Purification of Bovine Estrus-Associated Protein and Localization of Binding on Sperm

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ABSTRACT

An oviduct-specific, estrus-associated glycoprotein (EAP) of 85–95 kDa is detectable in both conditioned medium (CM) from oviductal explants and cannula-derived oviductal fluid (ODF). The objectives of this study were to purify EAP from both ODF and CM, to characterize the glycosylation of EAP, and to localize binding of EAP on sperm. EAP was purified from ODF by ammonium sulfate precipitation and ammonium sulfate back-extraction followed by electroelution from one-dimensional SDS-PAGE gels. EAP was recovered from CM by electroelution from SDS-PAGE gels. Purified EAP was used as antigen to produce polyclonal antibodies (anti-EAP), and the specificity of anti-EAP was demonstrated as a single band in Western blots of ODF. N-linked sugar residues were enzymatically removed from EAP purified from ODF. The resulting molecule was 7 kDa smaller and was similar in molecular mass to EAP derived from CM. Sperm were incubated with 35S-proteins synthesized by oviductal explant cultures. Autoradiographs of solubilized sperm membranes contained a 90–95-kDa protein that was confirmed by Western blotting to be EAP. EAP was localized on permeabilized membranes of sperm incubated in ODF by immunocytochemistry using polyclonal anti-EAP. EAP was bound to the head and middle piece of 97% of the sperm incubated for 4 h in ODF. From these results, we concluded that N-linked sugars account for approximately 8% of the molecular mass of ODF-derived EAP and that EAP binds to the head and middle piece of sperm.

INTRODUCTION

The protein constituents of bovine oviductal fluid (ODF) have been evaluated by analysis of daily ODF collected from normally cycling dairy cows or supernatants of in vitro oviduct cultures [1–5]. Unique proteins secreted at estrus, when the serum estradiol concentration is highest and progesterone concentration is lowest, are referred to as estrus-associated proteins (EAP). An 85–95-kDa bovine estrus-associated glycoprotein is secreted maximally around estrus and then decreases to low levels during the luteal phase [5,6]. Monoclonal antibodies that specifically detect bovine oviductal glycoproteins of 85–97 kDa have been characterized recently [7]. EAP have not been purified, although a recombinant cDNA library was made using baboon oviductal poly(A) RNA from a primate estrogen-dependent protein of 120 kDa [8].

Gerena and Killian [5] observed that bovine EAP (95 kDa) reacted with periodic acid-Schiff reagent on SDS-PAGE gels of ODF, indicating the presence of complex carbohydrates. Specific sugar residues associated with the glycosylation of bovine EAP from ODF also have been identified by lectin blotting. Both wheat germ agglutinin and peanut agglutinin react with EAP, indicating the presence of N-acetylglucosamine and galactosyl (β-1,3) N-acetylgalactosamine residues, respectively [9].

Comparison of EAP in ODF with that in conditioned medium from oviduct explant cultures [9] indicates that the molecular mass of EAP synthesized by explant cultures (85–90 kDa) is less than that of EAP from ODF. This lower molecular mass may be due to less glycosylation of the in vitro-synthesized molecule. In vitro oviduct cultures have also been incubated with 35S-methionine to assess total in vitro protein synthesis [9]. Only one band (90–95 kDa) was seen on autoradiographs from these explant cultures.

Because EAP may be associated with events surrounding fertilization, additional information about the carbohydrate moieties may provide insights into the function and regulation of EAP. Neither the amount of glycosylation contributed by N-linked sugar residues nor the size of deglycosylated EAP has been determined.

The plasma membrane of sperm consists of regional domains demonstrated by antibody and lectin binding studies [10,11]. Binding of a protein to a specific region of the sperm membrane may provide insights into its biological function.

It has been reported that five ODF proteins (140, 95, 78, 66, and 55 kDa) bind to sperm from rams [12]. Through the use of indirect immunofluorescence, oviducin (54 kDa), a human oviductal glycoprotein, was observed to bind to the head of human sperm [13]. Five bovine ODF proteins (90, 66, 53, 34, and 24 kDa) were shown to bind to bull sperm. Western blots of membranes from washed sperm previously incubated in ODF and probed with polyclonal antibody to purified bovine EAP (anti-EAP) indicated that the 90-kDa protein is bovine EAP [14].

The objectives of the present study were to purify bovine EAP, to further characterize the carbohydrate components, and to localize EAP binding on sperm.

MATERIALS AND METHODS

Collection of Oviductal Fluid

ODF was collected daily from cows with normal estrous cycles via indwelling oviductal cannulae [15]. Polyethylene
tubing encased in silastic tubing was inserted into the lumen of the oviduct. The oviduct was ligated distally and proximally to the cannula to prevent influx of peritoneal and uterine fluids. Cannulae were exteriorized through puncture wounds made in the flank.

Stage of the ovarian cycle was determined by RIA of daily serum progesterone [4]. Daily blood samples were collected concomitantly with ODF samples. Serum progesterone concentrations > 1.5 ng/ml were considered to be luteal, whereas samples with serum progesterone ≤ 1.5 ng/ml were considered to be follicular. Visible signs of estrus also were noted. Follicular phase ODF was pooled across days and assayed for total protein [16]. Fluid samples were stored in liquid nitrogen until pooled for purification of EAP.

Culture of Oviductal Explants

Ampullae were excised from whole oviducts obtained from three cows slaughtered on the day of observed standing heat. Ampullae from each cow were cut into pieces approximately 1 mm³. Tissue pieces from each cow were placed in individual culture dishes containing 5 ml Dulbecco's Modified Eagle's Medium supplemented with D-glucose and L-glutamine (#D5648; Sigma Chemical Co., St. Louis, MO) plus RPMI 1640 with L-glutamine (#R7130; Sigma). After 48 h of culture at 39°C, conditioned medium (CM) from each dish was collected, individually dialyzed, and concentrated. Each preparation was then assayed for total protein [16] and frozen until purified as described below.

Electrophoresis and Western Blotting

One-dimensional SDS-PAGE was used to separate ODF proteins and assess protein purity [17]. Resolving gels were cast using 10% acrylamide; stacking gels contained 4% acrylamide. Polymerization of gels was induced by addition of 5% ammonium persulfate and 0.05% N,N',N"-tetramethylethylenediamine. To visualize protein bands after electrophoresis, gels were stained for 2 h with a 0.125% aqueous solution of Coomassie Brilliant Blue in 10% acetic acid plus 40% methanol. Gels were destained with an aqueous solution of 5% ammonium persulfate and 0.05% N,N',N"-tetramethylethylenediamine. After 30 min, the gel was washed with distilled water and stained for an additional 30 min after the desired concentration was reached. A pH of 8.0-8.2 was maintained during the precipitation and DEAE-cellulose column chromatography [19]. Total protein concentration was assayed and aliquots were frozen.

Specificity of anti-EAP was determined by Western blotting of on- and two-dimensional SDS-PAGE gels of whole ODF, with anti-EAP used as primary antibody. No cross-reactivity was observed with albumin or other ODF proteins, and EAP was detected in follicular ODF, but not in luteal ODF (Fig. 1, top).

Purification of EAP from Oviductal Fluid

All purification steps were performed at 0-4°C unless stated otherwise.

**Step 1.** Solid enzyme-grade ammonium sulfate was added slowly to approximately 30 ml ODF (10 mg protein/ml) until a concentration of 35% saturation was reached. The solution was stirred on ice for about 30 min at a slow speed while the ammonium sulfate was being added, and for an additional 30 min after the desired concentration was reached. A pH of 8.0-8.2 was maintained during the precipitation by addition of 6 M ammonium hydroxide as needed. The solution was centrifuged at 12 000 × g for 20 min; the supernatant was then retained and the pellet discarded. Additional solid ammonium sulfate was slowly added to the supernatant to a final concentration of 70% saturation. After centrifugation, the pellet was retained.

**Step 2.** Solid ammonium sulfate was dissolved in 10 mM Tris-HCl, pH 8.2, for each back-extraction described below. The pellet was added to 20 ml of 65% saturated ammonium sulfate solution, stirred on ice for 30 min, and then centrifuged at 12 000 × g for 20 min. The supernatant was retained; the pellet was suspended in 20 ml of 60% saturated ammonium sulfate and stirred on ice for 30 min. After centrifugation, the supernatant was refrigerated and
the pellet added to 20 ml of 55% saturated ammonium sulfate solution. This process was repeated through successive solutions of 50, 45, 40, and 35% saturated ammonium sulfate until the pellet was completely dissolved.

**Step 3.** Each supernatant obtained after centrifugation of ammonium sulfate back-extractions (65–35%) was dialyzed overnight in 4 L of 10 mM Tris-HCl, pH 8.2, plus 1% glycerol. After dialysis, each supernatant was filter-sterilized with a 25-mm, 0.2 µm cellulose acetate syringe filter (Nalge Co., Rochester, NY) and stored in a sterile plastic vial at −20°C.

**Step 4.** Presence of EAP in each supernatant was assessed by SDS-PAGE. Samples containing EAP (60, 55, and 50%) were dialyzed overnight in 50 mM ammonium bicarbonate to facilitate lyophilization. After dialysis, 1-ml aliquots of supernatant were pipetted into Eppendorf tubes, lyophilized to dryness, and stored at −20°C.

**Step 5.** A lyophilized sample was diluted to 50 µl with distilled water, and 20–25 µg protein/lane was loaded in 4 or 5 lanes of a 10% SDS-PAGE gel. Aliquots of each sample were electrophoresed at 100 V for 3.5 h.

**Step 6.** Gel lanes designated for Western blotting were excised and proteins were transferred onto NCP as described above.

**Step 7.** After transfer of the proteins was complete, NCP was stained for 25 sec with 0.1% amido black and then destained for 2–3 min in 5% methanol plus 10% acetic acid to visualize protein bands. Bands in the standard lane on
NCP were aligned with bands in the standard lane on the gel to locate the position of EAP and excise the EAP band. Excised gel bands containing EAP were placed in dialysis tubing (molecular weight cutoff, 10,000; SpectraPor Membrane, Spectrum Laboratory Products, Houston, TX) with 0.5 ml SDS tank buffer and submerged in an electroelution chamber containing SDS tank buffer. Protein was electroeluted at room temperature from gel bands at 100 V for 1 h.

**Step 8.** SDS buffer containing eluted protein was removed from dialysis tubing and dialyzed overnight in 2 l of 50 mM ammonium bicarbonate. After dialysis, samples were lyophilized to dryness and then stored at −20°C.

**Step 9.** All electroeluted EAP samples were checked for purity by one-dimensional SDS-PAGE and Western blotting, as described above, using 10–20 µg of eluted protein.

**Purification of EAP from Explant Culture Medium**

**Step 1.** Medium from 48-h oviductal explant cultures was dialyzed overnight in 4 l of 50 mM ammonium bicarbonate; it was then lyophilized to dryness and stored at −20°C.

**Step 2.** Each sample was diluted to 50 µl with distilled water, and 20–25 µg protein was loaded in 4 or 5 lanes of a 10% SDS-PAGE gel and electrophoresed at 100 V for 3.5 h.

**Step 3.** Gel lanes designated for Western blotting were excised and proteins were transferred onto NCP at 15–20 V (about 150 mAmp) for 30 min.

**Step 4.** After protein transfer was complete, NCP was stained for 25 sec with 0.1% amido black and then destained for 2–3 min in 5% methanol plus 10% acetic acid for visualization of protein bands. Bands in the NCP standard lane were aligned with bands in the standard lane on the gel to locate the position of EAP and excise the EAP band. Excised gel bands containing EAP were placed in dialysis tubing with 0.5 ml SDS tank buffer and submerged in an electroelution chamber containing SDS tank buffer. Protein was electroeluted from gel bands at room temperature at 100 V for 1 h.

**Step 5.** SDS buffer containing eluted protein was removed from dialysis tubing and dialyzed overnight in 2 l of 50 mM ammonium bicarbonate. After dialysis, samples were lyophilized to dryness and then stored at −20°C.

**Step 6.** All electroeluted EAP samples were checked for purity by one-dimensional SDS-PAGE and Western blotting, as described above, using 10–20 µg of eluted protein.

**Deglycosylation of Bovine EAP**

The amount of N-linked sugars contributing to the molecular mass of EAP was assessed by enzymatically deglycosylating bovine EAP purified from whole ODF. An 85-µg (40 µl) sample of pure EAP was mixed with 29.2 µl of 0.55 M potassium phosphate buffer (pH 8.6), 12 µl of 10% SDS, and 4.0 µl of β-mercaptoethanol. The mixture was boiled for 3 min and then cooled to room temperature. Nonidet-P-40, a nonionic detergent, was added (8.52 µl). Finally, N-glycanase, isolated from Flavobacterium meningosepticum (Genzyme Corp., Cambridge, MA), was added (1 U/10 µg protein), and the mixture was incubated at 37°C. At 0, 4, 8, 18, and 24 h, a 15-µl sample was taken and boiled for 5 min. The cleavage site for N-glycanase is the β-aspartylglycosamine bond between the innermost GlcNAc and the asparagine residue of the glycoprotein. All common classes of Asn-linked oligosaccharides (including sialylated, phosphorylated, and sulfated sugar chains) are hydrolyzed by N-glycanase (product information, Genzyme Corp.).

Enzyme-digested samples were run on SDS-PAGE and transferred to NCP, and a Western blot was probed with anti-EAP. This entire procedure was repeated a second time on a different day. Deglycosylated EAP was not used in localization studies.

**Incubation of Sperm with 35S-Protein Synthesized by Oviductal Explant Cultures**

A qualitative study was performed to determine whether EAP synthesized in vitro could bind to sperm membrane. Ampullae were excised from oviducts taken from three cows at standing heat. Ampullae from each cow were cut into 1-mm² pieces and placed in culture dishes containing 2 ml Dulbecco’s Modified Eagle’s Medium supplemented with D-glucose and L-glutamine (#D5648, Sigma) plus RPMI 1640 with L-glutamine (#R7130, Sigma). L-[35S]-Methionine (4 µCi/ml) was added to each culture dish. CM was collected after 48-h culture of the explants at 39°C. Samples of CM were pooled (CMP), dialyzed, concentrated by ultrafiltration, and assayed for total protein [16]. Sperm were incubated (5 × 10⁷ sperm/ml) in CMP at 39°C for 4 h. Sperm membranes were solubilized by incubating for 1 h in sodium deoxycholate buffer [20]. Each membrane sample was electrophoresed on two SDS-PAGE gels. One gel was stained with Coomassie Brilliant Blue, and an autoradiograph was made by exposing x-ray film to the duplicate gel.

**Immunocytochemical Localization of EAP on Sperm Membrane**

Semen was collected from two fertile bulls. Sperm in 1 ml of semen from each ejaculate were washed twice with 9 ml of protein-free modified Tyrode’s medium (MTM) and centrifuged at 1000 × g for 20 min. Sperm (1 × 10⁸/ml) were pooled and added to each of the following treatments: 1) MTM, 2) MTM + heparin (100 µg/ml), and 3) follicular-stage ODF (5 mg protein/ml). The samples were incubated at 39°C. Sperm incubated in MTM alone served as a negative control. Sperm incubated in MTM + heparin served as a negative control for EAP binding and a positive control for capacitation. At 0 and 4 h, 10 µl of sperm was pipetted onto a glass slide, fixed in methanol, and frozen until used. Slides containing sperm from 0 h served as a negative control for each treatment.
Slides were thawed at room temperature. Membranes were partially permeabilized by incubation for 30 min at 4°C in a 1:100 dilution of sodium deoxycholate buffer [20]. Slides were washed twice with PBS containing 0.5% Tween 20, pH 7.2. To visualize the site of EAP binding, immunocytochemistry was performed; anti-EAP was used as primary antibody (5 μg/ml) and DAB as peroxidase substrate. To coverslip slides temporarily, one edge was fastened with rubber cement. At least 100 sperm per slide were evaluated by light microscopy. Bound EAP was detected by a positive reaction, which produced a brown color.

RESULTS

Purification of Bovine EAP

Purified bovine EAP was obtained from cannula-derived follicular-stage ODF by use of ammonium sulfate precipitation and electroelution from SDS-PAGE gels. Ammonium sulfate precipitation was performed at 35% and 70% saturation (step 1). Because EAP is a glycoprotein, reverse ammonium sulfate extractions were performed from 65 to 35% saturation to maximize separation of the ODF proteins (step 2).

Reverse ammonium sulfate extractions yielded EAP in highest concentrations at 60, 55, and 50% saturation, along with five or six other, contaminating proteins, including albumin. Supernatants containing EAP were dialyzed (step 3), lyophilized (step 4), and run on one-dimensional SDS-PAGE (step 5). Bands containing EAP, at 95 kDa, were excised (step 6) and electroeluted (step 7), yielding pure EAP. Purity was assessed by both two-dimensional (Fig. 1, top) and one-dimensional (Fig. 1, bottom) SDS-PAGE, and a single protein band was detected at approximately 95 kDa.

Bovine EAP was purified from oviductal explant culture medium after 48-h incubation of the tissue (step 1). Medium was dialyzed, lyophilized, and run on SDS-PAGE (step 2). Bands containing EAP, at 85–90 kDa, were excised (step 3) and electroeluted (step 4) to yield pure EAP. This preparation of EAP migrated as a single Coomassie blue-stained band at 85–90 kDa on SDS-PAGE gels (Fig. 1, bottom).

Enzymatic Deglycosylation of EAP

Bovine EAP derived from ODF was incubated with N-glycanase to remove N-linked sugar residues. SDS-PAGE and Western blotting of EAP and enzymatically deglycosylated EAP showed no decrease in size until 18 h of digestion, with no further decrease in size after 24 h (Fig. 2). The apparent molecular mass of glycosylated EAP was 96 kDa at 0 and 8 h. After 24 h of exposure to N-glycanase, the molecular mass of EAP had decreased to 88.5 kDa. This relative decrease in molecular mass indicated that approximately 8% of the total molecular mass of EAP could be attributed to N-linked sugar residues.
**Association of EAP from Oviduct Cultures with Sperm Membrane**

Bovine sperm were incubated with $^{35}$S-proteins synthesized by in vitro-cultured oviductal explants (Fig. 3, top). Autoradiographs of the solubilized sperm membranes contained one predominant band at 90–95 kDa (Fig. 3, bottom), corresponding to EAP, and three or four lower-molecular-mass bands of low intensity. The 90–95-kDa band
was confirmed by Western blotting using anti-EAP as primary antibody.

Localization of EAP on Sperm

To localize EAP binding on sperm membrane, immunocytochemistry was performed on incubated sperm that were methanol-fixed onto slides. No binding of EAP was detected on sperm unless slides containing sperm were incubated in sodium deoxycholate buffer to partially permeabilize the membranes. Furthermore, 10 min of permeabilization was not sufficient to reveal EAP binding on the membrane, but 30-min incubation in deoxycholate buffer allowed detection of EAP binding.

At 0 h of incubation, sperm did not react for binding of EAP in any treatment. After 4 h of incubation, sperm from the ODF treatment reacted with anti-EAP, while sperm from treatments without EAP showed no reaction (Fig. 4a). Binding was detected in 96–97% of the ODF-treated sperm, which showed brown staining over the entire sperm head and over the middle piece of the tail (Fig. 4b).
DISCUSSION

Although purification of bovine EAP from ODF was accomplished by the procedures outlined above, several unsuccessful attempts were also made. These included purification of dialyzed ammonium sulfate back-extraction fractions containing EAP via column chromatography. However, anion-exchange columns (DEAE and ecteola cellulose) yielded EAP with albumin. Because recovery from DEAE was low (50%), ecteola was used, allowing about 70% recovery of EAP with albumin.

Other types of column chromatography, such as Sephadex G200, Sephacryl P200, and cation exchange, were used in earlier purification trials. Purification of EAP was not accomplished with these types of column chromatography. Two problems consistently encountered were tight binding of EAP to column materials and inability to remove albumin. The pI of EAP is acidic (about 4.7), nearly identical to the pI of albumin, contributing to this problem. For these reasons, electroelution of EAP from SDS-PAGE gels was performed.

The association of EAP and albumin may be of physiological significance. Bovine EAP has been shown to facilitate sperm capacitation and fertilizing ability in vitro [21]. Cholesterol efflux from sperm membrane is believed to be an important part of the capacitation process [22]. Albumin, the most abundant protein in ODF, can act as a sterol acceptor [23] and therefore may mediate sperm capacitation.

To produce antibodies with specificity for one protein, the protein serving as the antigen must be pure. Purification of bovine EAP from ODF and CM by the procedure described above yielded a single band at 85–95 kDa on Coomassie-stained one-dimensional SDS-PAGE gels. The specificity of polyclonal antibodies to bovine EAP was tested by probing Western blots of whole ODF using anti-EAP as the primary antibody. A single band at 85–95 kDa was detected by the anti-EAP. With the use of anti-albumin as primary antibody, a single band at 65 kDa was detected on Western blots. This confirmed specificity for EAP, showing no cross-reactivity with other ODF proteins. The purity of EAP was also confirmed by Western blotting on two-dimensional SDS-PAGE gels. Western blots of whole ODF were probed using anti-EAP as the primary antibody. A single spot corresponding to EAP was detected by the anti-EAP.

Carbohydrate moieties associated with EAP may be biologically important. Carbohydrates are known to perform a crucial role in cell surface recognition, antigen binding, and maintenance of the biological activity of a molecule. The amount of glycosylation may affect the half-life of proteins and may regulate the bioactivity of a hormone or mediator molecule. After deglycosylation, there often is a change in bioactivity [24].

Carbohydrate groups on cell surface molecules have potential for great structural diversity and may be important in cell-to-cell recognition. This is possible because different monosaccharides can be joined at different hydroxyl groups, C-1 linkages can have alpha or beta configurations, and branching also may occur [25]. Glycosylation often is related to the bioactivity of a molecule. It has been demonstrated that carbohydrate content, particularly sialic acid, is essential for the biological activity of FSH and LH [26].

Because N-glycanase hydrolyzes oligosaccharide chains by cleaving between asparagine and GlcNAc, removal of N-linked sugars by this enzyme is believed to be complete. The molecular mass of EAP decreased after 18 h of incubation with N-glycanase, but no further decrease was observed after 24 h of incubation. This indicated that enzymatic removal of N-linked sugars had gone to completion. Glycosylation of EAP by O-linked sugar residues, which would remain after incubation with N-glycanase, also may contribute bioactivity to the molecule.

Bovine EAP from whole ODF is slightly larger (95 kDa) than that synthesized by explant cultures (85–90 kDa). The difference in size is presumably attributable to less glycosylation of the protein synthesized in vitro. Enzymatic hydrolysis of N-linked sugars from bovine EAP derived from ODF decreased the molecular mass from 96 kDa to 88.5 kDa. The resulting molecule was similar in size to EAP synthesized by in vitro explant cultures (85–90 kDa). This suggests that EAP synthesized in vitro may be less glycosylated than EAP from ODF. Studies comparing EAP from ODF with EAP from explant cultures showed that the two molecules had similar biological activities for promoting in vitro capacitation of bull sperm [21]. Therefore, the smaller molecular mass of in vitro-synthesized EAP does not appear to correspond to a lower biological activity of the protein.

Binding of oviducal fluid proteins to sperm has been demonstrated in rams [12], bulls [14], and men [13, 27]. Localization of oviductin from human ODF was shown to bind to sperm heads [13]. A previous study from this laboratory reported that solubilized sperm membranes contained ODF proteins, including EAP, after sperm were incubated in ODF [14].

Incubation of 35S-protein synthesized by explant cultures with sperm clearly showed specific binding of EAP to the sperm membrane. Among proteins synthesized by explant cultures, EAP was the main protein that bound to sperm. No change in molecular mass was observed in the protein bound to sperm. These results suggest that differences in the molecular mass of EAP from ODF and in vitro explant cultures do not affect binding to sperm. Therefore, differences in molecular mass of the two molecules may not affect biological activity of EAP related to sperm.

Specific binding of bovine EAP from ODF to the sperm surface was visualized by using immunocytochemistry to detect the presence of EAP on capacitated sperm. The results clearly showed staining of the head and middle piece of sperm capacitated in MTM + ODF, whereas staining was not detected on the head, middle piece, or tail of sperm incubated in MTM alone (uncapacitated) or sperm capaci-
tated with MTM + heparin. Anti-EAP does not react with heparin, indicating that EAP does not contain antigenic sugar residues similar to those of heparin. In addition, anti-EAP does not detect other ODF proteins or sperm membrane proteins.

Detection of EAP binding to sperm was possible on intact sperm after the sperm membrane was partially permeabilized. Sperm from all treatments were subjected to permeabilization followed by immunocytochemistry. Because EAP binding was detected only on the heads and middle pieces of sperm incubated in ODF, it is possible that EAP binding to sperm is cryptic, rather than on the membrane surface; this may involve the loss of membrane cholesterol that occurs during capacitation [28]. Cholesterol efflux from the membrane decreases the cholesterol:phospholipid ratio and increases membrane fluidity. Changes in conformation and distribution of membrane proteins occur with changes in membrane fluidity and permeability. These membrane changes could result in redistribution and cryptic localization of previously surface-bound proteins. EAP may bind initially to the membrane surface and then become localized within the membrane, possibly associating with phospholipids, as cholesterol is lost. Proteins that bind specifically to the head may be involved in regulation of metabolism, motility, or hyperactivation.

Bovine EAP was purified from whole ODF and from oviductal explant culture medium. Purified bovine EAP was used to produce polyclonal antibody against the 95-kDa protein and was used in studies to evaluate the biological function of EAP. Polyclonal antibodies to EAP were specific, showing no cross-reactivity with other ODF proteins on Western blots.

Enzymatic removal of N-linked sugar residues from bovine ODF-derived EAP yielded a molecule with an 8% decrease in molecular mass. This smaller molecule was similar in size to EAP secreted by in vitro tissue cultures. The smaller size of the molecule synthesized by in vitro oviductal cultures may be due to a smaller amount of glycosylation of the protein.

Specific binding of bovine EAP to sperm membrane was visualized by immunocytochemistry. Intact sperm membrane did not reveal protein binding; however, with partial membrane permeabilization, EAP binding was detected on the head and middle piece of sperm incubated with ODF for 4 h. EAP was not detected on sperm membrane from either control treatment at 4 h or on sperm from any treatment at 0 h.

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