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Fertilization in *Sepia officinalis*: the first mollusk sperm-attracting peptide

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Abstract

Egg mass extract was used to characterize regulatory peptides, involved in the successive steps of egg-laying of the cuttlefish *Sepia officinalis*. Among these peptides, a C-terminally amidated hexapeptide revealed a sperm-attracting activity. MALDI-TOF MS (matrix-assisted laser desorption ionization-time of flight mass spectrometry) and Edman degradation led to a peptide of *m*/*z* 596.6 and the following primary sequence: Pro–Ile–Asp–Pro–Gly–Val–CO_{NH2}. From concentrations as low as 10^{-17} M, the PIDPGVamide was able to attract freshly dissected spermatozoa. Nano-ESI-Q-TOF MS (nano-electrospray ionization-quadrupole-time-of-flight mass spectrometry) analysis established the quantitative occurrence of this peptide in different egg structures. The PIDPGVamide appears to be synthesized in oocytes during vitellogenesis and released by the embedded oocytes in the external media during egg-laying to facilitate fertilization by increasing chances of gamete collision. This novel peptide called SepSAP for *Sepia* sperm attracting peptide is the first sperm-attracting peptide, identified in mollusks or even in protostomians.

Keywords: Chemotaxis; Fertilization; Sperm; Oocyte; Sperm-attracting peptides; Mollusks; Cephalopods; *Sepia officinalis*; Mass spectrometry; In vitro bioassays

In the cuttlefish *Sepia officinalis*, fertilization is achieved by the female in the external medium after the secretion of gelatinous envelopes by accessory sex glands that constitute the egg capsule. As shown in Fig. 1, the full grown oocytes (B) pass through the oviducal gland where the first egg capsule is secreted (C). The partially embedded oocyte is released by the distal oviduct into the mantle cavity where a second envelope, stained with ink, is secreted by the nidamental glands (D). The spermatozoa stored in the copulatory pouch located under the buccal mass are released in the external medium around the embedded oocyte (E). The occurrence of sperm attractant in the oocyte and/or in the capsule is essential to increase chances of collision and, thus, to allow fertilization.

In prostostomes, although no factor had been identified until now, evidence for sperm chemotaxis had been reported for the chiton (mollusk polyplacophora), as its sperm deviates in the direction of a pipette injecting an egg extract [1]. Clapper and Brown [2,3] observed that *Limulus* sperm, as many arthropod sperm, were almost completely inactive when spawned and were activated probably by some agent diffusing from the egg.

In the Deuterostome phyla, many sperm-attracting and/or activating factors have been identified. These factors are usually small peptides ($M_r < 2000$) in invertebrates and larger peptides ($M_r > 8000$) or protein in vertebrates. Their origin can be the ovarian follicles in human [4], the jelly coat in the Xenopus [5], the oocytes in the Ascidies [6] or the follicular cells in Herring and in Echinids [7].

More than 70 sperm-activating peptides (SAPs) with slight differences in amino acid sequence have been identified in sea urchins [8–13] and 12 in starfish [14]. Amino acid sequences were different for sea urchins and starfish peptides, suggesting a species specificity. These

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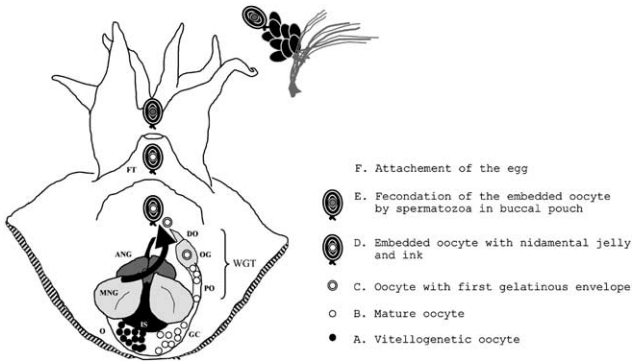

Fig. 1. Illustration of the successive steps of egg-laying in *S. officinalis*. A ventral view of the animal is presented.

peptides stimulate sperm motility and respiration, attract spermatozoa to the eggs [15], and facilitate the acrosome reaction [16].

In teleosteans, five herring sperm-activating proteins (HSAPs) have been purified from egg-conditioned sea water [17]. The similar sperm-activating capacity and N-terminal amino acid sequence suggested that these HSAPs were isoforms. Recently, a secretory protein of 73 amino acids has been deduced from a herring egg cDNA [7]. This HSAP matched the sequence of the N-terminal portion for the residues 22–61 of the purified HSAPs exactly.

In amphibians, if a 10 kDa heat-stable protein from the egg jelly of *Xenopus laevis* induced a dose-dependent chemoattractant activity [5] in sperm, a 21 kDa protein has been recently characterized from *Xenopus* egg jelly [18].

In pigs, sperm membrane proteins of 105, 56–62 and 45 kDa able to bind the egg extracellular matrix have been identified [19].

In humans, ovarian follicles are suspected to release sperm factors, which could stimulate the spermatozoid motility. These non-identified factors could play a role in the orientation of spermatozoa toward an ovulated egg to increase the chances of collision and to prevent fertilization by defective sperm [20].

In recent years, we have focused on the *S. officinalis* regulatory factors involved in the successive steps of egg-laying: first, the storage of full grown oocytes in the genital coelom induced by the release of 5-HT in the lumen of the genital tract to inhibit the proximal oviduct until mating [21], second, the transport of oocytes into the genital tract by oviduct contractions modulated by neuropeptides related to APGWamide and FMRFamide families [22,23] and by ovarian peptides, which induced oviduct cyclic contractions and stimulated the contractions of accessory sex glands [24,25], and third, the attraction of mature adults in the egg-laying coastal areas by the release of water-borne pheromonal peptides by freshly spawned eggs [25].

In this context, the recent isolation of the water-borne pheromonal peptide ILME from *S. officinalis* egg mass [25] led to the characterization of two C-terminally amidated peptides (PIDPGVamide and FQamide) among five novel primary structures. The putative activity of these two peptides was successively verified on two in vitro bioassays: the contraction of the oviduct and the chemotaxis of spermatozoa.

In this report, we demonstrate that the C-terminally amidated hexapeptide PIDPGVamide is released in the external medium by the full grown oocytes and that in appropriate concentration, it can direct sperm to increase the probability of contact between gametes.

Experimental procedures

Animals. All mature cuttlefish were trapped in the Bay of Seine in May and June. They were maintained in 1000 L outflow tanks at the Marine Station of Luc sur Mer (University of Caen, France). The eggs released by several fertilized females in rearing tanks were collected during the three days following egg-laying.

For HPLC purification. 1.422 kg eggs were crushed in 5000 ml of methanol:water:acetic acid (90:9:1, v/v) and centrifuged 20 min at 25,000*g* at 4 °C. The supernatants were evaporated in a rotavapor and concentrated on chromafix C18.

For the nano-ESI-Q-TOF analysis. Ten vitellogenic follicles, 10 full grown oocytes, five whole newly spawned eggs, and five egg capsules were submitted to the extraction described above for purification. Moreover, 50 full grown oocytes were incubated 10 h in 20 ml sea water and 1000 freshly spawned eggs in 2 L sea water. The incubation media were concentrated on chromafix C18.

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 dry egg extract was successively separated onto a Macherey–Nagel RP18ec column (4.6×250, 5 μm) with an acetonitrile linear gradient of 1.33% per minute in TFA (0.1%). The second and last steps of purification were performed onto a Nucleosil C18 column (250×3, 7 μm), with an acetonitrile linear gradient of 1.33% per minute in ammonium acetate (25 mM).

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.

ESI-MS/MS analyses were done on a Q-TOF2 instrument (Micromass, UK) equipped with an orthogonal electrospray source (*Z-Spray*) operated in the positive electrospray ionization mode (ESI+). Instrument operation, data acquisition, and analysis were performed using MassLynx/Biolynx 3.5 software (Micromass), on a Windows NT server. Nal/CS mixture was used for mass calibration checks. Dried samples were resuspended in 5 μl MeOH/H_2O/AcOH (50:50:0.1%, v/v/v) and loaded into Au/Pd-coated nanoES capillaries (Protana, Odense, Denmark). The capillaries were positioned into the nanoflow Z-spray source using a binocular. Argon was used as collision gas at a pressure of 1 bar and a collision energy set at 35 eV. The mass range for acquisition was from *m/z* 400 to 700 and from *m/z* 50 to 620 in full scan MS and MS/MS, respectively. The quantities of peptide in unknown samples were estimated in MS/MS mode by selecting *m/z* 596 as product ion during an acquisition time of 2 min.

Sample preparation for nano-ESI-Q-TOF analysis was performed from tissue extracted concentrated as described above for purification, and submitted to the first-step HPLC.

The oviduct was dissected out from mature females and 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 the flow rate of 0.5 ml min^{-1} with synthetic sea water (Instant Ocean) containing 1 mM glucose and maintained at 15 °C. The synthetic peptides were injected in the perfusing flow using a three-way valve to avoid mechanical and thermal stresses. The flow of the fractions into the muscle chamber was traced by addition of phenol red (1 μM).

The spermatophores (complex system tubular containing spermatozoa) were removed from spermatophoric sacs or Needham's organs of male cuttlefish. The spermatophore, placed in artificial sea water (Instant Ocean) at 15 °C during 1 h, released spermatozoa. A swim-up spermatozoa suspension was collected and diluted to a working concentration of 4×10^6 cells/ml or 2×10^6 cells/ml in artificial sea water (ASW).

Three distinct sperm-attracting assays were developed. For the first one, the sealed chamber was a 140 mm × 21 mm petri dish. Four discs of agar 1% (20 mm × 10 mm) were placed in the chamber (Fig. 2A). ASW was disposed in such a way that all discs were recovered. The discs A–C served as control, while the disc D contained the synthetic peptide. The spermatozoa suspension (4×10^6 cells/ml) was placed in the centre of the chamber. After 2 h, the spermatozoa repartition was observed using inverted microscope.

The second one was performed in a T sytem. In a typical experiment (Fig. 2B), 2 ml ASW was placed in a cleaned and air-dried T system. The potential sperm-attractant (oocyte or synthetic peptide) was placed in the *Z* well determined in random manner. The spermatozoa suspension (4×10^6 cells/ml) was placed in the *X* well. After 10, 20, or 30 min at 15 °C, the point of intersection of T-system was made watertight and the contents of each zone were collected for sperm quantification. The number of spermatozoa was calculated for each sample by protein content determination [26] after a trichloroacetic extraction (10%). A relationship was established between the protein concentration and the number of spermatozoa per ml, using sperm suspensions of known concentration. Five replicates were performed for these experiments.

The third one was realized in a 96-well micro-chemotaxis system (chemoTx 116-8, Neuroprobe, Cabin John, MD). The lower wells were filled with a range of increasing concentrations of PIDPGVamide from 10^{-19} to 10^{-9} M or ASW for control. The upper wells were separated from the lower ones by polycarbonate nucleopore filter (8 μm pore diameter). The upper wells were filled with 50 μl swim-up spermatozoa suspended in ASW at a density of 2×10^6 cells/ml (Fig. 2C). The chamber was then incubated for 10 min at 15 °C. The accumulation of spermatozoa in the lower wells was measured, as described above for the T-system. Eight controls and eight replicates were performed for each PIDPGVamide concentration. All the experiments were submitted to a Student's *t* test.

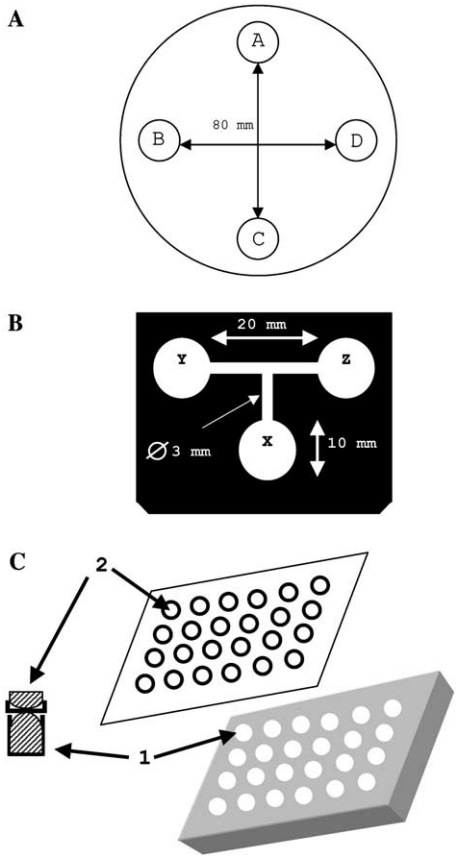

Fig. 2. Chemotaxis bioassays. (A) Sealed chamber. Four discs of agar corresponding to three controls and one experimental. The sperm suspension is deposited in the cross centre. After two hours of incubation at 15 °C, the spermatozoa repartition is observed with an inverted microscope. (B) T-system. Sperm suspension is deposited in the *X* well, control, and synthetic peptides (or oocytes) in the *Y* and *Z* wells, respectively. After 10, 20, or 30 minutes of incubation at 15 °C, the sperm content of each well is indirectly determined by the protein concentration. (C) Chemotaxis assay. (1) The 96 wells are filled with sea water (controls) or increasing concentrations of PIDPGVamide ($n = 8$). The sperm content is determined, as described above. (2) Sperm suspension is deposited on the top of the polycarbonate membrane (8 μm mesh).

Results

The purification of PIDPGVamide and FQamide was performed, as described for the peptide ILME [25]. PIDPGVamide, FQamide, and ILME were coeluted in an apparent pure HPLC fraction with three other peptides. The analysis of MALDI-TOF-MS spectrum associated to Edman degradation allowed elucidation of the primary sequence of the six peptides: ILME, PIDPGVa, FQa, AVLTPI, DYGLNF, and VQDPMR. The present study was focused onto the two C-terminally amidated peptides.

Oviduct bioassay

Injected in the perfusing flow from 10^{-6} to 10^{-12} M, PIDPGVa and FQa did not modify the spontaneous contractions of the distal oviduct dissected out from a mature female. Thus, these peptides were probably not involved in the transport of full grown oocytes into the female genital tract.

Sperm-attracting bioassays

Sealed chamber: a qualitative bioassay to study putative sperm-attracting activity of the peptides ILME, PIDPGVamide, and FQamide

The activity of ILME, PIDPGVamide, and FQamide was successively tested at increasing concentrations ranging from 10^{-6} to 10^{-10} M. The various concentrations of FQamide and ILME did not modify the random repartition of spermatozoa observed for the control in the sealed chamber. However, after two hours of incubation,

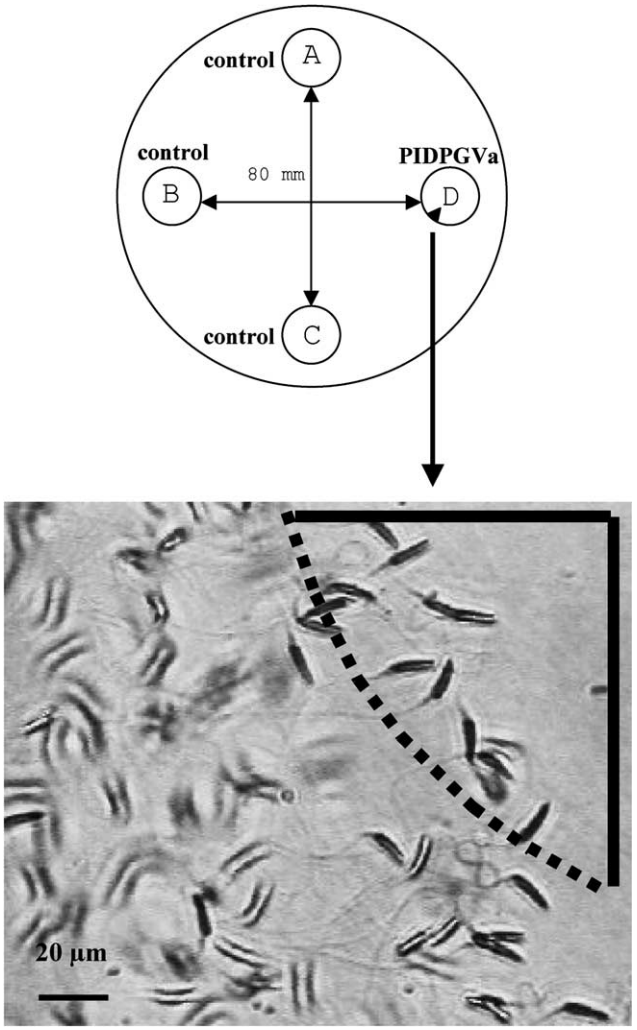

Fig. 3. Sealed chamber. The disc of agar containing PIDPGVamide is surrounded by a high concentration of spermatozoa.

the disc of agar containing 10^{-8} M PIDPGVamide appeared to be surrounded by a high concentration of spermatozoa (Fig. 3), suggesting a potential sperm-attracting activity.

T system: a quantitative bioassay for sperm attraction measurement

The T system was used to get quantitative data on the attracting power of the oocyte and the peptide PIDPGVamide. We observed that a full grown oocyte was able to attract spermatozoa. After 10 minutes of incubation, the sperm concentration of the Z wells containing the oocyte was significantly superior to the sperm concentration of the control in the Y well. The kinetic study showed that the difference between Y and Z well increased with the incubation time (Fig. 4). Following this first experiment, increasing concentrations of PIDPGVamide ranging from 10^{-19} to 10^{-9} M and applied in the Z well revealed a statically significant

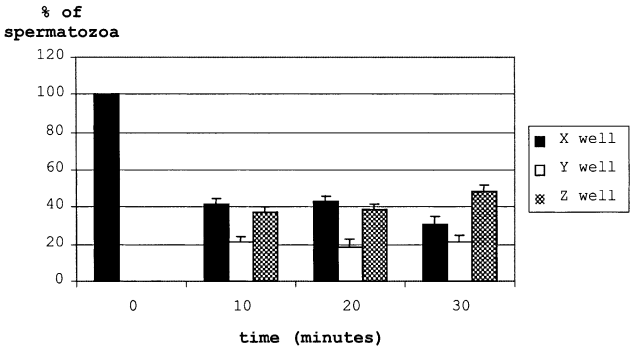

Fig. 4. Attraction of spermatozoa by full grown oocytes in the T-system. The difference of sperm concentrations between Y well (control) and Z well (oocyte) is statistically significant after 10 minutes of incubation. $P < 0.01$ for 10 min, $P < 0.006$ for 20 min, and $P < 0.006$ for 30 min ($n = 5$).

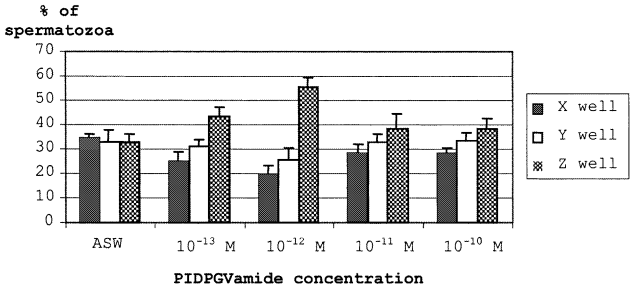

Fig. 5. T-system. Dose-dependent attraction of spermatozoa by increasing concentrations of PIDPGVamide. In the control (ASW), we observed a random distribution after 30 minutes of incubation. In the experimental series, the sperm concentration is statistically significant between control (Y well) and experiment (Z well) for 10^{-13} M ($P < 0.01$) and 10^{-12} M ($P < 0.001$) ($n = 5$).

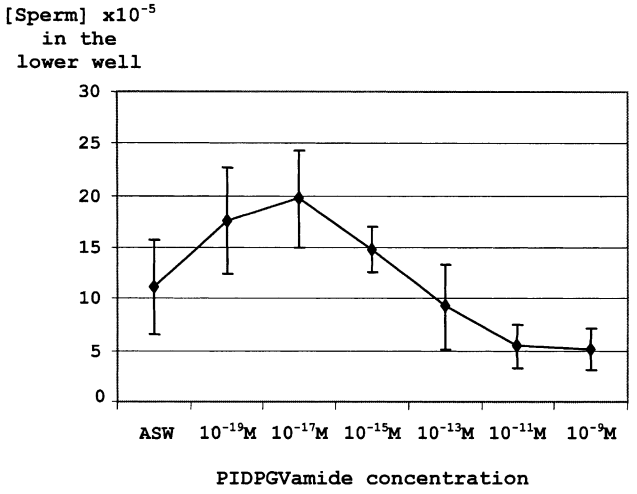

Fig. 6. Chemotaxis assay. Dose-dependent attraction of spermatozoa by increasing concentrations of PIDPGVamide. After 10 minutes of incubation, the sperm concentration is statistically different from that of the control (ASW) for 10^{-19} M ($P < 0.05$) and 10^{-17} M ($P < 0.01$) of PIDPGVamide ($n = 8$).

chemotaxis activity for 10^{-13} and 10^{-12} M. After 30 min of incubation, 56% of the total spermatozoa was in the well *Z*, against 25% in the control (well *Y*) and 19% in the well *X* where the sperm suspension was deposited (Fig. 5). For concentrations of 10^{-11} and 10^{-10} M, the attractive power of PIDPGVamide was still measurable but decreased and differences were not statistically significant (Fig. 6).

Chemotaxis assay: a quantitative bioassay, which partially reproduced natural fertilization conditions

In natural conditions, the fertilization of embedded oocyte is performed very close to the copulatory pouch in which the spermatozoa are stored since mating. The neuroprobe chemotaxis system allowed the reproduction of natural fertilization conditions. In each experimental well, the sperm suspension is just separated from the attractant by a porous membrane, which can be crossed by spermatozoa and soluble molecules.

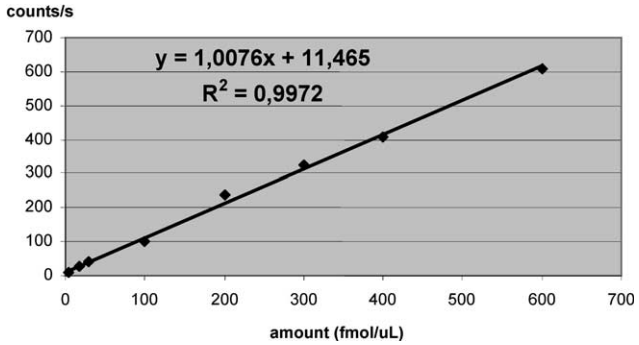

Fig. 7. PIDPGVamide concentration determination using nano-ESI-Q-TOF. The counts of the *m/z* 70, 326.1, and 596 obtained from samples of increasing concentrations of synthetic PIDPGVamide were used to elaborate a standard curve.

From 10^{-19} to 10^{-15} M PIDPGVamide, we observed a chemotaxis after only 10 minutes of incubation. For 10^{-11} and 10^{-9} M, the PIDPGVamide repelled the

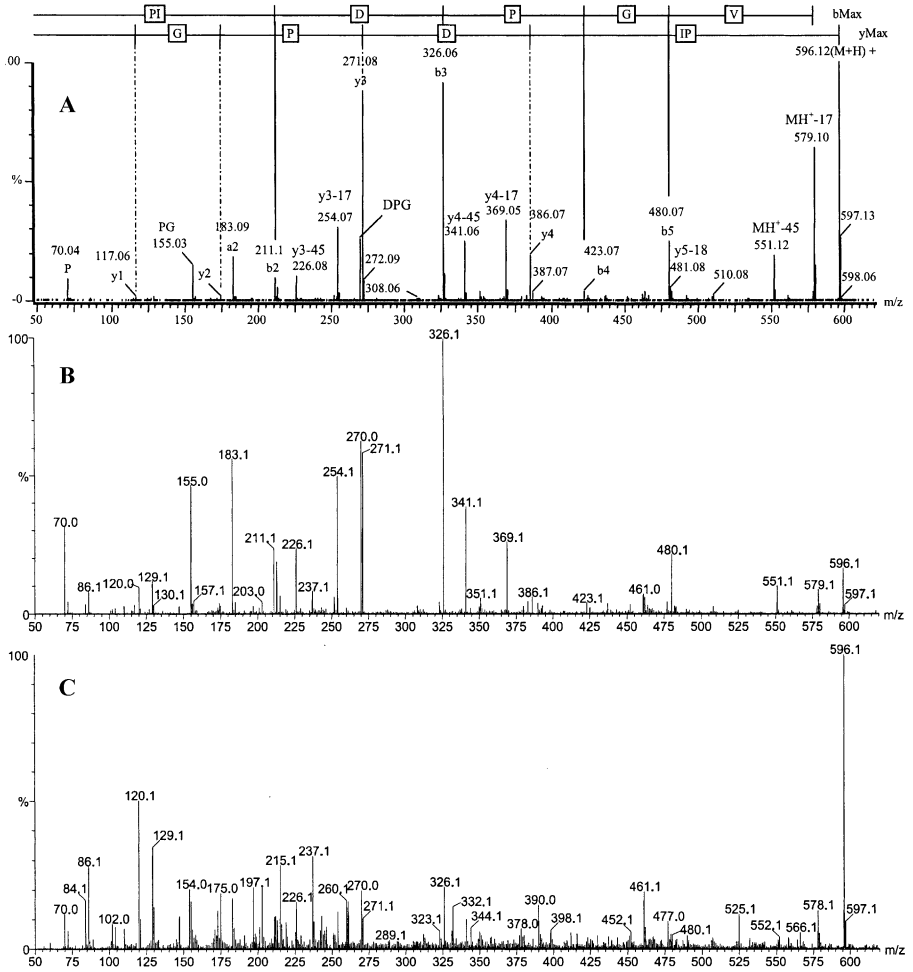

Fig. 8. MS/MS spectrum: synthetic PIDPGVamide (A), vitellogenic follicles (B), and full grown oocytes (C). The PIDPGVamide is identified in the samples by the occurrence of C-terminally fragments (*y* ions) and N-terminally fragments (*b* ions). The ions of *m/z* 70, 326.1, and 596 used for concentration determination were present in each sample.

Table 1
PIDPGVamide mapping and quantification

	Vitellogenic follicles (VF)	Full grown oocytes (FGO)	Freshly spawned eggs (< 24 h)	SW containing FGO	Egg capsule from eggs (> 24 h)	SW containing eggs (> 24 h)
PIDPGVa concentration	16 fmol/VF	30 fmol/FGO	60 fmol/egg	5 fmol/FGO/h	191 fmol/capsule	Not detected

spermatozoa. The maximum chemotaxis was obtained for 10^{-17} M and the threshold was inferior to 10^{-19} M.

Nano-ESI-Q-TOF

The mass parameters were optimized using the synthetic peptide. The occurrence of the peptide in the sample was established by the comparison between the synthetic peptide MS/MS spectrum and the sample MS/MS spectra for the *m*/*z* 596. This method allowed the detection of quantities nearer to the femtomole even from a complex matrix.

The detection was completed by the estimation of peptide quantities from the count number of the *m*/*z* 596, 326, and 70, respectively, corresponding to the molecular ion of the peptide, the fragment Pro–Ileu–Asp and the immonium ion of the proline. The standard curve is presented in Fig. 7.

The MS/MS spectra of two samples are presented (Figs. 8B and C), associated to the standard MS/MS spectrum of the synthetic PIDPGVamide (Fig. 8A).

In Table 1, the summarized results of the MS/MS analysis demonstrated that the expression of PIDPGVamide started during vitellogenesis in the ovarian follicles (Fig. 8B). The peptide was then accumulated in the oocytes (Fig. 8C) before being released during fertilization (Table 1). The concentrations of PIDPGVamide suggest that the expression of the peptide continued in the embedded oocyte. After fertilization, the newly spawned eggs did not release any detectable quantity of PIDPGVamide probably because of the egg capsule polymerization. The peptide is then accumulated in the egg capsule after fertilization (Table 1).

Discussion

The sperm-attracting peptide (SepSAP) characterized in the mollusk cephalopod *S. officinalis* represents the first molecule discovered in protostomians that are able to direct spermatozoa. The activity of this C-terminally amidated hexapeptide has been verified by three successive in vitro bioassays based on the establishment of a concentration gradient between the SepSAP source and the sperm suspension. Molecular, qualitative, and quantitative data clearly demonstrated the existence of a sperm–oocyte communication during fertilization in the cuttlefish. The attracting peptide is expressed early in the vitellogenic follicle, as show by mass spectrometry. It remains to be determined which tissue(s) are responsible for the peptide expression: the follicular cells, the oocyte or the both. Micro-dissection followed by mass spectrometry may elucidate this point. The expression and, thus, the accumulation of the peptide seems to continue during the storage of full grown oocytes in the genital coelom before mating. The SepSAP is released by the full grown oocytes in external medium (sea water in the case of cuttlefish). Also, to enable fertilization, embedded oocyte could release the SepSAP in the cavity bordered by the arms of the animal. Observation of the *Sepia* egg-laying demonstrated that female before attaching the egg by the arm tips to a solid substrate forms a cavity with arms to maintain embedded oocyte near copulatory pouch. During this stage of 3–4 min, we can suspect that the SepSAP diffusing across the soft egg capsule allows the sperm to reach the oocyte.

The quantification performed in mass spectrometry revealed that one oocyte released approximately 5 fmol/h and then 0.25 fmol for 3′, which is the time corresponding to the natural fertilization. Thus, the results obtained from the in vitro bioassay with the neuroprobe system, which gave a maximum of chemotaxis for a concentration of 0.01 fmol, appear to be consistent with the mass spectrometry data and confirm the existence of a concentration gradient, allowing the spermatozoa to reach the source of SepSAP release.

After fertilization, the polymerization of egg capsule (induced by the sea water pH) is probably responsible for SepSAP trapping. As showed by the MS data, the trapping triggers a local peripheral increase of SepSAP concentration, which probably repels spermatozoa left inside and around the capsule after fertilization. Anyway, the bioassay performed with the Neuroprobe system revealed from SepSAP 10^{-11} M a slight repellent effect (not statistically significant). The fertilization could induce a massive release of SepSAP in the smooth not yet polymerized envelope surrounding the oocyte and, thus, prevents polyspermy phenomena. In addition, associated to this mechanism, we cannot exclude the hypothesis of a release of repellent substances by the spermatozoa after fertilization.

Moreover, the bioassays showed that only a fraction of the sperm suspension is attracted by the SepSAP. As suggested by Ralt et al. [27], and Cohen-Dayag et al. [28] for human sperm in which only one spermatozoan per million is found in the fertilizing site [29], the attraction

of sperm by a sperm-attracting factor may be a means for selecting spermatozoa more competent for fertilization. The spermatozoa that reach the oocyte may be attracted to the oocyte because they are able to fertilize it whereas the rest are repelled by the same or other factors. Just as the fertilizability of human female gametes is strongly correlated with their capacity to attract spermatozoa [27]. The structure of the SepSAP (PIDPGVamide) revealed a sequence homology with the schistostatin I (LCDFGVamide), a neuropeptide characterized from the cDNA of *Schistocerca gregaria* and that exerted a myotropic inhibitory activity on the locust oviduct [30]. The two C-terminal amino acids appeared to be conserved with the C-terminal amidation as well as the third amino acid. We can speculate that the sequence Gly–Val–CO_{NH_2} plays an important role in the conservation of biological activity. This C-terminal sequence is recovered in most of the echinid SAPs, in which the C-terminal amide is substituted by a C-terminal glycine. Thus, we cannot rule out the fact that the cephalopod and arthropod peptides could be related to the echinids sperm-activating (attracting) peptides.

The SepSAP could belong to a large peptide families as described in teleosteans (HSAPs) [17] and echinids (speracts). The characterization of the cDNA should allow to elucidate this point.

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