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## Identification of Bovine Sperm Acrosomal Proteins that Interact with a 32kDa Acrosomal Matrix Protein

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### Abstract

Mammalian fertilization is accomplished by the interaction between sperm and egg. Previous studies from this laboratory have identified a stable acrosomal matrix assembly from the bovine sperm acrosome termed the outer acrosomal membrane-matrix complex (OMC). This stable matrix assembly exhibits precise binding activity for acrosin and N-acetylglucosaminidase. A highly purified OMC fraction is comprised of three major (54, 50, and 45kDa) and several minor (38–19kDa) polypeptides. The set of minor polypeptides (38–19kDa) termed “OMCrpf polypeptides” is selectively solubilized by high-pH extraction (pH 10.5) while the three major polypeptides (55, 50 and 45kDa) remain insoluble. Proteomic identification of the OMC32 polypeptide (32kDa polypeptide isolated from high-pH soluble fraction of OMC) yielded two peptides that matched the NCBI database sequence of acrosin-binding protein. Anti-OMC32 recognized an antigenically related family of polypeptides (OMCrpf polypeptides) in the 38–19kDa range with isoelectric points ranging between 4.0 and 5.1. Other than glycohydrolases, OMC32 may also be complexed to other acrosomal proteins. The present study was undertaken to identify and localize the OMC32 binding polypeptides and to elucidate the potential role of the acrosomal protein complex in sperm function. OMC32 affinity chromatography of a detergent soluble fraction of bovine cauda sperm acrosome followed by mass spectrometry-based identification of bound proteins identified acrosin, lactadherin, SPACA3, and IZUMO1. Co-immunoprecipitation analysis also demonstrated the interaction of OMC32 with acrosin, lactadherin, SPACA3, and IZUMO1. Our immunofluorescence studies revealed the presence of SPACA3 and lactadherin over the apical segment; whereas, IZUMO1 is localized over the equatorial segment of Triton X-100 permeabilized cauda sperm. Immunoblot analysis showed that a significant portion of SPACA3 was released after the lysophosphatidyl choline (LPC)-induced acrosome reaction; whereas, the IZUMO1 and lactadherin polypeptides remain associated to the particulate fraction. Almost entire population of bovine sperm IZUMO1 relocates to the equatorial segment during the LPC-induced acrosome reaction. We propose that the interaction of OMC32 matrix polypeptide with detergent soluble acrosomal proteins regulates the release of hydrolases/ other acrosomal protein(s) during the acrosome reaction.

## Keywords

Bovine sperm; Acrosome; Acrosomal Protein; Lactadherin; SPACA3; IZUMO1

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## Introduction

In mammalian fertilization, the male germ cell delivers the paternal genome to the oocyte. The transfer of the paternal genome to the egg is mediated by the acrosome reaction. An array of proteins, signaling pathways, cell-cell interactions, and cellular components are also involved. During the acrosome reaction, plasma membrane, forming a fenestrated hybrid membrane complex, which permits the release of acrosomal hydrolases that are thought to function in zona penetration. This process is a receptor-regulated, calcium-dependent process. The acrosome reaction occurs once in the lifespan of the spermatozoa and is a criterion for sperm fusion with the egg membrane [1–3]. The mammalian sperm acrosome is a membrane-bounded, cap-shaped organelle attached to the anterior pole of the sperm head [4, 5]. The acrosome exhibits similar properties to regulated secretory vesicles of somatic cells [6], but it is distinguished from typical secretory granules by its highly polarized architecture [5]. The sperm acrosome consists of three morphologically distinct regions termed: the apical, principal and equatorial segments. The acrosome is considered essential for fertilization; men and mutant animals that produce sperm lacking acrosomes are infertile [7–9]. The acrosome contains glycohydrolases that function in sperm binding and penetrating the zona pellucida [1, 4]. In addition to its constituent hydrolases, the acrosome also contains an insoluble structural framework termed the acrosomal matrix [2, 10, 11]. A variety of evidence suggests that the acrosomal components may participate in sperm-zona adhesion as well as penetration of the zona pellucida [12, 13]. Some hydrolases are compartmentalized within morphologically distinct domains of the acrosomal matrix [10, 14–19]. The insoluble matrix assemblies are structural elements that define acrosomal shape, but they also bind specific hydrolases [14, 17, 18, 20–22]. The association of acrosomal enzymes with domain-specific matrix proteins has been suggested to function in enzyme stabilization prior to the acrosome reaction and in regulation of the sequential enzyme release during the acrosome reaction [10, 16, 19–21, 23–25]. In addition, specific elements of the acrosomal matrix remain intact and associated with the hybrid membrane complex after the acrosome reaction [26]. Thus, the identification of proteins localizing to the acrosome is fundamental to the understanding of its contribution to fertilization.

We have previously isolated and characterized a localized, stable acrosomal matrix assembly from the bovine acrosome termed the outer acrosomal membrane-matrix complex (OMC) [21, 27]. This stable matrix assembly exhibits specific binding activity for acrosin [21] and N-acetylglucosaminidase (NAGA) [28] and is restricted to the apical and principal segments of the acrosome where it is associated with the luminal surface of the outer acrosomal membrane [21, 27]. A highly purified OMC fraction is comprised of three major (54, 50, and 45kDa) and several minor (38–19kDa) polypeptides. The set of minor polypeptides (38–19kDa) termed “OMCrpf polypeptides” is selectively solubilized by extraction of OMC in 0.1M CAPS buffer (3-[cyclohexylamino]-1-propane sulfonic acid), pH 10.5, while the three major polypeptides (55, 50 and 45kDa) remain associated with the

sedimentable “stripped” OMC [26]. N-terminus amino acid sequencing of OMC32 (32kDa polypeptide isolated from high-pH soluble fraction of OMC) indicated sequence homology with the family of acrosomal SP-10 proteins described in baboon spermatozoa [29] as well as human [30, 31], mouse [32], and fox [33] spermatozoa. The potential of utilizing SP-10 antigen as targets for immunocontraceptive vaccines has previously been suggested [34]; however, the biological function of these proteins has not been resolved. Acrosin and NAGA bound the OMC32 polypeptide in a concentration-dependent fashion [28]. The binding specificity of acrosomal matrix proteins to hydrolases strongly suggests that the matrix polypeptides play an important role in the regulation of hydrolases released during the acrosome reaction and could also function during acrosome assembly to target and/or segregate hydrolases within the acrosome interior. Other than glycohydrolases, OMC32 may also be complexed to other acrosomal proteins. It is likely that the interaction of OMC32 polypeptide to other polypeptides may have a high impact on sperm function. The objective of the present study is to identify, characterize and localize the OMC32 binding polypeptides and to elucidate the potential role of the acrosomal protein complex (which consists of OMC32 and other characterized polypeptides) in sperm function.

## Materials and Methods

### Sperm Preparation

Bovine epididymides were obtained from Randolph Packing Co., Inc., Asheboro, NC. Cauda epididymides were incubated in Hanks' balanced saline, pH 7.4, at 37°C for 5 min. To permit sperm release, the epididymides were minced in 37°C Hanks' saline. The sperm suspension was collected and centrifuged at 1,000 X g for 10 min at 4°C. The pellets were washed three times by re-suspension in Hanks' saline followed by centrifugation as above. The final pellets were re-suspended in a Tris-saline-protease inhibitor solution (TNI), composed of 150mM NaCl, 25mM Tris-HCl (pH 7.5), 2mM benzamidine, 1µg/ml leupeptin, 1µg/ml pepstatin, 1mM NaF, 1mM sodium orthovanadate and 0.05% sodium azide, and centrifuged at 1,000 X g for 10 min at 4°C. Pellets were washed twice more with TNI as described above. The TNI-washed sperm pellets were used in the extraction and fractionation protocols described below. Protein concentration was estimated by Bradford [35] assay.

### Isolation of Outer Acrosomal Membrane-Matrix Complex (OMC)

The published procedure of Nagdas *et al.* [21] was followed to isolate the heads from bovine cauda epididymal spermatozoa. The sperm head pellet was re-suspended into 20 ml of TNI containing 0.6% Triton X-100. After overnight extraction at 4°C with constant agitation, the suspension was homogenized with 50 strokes of a glass-Teflon homogenizer (Knotes, Vineland, NJ) to detach the detergent-insoluble OMC from the heads. A Percoll gradient; consisting of 20 ml sperm heads suspension and 80 ml of a solution made up of 50% Percoll, 0.25 M sucrose, and 0.05 M Tris-HCl, pH 7.5; was used with centrifugation to separate the acrosomal components from the sperm heads [21]. The centrifugation took place at 60,000 X g for 35 min in a Beckman 70Ti rotor. This OMC band was collected and diluted in a 1:1 suspension with TNI and then pelleted in a Beckman SW40 rotor by centrifugation at 100,000 X g for 1 hr.

### Fractionation of OMC Polypeptides

The OMC fraction was extracted overnight at 4°C with 100 mM CAPS buffer (3-[cyclohexamino]-1-propanesulfonic acid; Sigma Chemical Co., St. Louis, MO), pH 10.5. The extracted solution was centrifuged for 30 min at 100,000 X g in a Beckman SW40 rotor which resulted in high-pH soluble and insoluble fractions. SDS-PAGE revealed that the 38–19kDa polypeptide family (termed as OMCrpf polypeptides) was solubilized by high-pH extraction whereas the 54, 50, and 45 kDa polypeptides remained associated with the high-pH insoluble particulate fraction [21, 27]. The high-pH soluble, supernatant fraction was dialyzed and lyophilized. The purification of the 32kDa polypeptide (OMC32) from the high-pH soluble fraction was performed by continuous-elution SDS-PAGE on 12% acrylamide gels using a Model 491 Prep Cell (Bio-Rad Laboratories, Hercules, CA) following the method of Olson *et al* [26].

### Identification and Characterization of OMC32 Binding Proteins

The OMC32 polypeptide (0.5mg), which was purified by continuous-elution SDS-PAGE from the high-pH soluble OMC fraction, was coupled to an AminoLink Plus resin at pH 10.0, following the manufacturer's procedure (Pierce Chemical Co., Rockford, IL). As a control column, bovine cauda sperm tails were isolated following our method [21]. The isolated tails were extracted in TNI containing 0.1% Triton X-100 (TNI-TX) at 4°C for 1 hr and centrifuged at 100,000 X g for 30 min in a Beckman SW40 rotor. The pellet obtained after centrifugation was extracted overnight at 4°C with 100 mM CAPS buffer, pH 10.5, followed by centrifugation for 30 min at 100,000 X g in a Beckman SW40 rotor. The high-pH extracted supernatant was coupled to an AminoLink Plus resin at pH 10.0, following the manufacturer's procedure and used as a control column to examine the specificity of the binding of OMC32 polypeptide to other proteins. Bovine cauda sperm was extracted in TNI containing 0.1% Triton X-100 (TNI-TX) at 4°C for 1 hr and centrifuged at 100,000 X g for 30 min in a Beckman SW40 rotor. The supernatant obtained after centrifugation was applied to both OMC32 coupled and control columns. The columns were washed with 10 column volumes of TNI-TX solution and then eluted with 0.1M Glycine-HCl, pH 2.5. The acid eluted fractions were neutralized to pH 7. After neutralization, an aliquot of the acid eluted fractions of the OMC32 affinity column was analyzed using 12% SDS-PAGE under reducing conditions and stained with Coomassie blue and silver.

### Proteomic Analysis

Proteomic identification of OMC32 polypeptide was performed at the Mass Spectrometry Facility of UNC School of Medicine Proteomic Center, Chapel Hill, NC. The 32kDa band and OMC32 binding polypeptides were subjected to MALDI-TOF-TOF analysis to obtain internal amino acid sequences of several tryptic peptides. Derived peptide sequences were analyzed in the National Center for Biotechnology Information (NCBI) database to determine if a full length sequence has been reported and to identify potential functional motifs such as a transmembrane hydrophobic domain or an extracellular domain with consensus glycosylation sites and to define potential phosphorylation sites as well as protein interaction domains on its cytoplasmic segment.

### SDS-PAGE and Immunoblot

SDS-PAGE was performed on 12% or 7.5% continuous or 7.5% to 15% gradient polyacrylamide gels [36]. Polypeptide bands were visualized either by Coomassie Brilliant blue R (CBBR) [37] or silver [38] staining. Western blots were prepared on PVDF membranes [39]. Two-dimensional PAGE (2D-PAGE) was performed using a Bio-Rad precast immobilized (pH 3–10) gradient gel ready for isoelectric focusing (IEF). Immunoblots were blocked in phosphate buffered saline (PBS) blocking buffer containing 5% heat inactivated normal goat serum (NGS), 2.5% bovine serum albumin (BSA), 0.1% Tween-20, and 5% nonfat dry milk at room temperature for 1 hr. After blocking, the membranes were washed in PBS containing 0.1% Tween-20 (PBS-TW) three times. Membranes were incubated with primary antibodies (anti-SPACA3 from Abcam, Inc., Cambridge, MA; anti-acrosin antibody and anti-IZUMO1 from Antibodies-Online Inc., Atlanta, GA; anti-lactadherin from Thermo Scientific, Waltham, MA) or preimmune sera in PBS containing 0.1% Tween-20 and 1% goat serum (PBS-TW-GS), respectively at room temperature for 1 hr. We have used our hamster acrosin polyclonal antibody raised in guinea pigs [20]. Following three washes in PBS-TW at ambient temperature, membranes were incubated with affinity-purified horseradish peroxidase-conjugated secondary antibody (KPL Inc., Gaithersburg, MD) diluted in PBS-TW-GS (1:5000) at room temperature for 1 hr. Membranes were washed three times in PBS-TW. Immunoreactive bands were visualized using diaminobenzidine for color development or by enhanced chemiluminescence (ECL) detection (Pierce Super Signal, Rockford, IL).

### Immunoprecipitation

For co-immunoprecipitation (Co-IP) experiments, cauda sperm were extracted in RIPA buffer (RIPA buffer contains 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 0.15M NaCl, proteases inhibitors in 10mM sodium phosphate buffer, pH 7.2) for 2 hr at 4°C and then centrifuged at 40,000 rpm for 30 min in a TL55 rotor (Beckman). To remove endogenous IgG, the supernatant fraction was incubated with protein A-sepharose beads at 4°C for 30 min and centrifuged at 12,000 X g for 3 min. The supernatant obtained after RIPA buffer extraction of cauda sperm was dialyzed against PBS (phosphate buffered saline) and subjected to cross-linking chemically using the homobifunctional cleavable cross-linkers, dithiobis[sulfosuccinimidyl propionate] (DTSSP). Cross-linking was terminated by the addition of 10mM lysine. Then, the cross-linked sample was subjected to Co-IP analysis to identify protein(s) associated with the OMC32 polypeptide. The OMC32-protein complexes were immunoprecipitated with affinity-purified anti-OMC32 using Pierce Immunoprecipitation Kit. Immunoprecipitated proteins were incubated with SDS sample buffer using reducing conditions and analyzed by Western blotting to identify co-precipitating proteins. The anti-OMC32 immunoprecipitation pellet was immunostained with the respective antibodies of OMC32, lactadherin, SPACA3, acrosin, and IZUMO1.

### Immunofluorescence Microscopy

Spermatozoa were fixed in 4% formalin in 0.1 M sodium phosphate buffer (PBS), pH 7.4, at 4°C for 30 min and plated on poly-L-lysine-coated coverslips. Following a wash in PBS, cells were permeabilized in PBS containing 0.1% Triton X-100 at 4°C for 30 min or in

–20°C acetone for 10 minutes. After three rinses in PBS, samples were blocked with 1% normal goat serum in PBS (PBS-NGS). Coverslips were incubated with equal dilutions of immune (anti-SPACA3 from Abcam, Inc., Cambridge, MA; anti-acrosin antibody and anti-IZUMO1 from Antibodies-Online Inc., Atlanta, GA; anti-lactadherin from Thermo Scientific, Waltham, MA) or preimmune serum in PBS-NGS for 1 hr, and washed three times with PBS. Cells were then incubated with Cy3-conjugated goat anti-rabbit IgG (KPL Inc., Gaithersburg, MD) in PBS-NGS for 1 hr. Coverslips were then washed with PBS and were examined both by phase contrast and epifluorescence microscopy.

### Capacitation and Acrosome Reaction

Sperm ( $4-6 \times 10^7$ /ml) were capacitated in the presence of heparin (10 mg/ml) in a modified Tyrode's medium (pH 7.4) for 4 hr at 39°C with a 95% air: 5% CO<sub>2</sub> atmosphere. [40]. To initiate the acrosome reaction sperm were incubated with 100 µg/ml lysophosphatidyl choline (LPC) for 15 min following the end of the 4 hr incubation. Noncapacitated, capacitated, and acrosome-reacted sperm were fixed for immunofluorescence staining or centrifuged at 10,000 X g for 10 min at 4°C. The pellets and supernatants were adjusted to equal volumes and used for SDS-PAGE and acrosin determination. For IZUMO1 immunostaining, sperm were fixed in 4% formalin in 0.1 M sodium phosphate buffer (PBS), pH 7.4, at 4°C for 30 min and plated on poly-L-lysine-coated coverslips. Following a wash in PBS, cells were permeabilized in –20°C acetone for 10 minutes. After three rinses in PBS, samples were blocked with 1% normal goat serum in PBS (PBS-NGS). Acrosome-reacted spermatozoa was then incubated in anti-IZUMO1 in PBS-NGS for 1 hr, and washed three times with PBS. Cells were then incubated with Cy3-conjugated goat anti-rabbit IgG in PBS-NGS for 1 hr. Coverslips were then washed with PBS and were examined both by phase contrast and epifluorescence microscopy. For the assessment of sperm acrosomal status, noncapacitated and acrosome-reacted sperm were stained with FITC-conjugated PSA (Pisum Sativum agglutinin) (Vector Laboratories, Inc., Burlingame, CA) [41]. Aliquots of noncapacitated and acrosome-reacted spermatozoa were fixed with 2% formalin in PBS, pH 7.4, washed in PBS, smeared onto slides, and air-dried. Spermatozoa were incubated with 50µg/mL FITC-PSA in PBS for 1 hr, washed, mounted, and analyzed by epifluorescence microscope. Acrosome-intact spermatozoa exhibited a homogenous signal over the entire acrosomal region whereas the acrosome-reacted sperm displayed a signal over the equatorial segment or no fluorescence over the acrosomal region.

## Results

### Purification of OMC32 Polypeptide

We have previously isolated and characterized a localized, stable acrosomal matrix assembly from the bovine acrosome termed the outer acrosomal membrane-matrix complex (OMC) [21, 27]. A highly purified OMC fraction is comprised of three major (54, 50, and 45kDa) and several minor (38–19kDa) polypeptides [21]. The set of polypeptides (38–19kDa) termed “OMCrpf polypeptides” (antigenically related polypeptide family) is selectively solubilized by extraction of OMC in 0.1M CAPS buffer (3-[cyclohexylamino]-1-propane sulfonic acid), pH 10.5 (Fig.1A, lane 1), while the three major polypeptides (55, 50 and 45kDa) remain associated with the sedimentable “stripped” OMC [26]. The 32kDa

polypeptide, termed OMC32 (Fig.1A, lane 2) was purified from the high-pH soluble fraction of OMC by continuous-elution SDS-PAGE following our published protocol [26]. OMC32-containing fractions were pooled and utilized for the proteomic identification and for the coupling of OMC32 polypeptide to an AminoLink Plus resin. Proteomic identification of the OMC32 polypeptide by MALDI-TOF-TOF analysis yielded 2 peptides (Fig.1A, shown in box) that matched the NCBI database sequence of acrosin-binding protein (Bos Taurus; GI: 194666681). Previously, we have shown that OMC32 exhibits strong immunological reactivity with the entire 38–19kDa set of polypeptides [26]. Two-dimensional PAGE was performed to examine charge variant isoforms of OMC32 polypeptides. The total OMC fraction was fractionated by two-dimensional PAGE and transferred to PVDF membranes for immunoblot analysis. Blots stained with anti-OMC32 exhibited a set of intensely stained polypeptides in the 38–19kDa range with isoelectric points ranging between 4.0 and 5.1 (Fig.1B). A duplicate blot stained with preimmune serum was used as the control and exhibited no positive spots (data not shown).

### Identification and Characterization of OMC32 Binding Proteins

To identify candidate proteins that are associated with OMC32 polypeptide other than acrosin, a Triton X-100 lysate of bovine cauda sperm was prepared (Fig.2, lane 1) and fractionated on an OMC32 affinity column. After the removal of unbound polypeptides (Fig. 2, lane 2) and extensive washing, the OMC32 binding polypeptides were eluted in Glycine-HCl, pH 2.5, and analyzed by SDS-PAGE followed by silver staining (Fig.2, lane 3). The acid eluted fraction of the OMC32 affinity column revealed the presence of an array of polypeptides whereas no band was seen in the acid eluted fraction of the control column (data not shown) strongly confirms the specificity of binding four polypeptides to the OMC32 polypeptide. We performed the proteomic identification of four individual polypeptides (two major bands, 1 & 4, and two minor bands, 2 & 3, as shown in Fig.2, lane 3) by MALDI-TOF-TOF analysis. Proteomic data are shown in Table 1. Band 1 yielded four peptides matching PAS-6 and PAS-7 proteins (Lactadherin; Bos Taurus; GI:1632779), band 2 generated one peptide matching acrosin (Bos Taurus; GI:1888363), band 3 yielded four peptides matching izumo sperm-egg fusion protein 4 isoform1 (IZUMO1) (Bos Taurus; GI: 156120505), and band 4 yielded one peptide matching SPACA3 protein (Sperm Acrosome Membrane-Associated Protein, Bos Taurus; GI: 151555932). These experiments demonstrate that OMC32 matrix polypeptide interacts with detergent soluble acrosomal membrane proteins in a receptor-ligand type of interaction to establish the outer acrosomal membrane-matrix complex assembly.

### Biochemical Characterization of SPACA3, IZUMO1, Lactadherin, and Acrosin

Western blots of the detergent-soluble fraction of bovine cauda sperm stained with anti-SPACA3 demonstrated the presence of a 25kDa SPACA3 (Fig.3A, lanes 1 and 2). No band was seen on identical lanes stained with preimmune IgG (Fig.3A, lanes 3 and 4). Another blot stained with anti-IZUMO1 revealed the presence of a 40kDa IZUMO1 immunoreactive band (Fig.3B, lanes 1 and 2); no stained band was present in identical lanes probed with preimmune IgG (Fig.3B, lanes 3 and 4). Western blot of the detergent-soluble fraction of bovine cauda sperm stained with anti-lactadherin (Fig. 3C, lane 1) revealed the presence a 45kDa lactadherin. No band was seen with preimmune IgG (Fig.3C, lane 2). Immunoblots

of the detergent-soluble fraction of bovine cauda sperm stained with anti-acrosin (Fig.3D, lane 1) exhibited the presence of two forms of acrosin (44kDa and 42kDa). No band was seen on an identical lane stained with preimmune IgG (Fig.3D, lane 2).

### Identification of OMC32-Associated Proteins by Co-immunoprecipitation (Co-IP) Analysis

Utilizing protein-targeting strategy, we illustrated that acrosin, lactadherin, SPACA3, and IZUMO1 bind to OMC32 (Table 1). The objective of this experiment is to examine the interaction of OMC32 to above mentioned four proteins by Co-IP analysis. This technique permits the identification of physiological protein complexes. The supernatant obtained after RIPA buffer extraction of cauda sperm was immunoprecipitated with anti-OMC32. The “rpf” polypeptides (38–19kDa-Fig.4A, lane1) was completely recovered in the anti-OMC32 immunoprecipitation pellet (Fig.4A, lane 3). Previously, we had shown that Western blots of total sperm lysates stained with anti-OMC32 recognized an antigenically related family of polypeptides between 38–19kDa, termed “rpf”; OMC32 is a major polypeptide of the “rpf” family [26]. No immunoreactive band was observed when an identical lane of the anti-OMC32 IP pellet stained with preimmune rabbit IgG (data not shown). As shown in Figure 4B, IZUMO1 (lane 1) was also totally recuperated in the anti-OMC32 immunoprecipitation pellet (Fig.4B, lane 3). A significant portion of total SPACA3 (Fig.4C, lane 1) was co-precipitated with OMC32 (Fig.4C, lane 3) and the remaining SPACA3 was present in the unbound fraction (Fig.4C, lane 2). It is also noted that a major portion of total lactadherin (Fig.4D, lane 1) present in the anti-OMC32 IP- pellet (Fig.4D, lane 3) and the remaining lactadherin was present in the unbound fraction (Fig.4D, lane 2). On the contrary, almost a complete recovery of total acrosin (Fig.4E, lane 1) was illustrated in the anti-OMC32 IP-pellet (Fig.4E, lane 3).

### Immunofluorescence Localization of OMC32, IZUMO1, Acrosin, Lactadherin, and SPACA3

Triton X-100 permeabilized cauda sperm exhibited intense staining of the acrosomal segment with anti-OMC32 (Fig. 5A and A’). Both the apical and principle segments of the acrosome stained intensely as we had reported previously [26]. Triton X-100 permeabilized cauda sperm were stained with anti-IZUMO1 antibody to define the localization of IZUMO1. The equatorial segment of the bovine cauda sperm revealed intense staining, whereas a few sperm heads showed moderate staining over the principal segment (Fig.5B and B’). Using acrosin antibody, we examined the immunological localization of bovine sperm acrosin. Triton X-100-permeabilized cauda sperm exhibited intense staining of the acrosomal segment with anti-acrosin (Fig.5C and C’). Interestingly, detergent-permeabilized bovine cauda sperm stained with anti-lactadherin antibody, displayed intense crescent-like staining over the apical segment and faint staining over the principal segment (Fig.6A and A’). In contrast, SPACA3 exhibited sprinkle-type intense staining over the apical segment when stained with anti-SPACA3 antibody (Fig. 6B and B’). No stain was evident with rabbit preimmune serum (Fig. 6C and C’).

### Fate of IZUMO1, Lactadherin, and SPACA3 during Capacitation and Acrosome Reaction

Immunoblot analysis was utilized to determine whether processing and/or release of IZUMO1, lactadherin, and SPACA3 occurred during capacitation and the acrosome reaction. Immunoblot analyses revealed that under all treatment conditions the entire



IZUMO1 was retained in the sperm pellets (Fig.7A, lanes 1, 2 and 4); no immunoreactive polypeptide was detected in the supernatant fractions (Fig.7A, lanes 3 and 5). On the contrary, as demonstrated in Fig.7B (lanes 1 and 2), the entire SPACA3 polypeptide was retained in the pellet before and after capacitation and a significant portion of SPACA3 was released after LPC-induced acrosome reaction (Fig.7B, lane 5). The remaining portion of SPACA3 was retained in the sperm pellet (Fig.7B, lane 4). It is also noted that lactadherin was not released during the capacitation and the acrosome reaction (Fig.7C, lanes 3 and 5) but remained in the particulate cell subfraction (Fig.7A, lanes 1, 2 and 4). In addition, no differences in the electrophoretic mobility pattern of both IZUMO1, lactadherin, and SPACA3 were noted between the various incubation conditions (Figs.7A–C). Protein tyrosine phosphorylation is a key biochemical event accompanying sperm capacitation [42–44]. To examine whether capacitation successfully occurred under our current conditions, heparin-capacitated sperm were immunostained with anti-phosphotyrosine antibody (anti PY-20; BD Transduction Laboratories, Lexington, KY) for the immunofluorescence localization of tyrosine-phosphorylated polypeptides. The flagellum of capacitated spermatozoa exhibited an intense immunostaining with anti-PY20, while the head was unstained (Fig.7D, panel a). The principal piece segment of the flagellum displayed brighter fluorescence than the mid-piece. In contrast, noncapacitated spermatozoa stained with anti-PY20 displayed no fluorescence (Fig.7D, panel b). When anti-PY20 was preabsorbed with o-phospho-DL-tyrosine and used for staining, no fluorescence of the flagellum of the capacitated spermatozoa was observed (data not shown). This experiment demonstrates that capacitation-dependent tyrosine-phosphorylated proteins localized to the flagellum of bovine spermatozoa. Next, we analyzed our capacitated sperm with immunoblots stained with anti-PY20 (Fig.7E). Both 29 and 26 kDa polypeptides were phosphorylated during capacitation (Fig. 7E, lane 2). Since the 20 kDa phosphorylated polypeptide was present in both the control and capacitated sperm fractions, it suggests that the 20 kDa is a basal phosphorylated protein. Phosphorylation of the bovine cauda sperm polypeptide during capacitation confirms the efficacy of our capacitation conditions. Acrosin levels were analyzed in the pellet and supernatant fractions of non- capacitated and acrosome-reacted spermatozoa to confirm the occurrence of an LPC-induced acrosome reaction. As shown in Table-2, almost 94% acrosin activity remained in the pellet fraction of non-capacitated spermatozoa whereas the addition of LPC to heparin-capacitated sperm exhibited the release of ~85% of total acrosin into the supernatant fraction. Previously, comparable results were also reported in bovine ejaculated and cauda epididymal spermatozoa [26, 45]. To define the fate of IZUMO1 following the LPC-induced acrosome reaction, acrosome reacted bovine sperm were immunostained with anti-IZUMO1. By immunofluorescence all positive staining was observed over the equatorial segment of the acrosome-reacted sperm (Fig.8A) whereas non-capacitated cauda sperm revealed the presence of IZUMO1 over the equatorial and principal segments (Fig.5B). To confirm the effectiveness of the LPC-induced acrosome reaction morphologically, we stained the noncapacitated (Fig.8B) and acrosome-reacted (Fig.8C) sperm stained with FITC-PSA. As shown in Fig.8C, a strong signal was observed over the equatorial segment of the acrosome-reacted sperm whereas noncapacitated spermatozoa (Fig.8B) displayed a homogenous signal over the entire acrosomal region. The release of acrosin (Table-2) and the presence of FITC-PSA stain over the equatorial region (Fig.8C) confirm the efficacy of the LPC-induced acrosome reaction conditions both by

biochemically and morphologically. These studies indicate that the IZUMO1 and lactadherin polypeptides remain associated to the particulate fraction even after the release of acrosomal contents (acrosomal exocytosis), whereas the addition of LPC to heparin-capacitated sperm leads to the release of a significant portion of SPACA3. The relocation of bovine sperm IZUMO1 polypeptide to the equatorial segment occurs during the LPC-induced acrosome reaction.

## Discussion

Mammalian spermatozoa must undergo several morphological and biochemical modifications within the female reproductive tract before they become fully fertilization competent. Two important physiological alterations are capacitation and the acrosome reaction. The spermatozoan acrosome is a modified secretory granule containing a number of hydrolytic enzymes that assist sperm to penetrate the egg. Successful completion of the acrosome reaction is an elementary prerequisite for mammalian fertilization. Acrosomal membrane and matrix are thought to provide a stable scaffold that allows the controlled and sequential release of matrix-associated proteins during the acrosome reaction, as well as to facilitate interactions between the sperm and oocyte [19, 46]. We have previously isolated and characterized a localized, stable acrosomal matrix assembly from the bovine acrosome termed the outer acrosomal membrane-matrix complex (OMC) [21, 27]. This stable matrix assembly exhibits specific binding activity for acrosin [21] and N-acetylglucosaminidase [28]. In the present study we identified and characterized OMC32 binding proteins localized to the acrosomal membrane of cauda spermatozoa. Utilizing protein-targeting strategy, we identified acrosin, lactadherin, SPACA3, and IZUMO1 as novel binding partners for OMC32.

The binding specificity of OMC32 to acrosin strongly suggests that the OMC32 polypeptide plays an important role in the regulation of acrosin release during the acrosome reaction and in maintaining a hydrolase pool at the site of sperm-zona interaction. Previously, we had shown by enzymatic assay that acrosin binds to OMC32. In the current study, we reconfirmed our previous data utilizing proteomic and immunological approaches. Our immunoblot results are in agreement with our previous data; the presence of two forms (44 and 42kDa) of bovine cauda sperm acrosin [21]. Other investigators have previously identified proacrosin-binding proteins in the 28 to 32kDa range that are present in sperm acid extracts of different species [22, 47–53], but neither the distribution of these proacrosin-binding proteins within the acrosome nor their relationship to the acrosomal matrix have been examined. Our previous [21, 28] and present studies demonstrate that acrosin binds to a domain-specific, detergent-resistant, stable acrosomal matrix polypeptide (OMC32). It has been shown that the knock-out acrosin-deficient mice are still fertile, but experience delayed fertilization [54]. Although several studies dispute the critical role of acrosin in mammalian fertilization, the role of acrosin in sperm physiology is not known exclusively. During the acrosome reaction, the acrosome matrix polypeptides are not only activated but also released. Various acrosome matrix components may serve as substrates of activated acrosin and then undergo release from the matrix. The acrosin-deficient mice demonstrated a delayed release of several acrosome proteins (e.g., sp56, MC101) compared to wild type mice [55]. These studies suggest that acrosin accelerates the dispersal of the acrosomal

proteins. Acrosin may participate in fertilization in the processing and/or releasing of other acrosomal proteins or sperm cell membrane proteins during acrosome exocytosis rather than directly hydrolyzing the zona pellucida.

In the present study, we found the presence of a 45kDa lactadherin in the detergent-soluble fraction of bovine cauda sperm (Fig.3C) that interact with OMC32 polypeptide. Our immunolocalization study revealed the presence of lactadherin mainly over the apical segment of detergent permeabilized bovine cauda sperm (Fig.6A). Petrunkina *et al.* [56] showed that in boar spermatozoa the 47kDa lactadherin is present at the apical ridge of sperm head of testicular, epididymal, and ejaculated boar spermatozoa. They proposed that the C-terminal peptide of the C2-like domain lactadherin interacts with the sperm membrane during capacitation and the C2-like domain is homologous to the phosphatidylserine-binding protein region of factor VIII [57] and is highly conserved within the lactadherin family [58]. Utilizing proteomic and co-immunoprecipitation analyses, Miles *et al* [59] presented that green fluorescent protein (GFP) tagged 20S proteasomal core subunit  $\alpha$ -type1 (PSMA1-GFP) interacts with several acrosomal membrane-associated proteins of pig sperm; one of the proteins is lactadherin. They proposed that lactadherin anchors proteasomes to the acrosomal structure and exhibits in proteasome-assisted acrosomal remodeling during sperm capacitation and acrosomal exocytosis. The potential role of boar sperm lactadherin as an integrin RGD-dependent ligand was suggested by Ensslin *et al.* [60] and Anderson *et al.* [61]. Anderson *et al.* [58] also demonstrated that lactadherin can act as a connector between two surfaces by binding to integrin receptors through its N-terminal RGD-binding sites in the second EGF-like domain and to phospholipids through its C-terminal C1/C2-like domains. However, in other cell types, Taylor *et al.* [62] demonstrated that human lactadherin expressed in human milk and breast carcinomas promotes RGD-dependent cell adhesion via integrins. Several studies proposed that lactadherin acts as an anchoring molecule and/or may be involved in other aspects of sperm physiology. The function of lactadherin in association with spermatozoa still remains unclear. It is possible that the interaction of bovine sperm lactadherin to a detergent-insoluble acrosomal matrix protein (OMC32) will elicit a further signal cascade priming the capacitation and/or acrosome reaction. Additionally, it may be involved in preparation/organization of the sperm-egg fusion. Our future studies will address these possibilities.

SPACA3 is one of the four polypeptides that binds to OMC32. Mandal *et al.* [63] first reported a unique, non-bacteriolytic, c lysozyme-like protein, SLLP1, encoded by the gene SPACA3 at locus 17q 11.2 and localized in the acrosome of human spermatozoa. They also showed the presence of SLLP1 on the luminal surface of both inner and outer acrosomal membranes. In the human male reproductive system, four c-type lysozyme genes have been identified [64], one of them is SPACA3. All are highly expressed in the testis and epididymis. SPACA3 plays an important role in fertilization of mice. A human lysozyme-like protein, SLLp1, was identified as an intra-acrosomal sperm protein, and its encoded gene is SPACA3 [65]. In the present study, we showed the presence of a 25kDa SPACA3 immunoreactive band in the detergent-soluble fraction of bovine cauda sperm (Fig. 3A) and SPACA3 exhibited sprinkle-type intense staining over the apical segment when stained with anti-SPACA3 antibody (Fig.6B). Mandal *et al.* [63] reported that the treatment of

capacitated sperm with anti-SLLP1 serum showed a decrease in the number of sperm fused per egg, suggesting the role of SLLP1 in sperm/egg adhesion. They proposed that after the acrosome reaction, SLLP1 could be a potential receptor for the egg oligosaccharide residue N-acetylglucosamine, which is present in the extracellular matrix over the egg plasma membrane and within the perivitelline space, pores of zona pellucida, and cumulus layers [66–68]. In the present study, we have also shown that LPC-induced heparin-capacitated sperm exhibit the release of a significant portion of SPACA3. Previously, it had been shown that several acrosomal proteins are proteolytically processed and released during the acrosome reaction [16, 18–19, 24–25, 69–73]. We propose that acrosin participates in the release of SPACA3 during acrosome exocytosis. Future studies will address the mechanism of release of bovine sperm SPACA3 during the acrosome reaction.

OMC32 also binds to a 40kDa IZUMO1 of bovine cauda sperm. IZUMO1 (named after a Japanese shrine dedicated to marriage), a protein that spans the sperm's plasma membrane, a novel member of immunoglobulin superfamily, is essential for fertilization in mammals [74]. It is localized on the mouse sperm head and is encoded by a single-copy gene on mouse chromosome 7. The roles of N-glycan (~6kDa N-linked oligosachharides) in the mouse sperm IZUMO1 is to protect IZUMO1 from fragmentation during sperm maturation in the epididymis [75]. IZUMO1-deficient male mice and CD9-deficient female mice exhibited infertility resulting from a severe impaired sperm-egg fusion [76]. Utilizing co-immunoprecipitation studies, Ellerman *et al.* [77] showed that other mouse sperm proteins interact with IZUMO1, suggesting formation of a multiprotein membrane complex. It has been shown that IZUMO1 interacts with ACE3 (angiotensin1-converting enzyme 3) [76]. Our both protein targeting strategy and co-immunoprecipitation studies revealed that IZUMO1 interacts with a 32kDa detergent insoluble acrosomal matrix polypeptide (OMC32). Miranda *et al.* [78] observed the localization of IZUMO1 on regions adjacent to the equatorial segment of acrosome-reacted mouse sperm, whereas Ellerman *et al.* [77] observed IZUMO1 localized to either the entire acrosomal region or on the anterior acrosome. Our immunofluorescence localization results displayed the presence of IZUMO1 at the equatorial segment of the bovine cauda sperm, whereas a few sperm heads showed moderate staining over the principal segment (Fig.5B). The different results for localization of IZUMO1 on acrosome-reacted sperm using different antibodies raise the possibility of the existence of separate subpopulations of the molecule, further studies are required to clarify the localization of IZUMO1 on fusion-competent sperm. Yanagimachi [79] strongly suggests that sperm fuses with egg through a region overlapping either the equatorial segment or the post acrosomal region depending on the species. In order for fusion to occur, IZUMO1 needs to relocate from the anterior head to equatorial segment or the post acrosomal region during the acrosome reaction. Testis-specific serine kinase 6 (Tssk6)-null sperm fail to relocate IZUMO1 during the acrosome reaction. Based on the results, they proposed that Tssk6 plays a role in the changes of IZUMO1 localization and that these changes are essential for gamete fusion [80]. In the present studies, we have shown that bovine sperm IZUMO1 polypeptide remains associated to the particulate fraction even after the release of acrosomal contents (acrosomal exocytosis). Ellerman *et al.* [77] found that the 55kDa form of IZUMO1 was retained on the acrosome-reacted mouse sperm. They proposed that IZUMO1 plays a role in gamete fusion by organizing or stabilizing a

molecular complex on the sperm membrane. The relocation of IZUMO1 from the acrosomal cap to the equatorial segment occurs during mouse sperm acrosome reaction and further over the whole sperm head during spontaneous acrosome reaction [81]. Rat sperm IZUMO1 undergoes a significant phosphorylation during capacitation [82]. Utilizing a fluorescent tag, they reported the dynamics of diffusion of mouse sperm IZUMO1 from the acrosomal membrane to the surface at the time of the acrosome reaction. IZUMO1 localized to the equatorial segment of the sperm surface after the acrosome reaction. This region is considered to initiate fusion with the oolemma. The proteins CD9 on egg and IZUMO1 on spermatozoa are the only two factors proven so far to be essential in sperm-egg fusion [83]. For the relocation of IZUMO1, it is likely that rat sperm IZUMO1 becomes associated with other proteins. These protein-protein interactions might be regulated by the phosphorylation status of the molecule. TSSK6 may phosphorylate IZUMO1 as these cells prepare for fertilization. These phosphor-regulations are likely to act as a scaffold [84]. A putative functional site of IZUMO1 exists in the N-terminal region (Asp5-Leu113). The formation of a helical dimer at the N-terminal region is essential for the function of IZUMO1. IZUMO1 alone cannot cause sperm-egg membrane fusion; rather it induces cellular surface interactions such as membrane tethering [85]. Bianchi *et al.* [86] showed that recombinant IZUMO1 binds both wild-type and CD9-deficient eggs, suggesting that IZUMO1 interacts with an egg receptor other than CD9. They identified the folate receptor (Folr4) as the receptor for IZUMO1 expressed on the mouse egg surface, a GPI-anchored protein, and is essential for female fertility. Folr4 cannot bind to folate and is named as “Juno”. IZUMO1-Juno interaction is conserved within several mammalian species. Female mice lacking Juno are infertile and Juno-deficient eggs do not fuse with normal sperm. The rapid shedding of Juno from the egg membrane within vesicles after fertilization suggests a mechanism for the membrane block to polyspermy, ensuring eggs normally fuse with just a single sperm [87]. Wassarman [88] proposed that local clustering of Juno on the egg’s membrane, possibly organized by another membrane-spanning protein such as CD9, might occur to increase the strength of sperm binding. Our data demonstrate the presence of an entire pool of bovine sperm IZUMO1 (Fig.6A) in the particulate cell subfraction, associated with the hybrid membrane complex following the acrosome reaction. Previously, we had shown that OMCrpf polypeptides, OMC32 polypeptide is one of the OMCrpf polypeptides, exclusively localized to a stable acrosomal matrix assembly associated with the outer acrosomal membrane and remain particulate succeeding the acrosome reaction. Our present study reveals that bovine sperm IZUMO1 relocates to the equatorial segment during the LPC-induced acrosome reaction (Fig. 8C). We propose that the OMC32- IZUMO1 complex in the acrosome may function to maintain an IZUMO1 pool at the equatorial segment after the acrosome reaction for the successful completion of sperm-egg fusion. We hypothesize that the interaction of OMCrpf polypeptides/OMC32 polypeptide with other acrosomal proteins regulate sperm function in mammalian fertilization. Additional studies are needed to address this issue.

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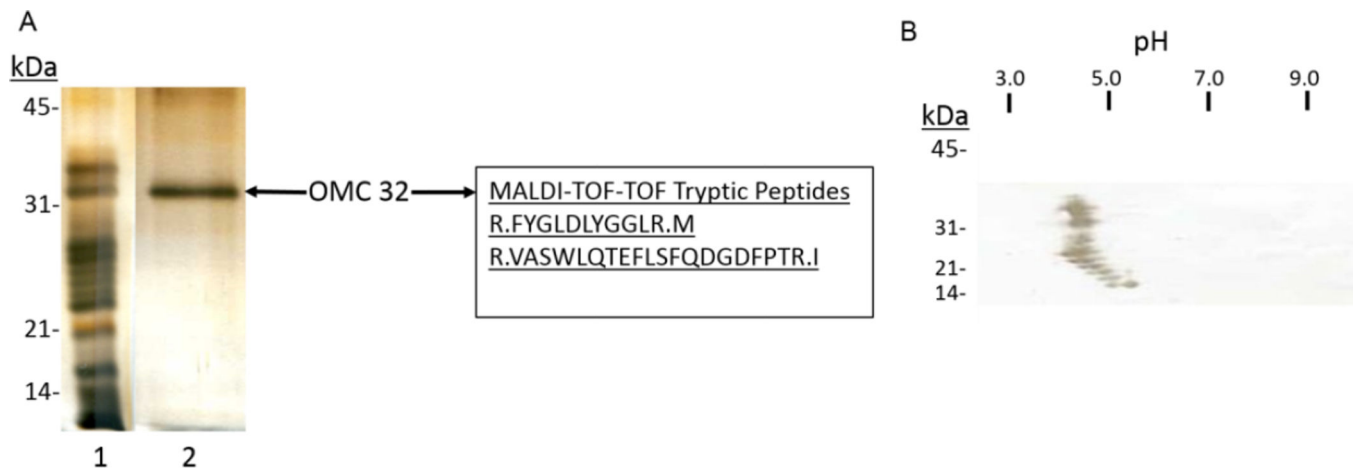
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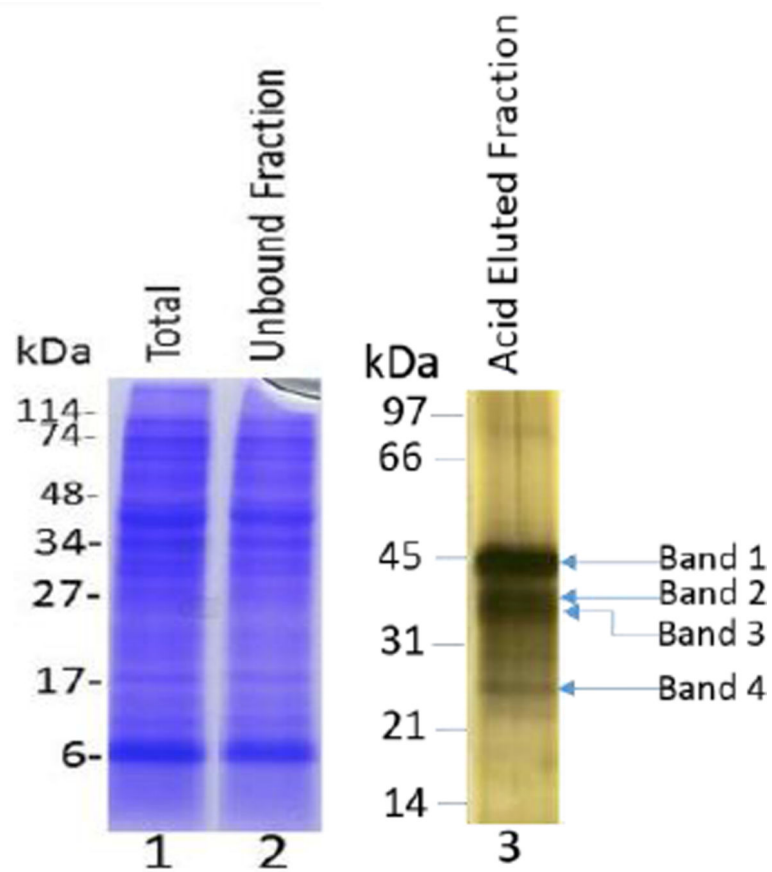
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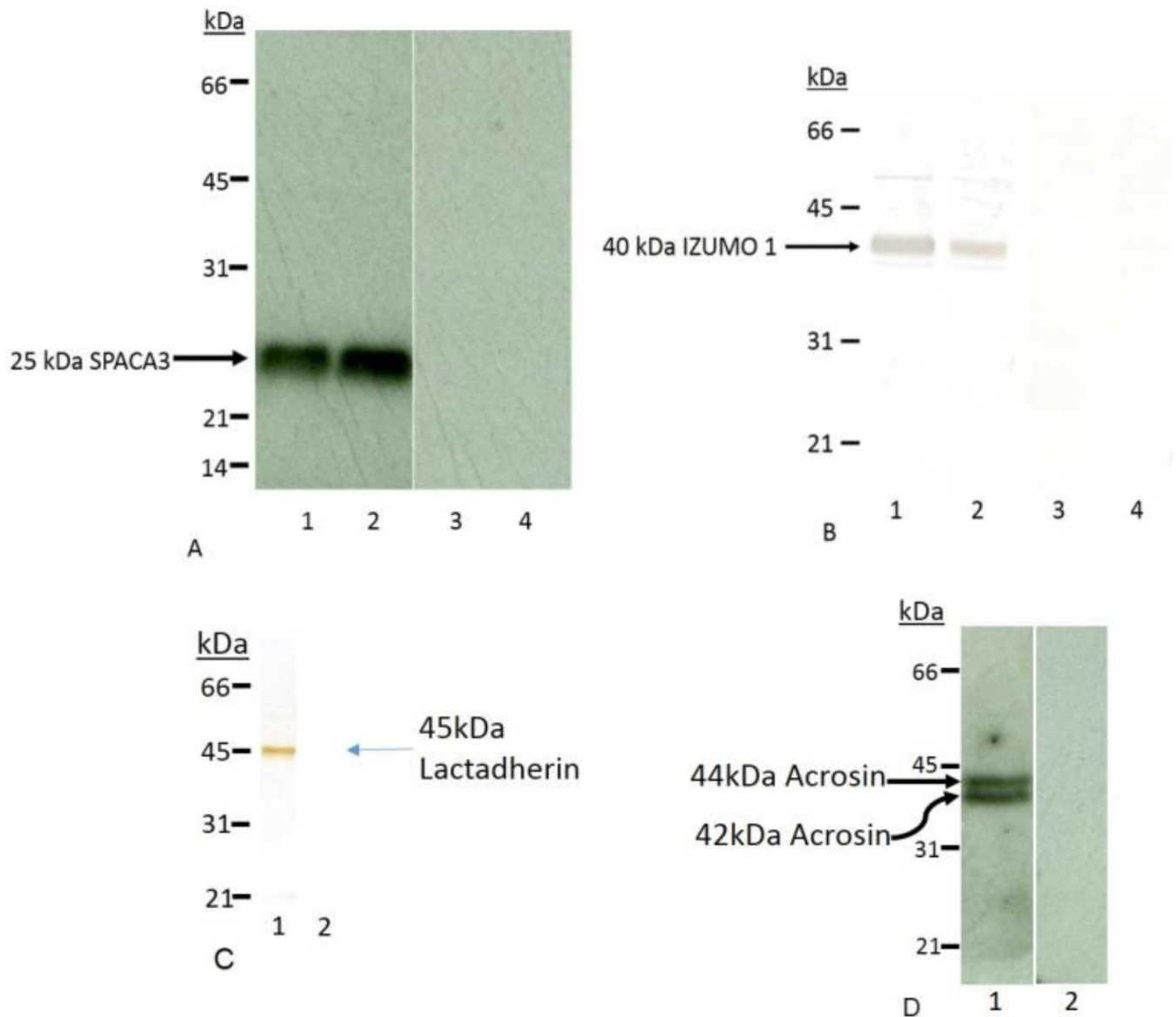
**Figure 1.**

A: Silver-stained SDS-polyacrylamide gel. Lane 1 shows that the 38–19 kDa family of polypeptides are released by high-pH extraction. Lane 2 shows the purity of the 32kDa polypeptide that was characterized by proteomic identification utilizing MALDI-TOF-TOF analysis (shown in box).

B: Immunoblot, stained with anti-OMC32, of total cauda sperm OMC fractionated by two-dimensional PAGE.

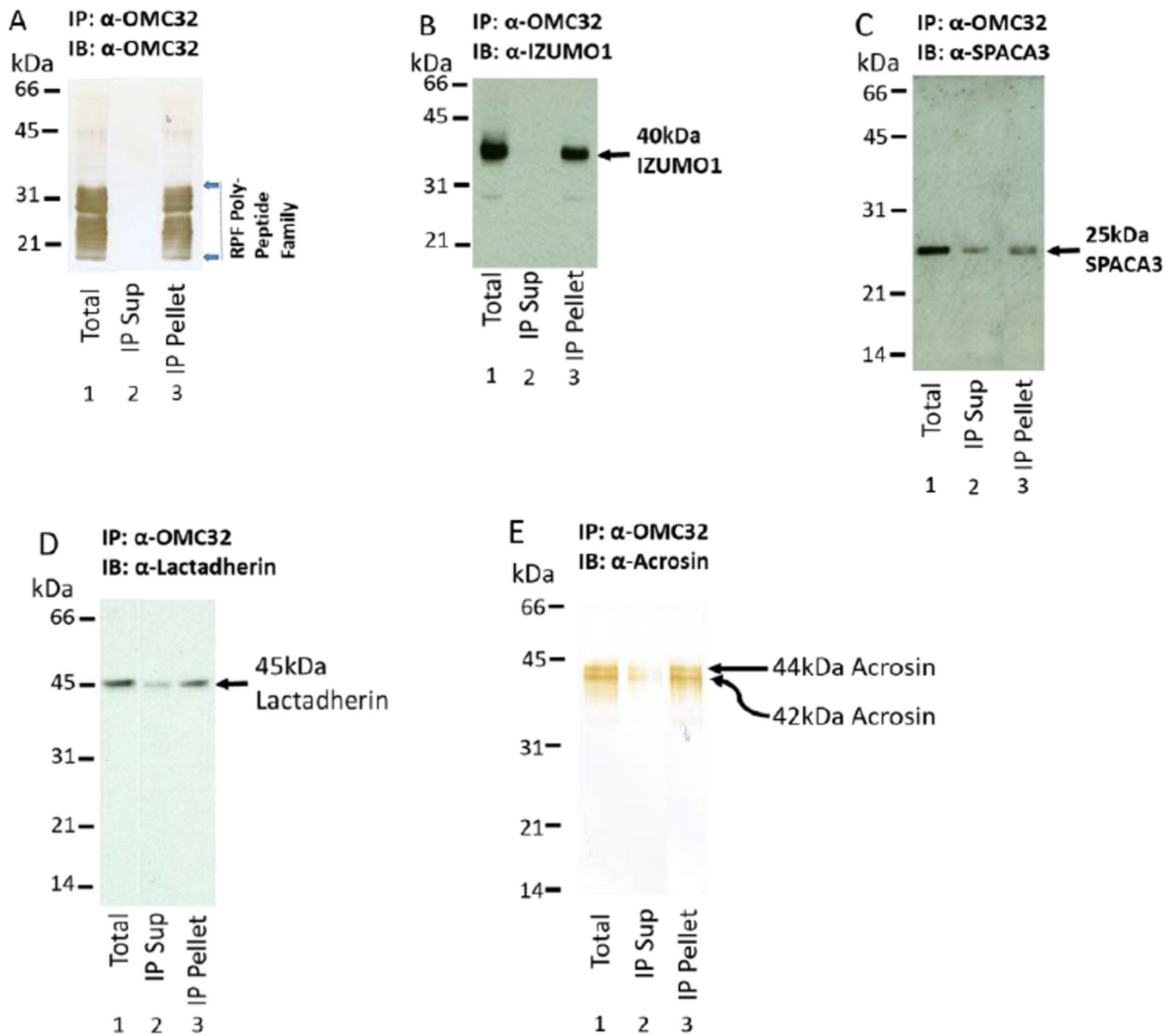


**Figure 2.** OMC32 (0.5mg) polypeptide was coupled to an AminoLink Plus resin at pH 10.0. Bovine cauda sperm was extracted in TNI containing 0.1% Triton X-100 (TNI-TX) at 4°C for 1 hr and centrifuged at 100,000 X g for 30 min in a Beckman SW40 rotor. The supernatant obtained after centrifugation was applied to the OMC32 coupled column. The column was washed with 10 column volumes of TNI-TX solution and then eluted with 0.1M Glycine-HCl, pH 2.5. Lane 1 shows the pattern of polypeptides in the detergent soluble fraction of bovine cauda sperm. Lane 2 reveals the unbound polypeptides to the OMC32 coupled column. The acid eluted fraction of the OMC32 affinity column was stained with silver (lane 3). Two major bands, 1 & 4, and two minor bands, 2 & 3, as shown in Fig.2, lane 3, are subjected to MALDI-TOF-TOF analysis for the proteomic identification.



**Figure 3.**

The detergent-soluble fraction of bovine cauda sperm was separated by SDS-PAGE and transferred to PVDF membranes for immunoblot analysis. Western blots were stained with anti-SPACA3 (Fig. A, lanes 1, 2), anti-IZUMO1 (Fig. B, lanes 1, 2), anti-lactadherin (Fig. C, lanes 1, 2), and anti-acrosin (Fig. D, lane 1). Lanes 3 and 4 of Figures A and B and lane 2 of Figures C and D were stained with pre-immune IgG. 5 $\mu$ g protein was loaded into lanes 1 and 3, and 10 $\mu$ g protein was loaded into lanes 2 and 4 of Figure A whereas lanes 1 and 3 and lanes 2 and 4 of Figure B represent 10 $\mu$ g and 5 $\mu$ g protein, respectively. In Figures C and D, 10 $\mu$ g protein was loaded into lanes 1 and 2.

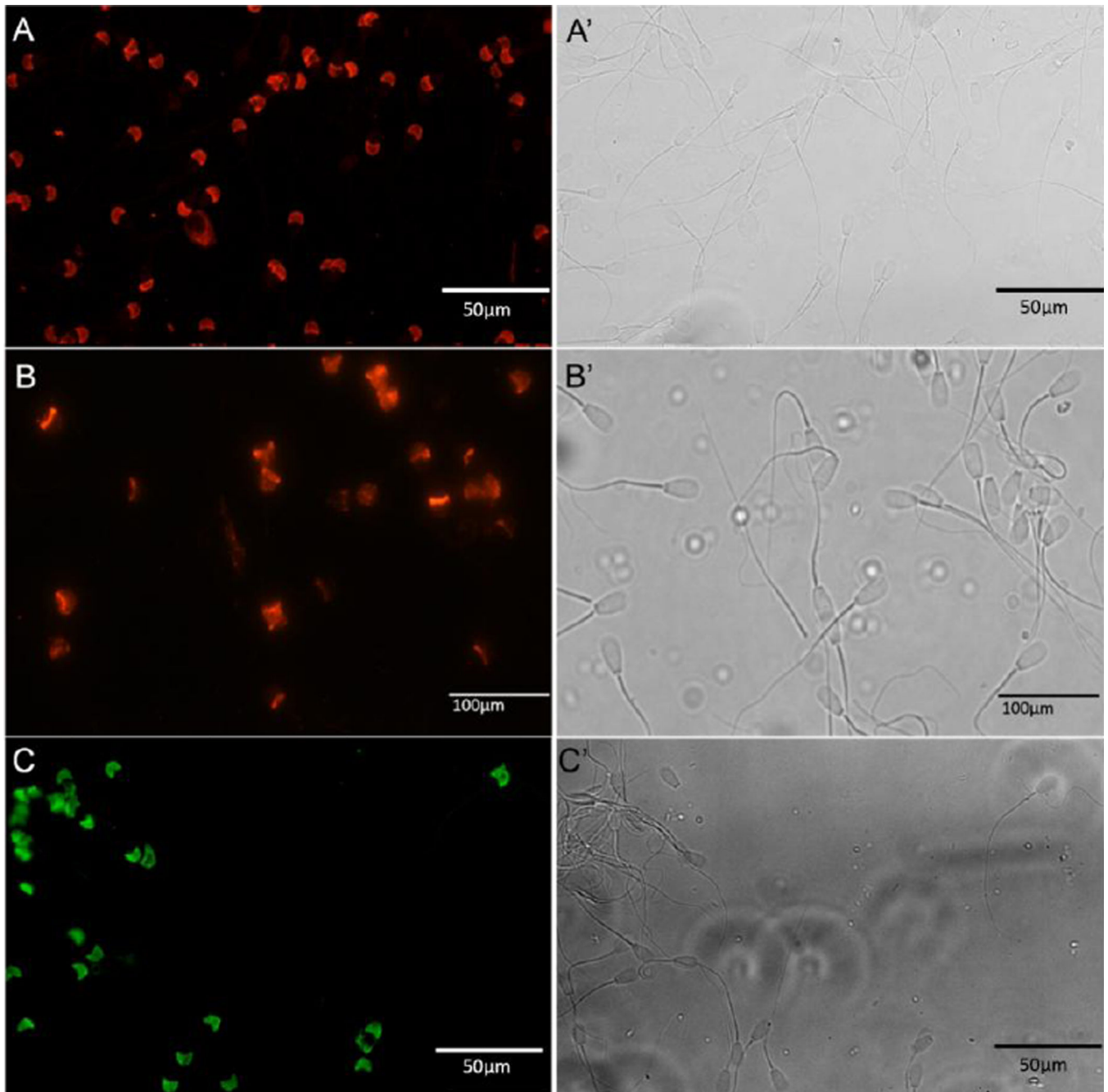
**Figure 4.**

Identification of OMC32-associated proteins by co-immunoprecipitation analysis. The supernatant (~200 $\mu$ g protein) obtained after RIPA buffer extraction of cauda sperm was immunoprecipitated with anti-OMC32 and analyzed by reducing SDS-PAGE on a 12% gel. **A-** Immunoblot of soluble fraction of cauda sperm after RIPA buffer extraction (Total-lane 1), immunoprecipitation supernatant (lane 2), and anti-OMC32 IP pellet (lane 3) immunostained with anti-OMC32. Note that a complete recovery of “rpf” polypeptides (38–19kDa) was observed in the anti-OMC32 immunoprecipitation pellet (lane 3). **B-** Western blot of soluble fraction of cauda sperm after RIPA buffer extraction (Total-lane 1), immunoprecipitation supernatant (lane 2), and anti-OMC32 IP pellet (lane 3) immunostained with anti-IZUMO1. As shown in lane 3, IZUMO1 was completely recovered in anti-OMC32 immunoprecipitation pellet.

**C-** Total RIPA buffer soluble fraction of cauda sperm (lane 1), immunoprecipitation supernatant (lane 2), and anti-OMC32 IP pellet (lane 3) immunostained with anti-SPACA3. A substantial portion of total SPACA3 was present in the IP pellet (lane 3) and the remaining SPACA3 was present in the IP-Sup (lane 2).

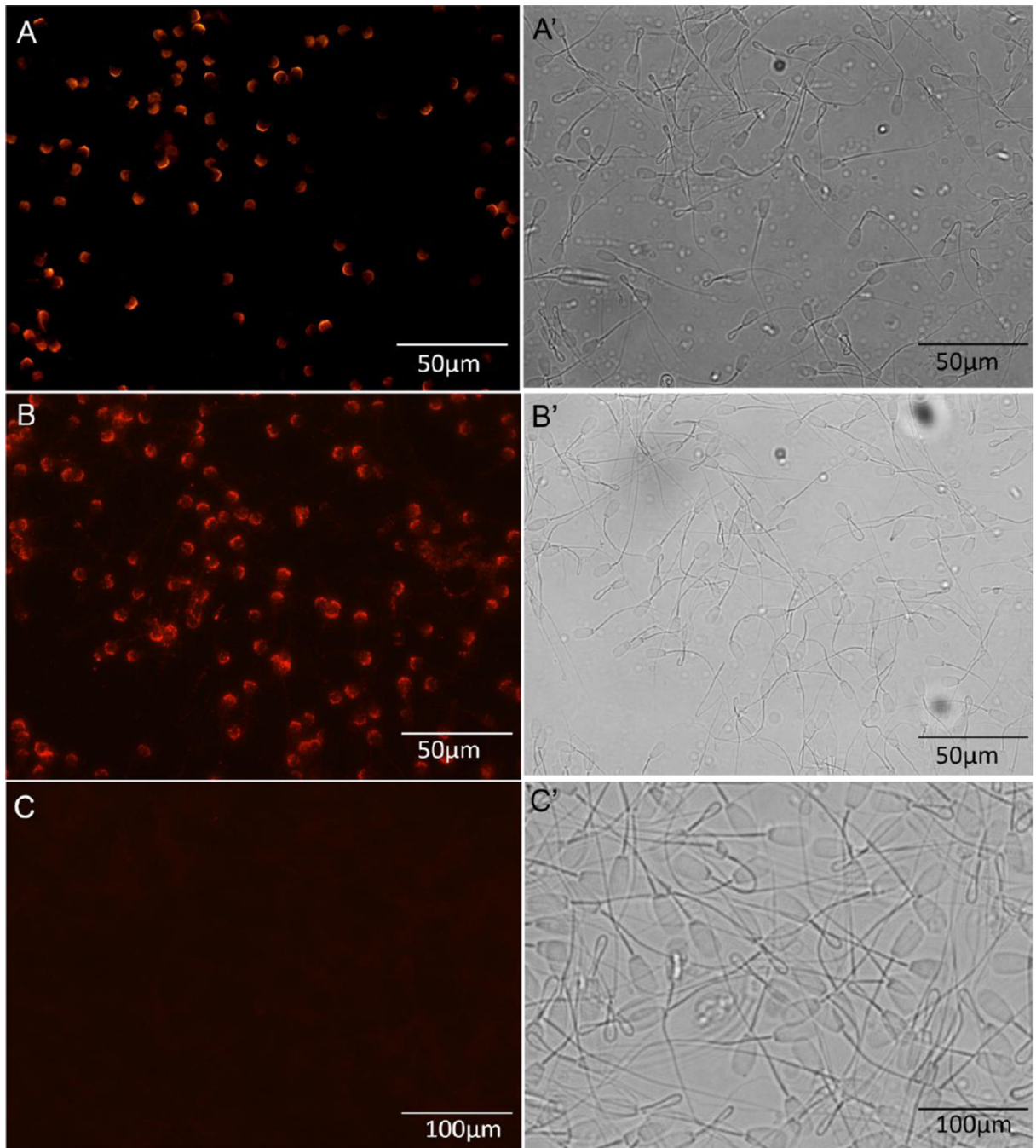
**D-** Western blot of soluble fraction of cauda sperm after RIPA buffer extraction (Total-lane 1), immunoprecipitation supernatant (lane 2), and anti-OMC32 IP pellet (lane 3) immunostained with anti- lactadherin. In the IP pellet (lane 3), a major portion of total lactadherin is achieved and the remaining lactadherin was present in the IP-Sup (lane 2).

**E-** Immunoblot of soluble fraction of cauda sperm after RIPA buffer extraction (Total-lane 1), immunoprecipitation supernatant (lane 2), and anti-OMC32 IP pellet (lane 3) immunostained with anti-acrosin. Note that almost a complete recovery of acrosin was observed in the anti-OMC32 immunoprecipitation pellet (lane 3).

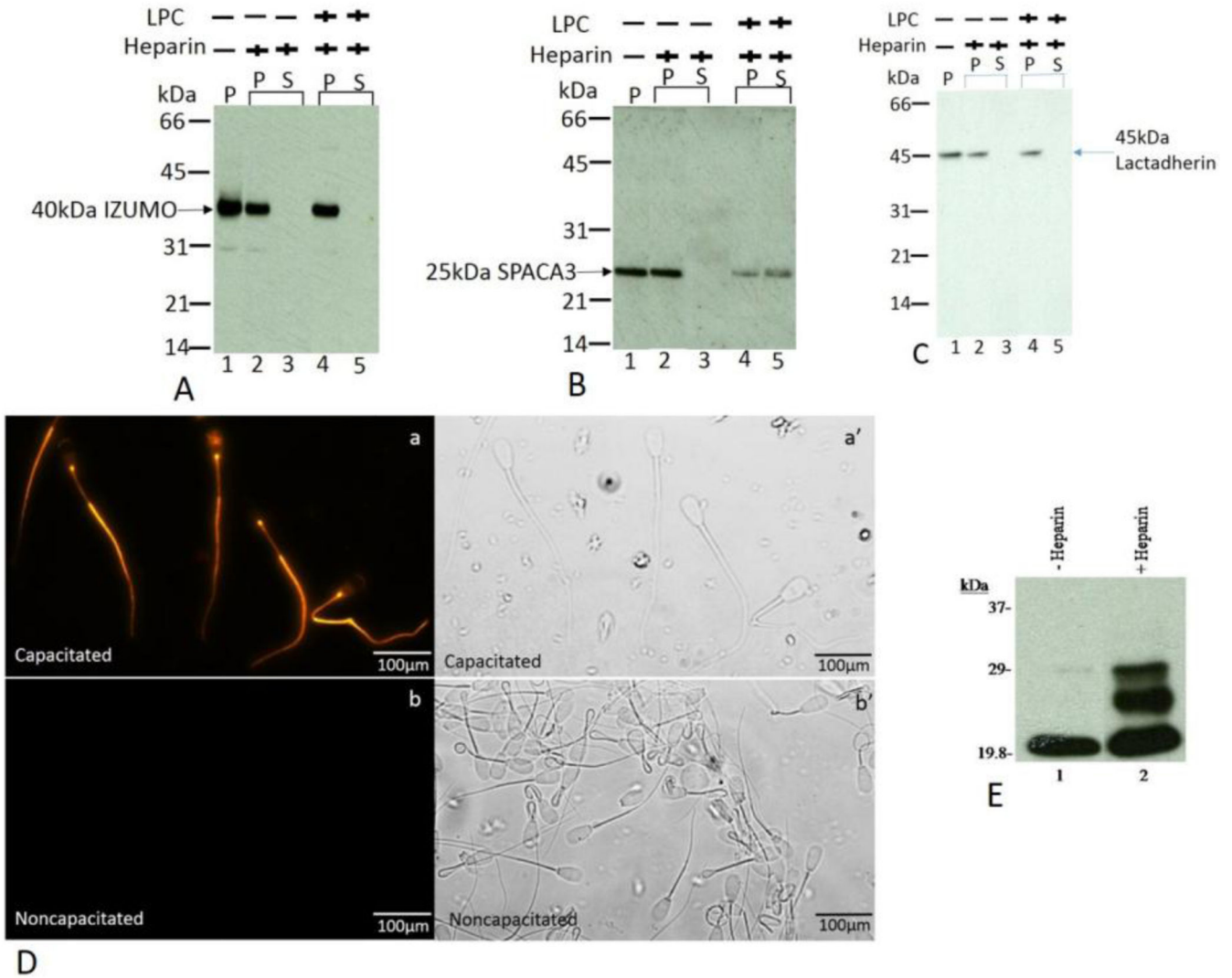


**Figure 5.** Immunocytochemical localization of OMC32 (panel A), IZUMO1 (panel B), and acrosin (panel C) in bovine cauda epididymal spermatozoa. Matched phase-contrast (A', B', and C') and fluorescence photomicrographs (A, B, and C) of spermatozoa stained with anti-OMC32 (A and A'), anti-IZUMO1 (B and B'), and anti-acrosin (C and C') are shown.





**Figure 6.** Paired phase-contrast (A', B', and C') and fluorescence images (A, B, and C) of bovine cauda sperm immunostained with anti-lactadherin (A and A'), anti-SPACA3 (B and B') antibodies and preimmune serum (C and C').



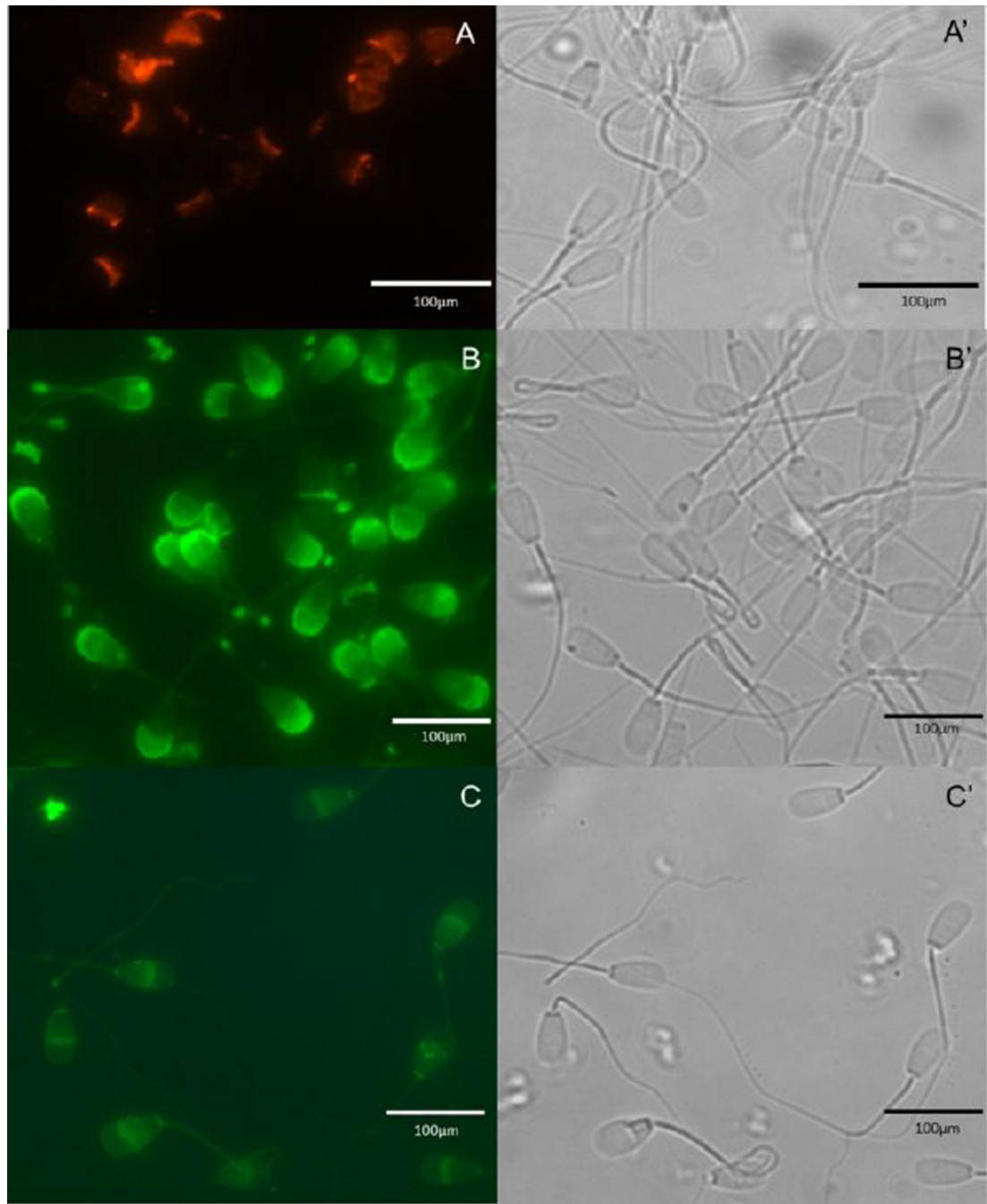
**Figure 7.**

A, B, and C: Immunoblot analysis of the pattern of IZUMO1 (Fig. A), SPACA3 (Fig. B), and lactadherin (Fig. C) polypeptides in the particulate (P) and supernatant (S) fractions of spermatozoa after a 4 hr incubation in the presence or absence of heparin and with or without a final 15 min exposure to LPC. The immunoblot was stained with anti-IZUMO1 (Fig. A), anti-SPACA3 (Fig. B), anti-lactadherin (Fig. C). Note that under each treatment condition the entire IZUMO1 and lactadherin pools remain in the pellets (Figs. 7A and 7C, lanes 1, 2 and 4); no immunoreactive band was detected in the supernatant fractions (Figs. 7A and 7C, lanes 3 and 5). On the contrary, as demonstrated in Figure 7B (lanes 1 and 2), the entire SPACA3 polypeptide was retained in the pellet of noncapacitated and capacitated sperm pellet fractions and a significant portion of SPACA3 was released after LPC-induced acrosome reaction (Fig.7B, lane 5). The remaining portion of SPACA3 was retained in the sperm pellet (Fig.7B, lane 4).

D: Immunocytochemical localization of tyrosine-phosphorylated proteins in capacitated (panel a and a') and noncapacitated spermatozoa (panel b and b'). Matched phase contrast

(panels a' and b') and fluorescence (panels a and b) photomicrographs of spermatozoa stained with (anti-PY20).

E: Protein tyrosine-phosphorylation pattern of cauda epididymal spermatozoa. Western blots of noncapacitated (lane 1) and capacitated (lane 2) spermatozoa fractionated by reducing SDS-PAGE and stained with anti-phosphotyrosine (anti-PY20).



**Figure 8.** Matched phase contrast (panel A') and fluorescence (panel A) photomicrographs of LPC-induced acrosome reacted bovine sperm immunostained with anti-IZUMO1. Note that the cells show specific staining at the equatorial segment, demonstrating the re-localization of entire pool of IZUMO1 over the equatorial segment in acrosome-reacted spermatozoa. Matched phase contrast (B' & C') and fluorescence (B & C') images of noncapacitated sperm (Fig. B & B') and acrosome-reacted sperm (Fig. C & C') stained with FITC-

conjugated PSA. PSA stained confirmed the LPC-induced acrosome reaction of bovine sperm.

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**Table 1**  
**Identification of OMC32 Associated Proteins by MALDI-TOF-TOF Proteomic Analysis**

Proteomic identification of four individual polypeptides (two major bands 1 & 4 and two minor bands 2 & 3 as shown in Fig.2, lane 3) by MALDI-TOF-TOF analysis are presented in Table-1

Bands from Lane 3	Protein	Matches Peptides
Band 1	PAS-6 and PAS-7 Proteins (Lactadherin)	R.QFQFIQVAGR.S K.NIFETPFQAR.F
		R.DFGHIQYVAAYR.V K.INLFDTPLETQYVR.L
Band 2	Acrosin	K.EVEWGSNKPKPPLQER.Y
Band 3	Izumo Sperm-Egg Fusion Protein 4 Isoform 1	K.LNQVANAVYQR.M K.ELHLAIPAEITR.E K.MYFPGYFPNELR.A R.EQVHLIQNAIIESR.I
Band 4	SPACA3 Protein (Sperm Acrosome Membrane Associated Protein)	R.VLQDFGLEGYR.G

**Table 2**  
**Distribution of Total Acrosin of Non-Capacitated and Acrosome-Reacted Spermatozoa**

The distribution of acrosin between pellet and supernatant fractions is shown in Table-2. Only acrosome-reacted sperm exhibit substantial acrosin release to the supernatant fraction. Values represent mean  $\pm$  SD of three experiments.

	Non-Capacitated Sperm	Acrosome-Reacted Sperm
Pellet	94 $\pm$ 6	15 $\pm$ 5
Supernatant	6 $\pm$ 4	85 $\pm$ 7

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