Progesterone Affects Vitellogenesis in *Octopus vulgaris*

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Abstract: The electrophoretic analysis of egg yolk proteins of the mollusc *Octopus vulgaris* by both SDS- and native-PAGE, showed two different patterns that mirror the two main periods characterizing the reproductive cycle of the female of *Octopus vulgaris*: the non-vitellogenic period (October-February) and the vitellogenic period (March-July). Since different levels of progesterone also characterize these stages, we performed *in vitro* incubations of previtellogenic ovary of *Octopus vulgaris* with this steroid in order to clarify its possible involvement in the regulation of egg yolk protein synthesis. Treatment of *Octopus* eggs with progesterone induced the appearance of a 70 kDa protein which cross-reacted with antibodies raised against crab vitellogenin. This protein was further identified as a component of *Octopus* egg yolk proteins of the vitellogenic period. Bromodeoxyuridine (BrdU) incorporation in the nuclei of follicle cells after treatment with progesterone brought about follicle cell proliferation, suggesting that this hormone may be involved in vitellogeneisis in *Octopus*.

Keywords: Cephalopods, vitellogenin, cell proliferation, progesterone, reproduction.

INTRODUCTION

Several types of sex steroids have been detected in various species of invertebrates [1-3] and evidence has been accumulating over the last decades that the so called "vertebrate sex steroids" (progesterone, estrogens, androgens) are also present in invertebrates, and their levels change according to certain phases of the life cycle often coinciding with reproduction [4]. In fact, "vertebrate sex steroids" are ancient molecules that are widespread in vertebrates, invertebrates and plants [5]. It has been proposed that estrogens are the ligands of an ancestral steroid receptor whose specificity may have changed through evolution [6, 7]. A role for "vertebrate sex steroids" in the regulation of invertebrate reproduction has been disputed recently in light of the discovery that estrogen receptors from several molluscs do not bind estradiol in *in vitro* binding assays [8-13]. However, there is general agreement that "vertebrate sex steroids" play a role in controlling gonadal growth and sex determination in invertebrates [14, 15].

Many studies have been carried out on the role of estrogens in reproduction in molluscs, particularly in the control of vitellogenesis: in bivalves 17β-estradiol is involved in vitellogenin synthesis [16] and recent studies corroborate estrogen involvement in controlling immune capacity and energy metabolism related to vitellogenesis [17]. *In vivo* and *in vitro* administration of 17β-estradiol brought about an increase in vitellins in the ovary of the Pacific oyster *Crassostrea gigas* and in the scallop *Patinopecten yessoensis* [16, 18] and the estrogen receptor cloned in *Crassostrea gigas* has been immunolocalized in the nuclei of follicle cells, the site of vitellogenin synthesis [12].

Conversely, direct evidence regarding a role for progesterone in regulating reproductive functions in invertebrates are scant. Information is currently available only in crustaceans about the role of progesterone in the endocrine control of vitellogenesis. Injections of progesterone and 17αOH-progesterone in the shrimp *Metapenaeus ensis* [19] induced ovarian maturation, and stimulated vitellogenin secretion in *Penaeus japonicus* [20]. In *Penaeus monodon* the levels of progesterone, as well 17β-estradiol, in the hemolymph, ovaries and hepatopancreas were closely related to the stage of ovarian maturity [21]. In *Procambarus clarkii*, 17α-hydroxyprogesterone produced a significant increase in the gonadosomatic index and oocyte diameter [22]. Progesterone also stimulated vitellogenin gene expression in *Metapenaeus ensis* [23]. Finally immunological characteristics and tissue localization of a putative progesterone receptor in *Austropotamobius pallipes* suggest an involvement in the modulation of reproductive functions by progesterone in this crustacean [24].

In cephalopods, the synthesis of yolk proteins takes place within the ovary [25-27]. *Octopus vulgaris* eggs (oocytes and follicle cells) are contained in a single ovary at different states of maturation. As a common feature in cephalopods (squid, cuttlefish and octopus), during the annual cycle, the follicle cells enfold into the oocyte and synthesize yolk components [28, 29]. Recently, the sex steroids progesterone and estradiol and their specific receptors have been identified in the cephalopod *Octopus vulgaris* [30-32].

Morphological changes in the oviduct and oviducal gland observed throughout the reproductive cycle in *Octopus vul-*

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garis, along with progesterone and estradiol fluctuations, suggest that both steroids might work in synergy to sustain the growth and differentiation of the female reproductive system in this species [4]. Moreover, the localization of both progesterone and 17β-estradiol receptors in the nuclei of the follicle cells in the ovary of *Octopus vulgaris*, sustains the theory that there is a role for these steroids in the regulation of the synthesis of yolk [31, 32].

The purpose of studies reported here was to investigate the role played by progesterone in *Octopus vulgaris* vitellogenesis. To reach this goal we performed *in vitro* incubations of *Octopus vulgaris* eggs with progesterone. Our findings indicate that progesterone induces yolk protein synthesis in *Octopus vulgaris* eggs. Only one protein was identified with immunological methods by using antibodies raised against crab vitellogenin that specifically recognises a 70kDa protein. Incorporation of bromodeoxyuridine (BrdU) in the nuclei of follicle cells after progesterone exposure showed that progesterone induced cell proliferation.

MATERIALS AND METHODS

Animals

Females of *Octopus vulgaris* (n= 40; body weight between 1,0 and 2,5 kg) were captured in the harbor of Naples. Sampling started in October and ended in July (at least 4 animals per month) and was carried out for two years (2006-2007). Based on a previous study [4], females collected from October to February were considered previtellogenic, and females collected from March to July were considered vitellogenic. Animals were maintained, for a maximum of two weeks, in aquarium tanks with a recirculating seawater system as elsewhere described [33, 34] and fed on crabs *Carcinus moenas*. Animals were kept under natural photoperiod. Water temperature was set at 13-15°C. Animals were anesthetized on ice and ovary was removed and processed as follows.

Tissue Preparation and Steroid Treatment

Strands of eggs from the ovary of females in the previtellogenic period (0,5-1 g) were excised, weighed, and incubated for 30-60 min in artificial sea water (ASW) at 20°C containing progesterone (0.2 ng/ml) or without steroid (control). The steroid concentration employed for the *in vitro* incubation is 10 times higher the physiological concentrations reported in [4]. After incubation, eggs were extensively washed and homogenized in 50 mM Tris-HCl, pH 7.5 containing 100 mM NaCl, 10 mM EDTA, 1 mM phenylmethanesulphonylfluoride (PMSF) (homogenisation buffer). The homogenate was centrifuged at 7500 x *g* for 20 min at 4°C. The pellet was discarded and the clear supernatant was centrifuged again at 5000 x *g* for 1 h at 4°C. The supernatant was indicated as cytosolic extract, collected and stored at –80 °C for further analysis.

Electrophoresis and Western Blotting

The cytosolic extract was subjected to Western blotting as described in [31]. Briefly, 20 µg of total proteins were electrophoresed on 4% polyacrylamide gel (native-PAGE) or 5-20% gradient (SDS-PAGE) [35], proteins were transblotted onto nitrocellulose (Schleicher and Shuell, Keene, NH) using a Trans-blot chamber (Bio-Rad) for 1 hr at 100 V. The nitrocellulose was blocked for 30 min at 37°C with 5% non-fat dry milk in Tris-HCl 50 mM/Tween 20 (TBS-T) buffer, pH 7.6 and then incubated with primary antibody anti-crab vitellin (85kDa subunit, kindly provided by Dr. L. Pateraki) or rabbit IgG as a control for two hours at 20°C. The incubation with the secondary antibody (goat anti-rabbit alkaline phosphate conjugated) was carried out at 20°C for 4 h. Immunoreactive bands were revealed by incubation with NBT/BCIP (Sigma, St. Louis, MO). Marker proteins for SDS/PAGE were from Sigma: Myosin (205 kDa); ß-galactosidase (116 kDa), Albumin (66 kDa), Ovalbumin (45 kDa), Glyceraldehyde-3-phosphate Dehydrogenase (36 kDa), Carbonic anhydrase (29 kDa). Marker proteins for native PAGE were from Sigma: urease hexamer (545kDa), urease trimer (272 kDa), bovine serum albumin dimer (132 kDa), bovine serum albumin monomer (66kDa) and chicken egg albumin (45 kDa). Negative controls were performed by omission of primary antibody.

Egg Yolk Protein Analysis

About 5 g of strands of eggs from mature females were homogenized in ten volumes of 50mM Tris-HCl, pH 7.5, containing 100 mM NaCl, 50 mM $MgCl_2$, 10 mM $CaCl_2$, 1 mM PMSF, and 0.02% sodium azide. The homogenate was centrifuged at 7500 x *g* for 20 min at 4°C. After centrifugation, the supernatant, was freed from the debris and the fat cap and desalted on a sephadex G25 column (8 x 0.6 cm) equilibrated with homogenisation buffer.

A partial purification of the egg yolk proteins was achieved by ultracentrifugation using the procedure of [36]. Two ml of the supernatant eluate from the sephadex G25 column were mixed with 0.9 g of KBr to a final density of 1.28 g ml^{-1} and placed into an SW41 Ti ultra clear centrifuge tube. The sample was overlaid with the following densities of KBr solutions: 2 ml of 1.23 g ml^{-1}, 2 ml of 1.15 g ml^{-1}, and 2 ml of 1.063 g ml^{-1}. Five ml of 0.9% (w/v) NaCl were placed on top of the gradient, and the tubes were centrifuged at 274,000 x *g* for 18 h at 4°C in a Beckman (Fullerton, CA) SW41 Ti rotor. After ultracentrifugation, the gradient was collected in 1 ml aliquots. Each aliquot was assayed for total protein content by measuring the absorption at 280 nm, and analysed by native- and SDS-PAGE.

Purification of egg yolk proteins was performed by ion exchange chromatography. A 10 x 0.8 cm column of CM Sepharose CL-6B (Sigma) was regenerated by elution with 3 volumes each of 1 M HCl and 1 M NaOH and then equilibrated with 0.1 M sodium acetate containing 10 mM EDTA, buffered at pH 5.8. The protein containing fractions from ultracentrifugation were pooled and dialyzed for over 2 h against 0.1 M sodium acetate buffer containing 10 mM EDTA, pH 5.8, with several changes, then applied onto the column. The sample was eluted with a continuous salt gradient (0–1 M NaCl). Fractions of 4 ml each were collected and assayed for total protein content by measuring the absorption at 280 nm. The composition of the fractions was assessed by SDS-PAGE.

Bromodeoxyuridine: Proliferation Experiments

Strands of eggs from previtellogenic animals [4] were excised, weighed and incubated for 30 min in ASW according to the following experimental design: group 1: 1 g of

eggs incubated with 1 ng of progesterone plus 10µM BrdU; group 2 (control): 1 g of eggs incubated with 10µM BrdU. After incubation, eggs were extensively washed in ASW and fixed in 4% paraformaldheyde in 0.1 M phosphate buffer, pH 7.4, containing 0.9% NaCl (PBS) for 18 hours at RT and dehydrated in ethanol, cleared in xylene, and embedded in paraffin (Paraplast). Serial sections (5 μm) were cut and placed on coated slides. The sections were deparaffinized, rehydrated and then washed in PBS and incubated for 1hour in 1N HCl. The sections were then treated with 0.3 % H_2O_2 to block endogenous peroxidases. After 20 min incubation with normal goat serum, to reduce background staining, the sections were placed overnight in a moist chamber at 4°C with the mouse monoclonal anti-BrdU (1:500) as the primary antibody. After incubation, the sections were rinsed in PBS and incubated for 1 hour at RT with goat anti-mouse IgG conjugated with peroxidase (Pierce, Milan, Italy) (1:500 in PBS) followed by DAB visualization. The sections were dehydrated and mounted with BioMount (British BioCell International, London, U.K.). Images of the sections were digitally captured using a CCD video camera mounted on a Nikon Eclipse E400 microscope and analyzed with LUCIA screen measurement (Laboratory Imaging Ltd) and Adobe PhotoShop ver.6.0 softwares for MAC.

Protein Determination

Protein concentration was determined using Lowry's method [37], with BSA as standard.

Statistical Analysis

All data are expressed as mean ± standard error (SE). Data were statistically analyzed by one-way ANOVA followed by the Duncan's test.

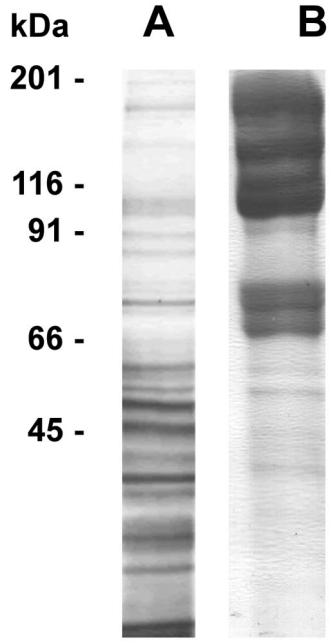

Fig. (1). SDS-PAGE analysis on a 5-20% gradient gel of ovarian extract of *Octopus vulgaris.* Lanes report electrophoretic analyses of ovarian extracts from animals captured in January (**A**), as representative of the non-vitellogenic period (October to February), and July (**B**), as representative of the vitellogenic period (March to July). Numbers on the left represent standard molecular weights.

RESULTS

Egg Yolk Protein Pattern Throughout the Annual Cycle

SDS-PAGE analysis of *Octopus vulgaris* egg yolk proteins showed that the protein pattern was similar from October to February and was characterized by numerous bands distributed mainly in a range of medium-low molecular weight (Fig. **1A**). The protein pattern from March to July was characterized by five bands ranging from 200 and 100 kDa and three bands ranging from 80 and 70 kDa (Fig. **1B**).

Native-PAGE analysis of *Octopus vulgaris* egg yolk proteins was also carried out in the same time frame and with the same frequency as SDS-PAGE and showed proteins that migrated from 750 kDa to 67 kDa in the months from October to February (Fig. **2A**), while in the months from March to July only one band of about 900 kDa was present (Fig. **2B**).

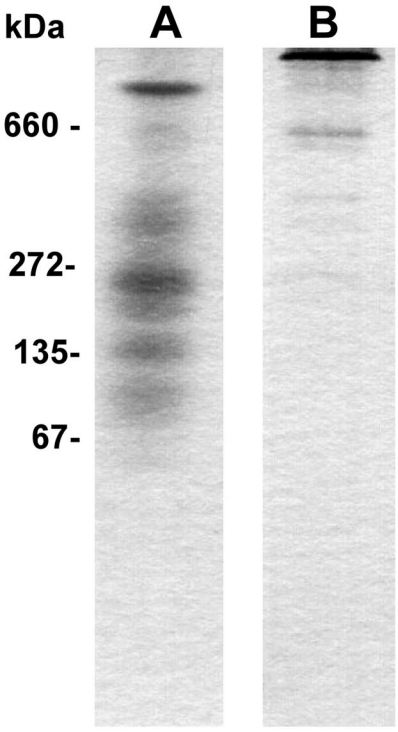

Fig. (2). Native-PAGE analysis on a 4% gel of *Octopus vulgaris.* Lanes report electrophoretic analyses of ovarian extracts from animals captured in January (**A**), as representative of the non-vitellogenic period (October to February), and July (**B**), as representative of the vitellogenic period (March to July). Numbers on the left represent standard molecular weights.

Immunoblotting of Egg Yolk Proteins

Western blot analysis was carried out on the egg yolk proteins subjected to SDS-PAGE. One immunoreactive band of 70 kDa was revealed in the months from March to July (Fig. **3**). No immunoreactive bands were visible in the months from October to February. An immunoreactive band of about 900 kDa was present in the months from March to July on egg yolk proteins subjected to native-PAGE (Fig. **3**). No or very faint immunoreactive bands were present in the remaining months. Negative controls did not show any immunoreactive band (Fig. **3**).

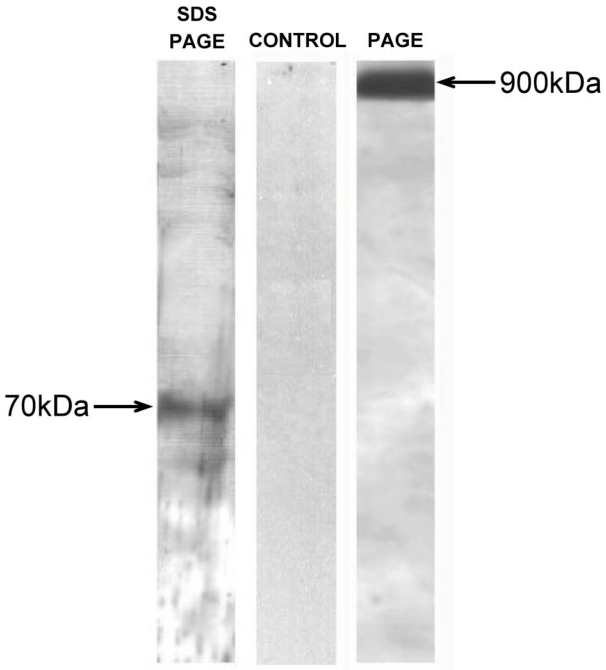

Fig. (3). Western blot analysis of *Octopus vulgaris* egg yolk proteins separated on SDS-PAGE and Native-PAGE using an antibody against crab vitellogenin. The arrow on the left indicates the 70kDa immunoreactive band. The arrow on the right indicates the 900 kDa immunoreactive band. No immunoreactive band is present in negative control lane.

Egg Yolk Protein Analysis

In order to clarify if the 70 kDa immunoreactive protein was an egg yolk protein, we partially purified egg yolk proteins by ion-exchange chromatography with NaCl gradient (0-1M) and analysed them by Western blotting. In Fig. (**4**) the ion-exchange chromatography profile is reported. One-peak was eluted at a 0.20 M NaCl. The peak was collected and run on both SDS- and native- PAGE. It resolved in several main bands and one band, respectively (Fig. **4** insert). When the proteins subjected to SDS-PAGE were blotted with the antibody raised against crab vitellogenin, one band of 70 kDa appeared (Fig. **5A**); when the proteins subjected to native-PAGE were blotted with the antibody raised against crab vitellogenin, one band of about 900 kDa was observed (Fig. **5A**). When the 900 kDa band was excised from the gel and subjected to SDS-PAGE an electrophoresis profile similar to the profile on SDS-PAGE of the ion-exchange chromatography peak was obtained (Fig. **5B**).

Effect of Progesterone on Egg Yolk Protein of 70 kDa

The 70 kDa protein was used as marker for evaluating the possible effect of progesterone on egg yolk proteins. After the *in vitro* incubation of strands of previtellogenic eggs, yolk proteins were extracted and analyzed by western blotting using the antibody raised against crab vitellogenin. An immunoreactive band of 70 kDa appeared in the electrophoretic profile of the proteins from eggs treated with progesterone (Fig. **6B**). A very faint immunoreactive band was also present in the control group (Fig. **6A**), but this may be due to the presence in the strand of eggs in advanced stage [see 4].

Effect of Progesterone on Cell Proliferation

The effect of progesterone on cell proliferation was monitored by *in vitro* incubation of previtellogenic eggs with progesterone and BrdU. The experiment was performed at least three times. BrdU incorporation was detected by using

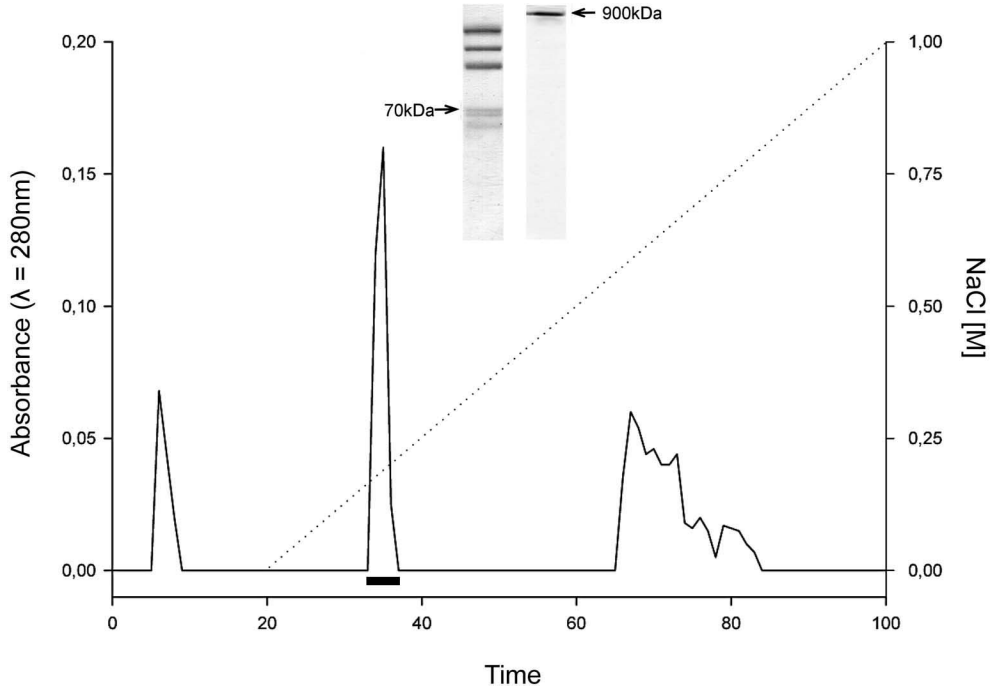

Fig. (4). Elution profile of *Octopus vulgaris* egg yolk proteins separated by a CM-Sepharose CL-6B cation-exchange chromatography column (10 x 0.8 cm). The buffer used was 0.1 M sodium acetate containing 10 mM EDTA, pH 5.8, and a gradient of 0–1 M NaCl was applied. Fractions (1 ml) were collected and monitored at 280nm. Insert shows SDS-PAGE and Native-PAGE analysis of fractions from the underlined peak stained with Coomassie blue. Proteins of about 70kDa and 900kDa are indicated by arrows on SDS-PAGE and Native-PAGE gels respectively.

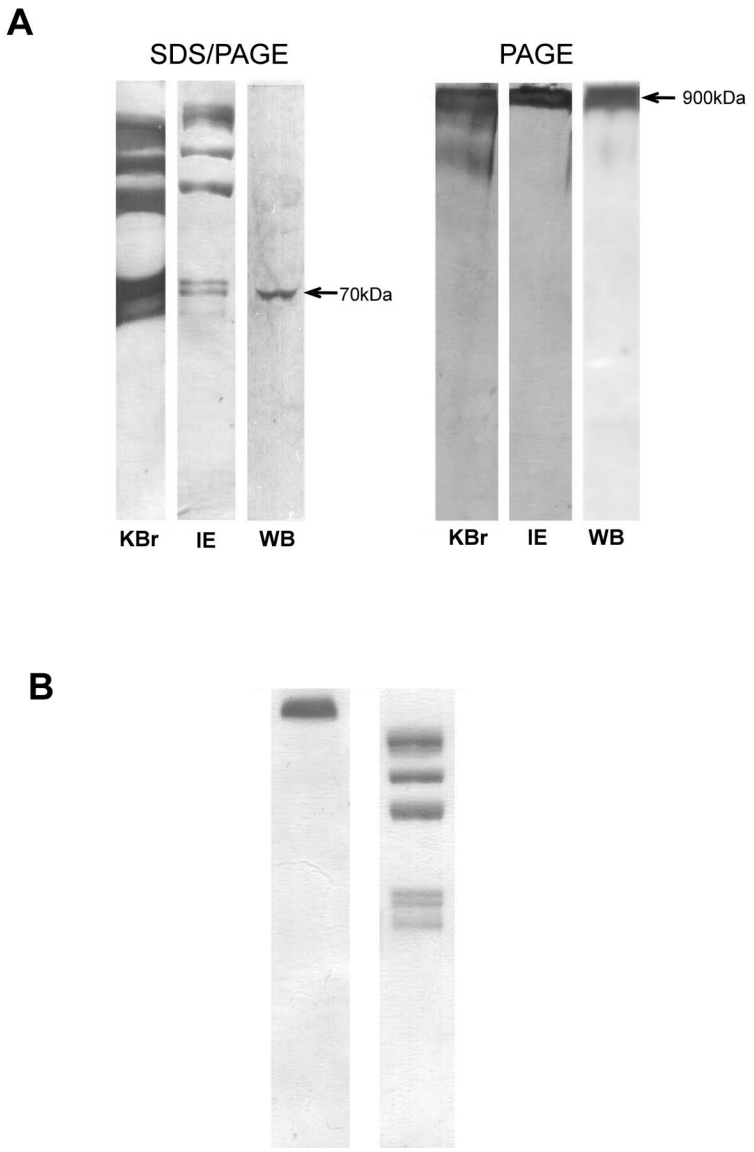

Fig. (5). Partial purification of *Octopus vulgaris* egg yolk proteins. (**A**) Protein containing fractions after ultracentrifugation (**KBr**) were subjected to ion exchange chromatography (**IE**) and then separated on either SDS-PAGE or Native-PAGE and subjected to Western blot analysis (**WB**). A single immunoreactive band is present on each blot (70kDa after SDS-PAGE and 900kDa after Native-PAGE). (**B**) When the 900 kDa band was excised from the gel (left) and subjected to SDS-PAGE an electrophoretic profile similar to the profile on SDS-PAGE of the ion-exchange chromatography peak was obtained (right).

an antibody against BrdU (Fig. **7**). In both the control (Fig. **7A**) and treated (Fig. **7B**) groups of eggs, immunoreactivity was confined to the nuclei of the follicular cells. In the control group, 35.50% ± 9.4 of eggs showed nuclear immunoreactivity. In the group of eggs treated with progesterone, 98.42% ± 1.2 of eggs showed nuclear immunoreactivity. The statistical analysis was performed on 150 eggs for each group. The difference between the number of immunoreactive eggs in the progesterone treated group of eggs was statistically different ($P<0.01$) from the control group (Fig. **7C**).

DISCUSSION

In this paper we show that progesterone incubation with eggs of non vitellogenic females of *Octopus vulgaris*, resulted in the appearance of a 70 kDa band that cross-reacted with antibodies against one egg yolk protein of the crab *Potamon potamios*.

Hormonal control of vitellogenin synthesis in liver is well documented in oviparous vertebrates [38, 39]. The precursor of yolk proteins is released into the bloodstream, sequestered by developing oocytes and proteolytically cleaved into the polypeptides subunits, lipovitellin and phosvitin, which are stored in the yolk granules of oocytes [40]. Instead, oviparous invertebrates are known to produce vitellogenin in different cell types. Nematodes synthesize vitellogenin in the gut and insects in the fat body [41]. In most crustacean species, vitellogenin is thought to be synthesized

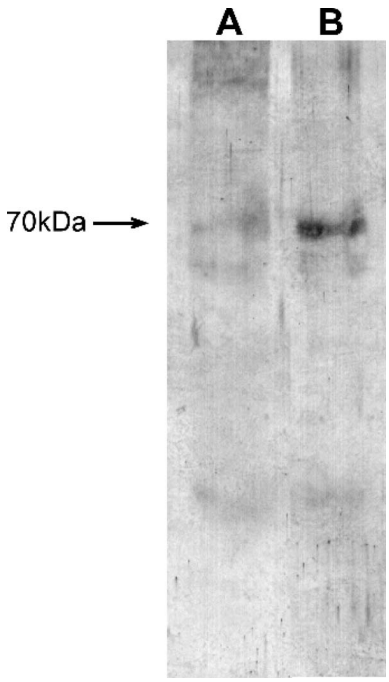

Fig. (6). Western blot analysis of *Octopus vulgaris* egg yolk proteins after treatment with progesterone. The 70kDa immunoreactive band is present in the electrophoretic profile of egg yolk proteins extracted from eggs treated with progesterone (**B**). A very faint immunoreactive band is also present in extracts from non treated eggs, may be due to the small percentage of eggs in advanced stage (**A**).

in both the ovary and non-ovarian tissues such as hepatopancreas and adipose tissue [42-44]. In cephalopods, follicle cells synthesize egg yolk precursors and release them into the growing oocyte [25-27].

A variety of hormonal and neuronal factors seem to be involved in the regulation of invertebrate vitellogenesis, including typical vertebrate sex steroids such as progesterone. Several lines of research suggest the involvement of progesterone in *Octopus vulgaris* reproduction. Indeed, progesterone and its specific receptor have been detected in the reproductive system of the female of *Octopus* [4, 31, 32]. In the ovary of *Octopus,* progesterone levels fluctuate according to the reproductive cycle, being very low during the non-vitellogenic period, and increasing at the onset of vitellogenesis [4]. This fluctuation seems to suggest a more specific involvement of progesterone in yolk synthesis, one of the most relevant reproductive functions in oviparous species characterized by telolecithic eggs.

In *Octopus vulgaris*, from October to February, oocytes increase in both diameter, and number of folds, but there is no evidence of yolk granules, while from March to July the ooplasm shows yolk granules, whose abundance increases up to the end of the vitellogenic period [4].

The electrophoretic analysis of the egg yolk proteins of *Octopus vulgaris* revealed two clearly different patterns, one corresponding to the vitellogenic period (March-July) and characterized by high molecular weight proteins and another corresponding to the non vitellogenic period (October-February), characterized by medium-low molecular weight proteins (present data). Since previous studies indicate that the vitellogenic period is characterized by high levels of progesterone and non vitellogenic period is characterized by low levels of progesterone [4] all these considerations suggest a role for progesterone in egg yolk protein synthesis.

In *Octopus vulgaris* as a common feature in cephalopods, follicle cells proliferate during the annual cycle, resulting in deep enfolding into the oocyte. This is a strategy to increase the surface of the epithelial layer responsible for egg yolk protein synthesis [28]. Thus, it is interesting to note that, the incubation of eggs of *Octopus vulgaris* with progesterone induced follicle cell proliferation, as demonstrated by bromodeoxyuridine incorporation in the nuclei of the follicle cells.

Our data along with the data present in literature [4, 29, 31] point at progesterone involvement in cephalopod vitellogenesis. Conversely, in vertebrates the main steroid involved in yolk synthesis is the 17ß-estradiol [45], although other factors have been proven to play an active role [46, 47]. Progesterone is regarded as the hormone of viviparity [48], and a modulator of vitellogenin synthesis, along with 17ß-estradiol in several species of vertebrates [49, 50].

In those invertebrates studied so far, mainly crustaceans and molluscs, both progesterone and 17ß-estradiol seem to play roles in reproduction, although the emerging picture still needs to be confirmed. 17ß-estradiol seems to be involved in the vitellogenesis of crustaceans [51-53], but a stimulatory effect of 17α-hydroxyprogesterone on vitellogenin secretion has been demonstrated in the shrimp *Penaeus japonicus* [20]. Progesterone stimulates ovarian development in the prawn *Parapenaeopsis hardwickii* [54] and in the shrimp *Metapenaeus ensis* [19] and, in the crab *Scilla serrata,* progesterone and 17ß-estradiol increased in the hepatopancreas and in the ovary throughout the reproductive cycle suggesting an involvement of these hormones in vitellogenesis [55], as also sustained by the presence of both progesterone and 17ß-estradiol receptors in the ovary as well as in the hepatopancreas of the crayfish *Austropotamobius pallipes* [24].

Several lines of evidence indicate that 17ß-estradiol is involved in the control of vitellogenesis in molluscs, although several recent papers report the cloning of molecules considered orthologs of estrogen receptors, but none of them bind efficiently 17ß-estradiol [8-13]. It has been reported that 17ß-estradiol stimulates vitellogenin in *Crassostrea gigas* [18] and in the scallop *Patinopecten yessoensis*, via estrogen receptors in the auxiliary cells associated with the oocyte [16]. On the contrary, direct evidence of progesterone action on egg yolk protein synthesis is still lacking. To our knowledge, this is the first evidence for a role for progesterone in egg yolk proteins synthesis in invertebrates.

CONCLUSIONS

In summary, our studies show that progesterone incubation with ovaries of *Octopus vulgaris* in previtellogenic phase, brings about the appearance of a 70 kDa protein which can be considered a yolk protein due to its immunological characteristics. Moreover, progesterone is responsible for follicle cell proliferation, a phenomenon naturally occurring during the vitellogenic growth of eggs in this species.

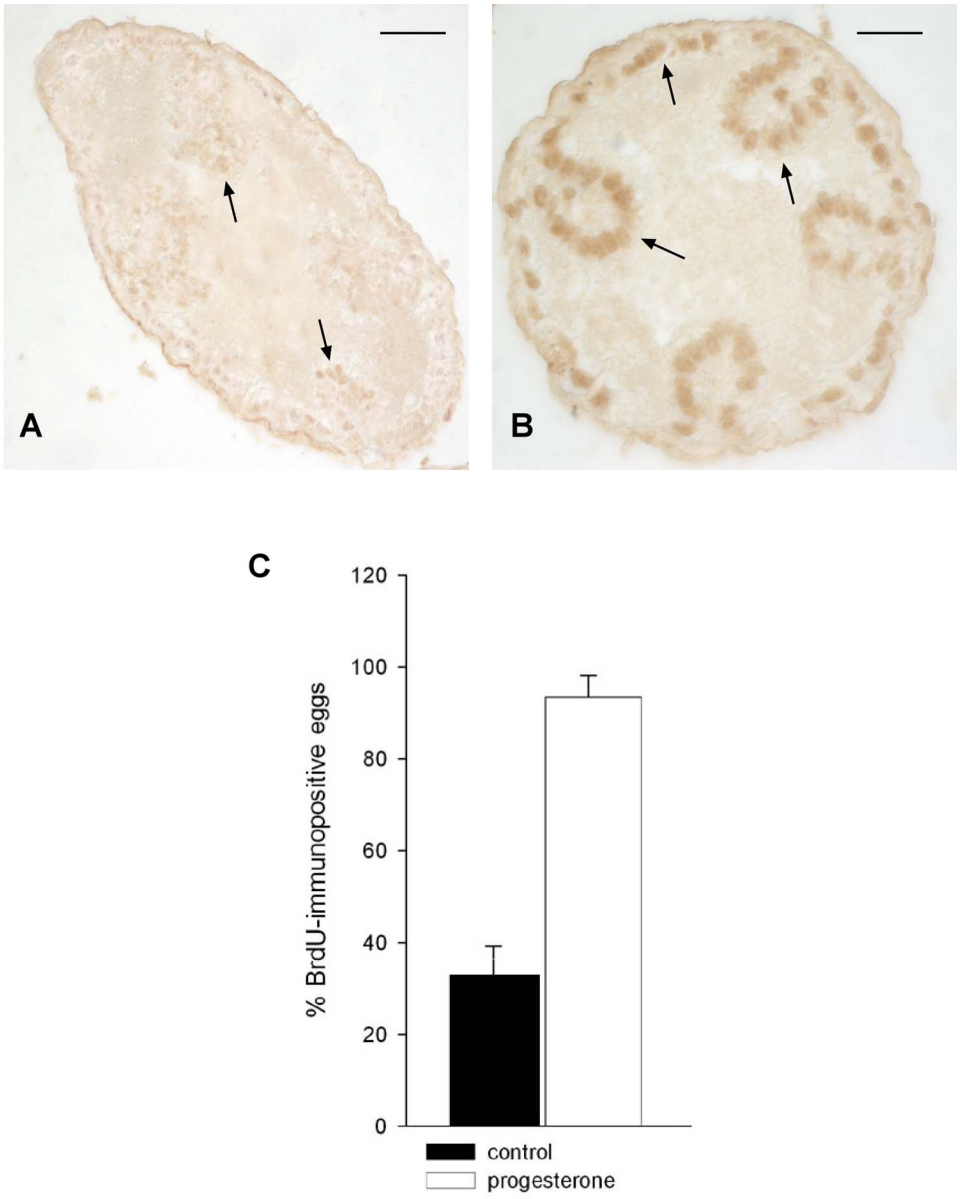

Fig. (7). Effect of progesterone on cell proliferation. BrdU immunoreactivity in control (**A**) and progesterone-treated (**B**) eggs. In both cases immunoreactivity is confined to the nuclei of follicle cells (arrows), but staining is less intense in the eggs form the control group. **(C**) Incorporation of BrdU, reported as percentage of BrdU immunoreactivity (% ± S.D.), after treatment with progesterone compared to the control. The values are the mean of three separated experiments. Scale bars: A = 50 μm; B = 35 μm.

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