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ILME: A Waterborne Pheromonal Peptide Released by the Eggs of *Sepia officinalis*

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A novel tetrapeptide modulating the oviduct contractions was characterized from egg mass of *Sepia officinalis*. After two purification steps by rpHPLC, an apparent pure fraction containing the biological activity was submitted to MALDI-TOF analysis. The mass spectrum revealed 6 peaks of *m/z* 293, 505, 596, 613, 728, and 745. The tissue peptide mapping performed in LC-MS demonstrated the occurrence of the *m/z* 505 peptide in the follicles, the full-grown oocytes, and in the eggs. This peptide was also recovered in the seawater after the incubation of full grown oocytes or eggs, demonstrating a release in the genital tract and in the environment. Edman degradation gave the following sequence: Ileu-Leu-Met-Glu. The synthetic peptide applied to the whole genital tract triggered a cyclisation of the contractions at 10^{-14} M. ILME appeared to be a chemical messenger released by the oocytes and the eggs, and was able to exert both paracrine and pheromonal activity. © 2000 Academic Press

Key Words: **pheromone; egg laying; LC-MS; HPLC; myotropin; peptide; ovary; oviduct; mollusc; cephalopod; invertebrate; *Sepia officinalis*.**

In the cuttlefish *Sepia officinalis*, the egg laying is made up of stereotyped successive steps. As shown in Fig. 1, at the beginning of egg laying, the full grown oocytes pass through the oviducal gland where they are embedded by a first gelatinous envelope. The release of each oocyte into the mantle cavity by contractions of the distal oviduct is followed by the secretion of the nidamental gland products which form the second egg capsule (1). The nidamental jelly is stained with ink simultaneously released from ink sac. The embedded oocytes are forced into the funnel tube, and brought to the base of the ventral arms. Spermatozoids are released from the copulatory pouch located under the buccal mass and they penetrate into the very soft oocyte envelopes. Finally the egg is attached by the arm tips to a solid substrate (2). Behavioural observations realised in rearing tanks showed that females usually laid eggs to form a single egg mass suggesting the occurrence of chemical attraction exerted by the freshly spawn eggs. The concentration of mature adults in particular areas of Normandy coast in spring for mating and egg laying (3) reinforced the existence of chemical messenger explaining this concentration. Such a phenomenon suggested that eggs may release pheromones transported by seawater as described for the marine mollusk *Aplysia californica* (4, 5). In this gastropod, the egg cordons are a source of both contact and waterborne pheromones such as the attractin, a peptide of 58 amino acids that attracts animals to an area and induces or facilitates reproductive activities.

With this in mind, the existence of specific peptides released from eggs mass and able to modulate the activity of the female oviduct was investigated in *Sepia officinalis*. The experimental approach was based on the use of HPLC and mass spectrometry, coupled with a sensitive *in vitro* biological assay (6, 7), allowing the identification of a peptide involved in the transport of oocyte in genital tract during egg laying in *Sepia officinalis*.

MATERIALS AND METHODS

Animals and eggs. All mature cuttlefish were trapped in the Bay of Seine in May and June. They were maintained in 1000-liter outflow tanks at the Marine Station of Luc sur Mer (University of Caen, France). The eggs released by several fertilised females in rearing tanks were collected during the 3 days following egg laying.

Recovery of material from tissues and seawater. For HPLC purification, 1.422 kg of 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 LC/MS analysis, follicles, full grown oocytes (smooth oocytes) and eggs were submitted to the extraction described above for purification. Moreover, the molecules released by 50 full grown oo-

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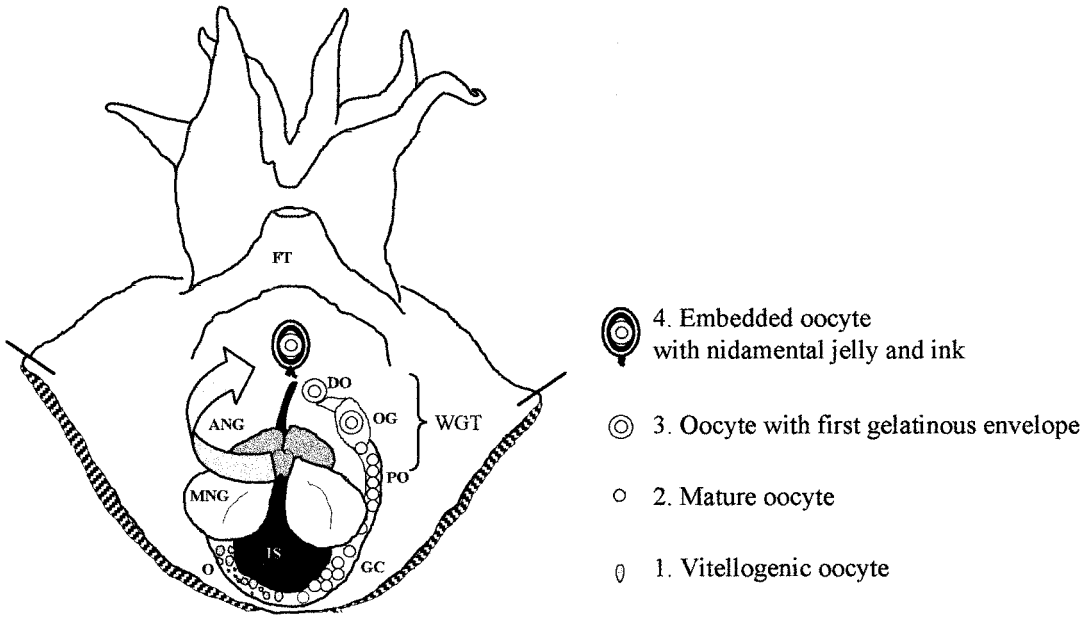


FIG. 1. Ventral view of mature *Sepia officinalis*. The successive steps of ovulation and embedding of eggs. ANG, accessory nidamental glands; DO, distal oviduct; FT, funnel tube; GC, genital coelome; IS, ink sac; MNG, main nidamental glands; O, ovary; OG, oviducal gland; PO, proximal oviduct.

cytes in 20 ml of seawater or by 1000 newly spawned eggs in 2 liters of seawater during the 10-h incubation were concentrated on chromafix C18.

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 21 nm. The dry eggs extract was resuspended in 200 μl of 0.1% TFA in water and injected onto a Macherey-Nagel RP18ec column (4.6 $\times$ 250, 5 μm) with an acetonitrile linear gradient of 1.33% per minute in TFA 0.1%. All fractions were tested on oviduct bioassay. The bioactive fractions eluted for 33 and 34 min were injected onto the Nucleosil C18 column (250 $\times$ 3, 7 μm) with acetonitrile linear gradient 1.33% per minute in 25 mM ammonium acetate. The bioactive fraction with a 29-min retention time was analysed by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF).

Biological assay. The myotropic bioassay was performed with several different organs. The genital tract (including the proximal oviduct containing full grown oocytes, the oviducal gland and the distal oviduct), the isolated distal oviduct, the ovarian stroma, the main nidamental gland and the esophagus were dissected from mature females. Each organ was suspended from a displacement tranducer (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 $\cdot$ 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 the fractions into the muscle chamber was traced by addition of phenol red (1 μM). Moreover the muscle chamber was perfused with seawater used for incubation of full grown oocytes or eggs.

Mass spectrometry analysis. The mass spectrometry analysis was performed with a Micromass TOF SPEC-E. The dry pellets of the apparent pure peak containing the activity with a 15.3-min retention time were concentrated and resuspended in 10 μl of 1:1 acetonitrile/01% TFA solution. One microliter of sample was mixed with an equal volume of the MALDI matrix prepared as follows: 10 mg of α-cyano-4-hydroxy cinnamic acid dissolved in 1 ml of a 1:1 acetonitrile/0.1% TFA solution. Sample (1 μl) was spotted into the wells of the MALDI target and air dried in a vacuum chamber prior to be submitted to multiple shots from the nitrogen laser (337 nm). The peptide of interest was identified in the spectrum by its monoprotonated form $[M + H]^+$.

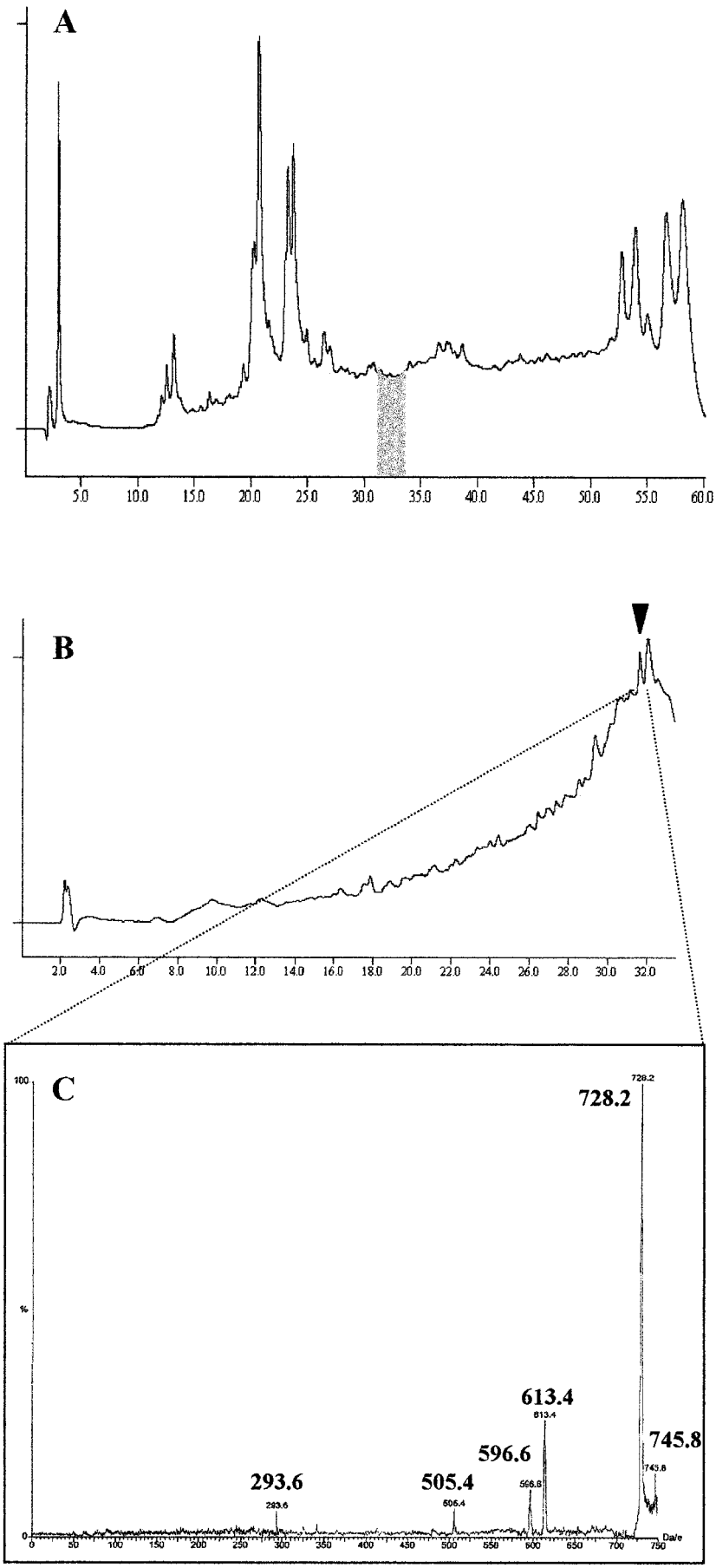
Mapping with LC/ESI-MS. The Liquid Chromatography/ElectroSpray Ionization Mass Spectrometry (LC/ESI-MS) analysis was performed with HP1100 series LC/MSD (Agilent Technologies) managed by the Chemstation software.

The organic fraction of each extract was injected onto a capillary C18 column (Vydac, 250 $\times$ 1.3 μm) with an acetonitrile linear gradient of 3% per minute in TFA 0.05%, with a flow rate of 1 ml/min. The outlet of the LC column was connected to the ESI interface with orthogonal sprayer. The MS data was acquired in scan mode considering the positive ion signal. The analysis was performed with a cone voltage of 80 V to obtain the monoprotonated molecules $[M + H]^+$.

Amino acid sequencing. 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.

Biological activity. Aliquots of synthetic Ileu-Leu-Met-Glu peptide (Genaxis) from 10^{-20} to 10^{-10} M were tested using the oviduct bioassay in order to obtain qualitative and quantitative data about

FIG. 2. Purification of a myotropic factor. (A) First step of purification on C18 column of the chromafix C18 eluate of the total egg extract. The myotropic fraction was detected by a bioassay (shaded part). (B) Second fractionation step on nucleosil C18. The myotropic fraction eluted at 31 min (arrow). (C) MALDI-TOF mass spectrum corresponding to the myotropic fraction.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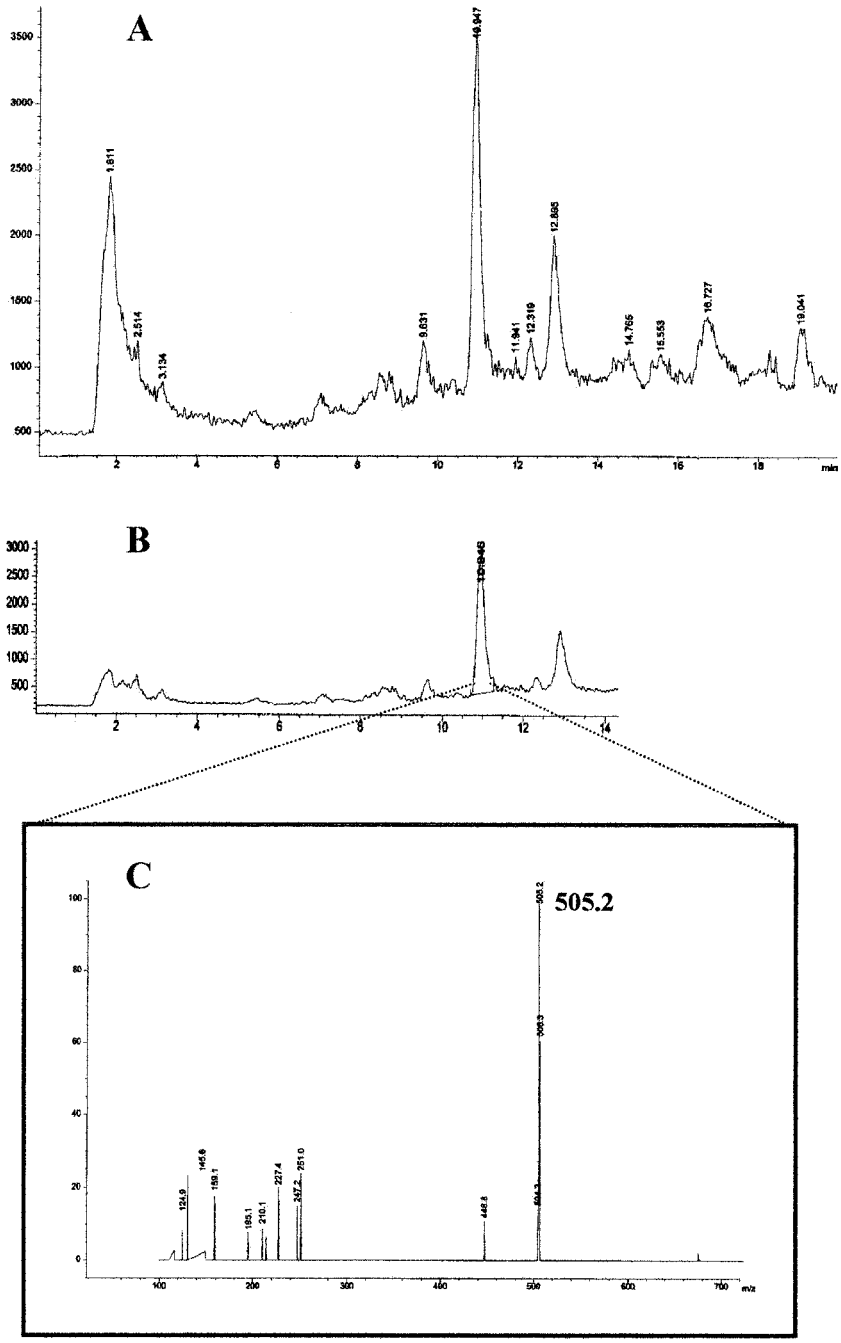

FIG. 3. LC/ESI-MS analysis from eggs extract for the peptide at *m*/*z* 505.4. (A) Total ion current. (B) Extracted ion chromatogram based on the mass of the single charged ion 505.4. (C) Extracted mass spectrum of the 10.84-min peak.

their myotropic activity. Moreover, to check the specificity of ILME, aliquots from 10^{-14} to 10^{-5} M were tested on different muscles. Each aliquot was resuspended in 100 μl of perfusion liquid and immediately injected as described above.

RESULTS AND DISCUSSION

Passage through chromafix C18 was used to prepurify the extract containing 1400 eggs of *Sepia officinalis*. The organic fraction obtained was fractionated using a RP18 column (Fig. 2A), all fractions were tested for motility of the cuttlefish oviduct. The 33 and 34 min fractions revealing a myotropic activity were further purified on the nucleosil C18 column. After the second step of purification, a myotropic fraction eluted at 31 min (Fig. 2B) was analysed by matrix-assisted laser desorption ionisation-time of flight (MALDI-TOF). The

TABLE 1

	m/z 293.6	*m/z* 505.6	*m/z* 596.6	*m/z* 613.8	*m/z* 728.2	*m/z* 745.8
Follicles	nd[a]	+[b]	+	nd	nd	nd
Full grown oocytes	nd	+	nd	nd	nd	nd
Eggs	+	+	+	+	nd	+
Incubation seawater with full grown oocytes	nd	+	nd	nd	nd	nd
Incubation seawater with eggs	nd	+	nd	nd	nd	nd

[a] nd, not detected.
[b] +, detected.

MALDI-TOF mass spectrum revealed six single charged ions at *m/z* 293.6, 505.4, 596.6, 613.4, 728.2, and 745.8 (Fig. 2C). The tissue mapping of these six molecules was performed using Liquid chromatography/electrospray ionisation-mass spectrometry (LC-MS). The results were summarised in the Table 1. The LC-MS analysis of seawater incubated with full grown oocytes or freshly spawned eggs revealed the occurrence of the peptide at *m/z* 505.4, suggesting the release of this peptide in the genital tract and in the environment. Moreover, the *m/z* 505.4 peptide appeared to be present in the eggs, the follicules, and the full grown oocytes. This is shown in Fig. 3 for the egg extract with the total ion current (TIC), the extracted ion chromatogram (EIC) and the extracted mass spectrum of the *m/z* 505.4. The seawater incubated with full grown oocytes induced the inhibition of contractions before triggering cyclic contractions (Fig. 4A). The seawater incubated with freshly spawned eggs induced a decrease in tonus and in frequency of the oviducal contractions (Fig. 4B), confirming the release of a bioactive molecule into the seawater by the full grown oocytes and by the eggs. A similar mechanism has already been described for the release of 5-HT by the full growth oocytes in *Sepia officinalis* (8). Sequence determination by Edman degradation of the peptide at *m/z* 505.4 gave the amino acid sequence Ileu-Leu-Met-Glu (ILME) with a calculated mass $[M + H]^+$ of 504.6. At a concentration of 10^{-10} M, the synthetic tetrapeptide induced a decrease of the tonus of the distal oviduct (Fig. 5A) and at 10^{-14} M, it triggered cyclic contractions of the whole genital tract containing full grown oocytes (Fig. 5B), confirming the observations realised for seawater incubated with these tissues. For 10^{-8} M, ILME decreased the basal tone and the frequency of the contractions of the main nidamental gland. Applied on the ovarian strom and on the esophagus, ILME did not exhibit any effect. Also the ovarian peptide ILME appeared to specifically target the genital tract and the nidamental glands which are

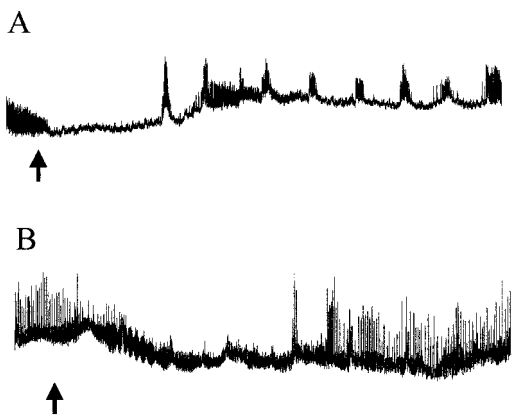

FIG. 4. Perfusion of distal oviduct with (A) seawater (10 ml) used for incubating full grown oocytes ($n = 25$); (B) seawater (10 ml) used for incubating newly spawn eggs ($n = 5$).

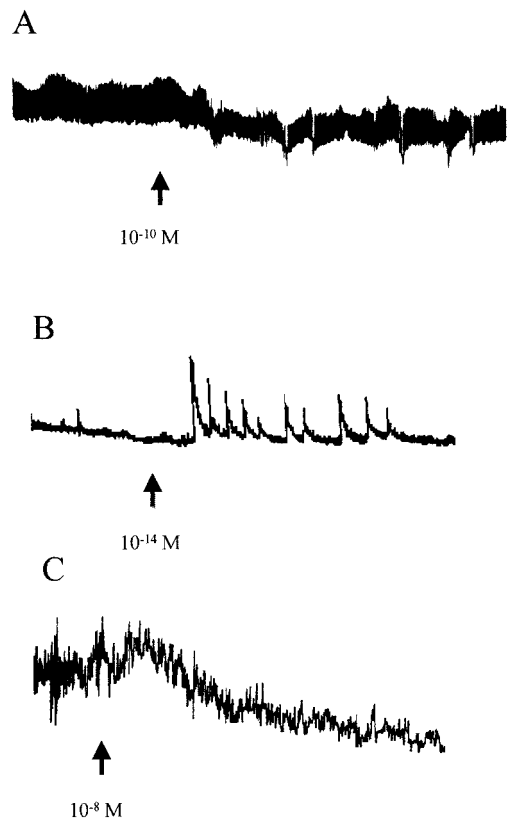

FIG. 5. Myotropic activity of ILME on distal oviduct (A), on the whole genital tract (B), on the main nidamental gland (C).

respectively involved in the transport of the oocytes during egg laying and the in mechanical secretion of egg capsules. The eggs of marine invertebrates were a source of peptides possessing biological activity. Indeed, the egg-conditioned medium of sea urchins contains peptides which stimulated sperm metabolism and motility. Speract was one of these chemoattractants purified from egg jelly (9). Similar peptide, asterosaps, were identified in starfish (10). In the marine gastropod, *Aplysia californica*, the egg cordons was equally a source of peptide pheromone such as attractin which establishes and maintains the adults aggregation in reproduction area (4). Hypotheses for chemical communication in cephalopods were already raised (11) even if the behavioural approach used as an assay appeared to be very delicate to perform. Nevertheless, the occurrence of the first water borne pheromone peptide involved in the transport of oocytes during egg laying has been clearly demonstrated in the cephalopod *Sepia officinalis* by means of LC-MS. Thus, the multifactorial regulation of egg-laying appeared to be extremely complex with the involvement of neuropeptides such as APGWamide-RPs, FMRFamide-RPs (6, 7), of neuromediator like 5-HT synthesized in ovarian follicles (8) and ILME expressed in vitellogenic ovarian follicles. Based on the approach used in this work, purification and characterisation of the egg-laying hormone of *Sepia officinalis* should become possible.

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