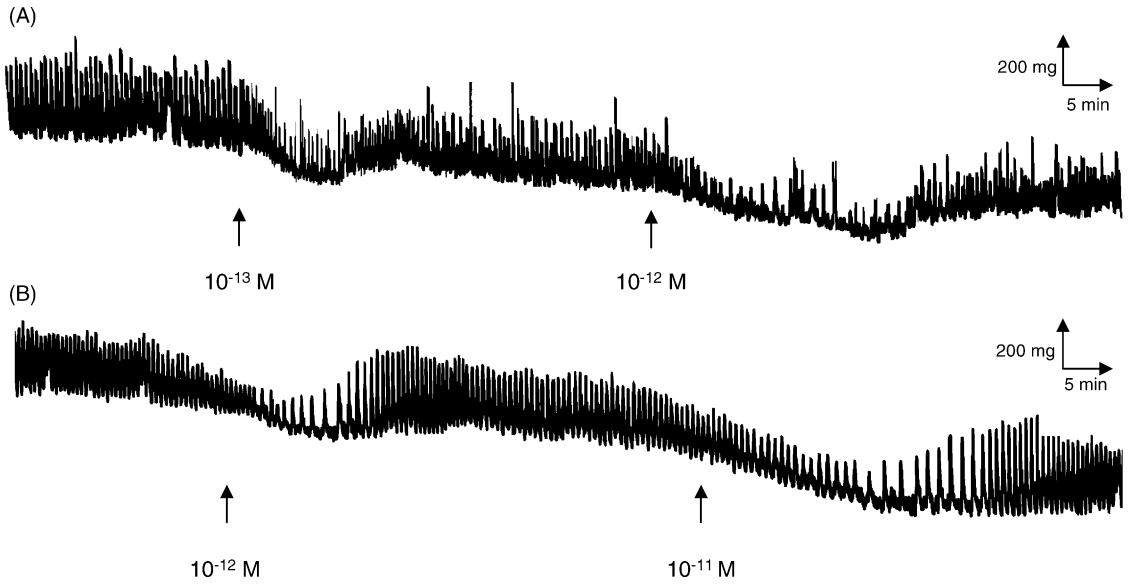

Fig. 10 – Contractions of the cuttlefish main nidamental gland in response to threshold concentrations of (A) DEVKIVL (10^{-13} M) and (B) DQVKIVL (10^{-12} M).

mechanical secretion of egg capsule products. The activity thresholds showed that OJPs modulate the contraction of whole genital tract and the main nidamental glands at concentrations as low as 10^{-12} and 10^{-11} M. The substitution of glutamic acid (E) by glutamine (Q) seems to reduce the activity of the peptide. Moreover, OJPs biological activity is restricted to the egg-laying period. Biological activity and tissue mapping very similar to those of SepCRP [2], suggest that OJPs and SepCRP act in concert to regulate oocyte release and egg capsule secretion. Observations performed in captivity showed that mating induces egg-laying behavior less than 1 h after sperm is deposited in the copulatory pouch. Modifications of the regulatory peptide cocktail targeting the oviduct and accessory sex glands are probably responsible for the stereotyped behavior leading to the formation of eggs (oocytes and capsules) and egg mass. This is illustrated by the fact that regulatory peptides released after mating, such as ILME [16], SepOvotropin [17], FMRFamide-related peptides (FaRPs) [21] and APGWamide-related peptides [20] would take over from peptides allowing the storage of FGO in the virgin females. The property to gel in water observed for synthetic peptides led to technical difficulties during purification steps and was exacerbated for DEVKIVLD. The peptide jelly, which can be compared to a reversible polymerization, could induce slow peptide diffusion in seawater which would contribute to the maintenance of a constant peptide concentration in the genital coelom, oviduct and mantle cavity, close to the targets.

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REFERENCES

[1] Bernay B, Baudy-Floc'h M, Zanuttini B, Gagnon J, Henry J. Identification of SepCRP analogues in the cuttlefish *Sepia officinalis*: a novel family of ovarian regulatory peptides. Biochem Biophys Res Commun 2005;338:1037–47.
[2] Bernay B, Gagnon J, Henry J. Egg capsule secretion in invertebrates: a new ovarian regulatory peptide identified by mass spectrometry comparative screening in *Sepia officinalis*. Biochem Biophys Res Commun 2004;314:215–22.
[3] Boletzky S. Fecundity variation in relation to intermittent or chronic spawning in the Cuttlefish, *Sepia officinalis* L. (Mollusca Cephalopoda). Bull Mar Sci 1987;40:382–7.
[4] Borovsky D, Carlson D, Griffin P, Shabanowitz J, Hunt D. Mosquito oostatic factor: a novel decapeptide modulating trypsin-like enzyme biosynthesis in the midgut. FASEB J 1990;4:3015–20.
[5] Bylemans D, Borovsky D, Hunt D, Shabanowitz J, Grauwels L, De Loof A. Sequencing and characterization of trypsin modulating oostatic factor (TMOF) from the ovaries of the grey fleshfly, Neobellieria (Sarcophaga) bullata. Regul Pept 1994;50:61–72.
[6] Cummins S, Nichols A, Amare A, Hummon A, Sweedler J, Nagle G. Characterization of aplysia enticin and temptin, two novel water-borne protein pheromones that act in concert with attractin to stimulate mate attraction. J Biol Chem 2004;279:25614–22.
[7] Cummins SF, Nichols AE, Warso CJ, Nagle GT. Aplysia seductin is a water-borne protein pheromone that acts in concert with attractin to stimulate mate attraction. Peptides 2005;26:351–9.
[8] Cummins SF, Schein CH, Xu Y, Braun W, Nagle GT. Molluscan attractins, a family of water-borne protein

pheromones with interspecific attractiveness. Peptides 2005;26:121–9.
[9] Dhainaut A, Richard A. Vitellogenèse chez les céphalopodes decapods. Evolution de l'ovocyte et des cellules folliculaires au cours de la maturation génitale. Arch Anat Micr Morph Exp 1976;65:183–208.
[10] Hansbrough J, Garbers D, Speract. Purification and characterization of a peptide associated with eggs that activates spermatozoa. J Biol Chem 1981;256:1447–52.
[11] 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.
[12] Henry J, Zatylny C, Boucaud-Camou E. Peptidergic control of egg-laying in the cephalopod *Sepia officinalis*: involvement of FMRFamide and FMRFamide-related peptides. Peptides 1999;20:1061–70.
[13] Hua Y, Bylemans D, De Loof A, Koolman J. Inhibition of ecdysone biosynthesis in flies by a hexapeptide isolated from vitellogenic ovaries. Mol Cell Endocrinol 1994; 104:R1–4.
[14] Jecklin L. Beitrag zur kenntnis der Laichgellerten und der biologie der embryonen decapoder cephalopoden. Rev Suisse Zool 1934;41:593–673.
[15] Karlson P, Luscher M. Pheromones': a new term for a class of biologically active substances. Nature 1959;183:55–6.
[16] Kikuyama S, Yamamoto K, Iwata T, Toyoda F. Peptide and protein pheromones in amphibians. Comp Biochem Physiol B 2002;132:69–74.
[17] Luporini P, Miceli C, Ortenzi C, Vallesi A. Ciliate pheromones. Prog Mol Subcell Biol 1996;17:80–104.
[18] Nishigaki T, Chiba K, Miki W, Hoshi M. Structure and function of asterosaps, sperm-activating peptides from the jelly coat of starfish eggs. Zygote 1996;4:237–45.
[19] Painter S, Clough B, Akalal D, Nagle G. Attractin, a water-borne peptide pheromone in *Aplysia*. Invert Reprod Develop 1999;36:191–4.
[20] Painter S, Clough B, Garden R, Sweedler J, Nagle G. Characterization of Aplysia attractin, the first water-borne peptide pheromone in invertebrates. Biol Bull 1998;194:120–31.
[21] Ram J, Muller C, Beckmann M, Hardege J. The spawning pheromone cysteine-glutathione disulfide ('nereithione') arouses a multicomponent nuptial behavior and electrophysiological activity in Nereis succinea males. FASEB J 1999;13:945–52.
[22] Sole N, Barany G. Optimization of solid-phase synthesis of [Ala8]-dynorphin A. J Org Chem 1992;57:5399–403.
[23] Suzuki N. Structure, function and biosynthesis of sperm-activating peptides and fucose sulfate glycoconjugate in the extracellular coat of sea urchin eggs. Zool Sci 1995;12:13–27.
[24] Suzuki N, Kajiura H, Nomura K, Garbers DL, Yoshino K, Kurita M, et al. Some more speract derivatives associated with eggs of sea urchins, *Pseudocentrotus depressus*, *Strongylocentrotus purpuratus*, *Hemicentrotus pulcherrimus* and *Anthocidaris crassispina*. Comp Biochem Physiol B 1988;89:687–93.
[25] Suzuki N, Kurita M, Yoshino K, Yamaguchi M. Speracts binds exclusively to sperm tails and causes an eletrophoretic mobility shift in a major sperm tail protein of sea urchins. Zool Sci 1987;4:641–8.
[26] Suzuki N, Nomura K, Ohtake H, Isaka S. Purification and the primary structure of sperm-activity peptides from the jelly coat of sea urchin eggs. Biochem Biophys Res Commun 1981;99:1238–44.
[27] Suzuki N, Shimomura H, Radany E, Ramarao C, Ward G, Bentley J, et al. A peptide associated with eggs causes a mobility shift in a major plasma membrane protein of spermatozoa. J Biol Chem 1984;259:14874–9.
[28] Toyoda F, Yamamoto K, Iwata T, Hasunuma I, Cardinali M, Mosconi G, et al. Peptides pheromones in newts. Peptides 2004;25.
[29] Yoshino K, Takao T, Shimonishi Y, Suzuki N. Sperm-activating peptide type-V (SAP-V), a fifth member of the sperm-activating peptide family, purified from the egg-conditioned media of the heart urchin Brissus agassizii. Comp Biochem Physiol B 1992;102:691–700.
[30] Yoshino K, Takao T, Suhara M, Kitai T, Hori H, Nomura K, et al. Identification of a novel amino acid, o-bromo-L-phenylalanine, in egg-associated peptides that activate spermatozoa. Biochemistry 1991;30:6203–9.
[31] Zatylny C, Durantou F, Boucaud-Camou E, Henry J. Evidence of 5-hydroxytryptamine synthesis in the follicles of *Sepia officinalis* and direct involvement in the control of egg-laying. Mol Reprod Dev 2000;55:182–8.
[32] Zatylny C, Gagnon J, Boucaud-Camou E, Henry J. The SepOvotropin: a new ovarian peptide regulating oocyte transport in *Sepia officinalis*. Biochem Biophys Res Commun 2000;276:1013–8.
[33] Zatylny C, Gagnon J, Boucaud-Camou E, Henry J. ILME: a waterborne pheromonal peptide released by the eggs of *Sepia officinalis*. Biochem Biophys Res Commun 2000;275:217–22.
[34] Zatylny C, Marvin L, Gagnon J, Henry J. Fertilization in *Sepia officinalis*: the first mollusk sperm-attracting peptide. Biochem Biophys Res Commun 2002;296:1186–93.
[35] Zeeck E, Müller C, Beckmann M, Hardege J, Papke U, Sinnwell V, et al. Cysteine-glutathione disulfide, the sperm-release pheromone of the marine polychaete *Nereis succinea* (Annelida: Polychaeta). Chemoecology 1998;8:33–8.