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Mussel MAP, a major gonad-duct esterase-like protein, is released into sea water as a dual constituent of the seminal fluid and the spermatozoon

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Summary

Our interest in the comparative analysis of male reproductive-tract esterases in different animal groups has led us to undertake a detailed study of the *Mytilus galloprovincialis* male-associated polypeptide (MAP) throughout the mussel gonad-duct tract and at spawning. The results of this work indicate that MAP is a major protein in *M. galloprovincialis* semen, with dual presence in both sperm cells and cell-free seminal fluid. Shortly after spawning, the released sperm mass is subdivided in diffused cloudy-like and thread-shaped 'clots', in which a soluble-phase MAP may persist as long as the clots keep their compact form. Additional experiments involving the incubation of spawned spermatozoa at increasing Triton X-100 concentrations demonstrated that MAP is also strongly associated with sperm cells. These results were further validated by immunofluorescent staining, which revealed that MAP is localized in the mid-piece region of spawned spermatozoa. This unexpected finding raises the possibility that MAP may play a role in sperm fertility in bivalves. Using whole-mount histology and micromanipulation techniques, we studied the structural patterning of the mantle gonad-duct network and assessed the sampling of luminal contents from the ducts. Of particular interest is the observation that MAP content in the luminal fluid increases from the lumen of the spermatogenic tubules to that of the collecting gonad ducts, where MAP is detected at a very high concentration. These high levels may lead to a significant presence of MAP in semen and consequently to a prolonged survival of sperm spawned at sea. In addition, data related to the potential structural similarity between mussel MAP and esterase S of the *Drosophila virilis* ejaculatory bulb are presented and discussed. Finally, we show that the 64 kDa protein of human semen reveals positive cross-reactivity with antibodies directed against *Mytilus* MAP and *Drosophila* esterase S. Taken together, the results reveal mussel MAP as the only esterase-like protein described so far whose distribution in the gonad and semen can be specifically associated with maturation, transport, emission and survival of spermatozoa outside.

Key words: esterase, external fertilizer, seminal fluid protein, spermatozoa, bivalve mollusc, fruitfly, mammal.

Introduction

Seminal fluid is defined as a cell-free fraction of the semen, which contains specific polypeptides and enzymes derived from excurrent gonad ducts and accessory sex glands. These proteins can mediate not only sperm transport but also functional maturation and fertilizing capacity of sperm. Analysis of seminal-fluid proteins is of interest for the study of cellular and molecular aspects of reproduction (Amann et al., 1993; Fazeli et al., 2000; Mikhailov and Torrado, 2000; Chapman, 2001).

The identification, characterization and potential functions of seminal-fluid proteins are mainly taken from studies on internal fertilizers, particularly from mammals (Robert and Gagnon, 1995, 1999; Syntin et al., 1996; Naaby-Hansen et al., 1997; Gerena et al., 1998; Mortarino et al., 1998). It has been shown that a sperm-coating protein acquired from secretions of the accessory sex gland can modulate the motility (Aumüller et al., 1997) and plasma membrane organization of mammalian spermatozoa (Soubeyrand and Manjunatu, 1997; Therien et al., 1999). In addition, fertilization-promoting peptides have been detected in mammalian semen (Kennedy et al., 1997). Sophisticated functional implications of seminal-fluid proteins in reproduction have been demonstrated in fruit flies. *Drosophila* seminal fluid contains a bulk of proteins secreted by the male accessory glands, the ejaculatory ducts and the ejaculatory bulb that affect the processes of sperm storage and sperm competition, and modulate female reproductive physiology after mating (reviewed in Chapman, 2001; Wolfner, 2002).

Some of the seminal-fluid proteins detected in fruit flies and mammals share the same (or similar) molecular mass, antigenic epitopes and enzymatic activity (see Torrado et al., 2000). An essential question in terms of the comparative physiology of reproduction is how similar seminal-fluid proteins become important for reproductive fitness in different animal groups (see Mikhailov and Torrado, 2000). We therefore decided that it would be interesting to trace the presence of such polypeptides identified in internal fertilizers in the seminal fluid of marine free-spawners. To date studies on seminal fluid components in seawater external-fertilizers are limited to the identification of the following compounds: a spawning pheromone in fish (Carolsfeld et al., 1997), arylsulphatase (Moriya and Hoshi, 1979, 1980) and proteins with esterase- and protease-like activities (Resing et al., 1985) in sea urchins, and a sperm-associated fucosidase in bivalve molluscs (Focarelli et al., 1997).

We focused our attention on protein composition of the gonad-duct and seminal fluids in the bivalve mollusc, *Mytilus galloprovincialis*. Species of the genus *Mytilus* are distributed worldwide and can be found attached by fibrous byssus threads to rock surfaces in open coasts. Their significant reproductive success is reflected not only in their wide occurrence along exposed coastlines, but also in their frequent predominance in intertidal rock habitats (see Gosling, 1992). Although these species are characterized by a strict gonochoric pattern of sexuality, their gonad system, as in a majority of bivalves, does not show any structural sign of sexual dimorphism. Neither accessory sex glands nor copulatory organs were found in these animals. During the reproductive period, males and females release their gametes in seawater where fertilization takes place.

In the *Mytilus* species, the tubular gonad is spread all along the mantle tissue. Published hand-drawn schemes (Wilson, 1886; White, 1937; Lubet, 1959) seem to suggest that the mussel reproductive-tract system consists of genital ducts, canals and follicles. However, a major limitation is that these schemes only mimic natural features of mantle-associated gonad organization. In this report we present a detailed structural patterning of the *M. galloprovincialis* tubular gonad and reproductive tract, required for gonad-duct manipulations and sampling.

Our previous studies led to the identification of the so-called ‘male-associated polypeptide’ (MAP), an abundant soluble protein in the male gonad with low levels in the female, which shows a marked male-predominant expression in the somatic gonad tissue of *M. galloprovincialis* (Mikhailov et al., 1995, 1996). MAP protein levels are related to male gonad development and the annual reproductive cycle, reaching maximum values during the spawning period (Mikhailov et al., 1998; Torrado and Mikhailov, 1998). Interestingly, MAP seems to be similar to esterase S, a seminal fluid protein of *D. virilis*, as judged by cross-reactivity with antibodies against esterase S, by its esterase enzymatic activity *in vitro*, and by its amino acid composition (Mikhailov et al., 1997; Torrado et al., 1997). Esterase S belongs to a family of diverse proteins in which related members are also expressed in the adult male reproductive tract of different *Drosophila* species (reviewed in Korochkin et al., 1990, Richmond et al., 1990; Oakeshott et al., 1995, 1999). Esterase S is expressed and secreted by the epithelium of the ejaculatory bulb and is transferred together with sperm to females upon copulation (Yenikolopov et al., 1983). Although a precise function of esterase S remains elusive, there is some evidence for a possible role in sperm storage and use in the female reproductive tract (see Korochkin et al., 1990).

Given our interest in the comparative analysis of male-reproductive tract esterases in different animal groups and the apparent lack of information on seminal-fluid proteins in marine free-spawners, we undertook a detailed study of MAP presence throughout the mussel gonad-duct tract and at spawning. We found that MAP is a major protein in *M. galloprovincialis* semen, present in both sperm cells and cell-free seminal fluid. Sperm-associated MAP is localized in the mid-piece region of spawned spermatozoa. This unexpected finding raises the possibility that in bivalves, MAP may play a role in sperm fertility. Of particular interest is the observation that MAP is detected in luminal fluids and sperm throughout the male gonad-duct system, from spermatogenic tubules to the terminal efferent duct. In addition, we show that human semen reveals positive cross-reactivity with antibodies directed against *Mytilus* MAP and *Drosophila* esterase S.

Materials and methods

Biological material

Sexually-mature adult, *Mytilus galloprovincialis* Lmk. (8–10 cm shell length) were obtained from commercial suppliers in La Coruña (Galicia, NW Spain) during the spawning period (March–April, 2001). 1-year-old juvenile mussels (6–8 mm shell length) were collected in June and July (2000) from the rocky shores of Balcobo beach (province of La Coruña, NW Spain). Transport of animals to the laboratory took no more than 1 h. Mussels were examined on the day of collection. Human ejaculates were obtained from healthy 30–35-year-old volunteers. After semen liquefaction, sperm cells were precipitated by centrifugation (500 ***g***, 10 min, 2°C). To obtain cell-free seminal fluid, the resulting supernatants were filtrated through a 0.22 μm membrane (Amicon, Beverly, MA, USA).

Induction of spawning and semen sampling

No method other than slightly increasing seawater temperature was used to induce sperm emission in mussels, so that no artificial alteration in the seminal fluid components, which are physiologically released at natural spawning, could be expected. Mussels were scrubbed thoroughly to remove fouling organisms, byssus threads were cut out, and the animals (15–20) were immersed in a 20 litre tank with filtered (0.45 μm) natural seawater (FSW) at room temperature (RT). Spawning was induced by increasing seawater temperature to 25–26°C, which was 2–4°C above RT. Once spawning was

initiated, males were easily identified and transferred to a separate smaller transparent tank at a lower density in FSW (2–3 animals per 10 liters FSW), where spawning continued. The transparent tank was located over a piece of black paper and spawning was monitored during 2–3 h. Photographs were taken and spawned sperm mass was recollected with minimum dilution in FSW. Microscopic examination of a small portion of each sample confirmed that they contained viable and motile spermatozoa. Semen samples were centrifuged (500 ***g***, 10 min, 2°C), and supernatants (i.e. semen-soluble fractions) were filtered (0.22 µm-membrane, Amicon, Beverly, MA, USA) and concentrated (3 kDa cut-off units, Amicon, Beverly, MA, USA). Sperm-containing sediments were resuspended in ice-cold FSW and precipitated again. Final sperm-cell pellets and cell-free supernatants were kept at –20°C.

Detergent treatment of spawned sperm

Spawned sperm were washed in FSW at 100 ***g*** for 1 min to remove any gross cell debris, assessed for motility and diluted for use. In all cases, large-orifice (1.5 mm diameter) pipette tips were used to minimize damage to the sperm membrane. Sperm suspension was divided into portions for detergent solubilization, and each sample was finally resuspended in the same volume of 500 µl of FSW, and incubated with a varying concentration (0.01–2% v/v) of Triton X-100 (Merck, Darmstadt, Germany) for 30 min at 20°C (each experiment was performed in triplicate). Sperm cells were sedimented (5,000 ***g***, 20 min), and the resulting supernatants were filtered and concentrated (3 kDa cut-off units, Amicon, Beverly, MA, USA). Precipitated sperm cells were resuspended and centrifuged again. The final pellets were immediately extracted by 2× SDS-Laemmli sample buffer, centrifuged (30,000 ***g***, 30 min, 12°C), and the supernatants kept at –80°C until use.

Gonad-duct manipulations

The male mantle lobe was removed and placed in a Petri dish. The mantle epithelium was dissected through a stereomicroscope (Nikon, Tokyo, Japan), and the content of the spermatogenic tubules and transversal gonad ducts (see Fig. 3) was aspirated using a fine capillary pipette or a 5 µl Hamilton syringe as previously described (Torrado and Mikhailov, 1998). The samples (10–20 µl) were centrifuged (500 ***g***, 15 min, 2°C), and the top 80% of the supernatants was subjected to a second centrifugation (60 000 ***g***, 30 min, 2°C). A small volume (1–2 µl) of the resulting supernatants was examined microscopically to confirm that all cells had been removed. The sperm-containing pellet (obtained after the first centrifugation) was resuspended in ice-cold FSW (1:100 v/v) and precipitated by centrifugation (20 000 ***g***, 15 min, 2°C). The resulting supernatants and sediments were stored at –20°C until use. For some experiments, fragments of transversal gonad ducts were microsurgically dissected from the mantle, and isolated samples were shaken in ice-cold FSW for 30 min in order to remove luminal fluid and sperm cells. Washed tube fragments were immediately extracted with 2× SDS-Laemmli sample buffer containing the protease inhibitor cocktail (Roche Diagnostic, Mannhein, Germany), centrifuged (30 000 ***g***, 30 min, 12°C) and supernatants stored at –80°C.

Protein sample preparation

Sperm pellets, seminal and gonad-duct-derived fluids were mixed with an equal volume of 2× SDS-Laemmli sample buffer containing a protease inhibitor cocktail (Roche Diagnostic, Mannhein, Germany), boiled for 5 min, cooled on ice and centrifuged (30,000 ***g***, 30 min, 12°C). The supernatants were stored at –80°C. Samples were subjected to SDS-PAGE followed by Coomassie gel staining, and protein concentration was measured by gel densitometry using bovine serum albumin (BSA) (Sigma, Madrid, Spain) as the reference protein. Mantle lobes were homogenized at a 1:1 (w/v) ratio in ice-cold deionized water (for isoelectrofocusing separations) or a 1:2 (w/v) ratio in 100 mmol l^{-1} Tris-EDTA buffer, pH 7.0 (for SDS-PAGE separations), both containing the protease inhibitor cocktail. After 30 min extraction on ice, homogenates were centrifuged at 60 000 ***g*** for 60 min at 2°C, and the supernatants used immediately or supplemented with glycerol (to a final concentration of 50% v/v) and stored at –80°C. Protein concentration in mantle extracts was measured according to the Bradford method.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Samples were separated using 5% stacking and 10% resolving Tris-glycine SDS-polyacrylamide gels (Bio-Rad, München, Germany). Proteins were visualized by Coomassie Blue staining. The apparent molecular masses (MW) of the bands were determined by comparing high and low MW calibration kits (Amersham Biosciences, Uppsala, Sweden) in the same gel.

Isoelectrofocusing (IEF)

Ultra-thin (0.4 mm) 5% polyacrylamide gels, containing a mixture of 5-7, 3-10 Bio-Lytes (Bio-Rad, München, Germany) in a proportion of 4:1 were electrophoresed in the mini IEF Cell (Bio-Rad, München, Germany) following the manufacturer's instructions. The visible marker, myoglobin (Sigma, Madrid, Spain), was loaded at opposite sides (near the cathode and anode contact zone) of the same gel run; IEF was finished when both myoglobin bands were colocated. After separation, proteins were detected by Coomassie Blue staining. The following pI markers (Sigma, Madrid, Spain) were used: β-lactoglobulin A (pI 5.1), carbonic anhydrase I (pI 6.6), carbonic anhydrase II (pI 5.9) and myoglobin (pI 6.8, 7.2).

Western immunoblot

Proteins resolved in SDS-PAGE gels were electrophoretically transferred to nylon or nitrocellulose membranes ('Nytran' or 'Optitran', Schleicher and Schuell, Dassel, Germany) by routine methods using the mini 'Trans-Blot' Cell (Bio-Rad, Hercules, CA, USA). Proteins resolved in IEF gels were transferred to nylon membranes as described by Heukeshoven and Dernick (1995). Protein loading and localization of MW and pI markers were verified by membrane

staining with Amido Black (Merck, Darmstadt, Germany) or Ponceau S (Sigma, Barcelona, Spain). Prior to immunodetection, the blots were incubated in a blocking solution containing 20% v/v normal horse serum (Sigma, Barcelona, Spain) in 100 mmol l^{-1} Tris-HCl (pH 7.6), 150 mmol l^{-1} NaCl, 0.1% Triton X-100, 0.01% NaN_3, at room temperature for 1 h. The following rabbit polyclonal antibodies were used: (1) anti-MAP raised against the protein isolated from the *M. galloprovincialis* male gonad (Mikhailov et al., 1995), at a dilution of 1:200; (2) anti-esterase S raised against a fusion-protein containing over 90% of the coding sequence of the *Esterase S* gene of *D. virilis* (Tamarina et al., 1991), at a dilution of 1:200, and (3) anti-pig esterase against the protein fraction isolated from porcine liver (Polysciences, Eppelheim, Germany), at a dilution of 1:200. Antibody dilutions were prepared in the blocking solution. All antibody incubations were followed by six washes (10 min in each) in the same solution without horse serum. Immunoblots were revealed as described (Mikhailov et al., 1997) using peroxidase-labeled anti-rabbit immunoglobulins (Sigma, Madrid, Spain), at a dilution of 1:3000, and diaminobenzidine tablets (Sigma, Madrid, Spain).

Densitometric analysis

Gels and blots were scanned using a model GS-700 densitometer (Bio-Rad, Hercules, CA, USA). Profile analysis of the fractions/bands including peak identification/quantitation, area integration and MW determination, was carried out with the software 'Molecular Analysis' (Bio-Rad, Hercules, CA, USA). The images were processed by computer software to remove image noise and background.

Protein purification and microsequencing

MAP was isolated from the *M. galloprovincialis* male gonad by IEF followed by SDS-PAGE. Briefly, a mantle water-soluble extract was separated by IEF in 5% polyacrylamide slab gels (Amersham Biosciences, Uppsala, Sweden). The MAP-containing fraction (pI 6.2) was isolated, subjected to SDS-PAGE, and MAP (39 kDa) was electro-eluted from SDS-gel fragments, using the electro-eluter model 442 (Bio-Rad, Hercules, CA, USA) following the manufacturer's instructions. Purified protein preparation was re-electrophoresed using a 10% resolving SDS-polyacrylamide gel, and MAP was subjected to in-gel digestion with sequencing grade modified trypsin (Promega, Madison, WI, USA) following recommendations described in Stensballe and Jensen (2001) and Soskic and Godovac-Zimmermann (2001). Random trypsinized peptides were microsequenced at the Laboratory of Mechanisms of Ocular Diseases of the National Eye Institute, NIH, Bethesda, USA.

Whole-mount histology

Juvenile mussels were fixed in Bouin's solution and extensively rinsed several times in 70% ethanol. To separate shells, the posterior adductor muscle was sectioned, internal organs (i.e. foot, hepatopancreas, gills) were removed and the whole (two-lobe) mantle sheet containing the gonad-tubular 'tree' was carefully micro-dissected and isolated. The isolated mantle preparation was stained with Hematoxylin-Eosin, dehydrated, mounted on slides, viewed and photographed with a Nikon stereomicroscope and a Nikon Eclipse 800 microscope.

Immunocytochemistry

Spawned sperm were washed and resuspended in FSW. The sperm suspension was dropped on slides precoated with 3-aminopropyltriethoxysilane (Sigma), air-dried and fixed in 4% neutral buffered formalin in FSW for 10 min at RT. Prior to immunostaining, fixed sperm cells were treated with 0.1% Triton X-100 (v/v) for 30 min, washed and further assayed as described in Torrado and Mikhailov (1998). Briefly, sections were pre-incubated with 20% normal horse serum in Tris-buffered saline, pH 8.0 (TBS: 50 mmol l^{-1} Tris-HCl, 50 mmol l^{-1} NaCl) for 1 h and incubated with rabbit anti-esterase S antibodies (at a dilution of 1:50) for 1 h at RT. After six washing steps in TBS (10 min each), sections were incubated with fluorescein-labelled anti-rabbit immunoglobulins (Sigma, Madrid, Spain) at a dilution of 1:1000 for 1 h. After six washing steps in TBS, sections were mounted in 100 mmol l^{-1} Tris-HCl (pH 8.6): glycerol (1:9, v/v) containing freshly added 0.1% *p*-phenylenediamine (Sigma, Barcelona, Spain) and viewed and photographed with a Nikon Eclipse 800 microscope (Nikon, Tokyo, Japan) equipped with epifluorescence. Control experiments included assessing a fluorescent background of sperm cells treated by anti-esterase S antibodies pre-absorbed by MAP.

Results

MAP is a major protein constituent of Mytilus *semen*

Laboratory-induced spawning was specially designed to observe and sample the semen at different times after the release of sperm masses. Sexually mature males were induced to spawn by a sequential increase of seawater temperature in the tank without shaking. When spawning begins, the sperm mass is actively ejected as a sharply defined white stream into the seawater from a well-defined point at the posterior part of the animal (Fig. 1A). It is possible that sperm ejection is assisted by ejaculatory bulb-like structures (see Fig. 3) as well as by muscular contractions of the spawning animal. Shortly thereafter, the released sperm mass subdivides into diffuse cloudy-like and compact thread-shaped aggregates (Fig. 1B). While the cloudy-like masses are progressively and spontaneously dispersed in the seawater, the compact sperm aggregates tend to precipitate at the bottom of the tank (Fig. 1C), remaining for a long time (2–3 h) if the seawater is not vigorously mixed. To the best of our knowledge, this is the first documentation of the compacted form of sperm emission (semen 'clots') in bivalve molluscs, a phenomenon rarely described in studies on free-spawning marine invertebrates (see Thomas, 1994).

To analyze the protein composition of the mussel semen, cloudy-like as well as compact sperm (before and after

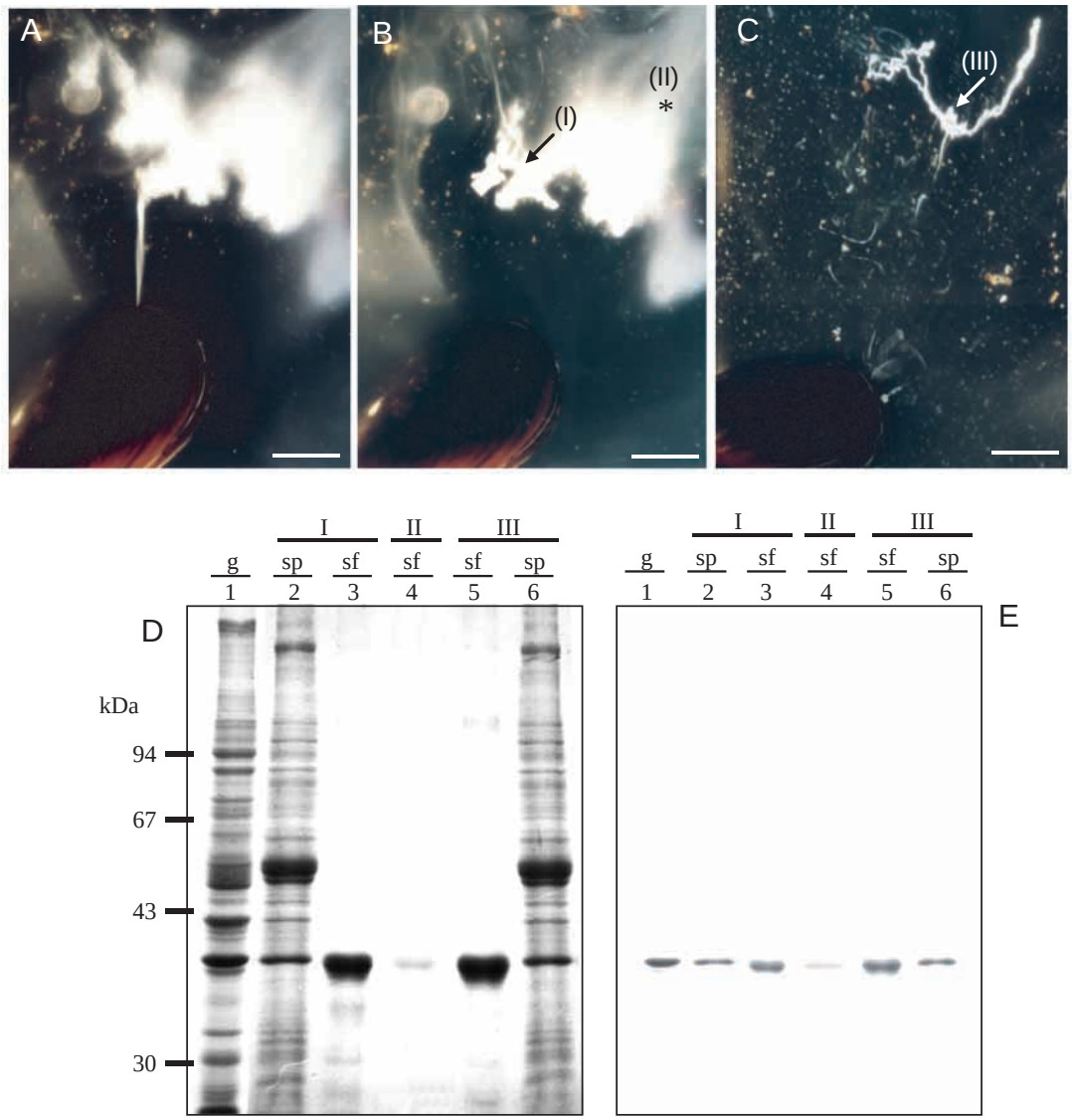

Fig. 1. The identification of MAP as a dual component of *M. galloprovincialis* semen. (A–C) Sequential phases of spawning (bars, 1.6 cm). (A) Whitish-colored semen streams are emitted by the male. (B) 5–10 min later, one portion of the sperm (II) is dispersed into seawater (asterisk), whereas the other portion (I) forms compact thread-shaped structures (black arrow), which are precipitated at the bottom of the tank (III, white arrow) 30–40 min after sperm emission (C). (D,E) The sperm suspension (II) and thread-shaped semen (I and III) were sampled, separated into cell-free seminal fluid (sf) and sperm (sp) fractions and studied by SDS-PAGE (D) followed by western blot (E) with anti-MAP antibodies. The protein loading for each lane is indicated. Lane 1, extract from the total male gonad (15 μg). Lanes 2 and 6, sperm fraction of the semen (15 μg each). Lanes 3–5, cell-free seminal fluid (2.0 μg, 0.3 μg and 2.0 μg, respectively). In both the soluble phase (Lanes 3–5) and in the cell fraction (Lanes 2, 6) of each semen sample, the 39 kDa band was detected on the gel (D) and it was positively stained by anti-MAP antibodies on the blot (E). The positions of marker proteins (kDa) are shown.

precipitation) (see Fig. 1B,C) were collected in a minimum volume of seawater. Cell-containing and cell-free soluble fractions of each semen sample were separated by centrifugation and filtration and analyzed by SDS-PAGE followed by western blot. On Coomassie Blue-stained gels, a band with an apparent molecular mass of 39 kDa (identical to that of the MAP isolated from the male gonad) was found in the sperm-cell fractions of each of the semen samples studied (Fig. 1D, lanes 2,6). A similar polypeptide detected in the cell-free soluble semen fraction was characterized by a slightly faster electrophoretic mobility (Fig. 1D, lanes 3–5). The identity of these bands was confirmed as MAP by western blot using anti-MAP antibodies (Fig. 1E). Of note, the cell-free soluble fraction of the cloudy sperm contains trace amounts of the 39 kDa protein although it was 2–3 times more concentrated than the seminal fluid obtained from both types of semen clots. These results suggest that after spawning, a portion of the seminal-fluid MAP, which is included in sperm aggregates, persists as long as the aggregates keep their compact form.

The densitometric analysis of Coomassie Blue-stained gels shows that MAP represents more than 90% of the protein in the soluble fraction of the spawned semen clots. These findings demonstrate that MAP is the predominant protein constituent of the mussel seminal fluid and that this male gonad-derived polypeptide is excreted together with sperm into seawater during spawning.

The sperm cells sampled at different time periods of spawning displayed positive immunoblot reactions with anti-MAP antibodies (see Fig. 1E, lanes 2 and 6). This raised the possibility that MAP could remain on the sperm surface long after sperm emission. To check this possibility, sperm suspensions obtained from the semen streams (see Fig. 1A) were diluted 50 times in seawater, and spermatozoa were kept in the container for 12 h at RT. Note that under these conditions any changes in sperm viability and motility could be detected. At different time-exposure periods, spermatozoa were sampled (using the so-called 'swim up' technique; see Graczykowski and Siegel, 1991), precipitated by centrifugation and analyzed by SDS-PAGE followed by western blot. Comparison of MAP immunoblot signals of 0 h- and 12 h-incubated sperm did not reveal any detectable reduction of MAP immunostaining intensity (data not shown). These results indicate that sperm cells do not lose MAP after prolonged swimming and survival in seawater, which suggests that MAP could be tightly associated with spermatozoa.

The association between MAP and sperm cells was evaluated according to its susceptibility to being removed from cells by the non-ionic detergent, Triton X-100. Spawned spermatozoa were treated with serial dilutions of Triton X-100 (from 0% to 2%) in seawater, sedimented by centrifugation and subjected to SDS-PAGE followed by western immunoblot with anti-MAP and anti-esterase S antibodies (see Fig. 2A–C, respectively). Although the same results were observed with both types of the antibodies used, the intensity of MAP immunodetection was higher in blots treated with anti-MAP in comparison to that in blots treated with anti-esterase S antibodies. It is likely that anti-esterase S recognizes only a part of MAP-specific epitopes, which are detected by anti-MAP antibodies.

A significant portion (up to 30%) of MAP immunoreactivity could be extracted from sperm cells using the lowest detergent concentration (0.01%), and 0.03% Triton X-100 treatment resulted in the removal of approximately 70% of the protein. Further increase in detergent concentration (0.06–0.6%) in the incubation medium did not result in a further significant decrease of the MAP-specific immunoblot signal of the treated sperm. Almost complete extraction (up to 95%) of the protein was achieved at a substantially higher concentration of Triton X-100 (2%) in seawater (Fig. 2E). Note that the observed kinetics of Triton X-100-mediated MAP extraction from sperm cells is relatively selective because a significant part of the other sperm proteins were not solubilised at any of the detergent concentrations used (see Fig. 2A). The reduced MAP signal of the Triton X-100-treated spermatozoa was not due to MAP proteolytic degradation as the protein was demonstrated in western blots of the resulting supernatants of untreated and detergent-treated sperm samples (Fig. 2D). In addition, no signs of protein degradation were observed on the gels or blots of the Triton X-100-treated sperm samples.

The simplest interpretation of these results is that there is more than one type of MAP association with spawned spermatozoa. Over 70% of MAP immuno-reactivity is extracted from the cells at a low Triton X-100 concentration, which could reflect adsorption of the protein on the sperm surface from MAP-containing luminal fluid during sperm transit through gonad collecting and efferent ducts (see below). However, another ('resting') portion of the protein (up to 20%) seems to be more tightly associated with spermatozoa because it could only be solubilized at a very high Triton X-100 concentration.

To study MAP localization in spawned sperm, these were washed, fixed, permeabilized with 0.1% Triton X-100 (v/v) and assayed by immunofluorescence. A MAP-specific signal was detected in the mid-piece of spermatozoa (Fig. 2F,G). Together with our previous findings that MAP can be detected on the surface of non-permeabilized spermatozoa, but not in sperm cells (Torrado and Mikhailov, 1998), the results suggest that seminal-fluid MAP may be internalized by mature sperm cells following its compartmentalization in the mid-piece region of spawned spermatozoa.

MAP is a major protein of the Mytilus *male gonad-duct luminal fluid*

The published data contains some contradictory conclusions on the structural and functional compartmentalization of the male tubular gonad in *Mytilus*, so we have studied gonad-duct branching morphogenesis in the mantle of juvenile mussels. The entire two-lobe mantle sheets were micro-dissected from post-metamorphic animals, fixed and stained. A representative whole-mount of the male paired tubular gonads and reproductive tract settled into a whole mantle sheet is shown in Fig. 3. Within each mantle lobe, the corresponding gonad-duct network includes: (1) a bulb-like structure with gonopore, (2) a major longitudinal gonad duct (LGD), (3) several (5–10) transversal gonad ducts (TGDs), (4) TGD-ramifications with numerous bud-like lateral extensions, and (5) terminal end tubules. Microscopic examination revealed that spermatogenesis takes place mainly in TGD-ramifications and terminal end tubules and so we propose to call these structures 'spermatogenic tubules' (STs). This is in contrast to the generally accepted interpretation of such structures as 'gonad acinus' or 'gonad follicles' (see, for instance, Gosling, 1992).

In adult mussels, the structural organization of the developed tubular gonad is not as clearly observable as in juveniles. In particular, STs form a complex overlapping tridimensional network, which occupies up to 90% of the mantle volume (consequently, mantle thickness increases significantly from 0.5–1 mm in juveniles to 5–6 mm in adults). Only at the ventral mantle edge is it still possible to see non-overlapping STs (Fig. 3E). Mature sperm was found in the lumen of STs, TGDs and LGD.

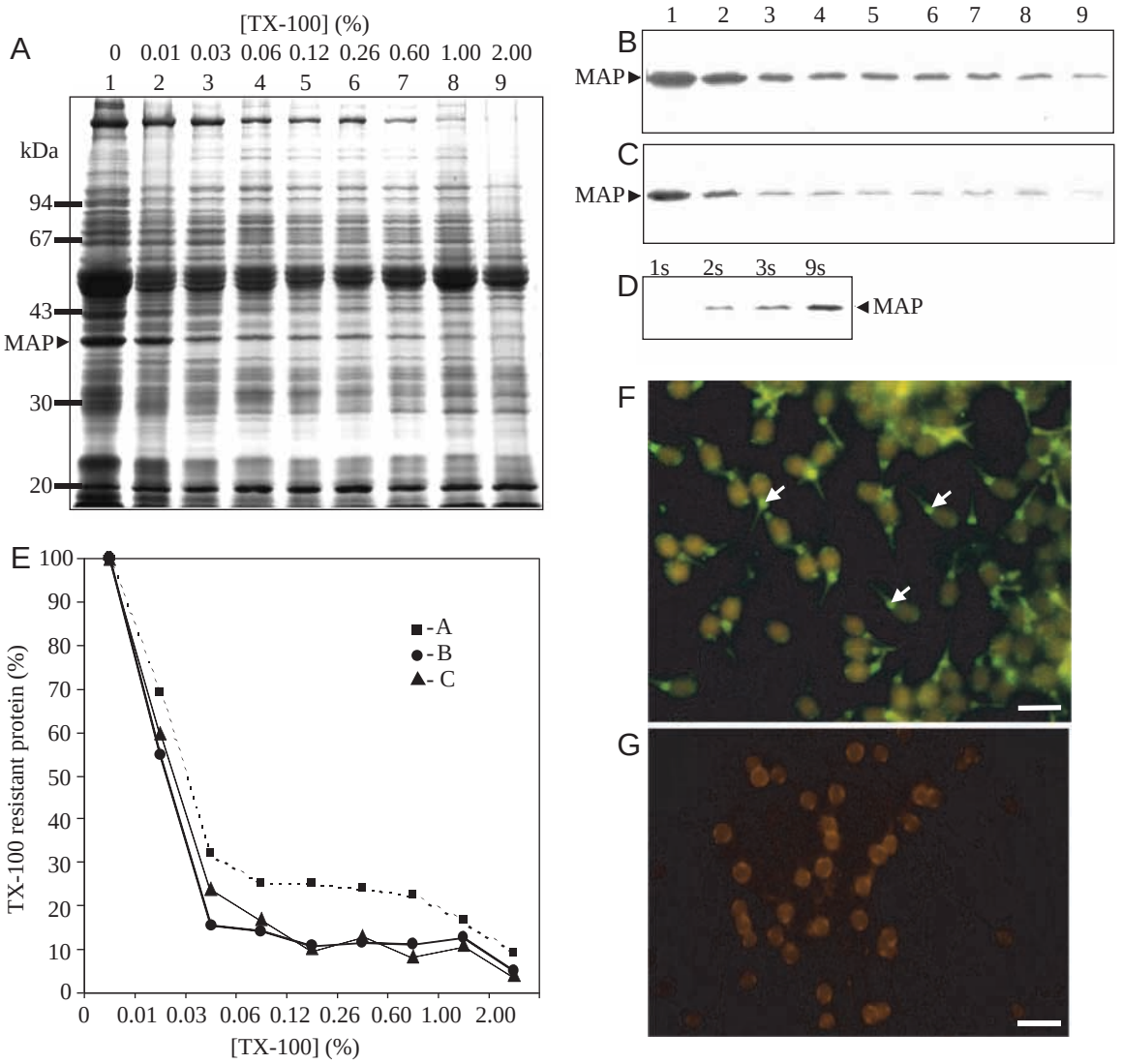

Fig. 2. MAP is a sperm-associated protein. Equal samples of spawned sperm (A, Lanes 2–9) were treated with Triton X-100 (TX-100) at final concentrations varying from 0.01% to 2.00%, respectively. Control (Lane 1) and detergent-treated spermatozoa (Lanes 2–9) were studied by SDS-PAGE (A) followed by western blot with anti-MAP (B) or anti-esterase S (C) antibodies. As shown in A, the 20 kDa band intensity of the treated sperm remains unchanged at all TX-100 concentrations used. (D) Resulting supernatants of control (Lane 1s) and TX-100-treated spermatozoa (Lanes 2s, 3s and 9s) were concentrated and subjected to SDS-PAGE followed by western blot with anti-MAP antibodies. (E) Graphical presentation of the MAP densitometric analyses of both the gel (A) and blots (B,C), showing the percentage of the protein resistant to extraction by TX-100. Note that the three profiles (A–C) have the same MAP retention dynamics. (F) Immunofluorescence localization of MAP in the mid-piece region of spawned spermatozoa (white arrows) using anti-esterase S antibodies. (G) Control reaction: no MAP-positive signals were detected in the sperm cells treated with the antibodies pre-absorbed by MAP. Bars, 3 µm.

Bulb-like structures were observed in the male gonad of both juveniles (Fig. 3D) and adults (Fig. 3F). An adult male was induced to spawn, and immediately following the first release of spermatozoa, its posterior adductor muscle and other tissues were sectioned, which allowed us to observe the sperm mass being ejected from the bulbs as white-colored streams (Fig. 3F).

We then studied the presence of MAP in luminal fluid extracted from different structural compartments of the male gonad-duct network. Using sexually mature adult males, luminal contents were extracted from LGD and TGDs and the corresponding cell-free fluids were subjected to SDS-PAGE. In all samples, a major 39 kDa polypeptide was detected (Fig. 4A). Western blot with anti-MAP antibodies showed that this polypeptide corresponds to MAP (Fig. 4B). Both the immunoblot and the electrophoretic MAP bands of the cell-free luminal fluid appeared as closely migrating doublets, while the male gonad and the sperm displayed clear sharp immuno-stained bands, which may suggest that different post-translationally modified forms of the MAP protein coexist in the luminal fluid.

Fig. 5 shows the results of a comparative analysis of the

proportion of MAP to total protein in the luminal and semen-derived fluids, and in the whole male gonad extract. Fluid was obtained from 3–5 animals, pooled and separated by SDS-PAGE. Stained gels were scanned by transmittance. For each gel, a total profile analysis was carried out, and the proportion of the MAP peak relative to the total protein scan value was calculated and expressed as a percentage. The MAP fraction percentage for the ST, LGD and seminal fluid profile was approximately 70%, 80% and 90%, respectively. We consider that these densitometric values reflect MAP proportions, taking into account that MAP is a major protein component of the scanned 40 kDa bands, as demonstrated by 2-D electrophoresis followed by western blot (Mikhailov et al., 1997). The kinetics of the MAP:total protein ratio can be ascribed to a progressive

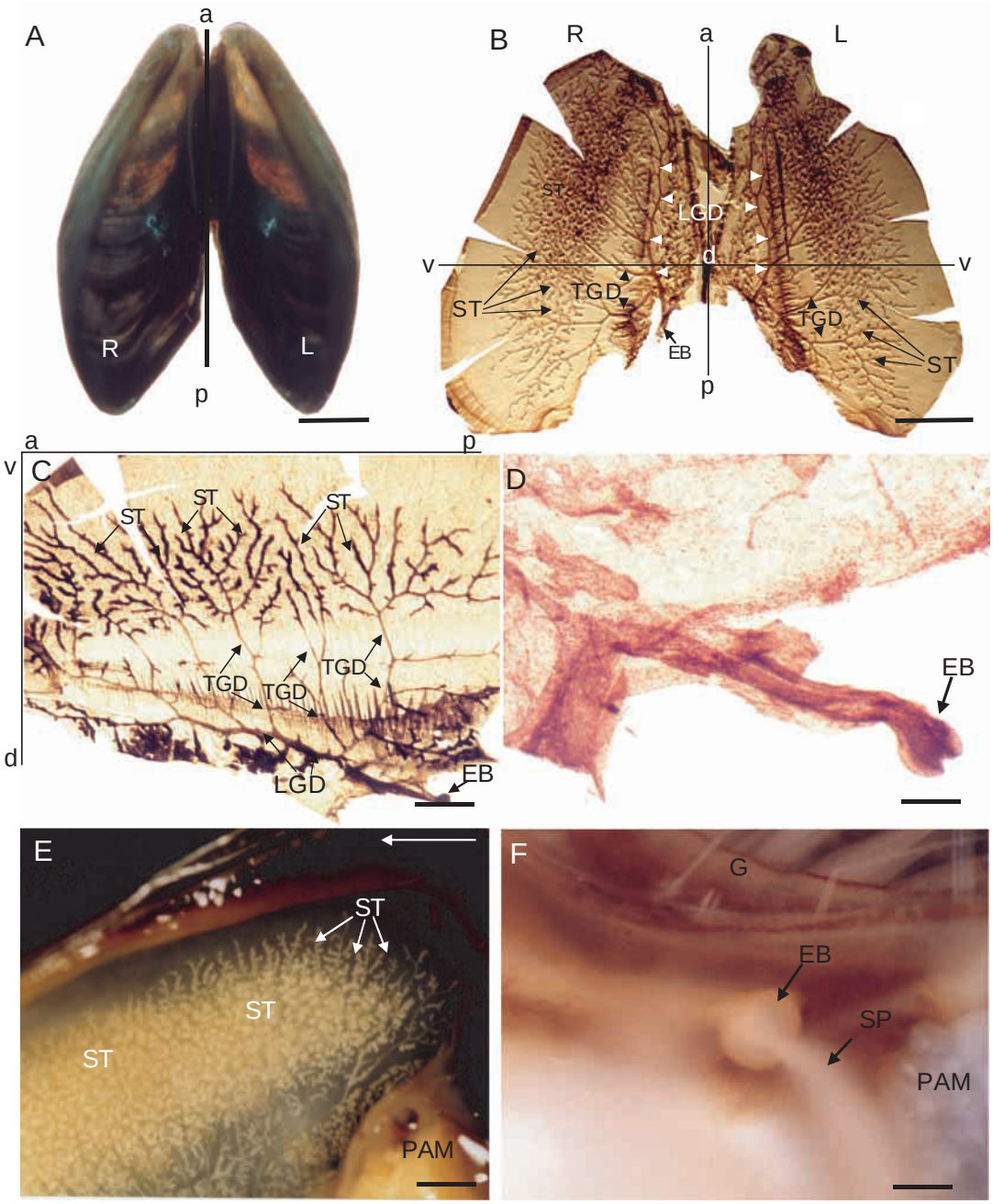

Fig. 3. Structural organization of the male reproductive system in the mantle of 1-year-old (A–D) and adult (E,F) mussels. (A) Dorsal view of the right (R) and left (L) shells; a–p, anterior-posterior axis; v–d, ventral-dorsal axis. (B) Paired tubular gonad and reproductive tract spreading into a two-lobe mantle sheet (whole-mount histology). (C) View of the tubular gonad network in one mantle lobe (whole-mount histology). (D) Detailed view of the ejaculatory bulb-like structure (whole-mount histology). ST, spermatogenic tubule; TGD, transversal gonad duct; LGD, longitudinal gonad duct; EB, 'ejaculatory' bulb-like structure. (E) The ST-network occupies almost the entire volume of the mantle lobe in adults. (F) Sperm emission (SP) through the EB-like structure. G, gills; PAM, posterior adductor muscle. Scale bars, 1 mm (A); 0.5 mm (B,E); 0.2 mm (C); 0.1 mm (D); 2 mm (F).

'simplification' of the protein composition of the samples studied, i.e. from a relatively complex protein mixture in the ST-sample to an almost single-protein pattern in the semen-derived fluid. This, in turn, suggests that MAP may be the only major protein in the gonad-duct luminal fluid that is excreted from the gonad at spawning. Note that it was necessary to concentrate the semen-derived fluid over 20 times to make it suitable for SDS-PAGE analysis, whereas protein content of the gonad-duct fluids was sufficient for use without concentration.

As we have previously suggested (Torrado and Mikhailov, 1998), MAP can be highly expressed in the gonad-duct wall. To investigate this possibility, fragments of the TGD (see Fig. 6A) were microsurgically dissected, extracted with Laemmli sample buffer, and analyzed by western blot with anti-MAP antibodies parallel to luminal sperm and luminal fluid samples obtained from the same TGD (Fig. 6B). The intensity of the MAP-specific immunoblot reaction in the TGD-wall sample was threefold higher than that in the luminal fluid and tenfold higher than in luminal sperm. The results indicate that the duct network may be a principal source of MAP in the ripe male gonad and strongly suggest that the protein is secreted by duct epithelial cells into duct lumen.

Mytilus *MAP shares sequence similarity with* Drosophila *esterase S*

The idea that MAP is a bivalve relative of *Drosophila* male-specific esterases is based on data from our previous studies on MAP amino acid composition, enzymatic activity and cross-reactivity with antibodies against *D. virilis* esterase S (Mikhailov et al., 1997; Torrado et al., 1997). Here we present structural evidence to support this suggestion.

A water-soluble extract of the *Mytilus* male gonad was subjected to IEF followed by western blot. On IEF gels, MAP was identified as a single band at pI 6.2 (Fig. 7A), recognized by three different antibodies against: (1) *Mytilus* MAP, (2) *Drosophila* esterase S, and (3) porcine esterase (Fig. 7B). The

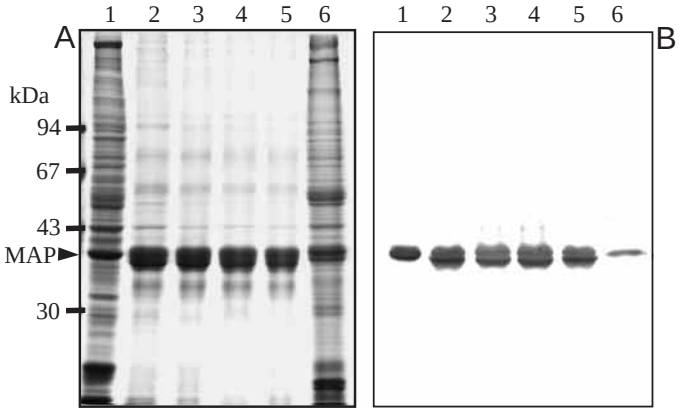

Fig. 4. MAP is a major protein constituent of the luminal gonad-duct fluid. Representative SDS-gel electrophoresis (A) and western blot (B) revealed with anti-MAP antibodies. The protein loading for each lane is indicated. Lane 1, extract from the total gonad/mantle (15 µg). Lanes 2 and 3, cell-free fraction obtained from the lumen of the longitudinal gonadal duct (LGD) (4 µg per lane). Lanes 4 and 5, cell-free fraction obtained from the lumen of the transversal gonadal ducts (TGDs) (4 µg per lane). Lane 6, sperm cells obtained from the lumen of the TGDs (15 µg). Note that the intensity of MAP immunostaining is much higher in the luminal fluid than in sperm and total gonad extract. The positions of marker proteins (kDa) are shown.

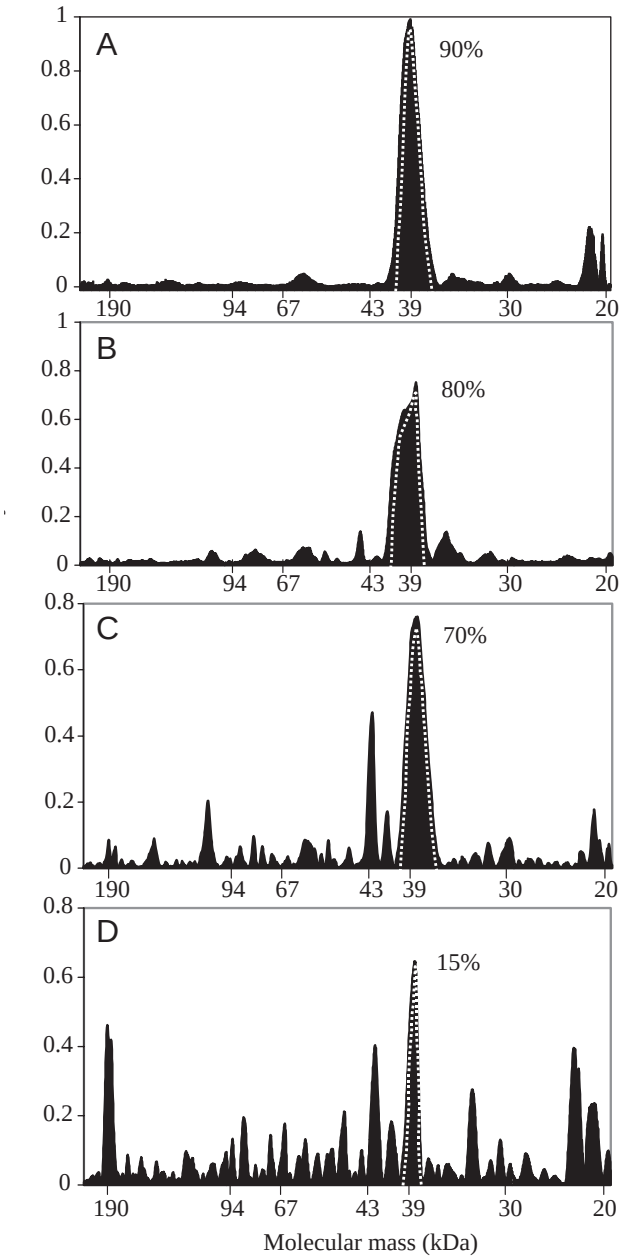

Fig. 5. The MAP:total protein ratio (%) is higher in the seminal fluid than in the spermatogenic tubule (ST) luminal fluid. Representative scan profiles of the Coomassie-stained proteinograms after SDS-PAGE. The protein loading for each lane is indicated. (A) Seminal fluid (1.5 µg). (B) Luminal fluid of the longitudinal gonadal duct (2.5 µg). (C) Luminal fluid of the STs (10 µg). (D) Extract from the whole gonad/mantle (10 µg). Dotted lines indicate the MAP fractions, 39 kDa.

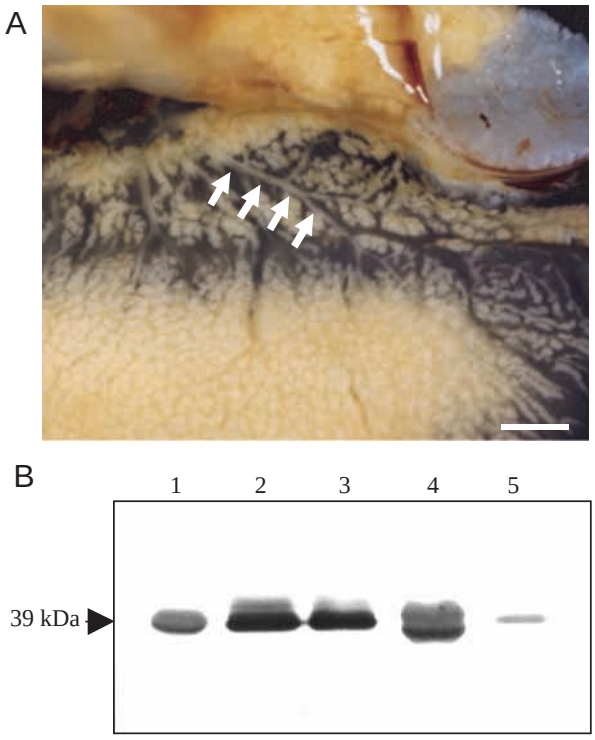

Fig. 6. MAP is highly detected in the gonad-duct wall of adult males. (A) One of the transversal gonadal ducts (TGDs; arrows) located in the posterior mantle region was microsurgically isolated and assayed as described in Materials and methods (bar, 0.4 cm). (B) Western blot (after SDS-PAGE) with anti-MAP antibodies. The protein loading for each lane is indicated. Lane 1, extract from the total male gonad/mantle (12 µg). Lanes 2 and 3, extract from the gonad-duct wall (12 and 6 µg, respectively). Lane 4, cell-free luminal fluid (5 µg). Lane 5, sperm cells from the lumen (10 µg). Note that the intensity of MAP imunostaining is higher in the gonad-duct wall (Lanes 2, 3) in comparison with that of the luminal fluid (Lane 4) and sperm (Lane 5).

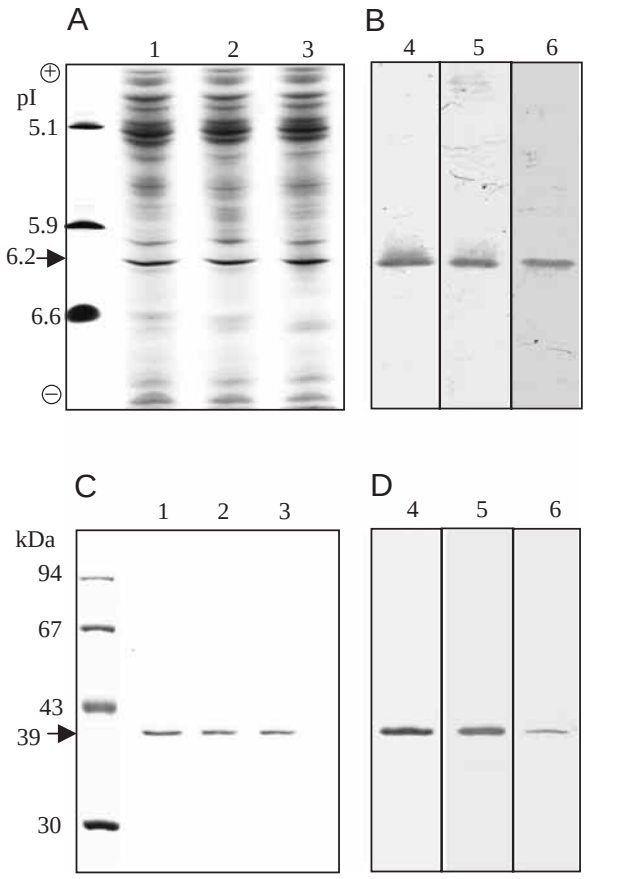

Fig. 7. Isolation of MAP by isoelectric focussing (IEF) followed by SDS-PAGE with immuno-identification of MAP fractions by western blot using three different antibodies. (A) IEF: Lanes 1–3, water-soluble extract from a whole gonad/mantle. (B) Western blots after IEF revealed with anti-MAP (Lane 4), anti-esterase S (Lane 5), and anti-porcine esterase (Lane 6) antibodies. Note that the same single band of pI 6.2 was detected by all antibodies. (C) SDS-PAGE of MAP fraction (Lanes 1–3) isolated from IEF-gels. Only 39 kDa bands were observed in the SDS-gel, which indicates the homogeneity of the MAP IEF-fraction. (D) Western blots after SDS-PAGE revealed with anti-MAP (Lane 4), anti-esterase S (Lane 5), and anti-porcine esterase (Lane 6) antibodies. The SDS-fraction contains the only 39 kDa mussel MAP, immunologically similar to both *D. virilis* esterase S and pig esterase.

pI 6.2 band contains a single protein of 39 kDa (Fig. 7C), which was recognized by all three antibodies (Fig. 7D).

Trypsinized peptides derived from the purified MAP protein were subjected to microsequencing. The sequences of seven peptides (5–7 residues each) were obtained (43 amino acids were identified in total). Using a multiple alignment, we were able to match all the MAP-derived peptide sequences over the *D. virilis* esterase S sequence; the resulting overall identity and similarity was 58% (25/43) and 67% (29/43), respectively (Fig. 8A). Note that none of the MAP-derived peptides are located in the conserved carboxylesterase domains (Fig. 8B). Taking into account an extreme structural divergence within the carboxylesterase protein multi-family (Torrado et al., 2000), the sequence similarity revealed between MAP and esterase S is considered highly significant.

It has been suggested that during evolution, carboxylesterase-like polypeptides could be recruited by the male reproductive tract to perform new sex-specific roles (Mikhailov and Torrado, 1999, 2000; Mikhailov et al., 1999). We decided to perform immunochemical screening of human semen using antibodies against MAP and esterase S. Human sperm and seminal fluid were separated by centrifugation and filtration, and the samples studied by SDS-PAGE followed by western blot. A single 64 kDa band was detected in the human seminal fluid and sperm by both types of antibodies used (Fig. 9).

Discussion

Through the use of electrophoretic assays and immunoblotting, the present investigation demonstrates that MAP, a major mussel gonad-duct protein similar to esterase S

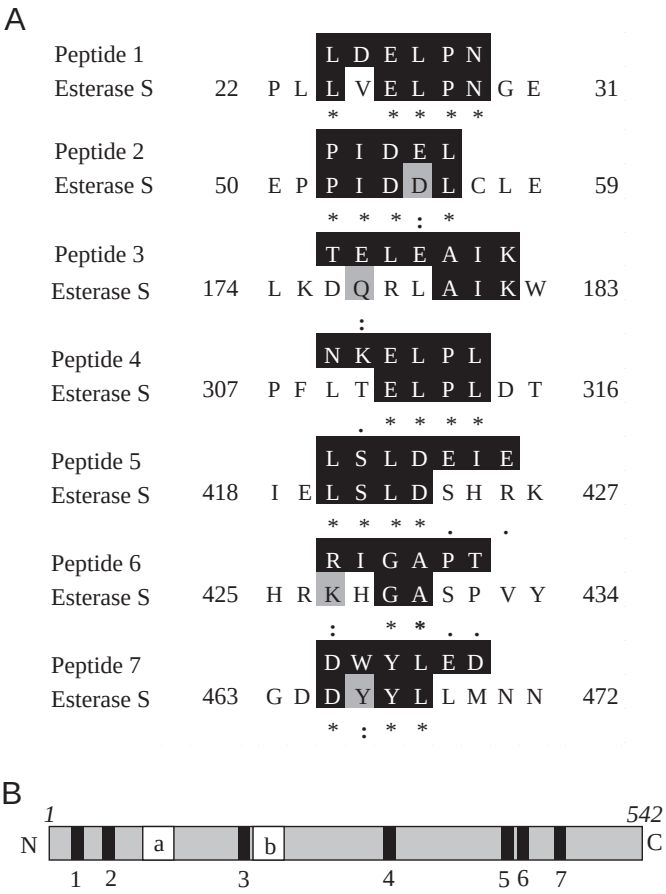

Fig. 8. Sequence alignments of the MAP peptides against the esterase S protein. (A) Alignments generated by ClustalW program (Thompson et al., 1994); identical (*), conservative (:), and semi-conservative (.) residues are indicated. Since the length of MAP peptides is short, no gaps were allowed in the alignments. (B) Localization of the aligned MAP regions (black) over the full-size esterase S sequence (grey). White boxes (a,b) indicate two relatively conserved amino acid domains of the *D. virilis* esterase S (for details, see Mikhailov and Torrado, 1999; Torrado et al., 2000).

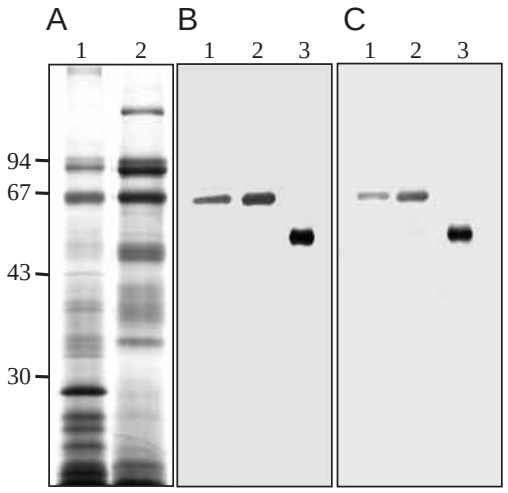

Fig. 9. A 64 kDa polypeptide is detected in human ejaculate by anti-*Mytilus* MAP and anti-*Drosophila* esterase S antibodies. Equal amounts (20 µg of total protein) of cell-free (Lane 1) and sperm-cell (Lane 2) fractions of human semen were separated by SDS-PAGE (A), blotted on membranes and treated with anti-MAP (B) or anti-esterase S (C) antibodies. Lane 3, rabbit liver carboxylesterase, 1 µg (Sigma). Note that the intensity of immunostaining of the 64 kDa band is slightly higher in the sperm than in the seminal fluid. The positions of marker proteins (kDa) are shown.

of the *D. virilis* ejaculatory bulb, is released in seawater as a dual component of mussel semen and that this component is the main protein of mussel seminal fluid and a constituent of spawned spermatozoon. To our knowledge, this is also the first characterization of the seminal fluid proteins in *Mytilus*.

We observed that mussel semen can form clots shortly after spawning, suggesting that clot-like sperm structures pre-exist in the gonad duct lumen. This seems to be an efficient way of collecting and transporting sperm mass from different branches of the reproductive system, and finally projecting the sperm mass outside. Of note, the sperm manually isolated from the STs exhibit lower motion activities than those of spawned sperm, as observed in *M. galloprovincialis* (Mikhailov and Torrado, 2000) and the zebra mussel, *Dreissena polymorpha* (Mojares et al., 1995). Emission of compacted sperm masses may represent an important feature for reproductive fitness in *Mytilus*. It has been shown that some marine free-spawners have evolved mechanisms to prevent or reduce sperm limitation at sea (Levitan and Petersen, 1995; Yund, 2000; Wedell et al., 2002). The release of sperm in viscous fluids was observed in sea urchins, annelids and algae, apparently to counteract sperm dilution effects (Thomas, 1994). We have been able to show that in *M. galloprovincialis* MAP is the only major protein in the fluid-phase fraction of the thread-shaped sperm clots, which suggests that MAP does indeed represent a protein component that may be implicated in maintaining such thread-shaped clots.

Indirect results do not support the interpretation that MAP is present in seminal fluid as a result of ‘shedding’ of the protein from the sperm surface, since the former displayed electrophoretic mobility slightly different from that of the sperm polypeptide (see Fig. 1D,E). It is interesting to note that electrophoretic mobility of the seminal-fluid MAP is similar to that of the faster migrating form of the luminal-fluid MAP (see Fig. 4). These electrophoretic patterns indicate that, in the duct lumen, MAP undergoes certain post-translational modifications rather than a proteolytic degradation because we did not observe the low molecular mass products (30 kDa or less) typical for MAP proteolysis on the corresponding blots.

In addition to being a protein component of mussel seminal fluid, MAP is also associated with spawned sperm. Before spawning, mature sperm cells are accumulated in the lumen of TGDs and LGD, where the luminal fluid is characterized by an extremely high level of MAP. We suggest that during transit through a MAP-enriched luminal environment, sperm cells may internalize MAP from the luminal fluid. In this way, MAP may become an integral component of the spermatozoon. The

immunofluorescence data on MAP localization in the mid-piece of the spawned sperm (Fig. 2) supports this suggestion. An uptake of MAP by sperm from ductal secretions can only be deduced if the protein is not in testicular sperm, as demonstrated by immunocytochemistry and immunocytoblotting (Torrado and Mikhailov, 1998).

Although MAP isolated from the male gonad is characterized by esterase activity (Mikhailov et al., 1997), we could not detect the enzymatic activity in mature sperm (M. Torrado and A. T. Mikhailov, unpublished observations), which may suggest that the MAP associated with the mid-piece region of spawned spermatozoa is an enzymatically inactive protein. Such protein changes have been shown to occur during mammalian sperm differentiation: mouse selenoprotein called PHGPx is expressed as a soluble active enzyme in round spermatids in early sperm development, and later turns into an enzymatically inactive protein, contributing to the structural integrity of the mid-piece of mature sperm (Ursuni et al., 1999).

It has been proposed that maturing sperm from luminal fluid internalizes MAP, so we speculated that relatively high MAP concentrations would be maintained in the luminal fluid along the male reproductive tract. As part of our continuing effort to investigate this issue, we studied the presence of MAP in luminal fluid sampled from different compartments of the mussel tubular gonad. Unlike most mussel organs, which have completed morphogenesis by the end of metamorphosis and whose subsequent development is mainly based on the enlargement of pre-existing structures, the gonad undergoes most of its morphogenesis in the adult state, depending on the annual reproductive cycle. Despite considerable efforts (reviewed in Bayne, 1976; Seed, 1969; Gosling, 1992), the structural organization of the *M. galloprovincialis* gonad has not been studied in detail. We re-evaluated the gonad pattern formation in mussel mantle tissue, using juvenile animals as the experimental model. Whole mounts revealed a clear compartmentalization of the tubular gonad network into STs, TGDs and the efferent LGD with terminal end bulb-like structures. In keeping with this pattern, we studied the luminal samples obtained from similar compartments of the adult male gonad and were able to show that MAP concentration in the lumen significantly increases *en route* from STs to the LGD. In addition, our immunoblot study revealed that the gonad-duct wall is highly enriched with MAP, which confirms our previous immunofluorescence results that MAP is localized in the epithelium of the male gonad ducts (Mikhailov et al., 1995; Torrado and Mikhailov, 1998). It seems likely that, in the mussel gonad, MAP is synthesized by duct epithelium and may be secreted into the duct lumen. The precise role of MAP and, in particular, the fluid-phase form of MAP that may influence sperm functional maturation and motility, merits further investigation.

Analysis of our previous results and the data of other research on similar expression patterns of esterases in the male reproductive tract of different animal groups (i.e. bivalve molluscs, fruitflies and rodents) has prompted us to formulate the hypothesis (Mikhailov and Torrado, 1999) that during evolution, esterase-like polypeptides are recruited by the male reproductive tract to perform new, adapted or modified, sex-specific roles, taking into account the requirements and limitations for reproduction in different species. In this paper, we present additional information on the structural similarity between mussel MAP and fruit fly esterase S and highlight MAP as a major protein in gonad duct fluid and spawned sperm. In this context, it should be noted that esterase S is also secreted into the lumen of the ejaculatory bulb and transmitted with the sperm to females upon copulation (see Korochkin et al., 1990).

Taken together these observations suggest that there is a general trend in esterase accomplishment of sperm emission in both free-spawners and internal fertilizers. On the basis of these assumptions, it is reasonable to expect that esterase-like proteins may be present in the seminal fluid of many sexually reproducing species. In the present study, the 64 kDa protein immunochemically similar to MAP and esterase S was identified in human seminal fluid and spermatozoa. Previous studies on enzyme profiles of human seminal fluid allowed the detection of uncharacterized protein fractions with broad esterase-like activities (reviewed in Mikhailov and Torrado, 1999, 2000). We still do not know whether the 64 kDa protein, observed by us in human seminal fluid, corresponds to any of the previously characterized human esterases. Of note, the protein product of the human liver carboxylesterase gene, HCE-2, has an apparent molecular mass of 64 kDa (Takai et al., 1997). More importantly, the fact that polypeptides similar to mussel MAP and fruit fly esterase S are also detected in seminal fluid in humans allows us to hypothesize that such male reproductive-tract-associated esterases may be essential for sperm fertility. Collectively, the results of this work as well as the data of other research (reviewed in Mikhailov and Torrado, 1999, 2000), reveal certain similarities between the mussel and mammalian reproductive system, such as the production of esterase-like proteins in the somatic gonad and excurrent duct network, an improvement in motility of sperm within the duct system, and association of gonad-derived esterases with sperm.

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