Ovarian and Sperm Regulatory Peptides Regulate Ovulation in the Oyster *Crassostrea gigas*

BENOÎT BERNAY,1 MICHELLE BAUDY-FLOC’H,2 BRUNO ZANUTTINI,3 CÉLINE ZATYLNY,1 STÉPHANE POUVREAU,4 AND JOËL HENRY1*

1Laboratoire de Biologie et Biotechnologies Marines, UMR 100 IFREMER Physiologie et Ecophysiologie des Mollusques Marins, Université de Caen, Caen Cedex, France
2Laboratoire SESO, UMR CNRS 6510, Institut de Chimie, Université de Rennes I, Rennes Cedex, France
3Groupe de Recherche en Informatique, Image et Instrumentation de Caen (GREYC) CNRS UMR 6072 Université de Caen (UFR des Sciences) et ISMRA, Caen, France
4Laboratoire de Physiologie des Invertebrés, UMR 100 IFREMER Physiologie et Ecophysiologie des Mollusques Marins, Site Expérimental d’Argenton, Presqu’île du Vivier, Argenton en Landunvez, France

ABSTRACT

For more than six decades, several studies have shown that genital products entering the mantle cavity via the incurrent siphon, initiate in oyster, strong and rhythmic contractions of the adductor muscle (AM). In order to characterize the regulatory peptides capable of triggering AM contractions, we focused on the identification of putative myotropic peptides from genital products. Two experimental approaches were developed. The first one, based on a mass spectrometry screening of the male genital products, led to the identification of the tetrapeptide APGWamide. This neuropeptide was also detected in the seminal secretions of the cephalopod *Sepia officinalis*. In this species, APGWamide is directly involved in the oocyte transport. In *Crassostrea*, in vitro bioassay demonstrated that APGWamide modulates the AM contractions that insure the release of oocytes in the external medium. Exposure of oysters to a physiological concentration of APGWamide triggered repetitive shell closures. The second experimental approach was based on the monitoring of HPLC purification by a myotropic bioassay using the cuttlefish oviduct contractions as a target. The successive purification steps of the acidic extraction of ovaries from mature female oysters, led to the characterization of the hexapeptide PIESVD. When applied to mature female oysters, this peptide triggered the increase of shell closure frequency. The activity of these two regulatory peptides is the first experimental evidence of a peptidergic control of egg-laying in oyster.

APGWamide and PIESVD could be used, in commercial and experimental hatcheries, for the identification of mature females to be selected for in vitro fertilization.

© 2006 WILEY-LISS, INC.
the transport of oocytes through the genital tract is performed by the ciliary motion of the epithelium (Hoek, 1883; Galtsoff, 1964). Oocytes are released and stored in the exhalent chamber. Then, they are forced through the gills to the inhalant chamber by highly characteristic AM contractions (Galtsoff, 1938a; Nelson and Allison, 1940). Finally, oocytes are released into the external medium, by the AM contractions, through a small spawning window opened between the inhalant chamber mantle margins (Nelson, 1921; Galtsoff, 1930, 1938a,b, 1961, 1964; Nelson and Allison, 1940; Rice et al., 2002). This activity is thought to result in a relatively wide and uniform distribution of oocytes, improving the chances of fertilization. In the cuttlefish, the first step of egg-laying is the release by the ovary of full-grown oocytes (FGO) into the genital coelom (Dhainaut and Richard, 1976). During this period, ovarian factors released by FGO inhibit the oviductal contractions and lead to the accumulation of FGO in the genital coelom (Zatylny et al., 2000a; Bernay et al., 2004). After mating, oocytes transport is performed through the oviduct by the whole genital tract (WGT) contractions, which are modulated by neuro- and ovarian peptides (Henry et al., 1997, 1999; Zatylny et al., 2000b,c). Oocytes then received two gelatinous envelopes and are fertilized by spermatozoas stored in the female’s copulatory pouch (Boletsky, 1983; Hanlon et al., 1999). Finally, the eggs are attached to a substrate until hatching. In both species studied, muscle contractions are responsible for the release of oocytes: AM contractions in oyster and WGT contractions in cuttlefish. In the last six decades, numerous studies have shown that oysters initiate a series of strong and rhythmic contractions of the AM in response to genital products entering the mantle cavity via the incumbent siphon (Galtsoff, 1938a,b, 1964; Nelson and Allison, 1940; Rice et al., 2002). In order to characterize myotropic peptides from male and female genital products in oyster, we developed two experimental approaches. The first one was based on the peptidomic analysis performed on male genital products. The peptidome was screened by means of mass spectrometry using specific sequence libraries. The second experimental approach consisted of the HPLC purification of putative ovarian peptides monitored by the cuttlefish oviduct contractions myotropic bioassay, which is a reliable and specific biological test.

MATERIALS AND METHODS

Animals

All the cuttlefish were trapped in the Baie of Seine between January and June. They were maintained in 1,000-L outflow tanks at 15°C ± 1°C at the Marine Station of Luc sur Mer (University of Caen, France) under a natural photoperiod. Oysters come from Aber Benoît (Bretagne, France) and Saint Vaast la Hougue (Normandie, France).

Micro LC-ESI-MS/MS Analysis

Analyses were 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 m/z 300 to 2,000. 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. An Xcalibur data system was used to acquire the data, which were further processed with 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 × 0.5mm, 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 was acquired in scan mode considering the positive ion signal.

In Vitro Bioassay

The myotropic bioassay was performed with several contractile organs: the WGT, the penis and the vas deferens from S. officinalis and the AM from C. gigas. 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 the flow rate of 0.5 ml/min with synthetic seawater (Instant Ocean) containing 1 mM glucose and maintained at 15°C. The samples 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.

In Vivo Bioassay: Shell Closure Frequency

Oysters were placed at 4°C out of water the night before the bioassay. During the experiment, each oyster was placed at 22°C ± 1°C, in a 450 ml tank containing an algal cocktail (25% Tetraselmis sp., 25% T. iso, 25% Chaetoceros calcitrans, and 25% Skeletonema costatum) adjusted to 30 cells/μl. Water was circulated in each tank to insure opening of the valves and filtration by the animals. Following addition of sample, the shell closure frequency was visually monitored during the subsequent assay period (10 min). Shell movements during female spawning period are so typical that they cannot be mistaken from any other type of muscular activity (Galtsoff, 1938a).

Synthetic Peptides

Peptides were synthesized using classical Fmoc (N-[9-fluorenylethoxycarbonyl) solid-phase chemistry and a commercially available automated peptide synthesizer by coupling Fmoc-α-amino acids on preloaded Wang resin. Protected amino acids were coupled by in situ activation with diisopropylcarbodiimide (DIPCIDI) and
N-hydroxybenzotriazole (HOBt). Nα-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 2h at 20 °C (Sole and Barany, 1992). 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 acetonitrile containing 0.08% TFA at a flow rate of 1 ml/min. Finally, integrity of each peptide was assessed by micro LC-ESI-MS/MS analysis.

Sperm Peptides

Recovery of material from tissues. Organs were harvested as follows: for \textit{S. officinalis}: central nervous system (supra-oesophageal mass, sub-oesophageal mass, optic lobes) and genital tract (vas deferens, seminal vesicles, penis, spermatophores dissected from mature male); for \textit{C. gigas}: oocytes, mantle edge, gills, labial palpe, smooth muscle, and striated muscle of the AM dissected from mature males and females. For micro LC-ESI-MS/MS analysis, 5 animal-equivalent of each organ studied, 80 animal-equivalent of visceral ganges, 150 g of oocytes, and 40 g of sperm from \textit{C. gigas} were successively homogenized in 10 volumes of 0.1N HCl at 100 °C and centrifuged 30 min at 35,000 g at 4 °C. The supernatants were concentrated using Chromafix C18 cartridges. In \textit{S. officinalis}, the spermatophores (complex tubular system containing spermatozoa) were removed from the spermatophoric sacs or Needham’s organs and separated into two aliquots. The first aliquot was washed with artificial SW (Instant Ocean) in order to remove seminal secretions. The two aliquots were then extracted as described above.

Identification of regulatory peptides from male genital products. A specific fasta library containing most invertebrates regulatory peptides was created to enable the identification of peptides by MS/MS. Screenings were performed using the Sequest software (Thermofinnigan) which can compare experimental and theoretical MS/MS spectra generated from fasta libraries. For each peptide, the amino acid sequence was definitely confirmed by the comparison between synthetic and endogenic peptide MS/MS spectra.

Bioactivity of APGWamide. Aliquots of synthetic APGWamide (Sigma Aldrich) ranging from 10^{-6} to 10^{-8} M were tested using the in vitro and in vivo bioassays in order to obtain qualitative and quantitative data associated with its myotropic activity.

APGWamide oysters in vivo bioassay. Two bioassays were performed with the APGWamide. The first bioassay was performed using 15 oysters and 10^{-7} M APGWamide for the first sample and 26 oysters and 10^{-6} M APGWamide for the second sample. The second bioassay was performed with 200 oysters and 10^{-6} M APGWamide. For each bioassay, the shell closure events were recorded during 10 min after injection of the peptide. The experiment was followed by the sacrifice of the animals for sex determination.

Tissue mapping by micro LC-ESI-MS/MS. The occurrence of peptides in the samples was established by the MS/MS data of the m/z 429.5 (APGWa), 459.6 (TPGWa), 486.7 (KPGWa), 514.7 (TPGWa), and 261.2 (GWa). This method detected small amounts of peptide. Peptide identifications were obtained from the comparison of MS/MS spectra of the selected m/z with the synthetic peptides.

Ovarian Peptides

Recovery of material from tissues. For HPLC purification and microLC-ESI-MS/MS analysis, 400 g of oyster ovaries were homogenized in 4 L of 0.1N HCl at 100 °C and centrifuged for 30 min at 35,000 g at 4 °C. Supernatants were concentrated on Chromafix C18 cartridges. Moreover, the female genital products released by five oysters were extracted in 0.1N HCl at 100 °C, centrifuged and concentrated on Chromafix C18 cartridges.

UV-HPLC purification. HPLC analyses were performed with a Varian 4050 integrator connected to a Varian 9012 solvent delivery system and a Varian 4005 wavelength UV-VIS detector set at 214 nm. The ovary extract was resuspended in 0.1% formic acid in water and injected onto a Nucleodur C18 column (250 × 10 mm, 20 μm) with an acetonitrile linear gradient of 1.33% per minute in 0.1% formic acid at a flow rate of 5 ml/min. A bioactive fraction eluted at 29 min was injected onto a Nucleosil C18 column (250 × 3 mm, 7 μm) with an acetonitrile linear gradient of 1.05% per minute in 10 mM ammonium acetate at a flow rate of 1 ml/min. The third purification step was performed on a Supelcosil C18 column (150 × 4.6 mm, 3 μm) with an acetonitrile linear gradient of 1.33% per minute in 0.1% formic acid at a flow rate of 1 ml/min. Finally, the last step of purification was performed in LC-ESI-MS/MS on a Nucleosil C18 column (120 × 4 mm, 3 μm) with an acetonitrile linear gradient of 0.66% per minute in 0.1% formic acid at a flow rate of 600 μl/min. A split ratio of 100:1 was used to perfuse the electrospray source at a flow rate of 10 μl/min. One minute fractions of each step were collected, dried, and stored at 4 °C until use.

Amino acid sequencing. Fasta libraries were generated with the software Aaseq 5.0 (Zanuttini and Henry, University of Caen). Screenings were performed using Sequest software (Thermofinnigan), which is able to compare experimental MS/MS spectra and theoretical MS/MS spectra generated from fasta libraries. The MS/MS spectrum of the amino acid sequence was then checked using MS-Tag and MS-Product (Protein Prospector 3.4.1, University of California). Finally, primary structures were verified by 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 online with a Model 120A HPLC system, as recommended by the manufacturer.

**PIESVD of Sydney oysters in vivo bioassay.** The shell closure bioassay was performed in the middle of June with oysters from Saint Vaast la Hougue, matured in SMEL (Syndicat Mixte pour l'Équipement du Littoral, Blainville). The first bioassay was performed with PIESVD $10^{-7}$ M on a sample of eight oysters and the second on a sample of 39 oysters with PIESVD $10^{-6}$ M. Shell closure events were visually recorded during 10 min. The experiment was followed by the sacrifice of animals for sex determination.

**Statistics.** Significant differences between treatments were tested by one-way analysis of variance (ANOVA) using the Statistica software. The method used to discriminate among the means was the Student's Newman Keuls procedure. Results were deemed significant for $P < 0.05$.

---

**RESULTS**

**Regulatory Peptides From Sperm**

Detection of APGWamide by Micro LC-ESI-MS/MS in male genital products of both studied species. Putative regulatory peptides were investigated by Sequest software using the sequence library of invertebrate regulatory peptides. This approach led to the identification of APGWamide in male genital products of both *S. officinalis* and *C. gigas*. The comparison of MS/MS data obtained from synthetic and endogenic peptides confirmed the amino acid sequence (Fig. 1). Furthermore, in the cuttlefish, APGWamide was detected in the seminal fluid coating of spermatophores but not in washed spermatophores demonstrating that APGWamide was associated with the seminal fluid.

**Bioactivity of APGWamide on *S. officinalis* male genital tract.** For concentrations ranging from 120 to 400 m/z, APGWamide was detected in the seminal fluid coating of spermatophores but not in washed spermatophores demonstrating that APGWamide was associated with the seminal fluid.
10⁻⁸ to 10⁻⁶ M, APGWamide had no effect on penis and proximal vas deferens contractions.

Bioactivity of APGWamide on *S. officinalis* female WGT. The synthetic peptide was tested on the WGT of mature and immature females. From a threshold at 10⁻⁷ M, APGWamide inhibited the contraction of this tract, decreasing the amplitude and the intensity of the contractions in mature females (data not shown). This biological activity was previously described in (Henry et al., 1997). In immature females, APGWamide had no effect on WGT contractions.

Bioactivity of APGWamide on oyster AM. The synthetic peptide was tested on the AM of mature female oysters. From 10⁻⁷ M, APGWamide decreased the intensity of the contractions. In a dose-response experiment, the increase of concentration appeared to be correlated with the increase of biological activity (Fig. 2).

Shell closure in vivo biological assay. Egg-laying was never triggered during experiments. For the first bioassay, oysters were exposed to 10⁻⁷ M or 10⁻⁶ M APGWamide and shell closure events were recorded during 10 min. After dissection, we noticed that APGWamide increased shell closure frequency from a threshold of 10⁻⁷ M on mature females. This peptide had no significant effect on mature males and immature oysters (Fig. 3). A second experiment was performed by exposing 200 oysters to 10⁻⁶ M APGWamide. Shell closures were recorded during 10 min. Shell closures (≥ 4) were displayed by 48% of oysters. Sex was confirmed after dissection. APGWamide induced shell closure events in 6% of immature oysters, 30% of males and 67% of females. Results are summarized in Figure 4.

APGWa-RPs tissue-mapping by microLC-ESI-MS/MS. Standard MS/MS spectra were acquired from the injection of 1 ng of each of the APGWamide-related peptides (APGWa-RPs). These spectra were used for the identification of APGWa-RPs in the tissues of *S. officinalis* and *C. gigas*. Table 1 summarizes the results obtained for the tissue-mapping performed in the male genital tract and central nervous system (CNS) of *S. officinalis*. In the male genital tract, APGWa was detected in the spermatophoric glands and TPGWa in the vas deferens. In the CNS, APGWa, TPGWa, and GWa were detected in the optic lobes, supra- and suboesophageal mass. No APGWamide-related peptides were detected in the hemolymph. Table 2 summarizes the results obtained for the tissue-mapping performed in males and females of *C. gigas*. Only APGWamide was detected in the sperm and visceral ganglia. APGWa-RPs were not detected in somatic tissues.

Ovarian Regulatory Factors

HPLC purification. The purification of oyster oocytes extract was monitored by the cuttlefish oviduct bioassay. After the first purification step, a myotropic activity was detected in the 28–29 min fraction. After two further steps of purification, the activity was concentrated in an apparently pure peak with a retention time of 17–18 min (Fig. 5). The last purification step was performed in LC-ESI-MS/MS.

LC-ESI-MS/MS purification. The 17–18 min HPLC fraction, containing the myotropic activity was submitted to LC-ESI-MS/MS purification. The spectrum revealed a pure peak at m/z 659 ± 0.5 (Fig. 6). This peak was concentrated to be submitted to Edman degradation.

Amino-acid sequence determination. Aaseq 5.0 software was used to build a sequence library ranging from 657 to 660 Da. The primary sequence of the peptide was partially determined by Sequest software, MS-Tag and MS-Product: PI/LESVD. The primary sequence of this peptide was definitively determined.
established by Edman degradation as PIESVD and checked by MS/MS analysis of the synthetic peptide.

**Bioactivity of synthetic peptide.** Increasing concentrations of synthetic PIESVD were tested on the WGT of cuttlefish. From a threshold of $10^{-6}$ M, the peptide decreased the intensity of the contractions (Fig. 7).

**In vivo bioassay: shell closure frequency.** Oysters were exposed to increasing concentrations of synthetic PIESVD. Shell closure frequency was visually recorded. From a threshold at $10^{-6}$ M, PIESVD induced a significant increase of shell closure frequency compared to the control in the population of mature females. The peptide had no significant effect on mature males (Fig. 8).

**Tissue-mapping by microLC-ESI-MS/MS.** Mapping was performed in MS/MS mode based on the m/z 659. Analysis performed in the male genital tract, hemolymph and nervous system (visceral ganglia) did not reveal any trace of this peptide. PIESVD was restricted to the ovary. Furthermore, the analysis of the genital products of spawning females clearly established the occurrence of a release of PIESVD during egg-laying.

**DISCUSSION**

Two regulatory peptides isolated from *C. gigas* were identified by mean of mass spectrometry screening and RP-HPLC purification and provide the first experimental evidence of a peptidergic control of egg-laying in oyster. The first approach, based on a peptidomic analysis, led to the identification of the tetrapeptide APGWamide in male genital products of *C. gigas*. Subsequently, this neuropeptide was detected in seminal secretions of the cuttlefish *S. officinalis*. MicroLC-ESI-MS/MS tissue mapping demonstrated that this peptide is located in the CNS and male genital tract in both oyster and cuttlefish, as well as, according to previous studies, in gastropods, bivalves, and cephalopods (Li et al., 1992; Smit et al., 1992; Smith et al., 1997; Favrel and Mathieu, 1996; Fan et al., 1997; Henry and Zatylny, 2002). In cuttlefish, APGWamide is directly involved in oocyte transport (Henry et al., 1997), whereas in gastropods, APGWamide detected in the male genital tract modulates the male mating behavior (Li et al., 1992; van Golen et al., 1995b). In *S. officinalis*, in vitro bioassays clearly demonstrate that APGWamide has no effect on penis and vas deferens contractions. The APGWamide detected in seminal fluid is deposited with spermatophores in the female copulatory pouch during mating. This pouch contains seminal receptacles surrounded by muscle fibers, which could be involved in the storage of spermatophores (Hanlon et al., 1999) and may be modulated by APGWamide. We speculate that the APGWamide detected in the oyster CNS (visceral ganglia) could target the AM of mature females, as is described in the sea scallop (Smith et al., 1997). Bioassays performed on the AM, which is responsible for the release of oocytes into the external medium, demonstrated that APGWamide induces AM contractions from $10^{-7}$ M. When oysters were exposed in vivo, a physiological concentration of APGWamide triggered repetitive shell closures. The in vivo bioassay also demonstrated that a majority of mature females appear to be sensitive to APGWamide (67% of females compared to only 30% of males). Moreover, when shell closures were observed, the frequency was higher in females.

**TABLE 1. Tissue Mapping of APGWamide-RPs in Male Sepia officinalis**

<table>
<thead>
<tr>
<th>Occurrence</th>
<th>APGWamide</th>
<th>RPGWamide</th>
<th>KPGWamide</th>
<th>TPGWamide</th>
<th>GWamide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sub-oesophageal mass</td>
<td>d</td>
<td>nd</td>
<td>nd</td>
<td>d</td>
<td>d</td>
</tr>
<tr>
<td>Supra-oesophageal mass</td>
<td>d</td>
<td>nd</td>
<td>nd</td>
<td>d</td>
<td>d</td>
</tr>
<tr>
<td>Optic lobes</td>
<td>d</td>
<td>nd</td>
<td>nd</td>
<td>d</td>
<td>d</td>
</tr>
<tr>
<td>Male genital tract</td>
<td>d</td>
<td>nd</td>
<td>nd</td>
<td>d</td>
<td>nd</td>
</tr>
<tr>
<td>Spermatophores</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Seminal fluid</td>
<td>d</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Penis</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Seminal vesicles</td>
<td>d</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Vas deferens</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>d</td>
<td>nd</td>
</tr>
</tbody>
</table>

*a* nd, not detected.  
*b* d, detected.

**TABLE 2. Tissue Mapping of APGWamide-RPs in Crassostrea gigas**

<table>
<thead>
<tr>
<th>Occurrence</th>
<th>APGWamide</th>
<th>RPGWamide</th>
<th>KPGWamide</th>
<th>TPGWamide</th>
<th>GWamide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male and female gills</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Male and female mantle edge</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Male and female smooth muscle</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Male and female striated muscle</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Male and female labial palpes</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Male and female visceral ganglia</td>
<td>d</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Female genital products</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Male genital products</td>
<td>d</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
</tbody>
</table>

*a* nd, not detected.  
*b* d, detected.
Contractions observed in the male population could be a consequence of the successive hermaphroditism of *C. gigas* (Amemiya, 1929; Galtsoff, 1961). Galtsoff demonstrated that although sex change was characterized by modifications in the spawning behavior, a developed female reaction could appear in sex-reversed males (Galtsoff, 1961). Furthermore, we suspect that the APGWamide detected in the visceral ganglia targets

**Fig. 5.** First (A), second (B) and third (C) purification steps of oyster oocyte extract. Myotropic fractions are indicated in black.
longitudinal and circular muscle fibers of the genital channel (Galtsoff, 1964). A similar observation was made in the sea scallop, where APGWamide immunoreactivity was found around the gonoduct (Smith et al., 1997). In this species, similar investigations led to the detection of 5-HT immunoreactivity around this channel (Matsutani and Nomura, 1986). In vivo experiments clearly demonstrated the involvement of 5-HT in the enhancement of oocytes release in bivalves (Matsutani and Nomura, 1982; Gibbons and Castagna, 1984; Braley, 1985; Khotimchenko and Deridovich, 1991; Ram et al., 1993; Fong et al., 1994, 1998). Thus, we speculate that APGWamide and 5-HT could be associated in the regulation of oocyte transport through this genital channel. APGWamide could also be involved in the regulation of egg-laying through a second pathway. APGWamide released with male genital products, and detected by mean of mass spectrometry screening, is suspected to target the AM of mature oysters through a pheromonal pathway. However, the effective concentration of 10^{-7} M established with synthetic peptide is much higher than those observed for other water-borne peptides identified in some invertebrates (Suzuki, 1995; Zatylny et al., 2000b,c, 2002; Bernay et al., 2004; Cummins et al., 2004, 2005a,b). Yet, it is similar to that observed in the polychaet worm Nereis for the water-borne peptide nereithione involved in male nuptial behavior and sperm release (Zeeck et al., 1998; Ram et al., 1999). Similarly, the proximity and high density of mature adults could lead to high concentrations of genital products in the environment surrounding mature females and participate in the regulation of egg-laying in oysters. Hence, the release of oocytes is probably induced by the action of multiple regulatory factors and APGWamide is only one of them. The results from this study using tissue mapping as well as biological activities as endpoints, clearly establish the involvement of APGWamide in the regulation of physiological mechanisms related to egg-laying in C. gigas and S. officinalis. In addition to sperm-linked regulatory peptides, we also focused on the identification of ovarian regulatory peptides, based on regulatory pathways established in the cuttlefish (Zatylny et al., 2000a,b,c). Following successive steps of UV-HPLC and microLC-ESI-MS/MS purification, a new hexapeptide was characterized from the ovary of mature females based on its ability to modulate the cuttlefish oviduct contractions. The primary structure of this ovarian peptide was established by MS/MS analysis and Edman degradation as PIESVD. MicroLC-ESI-MS/MS tissue mapping revealed that this peptide was restricted to the ovary. The occurrence of a release with female genital products in seawater was clearly established by MS/MS analysis. Furthermore, when synthetic PIESVD was
added to a rearing tank containing a population of oysters, shell closure frequency increases from a concentration of $10^{-6}$ M. This biological activity appears to be restricted to mature females. Thus, we speculate that PIESVD triggers AM contractions through two pathways: (i) when released with oocytes in the exhalant chamber, this peptide targets the AM and induces the release of oocytes into the external medium, and (ii) through a pheromonal pathway, the PIESVD released by spawning females with genital products could induce the egg-laying behavior of other mature females in the immediate vicinity. Although the activity threshold of PIESVD is relatively high, the proximity and density of mature oyster populations is consistent with pheromonal activity. The biological activity of PIESVD on the cuttlefish WGT demonstrates that some ovarian regulatory peptides could be highly conserved between cephalopods and bivalves and probably among the seven molluscan classes. Indeed, concerning oysters, in vitro bioassays are not reliable enough to monitor the purification of a peptide and in vivo bioassays require large quantities of biological material. Thus, the purification and characterization of regulatory peptides appears to be possible using interspecies bioassays. Finally, APGWamide and PIESVD could be used in commercial as well as in experimental hatcheries, for non-destructive sex and maturity stage determination in oysters. Statistical analysis clearly established that the main targets of APGWamide and PIESVD are the mature females in which they induce an important increase of shell closures. APGWamide and PIESVD, could be used to select mature females in order to provide pure gametes for use in genetic manipulation and in vitro fertilization.

ACKNOWLEDGMENTS

We thank Jean-Marc Nicolas and Darlene Mossman for correcting the English of the manuscript, Didier Rousseville the captain of the professional fishing boat “Père Arthur” and his crew for help in providing cuttlefish as well as Laurence Lafaitteur. We thank Aude Houdan, Bertrand Le Roy, Ian Probert and Algobank for microagal cultures and Jean Louis Blin, Bertrand Boucaud, and Stéphane Pacary from the SMEL for maturing oysters. We thank bean-Pierre Andrieu for Edman degradation.

REFERENCES


Galtsoff P. 1930. The role of chemical stimulation in the spawning reactions of *Ostrea virginica* and *Ostrea gigas*. Proc Natl Acad Sci USA 16:555–559.


