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Original Article

Eggshell *zona radiata*-proteins from cod *(Gadus morhua)*: extra-ovarian origin and induction by estradiol-17ß

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ABSTRACT Using Atlantic cod *(Gadus morhua)* as a model organism, the aim of this report was to delineate whether teleostean eggshell *zona radiata* proteins have their origin, i.e., site of synthesis, in gonadal or somatic tissues. Estradiol-17ß was administered intraperitoneally to one-year-old cod *(Gadus morhua)* with either undeveloped gonads or with differentiated gonads. By immunoblotting procedures estradiol-dependent protein induction was investigated using specific rabbit antisera directed against cod eggshell proteins and brown trout vitellogenin. No immunological cross-reactions were observed between the two antisera, and eggshell proteins and vitellogenin were detected in blood plasma and somatic tissues only in estradiol-treated cod. Three plasma-components were immunoreactive to antiserum directed against eggshell proteins, and these proteins possessed molecular weights of 78, 54 and 47 kDa, identical to the molecular weights of the cod eggshell α, ß and γ *zona radiata*-proteins. These three immunoreactive plasma-components were observed after administration of estradiol-17ß to both sexes, also in males having reached spermiation, and in juveniles of either sex without developed gonads. The data are interpreted to signify that cod eggshell *zona radiata*-proteins originate in an extra-ovarian tissue and are transported in the blood for deposition in the ovaries. We propose that oogenesis involves estradiol-17ß regulation of both eggshell *zona radiata*-proteins and vitellogenin synthesis.

KEY WORDS: *fish, zona radiata, extra-ovarian origin, estradiol-17β*

Introduction

In addition to the plasmalemma, oocytes in all vertebrates have characteristic extracellular envelopes (Ludwig, 1874; Dumont and Brummet, 1985; Hagenmaier, 1985), which are variously termed vitelline envelopes, eggshells or chorion. The distinction between primary acellular coats deposited in the follicle and secondary coats deposited after ovulation (Ludwig, 1874) is still relevant for contemporary investigations. These extracellular oocyte envelopes have also been defined as *primary* (from the oocyte), *secondary* (from the surrounding follicle or theca cells), or *tertiary* (from the oviduct and/or accessory urogenital system) according to their cellular origin, and all are held to be present in fish (Ginzburg, 1968). Ultrastructural studies (Hosokawa, 1985) on the growth of the eggshell in the teleost *Navadon modestus* suggested that the inner layer of the eggshell *(zona radiata)* seems to be derived from the oocyte, and hence is a primary oocyte envelope. This interpretation of eggshell biogenesis is supported by recent work of Begovac and Wallace (1989), where intact follicles incubated with (^{35}S)methionine *in vitro* synthesized the major eggshell proteins of pipefish. However, «isolated follicle cells or denuded oocytes failed to yield convincing evidence of such synthesis». Begovac and Wallace concluded «that the principal vitelline envelope proteins originate from within the follicle rather than elsewhere, such as the liver, and that the structural integrity of the follicle may be necessary for their elaboration.» This conclusion implies that the principal oocyte envelope is a primary or a secondary envelope, which is in agreement with numerous earlier reports that attributed synthesis of eggshell proteins to the ovarian follicular cells, or to the oocyte itself, or to both (Chaudhry, 1956; Kemp and Allen, 1956; Hurley and Fisher, 1966; Anderson, 1967; Rastogi, 1970; Wourms, 1976; Wourms and Seldon, 1976; Shackley and King, 1977; Sobhana and Nair, 1977; Tesoriero, 1977 and 1978; Erhardt, 1978; Lopes *et al.*, 1982; Guraya, 1986).

Abbreviations used in this paper: ABC-AP, Avidin-Biotin-Complex Alkaline phosphatase conjugate; BSA, bovine serum albumin; EDTA, ethylene diamino tetraacetic acid; LSI, liver somatic index; PBS, phosphate buffered saline; PAGE, polyacrylamide gel electrophoresis; RER, rough endoplasmic reticulum; TBS, tris/HCl buffered saline; zr, zona radiata.

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B in Fig. 2). This observation is confirmed by the data presented in Fig. 3 (compare lane Pe to Pc). Proteins with apparent molecular weights equal to the ß-and γ-monomers of the eggshell proteins are also detected in 0.02 µl blood plasma on a Coomassie-stained gel, while a component corresponding to the α-monomer appears as a faint band indicated with arrows (Fig. 2, panel B). These components are not detected in the control plasma (compare lanes Pe and Pc in Fig. 3).

An immunoblot of cod plasma probed with antiserum to monomeric cod eggshell proteins clearly demonstrates the presence of three major immunoreactive protein monomers in blood plasma, diluted 1:500, from estradiol-17ß treated juvenile cod, which are not detectable even in control plasma diluted 1:5 corresponding to 2 µl plasma (compare panel C to D in Fig. 2). Fig. 2 shows a Coomassie-stained gel and immunoblotting results of plasma from 7 individuals from the injected and control groups. All the injected fish (E_1-E_7) responded equally, and all the control fish (C_1-C_7) were negative. The detected immunoreactive components exhibit the same apparent molecular weights (78 kDa, 54 kDa, 47 kDa) as the three eggshell protein monomers (α, ß, γ) reported by Oppen-Berntsen *et al.* (1990)

In Experiment 1 (yearlings: males at spermiation, and females with immature ovaries), we observed that the three immunoreactive components were inducible in both sexes (data not shown). Experiment 2 involving younger yearlings without differentiated gonads, revealed that the synthesis of the three immunoreactive proteins occurs in the absence of developed gonads regardless of sex.

The data in Fig. 3 demonstrate the presence of the three immunoreactive components in somatic tissues such as muscle (M), brain (B), head-kidney (K), liver (L) and plasma (P). The presence of an additional immunoreactive component with an apparent molecular weight of 60 kDa (marked with an asterisk in Fig. 3, panel B) was demonstrated only in the liver fractions. Large amounts of several immunoreactive low molecular weight components are seen in the liver fractions. Such components are not detected in the other tissues.

Fig. 3 (panel C) demonstrates the appearance of an additional high molecular weight immunoreactive component after incubation with anti-brown trout vitellogenin antibodies. Since the blots presented in Fig. 3 panels B and C are identical except for the high molecular weight component in panel C, the large protein component is most probably vitellogenin. The data therefore verify the presence of vitellogenin in all tissues examined. Our antisera to cod monomeric eggshell proteins are demonstrated to be specific for eggshell protein monomers, and do not crossreact with vitellogenin (compare panels B and C in Fig. 3).

Discussion

The origin of monomeric eggshell proteins was studied by analyzing somatic tissues and plasma for the presence of such proteins after estradiol-17ß treatment of juvenile cod. The increased liver weight and plasma protein content (Table 1) suggests that estradiol-17ß has successfully induced hepatic vitellogenin synthesis in cod, as has been reported following high plasma content of estradiol-17ß in other species (Mommsen and Walsh, 1988). In plasma of estradiol-17ß-injected cod, vitellogenin was the major protein component as seen on a polyacrylamide gel (Fig. 2 B). This observation confirms the important role of estradiol-17ß in the regulation of reproduction in cod (Plack *et al.*, 1971) as it has in many other vertebrate species (Wallace, 1985)

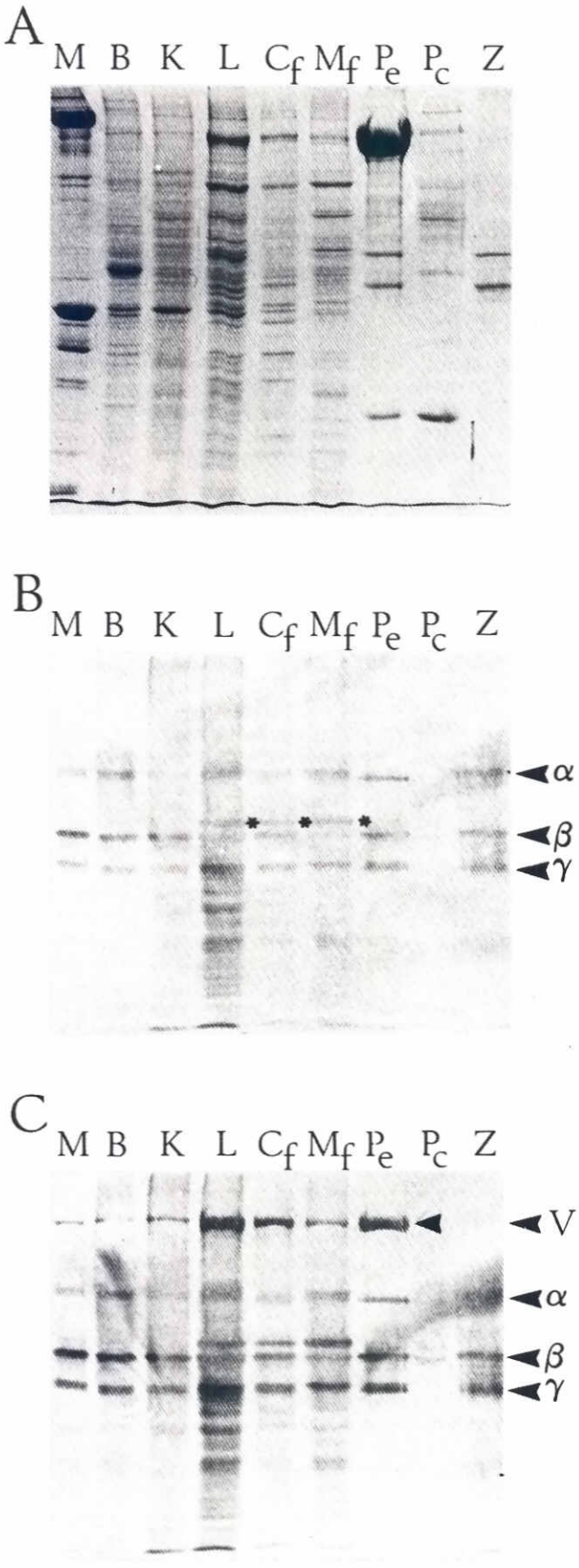
The antisera used in this study are specific for eggshell proteins as demonstrated in Fig. 1. This specificity is confirmed by the data shown in Fig. 3. Immunostaining is observed of the *zona radiata* (zr), confirming our previous conclusion that the three monomeric eggshell proteins are in fact components of the *zona radiata*. The organization of the eggshell in terms of its three constituent zr-proteins was not explored. The combined individual antisera to the three zr-proteins stain the *zona radiata* uniformly along its circumference, but concentric lamellae are seen. The resolution in our immunohistochemical data is not sufficient to address immunoreactivity of the basal lamina or the *zona pellucida*.

The immunoreactive components detected in plasma (Fig. 2) possess apparent molecular weights similar to the three zr-protein monomers (α, ß, and γ) from cod eggshell. We interpret these immuno-reactive components to be blood-borne zr-protein monomers. This finding strongly supports a «three-major-protein» nature of cod *zona radiata* (Oppen-Berntsen *et al.*, 1990). Eggshell (vitelline envelope) from rainbow trout *(Oncorhynchus mykiss)* also has an extraordinarily simple protein composition of three (Hyllner *et al.*, 1991) to four (Brivio *et al.*, 1991) major proteins.

The present data also demonstrate that cells capable of synthesizing zr-proteins are present in both sexes. This is in agreement with our recent finding of egg envelope proteins in the plasma of both sexes of rainbow trout after estradiol-17ß-treatment (Hyllner *et al.*, 1991). In addition, zr-proteins are produced in juveniles lacking developed gonads, and in sexually maturing males, showing that the zr-proteins originate in an extra-gonadal tissue. This strengthens our hypothesis that eggshell zr-proteins are not synthesized in a cell type confined to females such as follicular cells or the oocyte itself, which is the dominant view in the literature as reviewed in the introduction.

The possibility that eggshell proteins are synthesized in extra-ovarian tissue(s) is not envisioned in the nomenclature presently used (Ginzburg, 1968). However, one other oocyte protein is synthesized in the liver (e.g. vitellogenin, Wallace, 1985), and a similar hepatic origin for eggshell proteins would thus not violate basic principles of oogenesis. Glycosylation (Snider, 1984) of cod eggshell zr-proteins (Oppen-Berntsen, 1986) implies that these proteins are synthesized on rough endoplasmic reticulum (RER). In cod oocytes RER is reported to be very scarce (Kjesbu and Kryvi, 1988), and it seems unreasonable that the large amounts of zr-proteins in cod should be synthesized in a cell type extraordinarily poor in RER.

Follicular synthesis of estradiol-17ß is controlled by gonadotropin(s) according to the two-cell model proposed by Nagahama (1983). The multipotency of estradiol-17ß induction is well known (Wahli *et al.*, 1981), and Chen *et al.* (1987) have pointed out that vitellogenin mRNA is not the only mRNA that can be isolated from rainbow trout liver after exposure to estradiol-17ß. While gonadotropin(s) may control primarily follicular estradiol-17ß synthesis, estradiol-17ß controls multiple events related to oogenesis. This includes vitellogenesis, but our study adds to the growing evidence that estradiol-17ß induces several biosyntheses during oogenesis in teleosts (Hyllner *et al.*, 1991). Among the extra-ovarian tissues, only the liver has been shown to participate synthetically in oogenesis (vitellogenesis), as reviewed by Mommsen and Walsh (1988). The results presented in Fig. 3 (panels B and C) reveal the presence of zr-proteins in somatic tissues. However,



blood-borne vitellogenin will distribute all over an organism due to its presence in lymph and plasma, as is also shown for cod (Fig. 3, panel C). We interpret the presence of the three zr-proteins in these same tissues in a similar way. However, the immunoreactive components found only in the liver support a unique role of the liver with respect to zr-protein biosynthesis. The immunoreactive component with an apparent molecular weight of 60 kDa in the liver (Fig. 3, panel B, marked *) may be a not yet secreted precursor of a zr-protein. The numerous low molecular weight immunoreactive components may be partially degraded or partially translated zr-proteins. The data of Hamazaki *et al.* (1985, 1987 and 1989) show that one component of the fish eggshell may originate in the liver. Our data demonstrate that the major components of the eggshell of cod and rainbow trout derive from extra-ovarian proteins, and point to a more extensive oogenetic liver-ovary interdependency than previously described.

Begovac and Wallace (1989) concluded that pipefish have a follicular origin of the major *zona radiata* proteins and hence our data differ from their findings. This apparent paradox might be explained by differences between species reflecting the special anatomy of the pipefish ovary (Begovac and Wallace, 1987). Another possible explanation to the divergent findings might be the different eggshell protein extraction procedures used. Begovac and Wallace (1989) analyzed fresh as well as frozen material and extracted the proteins using a buffer containing calcium ions. The polymerization process that takes place in the eggshell after egg activation requires, or is stimulated by, the presence of calcium ions in the medium (Lønning *et al.*, 1984), pointing out the necessity for removing calcium ions as soon as ovulated unfertilized eggs have been obtained from the ovary (Oppen-Berntsen *et al.*, 1990). Ca^{2+}-dependent hardening of *zona radiata* is also recently demonstrated to occur in rainbow trout (Iuchi *et al.*, 1991). It is also our experience that extraction of *zr*-proteins from frozen material can be difficult. Since it is our experience that monomers are readily covalently ligated to dimers and oligomers which to some extent can be difficult to solubilize, their presence is however not reflected in an increased mechanical strength. We would like to suggest that *zr*-

Fig. 3. Immunodetection of monomeric eggshell proteins in somatic tissues of estradiol-17ß-treated juvenile cod. (Panel A) *9% Na-dodecylsulfate polyacrylamide gel electrophoresis (after Coomassie blue staining) of extracts from different tissues isolated from estradiol-treated fish. 10 µl of a 1:100 dilution of each tissue (10-15 µg) was applied to each well.* **(Panel B)** *Western blot of a gel identical to the one shown in panel A. Note the presence of the three immunoreactive proteins in all tissues. In the liver an array of different immunoreactive proteins of molecular weight is detected. The high molecular weight protein with apparent molecular weight of 60 kDa (marked with an asterisk *) is specific to the liver and not detected in other tissues.* **(Panel C)** *The same blot as in panel B after being additionally probed with rabbit anti-brown trout vitellogenin antiserum. An additional component much larger than the eggshell proteins is seen to react with the anti-vitellogenin antiserum (exact molecular weight was not determined), verifying in all tissues investigated the presence of vitellogenin. M, muscle; B, brain; K, kidney; L, liver; Cf, cytoplasmic liver fraction; Mf, membranous liver fraction; Pe, plasma from estradiol-treated fish; Pc, plasma from control fish; Z, Zr proteins. Detected proteins: V, vitellogenin with apparent molecular weight 135 kDa; α, ß and γ designates the three monomeric zr-proteins with apparent molecular weights of 78, 54 and 47 kDa, respectively.*

proteins possessing molecular weights of 110 kD or more as reported by Begovac and Wallace (1989), Brivio *et al.*, (1991) and Iuchi *et al.* (1991) may be dimers, oligomers or polymers and not monomers. These discrepancies obviously warrant closer examination.

The question whether an extra-ovarian origin of *zr*-proteins is unique to teleosts cannot be discussed in detail at the moment, mainly because animal species representing large groups such as amphibians, reptiles and birds have not yet been investigated. Furthermore, it is not clear if egg envelope proteins from different taxonomic classes are homologous structures. In mammals, the origin of the *zona pellucida*-proteins (zp-proteins) is different from teleostean zr-proteins (Wassarman, 1988), but might still be homologous to *zona pellucida* proteins in fish. Hence, further studies have to be performed in order to pursue this interesting question.

The presently used textbook terminology for egg envelopes (eggshells) is based on classification according to their tissue origin (see Balinsky, 1975). The dynamic concept of synthesis, secretion and transport of biomolecules revealed by modern cell biology has brought about a need for revision of such nomenclature. Although the nomenclature of Ludwig is still nominally applicable to liver-derived eggshell proteins, the commonly used textbook terminology does not seem to envision a hepatic origin for eggshell proteins, and hence does not seem to be applicable to the teleostean *zona radiata*. We suggest that the teleostean *zona radiata* originates not from the oocyte, follicle cells or accessory urogenital tract, but from an extra-ovarian tissue. Recently we reported estradiol-induced *in vitro* synthesis of zr-proteins in hepatocytes from rainbow trout (Oppen-Berntsen *et al.*, 1991). Deposition of prospective monomeric zr-proteins into the polymeric eggshell structure would appear to be a complex process. We are surprised by the close similarities in molecular weights of blood-borne zr-proteins and deposited zr-monomers, which indicate little molecular processing during deposition.

Materials and Methods

Fish stock and sampling

Domesticated Atlantic cod *(Gadus morhua)* were maintained at Austevoll Aquaculture Station near Bergen. The fish were fed dry pellets once a week. The stock of fish was maintained in net pens in the ocean under natural light, temperature and salinity conditions until selected for experiments. During experimentation different groups of fish were kept in separate tanks (1mx1mx0.5m) in the same room with identical light (natural photo period) and water conditions, 8°C and salinity 33‰. Sea water was pumped from 50 m depth, filtered and UV-treated before entering experimental tanks.

In the first experiment (Exp. 1), 6 cod yearlings were used, 3 males and 3 females with an average weight of 100 g (hatched in February 1988, used in experiments April 1989). Since the males had reached spermiation, while the females had immature ovaries, their sex could be determined by dissection. In the second experiment (Exp. 2) younger juvenile cod were employed (hatched in February 1989, used in experiments in November 1989). These individuals had not developed gonads, making sex identification impractical. Instead it was ensured that both sexes were represented in the experimental and control groups by selecting 28 individuals at random from several thousands in a net pen. A total of 14 individuals were injected with estradiol-17ß, while 14 individuals were kept as a control.

For both experimental series estradiol-17ß was dissolved in soybean oil and injected intraperitoneally (10 mg/kg body weight) as described by Haux and Norberg (1985). Control fish were injected only with soybean oil. Three injections were administered with 14 days between each injection. Two days after the last injection the fish were killed by a blow on the head, and samples were taken using heparinized syringes. Heparin and aprotinin (Sigma Chem. Co., USA) were added as anti-coagulant and protease inhibitor. The samples were cooled on ice and centrifuged. Plasma samples were frozen and stored at -80°C.

Sample preparation

Muscle, head-kidney, liver and brain were homogenized in 10 volumes (10 ml buffer/g tissue wet weight) of ice-cold extraction buffer (buffer B): 0.1 M Tris/HCl, pH 8.8, 8.0 M urea, 1% Na-dodecyl-sulfate, 0.3 M ß-mercaptoethanol (Merck), 0.1 M ethylene diamino tetraacetic acid (EDTA) (Merck) using Potter-Elvehjem homogenizer followed by sonication. After treatment with buffer B, only small amounts of cell debris were detected in the clear protein solution. The small amounts of unsolubilized material were removed by centrifugation (12000xg, 10 min). 100 µl of this 1:10 extract was diluted 10 times by adding 500 µl of sample buffer (Laemmli, 1970) and 400 µl distilled water. The diluted samples (1:100) were heated to 95°C for 2-3 min and centrifuged. The cytoplasmic and membranous liver fractions were prepared according to Förlin (1980). Subsequently 10 µl of each sample, equivalent to 10-15 µg protein, were subjected to Na-dodecyl-sulfate polyacrylamide gel electrophoresis (PAGE).

Analysis and detection of eggshell proteins

The blood samples were analyzed by Na-dodecyl-sulfate PAGE (stacking gel 4%; running gel 9%), and performed in a mini-gel system (Bio-Rad, US) according to Laemmli (1970). The prestained low molecular weight standard (Bio-Rad) was calibrated under these conditions by comparison to standard low molecular weight proteins (Bio-Rad) after Coomassie brilliant blue staining. Volumes corresponding to 2 µl and 0.02 µl plasma from, respectively, the control and experimental groups, were applied to each well. Two parallel gels were run, one to be stained for proteins with Coomassie brilliant blue, and the other subjected to Western blotting according to Towbin *et al.* (1979). The proteins in the gel were blotted electrophoretically overnight to a nitrocellulose filter using 400 V, 100 mA. The filter was incubated in 100 ml of Tris/HCl buffered saline, pH 7.5 (TBS) containing 1% (w/v) bovine serum albumin (BSA, Sigma Chem. Co.), and 100 µl mixed antisera corresponding to a total dilution of approximately 1:1000 for the combined antisera, so that each antiserum against individual zr-proteins is diluted 1:3000 (see below, *Antisera*).

Monomeric eggshell proteins were prepared according to Oppen-Berntsen *et al.* (1990). In short, ovulated eggs were removed from the female and homogenized immediately in buffer A: (0-4°C and pH 8.5) containing 100 mM EDTA, to prevent hardening, and 500 mM NaCl, to prevent precipitation of yolk proteins on the eggshells. The isolated eggshells were then subjected to an extensive washing procedure (4°C), before being solubilized in buffer B under conditions where unspecific aggregation between yolk proteins and eggshell proteins is not detected (Oppen-Berntsen *et al.*, 1990).

Antisera

Rabbit antisera were prepared against monomeric cod α-, ß-, γ- zr-proteins, solubilized according to the procedure described above, after purification of the three different *zr*-proteins by preparative Na-dodecyl-sulfate PAGE. The protein bands were visualized using an ice-cold solution containing 500 mM KCl. The opaque bands were excised from the gel, homogenized and the purified *zr*-proteins were extracted using extraction buffer as given above. The homogenized gel was pelleted by centrifugation, and the proteins were precipitated from the supernatant by the addition of five volumes of acetone. The rabbits were immunized three times with the first booster containing 80 µg in Freunds complete adjuvant, while the second and third contained 40 and 20 µg both in Freunds incomplete adjuvant, respectively. These antisera were demonstrated not to crossreact with yolk proteins (Fig. 1). Antisera were used in a 1:1:1 mix of antisera to the three individual zr-proteins both for immunohistochemistry and for immunoblotting. Antiserum against brown trout (*Salmo trutta*) vitellogenin (Norberg and Haux, 1988) reacted specifically with cod vitellogenin and showed no cross-specific reactivity with any of the cod eggshell proteins. Incubation with secondary antibodies was performed with goat anti-rabbit horseradish peroxidase conjugate obtained from Bio-Rad in 100 ml of TBS, 1% (w/v) BSA, dilution 1:2,000. The blot was developed for 1-5 min according to the standard

procedure with 4-chloro-1-naphthol (60 mg) dissolved 20 ml of methanol, and made up to 100 ml with TBS, by inspection of individual samples.

Preparation of tissue samples

Ovaries with unovulated eggs were fixed in phosphate buffered saline pH 7.4 (PBS) containing 2% glutaraldehyde and 3% formaldehyde. The tissue was rinsed in PBS to remove excess fixative and dehydrated in increasing concentrations of ethanol. Fixed specimens were mounted in Paraplast at 60°C, sectioned at 4 µm, and mounted on slides with glycerol or gelatin, dried overnight (37°C) and stored at room temperature until staining.

Immunohistochemistry

The (primary) antisera were diluted in a freshly prepared diluent of PBS, containing 1% BSA. Dilutions of mixed antisera (see above, *Antisera*) from 1:500 to 1:10,000 were tested for optimal staining on cod sections. The sections were stained and developed using Vectastain Avidin-Biotin-Complex Alkaline phosphatase conjugate (ABC-AP) commercial staining kit from Vector Labs., USA. The modified procedure of Nozaki *et al.* (1990) was used. Before immunostaining, sections were cleared in xylene for 2x10 min to remove Paraplast, and rehydrated in 100-70% ethanol followed by distilled water. When using the ABC-AP reagents, sections were incubated in 3% hydrogen peroxide for 10 min to remove endogenous peroxidase activity in the tissue before adding the enzyme substrate. Preincubation (blocking) of sections with non-immune goat serum (5%) was performed to avoid non-specific staining from adsorbed primary antibodies.

Dilutions of primary antibodies were carefully applied to sections and incubated for 1-2 h in a humidified chamber. Sections were washed with 3-4 changes of PBS (5 min each). One drop (50 µl) of goat anti-rabbit IgG with conjugated biotin was diluted in 10 ml PBS and applied as secondary antibody. Incubation time was one hour, followed by 3 rinses in PBS (5 min each). Next the avidin-biotin enzyme reagent was applied to the treated tissue sections, conjugated to alkaline phosphatase (ABC-AP kit, 3 drops each of reagent A and B in 10 ml PBS). This was incubated in a moist chamber for one hour and washed 3 times before adding substrate. Visualization of immunoreactive antigens in tissue was performed with Vector Red (an alkaline phosphatase substrate kit, Vector Labs) as the ABC-AP reagent, with 4 drops each of 1st, 2nd, and 3rd solution of Vector Red in 5 ml Tris, pH 8.2. The precipitating complex gives a pinkish red color.

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