Biochemical Characterization of Two Major Proteins of the Hamster Sperm Acrosomal Matrix

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Abstract

An acrosomal fraction, termed the ALM (acrosomal lamina complex) was isolated from hamster cauda epididymal spermatozoa that contain specific domains of the acrosomal matrix and an adherent detergent-insoluble complex, termed the acrosomal lamina, which is derived from the outer acrosomal membrane. By SDS-PAGE, the ALM fraction exhibited two major polypeptides of M_r=29,000 (ALM29) and M_r=22,000 (ALM22). We have also shown that an ALM complex binds both acrosin and N-acetylglucosaminidase (NAGA) in a dose-dependent manner and acrosin binds to the ALM29 polypeptide only. The objective of the present study is to investigate the binding efficiency of acrosin to the V8 protease-generated peptides of ALM29 and ALM22 polypeptides, to examine the presence of phosphate groups on both ALM29 and ALM22 polypeptides and to identify the binding competency of dephosphorylated ALM29 polypeptide to acrosin. Diagonal gel electrophoresis was performed to investigate whether both ALM29 and ALM22 polypeptides are joined by disulfide bridge(s). Both polypeptides migrate on a diagonal line with a comparable R_f in both non-reduced and reduced conditions suggesting that both polypeptides are not joined by disulfide bridge(s). All V8 protease-digested peptides of ALM29 and ALM22 showed immunoreactive bands when stained with anti-ALM22 antibody suggesting that all peptides are antigenically related family. Both ALM29 and ALM22 polypeptides were stripped out of the ALM complex by high pH (pH-11) extraction and were treated with alkaline phosphatase followed by immunoblot analysis. Both ALM29 and ALM22 polypeptides showed a reduction in size (~3 kDa) by alkaline phosphatase treatment. A sedimentation assay was employed to determine whether alkanine phosphate treated ALM29 polypeptide possesses acrosin binding. Dephosphorylated ALM29 polypeptide revealed a significant reduction (~50%) in acrosin binding in comparison with acrosin to a native ALM29 polypeptide. Our studies conclude that both ALM29 and ALM22 polypeptides are phosphorylated and demonstrate the role of phosphate group of ALM29 polypeptide in acrosin binding.

Keywords: Hamster Sperm; Acrosome; Acrosomal Protein; Acrosin

Introduction

Testicular spermatozoa are morphologically differentiated cells. They contain a highly condensed DNA, and transcription and translational processes are silent. Therefore, post testicular maturation of spermatozoa in the epididymis represents the main physiological event that develops both forward motility and fertilizing ability [1,2]. Among the several organelle modifications, the post-testicular changes of the acrosome in the epididymis include remodeling of acrosomal shape, development of distinct matrix domains, and the processing of specific acrosomal glycoproteins [3-10] and hydrolases such as proacrosin [11,12]. Spermatozoa exiting the epididymis are still fertilization incompetent. To achieve fertilizing competency, spermatozoa require residence in the microenvironment of the female tract for a finite period of time. This acquisition of functional competence is termed capacitation, during which, the membranes become more readily fusogenic [2,13-16]. Following capacitation, the interaction of sperm with specific zona pellucida molecules is thought to trigger acrosomal exocytosis through a ligand-receptor mediated signal transduction pathway. Recently, Jin et al. [17] showed that almost all fertilizing mouse sperm have undergone acrosomal exocytosis prior to the time they penetrate the zona pellucida utilizing genetically engineered mouse strains that express green fluorescent protein in the acrosomes. This study strongly raised the question of the acceptance of a well-established concept that intact mammalian sperm acrosomes are required to bind the zona pellucida prior to undergoing acrosomal exocytosis so that sperm can penetrate egg investments and fertilize the egg. During the acrosome reaction the periacrosomal plasma membrane and the underlying outer acrosomal membrane undergo irreversible extensive fusion, while the modulation of acrosomal matrix occurs which leads to both the dissemination of hydrolases within the acrosome and their release during the acrosome reaction [18-21].

Acrosin, a trypsin-like endoprotease derived from the enzymatically inactive precursor proacrosin, functions both in sperm binding and in penetration of the zonapellucida [18,22]. Natural and synthetic inhibitors of acrosin prevent fertilization both in vivo and in vitro [23,24]. Although Baba et al. [25] showed that the sperm of knockout acrosin-deficient mice penetrate the zona pellucida and fertilize the egg except for delayed fertilization, Yamagata et al. [26] demonstrated that the male Acrosin-/- mice delayed the release of several acrosomal components (e.g., SP56, Mc101) compared to wild type during acrosomal exocytosis suggesting the role of acrosin in accelerating the dispersal of the acrosomal matrix components. All these studies raise the possibility to re-examine the role of acrosin in mammalian fertilization. N-acetylglucosaminidase (NAGA) is a major acrosomal glycohydrolase which, in mouse sperm, exhibits a 20-fold higher activity than other acrosomal glycohydrolases [27]. NAGA is released during the acrosome reaction [27-29], and a specific NAGA inhibitor blocks the penetration of mouse sperm through the zonapellucida [27]. It has been proposed that, in the boar, NAGA functions before sperm interaction with the zonapellucida, facilitating sperm passage through the cumulus cell layer [28]. Thus, several studies suggest that mammalian fertilization is indeed a complicated process in which multiple hydrolases are involved. In several mammalian species, the acrosomal contents are segregated into spatially distinct domains of differing ultrastructural appearance [30-32]. In the guinea pig, specific acrosomal matrix polypeptides localized to restricted domains of the apical segment have been identified and characterized [32-36]. This suggests that regionally localized matrix assemblies may partition the acrosomal interior into distinct functional domains. It has also been proposed that binding interactions between the acrosomal matrix and specific hydrolases may account for the differences between hydrolases in their solubility and release rates during the acrosome reaction [20,37-41]. For example, while some acrosomal proteins such as hyaluronidase, dipeptidylpeptidase I, and AA 1 do not appear to be associated with the acrosomal matrix and are freely soluble and rapidly released upon cell disruption or during the acrosome reaction, other components, such as acrosin, are associated with a particular sperm function and are released slowly during the acrosome reaction [37-40,42-45]. Currently, we have shown 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 [46].

The acrosome reaction is a key control point in the fertilization process; the extent to which it impairs fertility in mammals is not well documented. The mechanism by which the acrosome and the acrosomal matrix polypeptides facilitate sperm-egg interactions remain unresolved. Previously, Nagdas et al. [21] identified and biochemically characterized an acrosomal lamina-matrix complex, termed ALM, from hamster cauda epididymal spermatozoa. This stable acrosomal matrix

fraction exhibits two major polypeptides of 29kDa (ALM 29) and 22kDa (ALM22). We have also shown that an ALM complex binds both acrosin and N-acetylglucosaminidase (NAGA) in a dose-dependent manner [21]. In a blot overlay assay, we have also shown that ALM29 polypeptide, but not ALM22 polypeptide, exhibits acrosin-binding activity [21]. In addition, we have previously demonstrated both ALM29 and ALM22 polypeptides are structurally related and exhibit sequence homology to mouse sperm acrosomal protein mSP10 [47]. The present study is focused on the binding efficiency of acrosin to the V8 protease-generated peptides of ALM29 and ALM22 polypeptides, to examine whether ALM29 and ALM22 polypeptides are phosphorylated, and to analyze the binding competency of dephosphorylated ALM complex to acrosin.

Material and Methods

Sperm Preparation

Mature male golden hamsters were housed in the Benedict College Animal care facility on a 14L: 10D cycle and given free access to food and water. Care and use of animals conformed to NIH guidelines for humane animal care and use in research, and all protocols were approved by the institutional Animal Care and Use Committee. Animals were asphyxiated with CO_2 and the cauda epididymides were removed, minced, and incubated for five minutes at 37oC in calcium-free Tyrodes medium to permit sperm release. The sperm suspension was centrifuged at 100 x g for 1 minute to sediment epididymal tubule fragments, and the supernatant was re-centrifuged at 1,500 x g for 10 minute at 4°C to obtain a sperm pellet for the following protocols.

Isolation of the acrosomal fraction

The ALM complex was isolated from cauda epididymal spermatozoa following the method of Nagdas et al. [21]. Sperm from the cauda epididymides were re-suspended in 10 volumes of ice-cold extraction solution (TX-TNI) composed of 0.1% Triton X-100 in TNI (TNI= 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 immediately centrifuged at 500 x g for 10 min. The pellet was extracted for 30 min at 4°C with TX-TNI and homogenized for 10-20 strokes with a glass silicone-coated homogenizer to detach the ALM complex from the sperm heads. Twenty milliliters of the sperm suspension were mixed with 100 mL of 45% Percoll, 0.25 M sucrose, 0.1% Triton X-100, and 25 mMTris-HCl (pH 7.0), and then centrifuged at 60,000 x g for 35 min in a Beckman 70Ti rotor (Beckman Instruments, Palo Alto, C.A). The ALM fraction formed a band at the top of the gradient which was collected, diluted with TNI, and pelleted at 100,000 x g for 20 min in a Beckman SW40 rotor.

Gel electrophoresis and western blotting

SDS-PAGE was performed on 12% or 15% polyacrylamide gels [48]. Polypeptide bands were stained with Coomassie blue [49]. Western Blot analysis was performed by the electrophoretic transfer of polypeptides to PVDF membranes [50]. Diagonal gel electrophoresis was performed to investigate whether both ALM29 and ALM22 polypeptides are joined by disulfide bridge(s) following the method of Wang and Richards [51]. For two-dimensional PAGE, Bio-Rad pre-cast immobilized pH (3-10) gradient strips (7cm) were used for isoelectric focusing (IEF) in a Bio-Rad Protean IEF cell following the manufacturer's instructions.

Immunoblot analysis

Immunoblots were blocked overnight at 4°C in PBS (150 mMNaCl, 20 mM sodium phosphate, pH 7.6) containing 0.1 % Tween-20, 2.5% BSA, 5% heat-inactivated normal goat serum (NGS), and 5% nonfat goat milk. After three rinses in PBS containing 5% NGS and 0.1% Tween-20 (PBS-NGS), blots were then incubated with preimmune and immune serum (anti-ALM22 polyclonal antibody) diluted in PBS-NGS for 1 hour. After three washes in PBS-NGS, the blots were incubated in an affinity-purified horseradish peroxidase-conjugated goat antiguinea pig IgG (KPL Inc., Gaitherburg, MD) in PBS-NGS for one hour. Blots were washed three times in PBS, and immunoreactive bands were visualized using diaminobenzidine for color development.

Assay of hydrolases

For the assay of hydrolases, sperm pellets were resuspended in TN (150 mM NaCl, 25 mM Tris-HCl, pH 7.5) and centrifuged at 5,000 rpm for 10 minutes at 4°C. The pellets were then extracted into a high-salt Triton X-100 solution (500mM NaCl, 0.1% Triton X-100, 25mM Tris-HCl, pH 7.5) for 1 hour at 4°C and centrifuged at 14,000 rpm for 10 minutes. The supernatant will be used as a source of acrosin. Acrosin activity was measured spectophotometrically at 410 nm in 0.05M Tris-HCl buffer (pH 8.0), 0.1% Triton X-100, 0.05 M $CaCl_2$, and 0.1 mM N-p-TosylGly-Pro-Arg-p-niroanilide (Sigma Chemical Co., St. Louis, MO) [20]. One unit of acrosin is described as the quantity of enzyme required to hydrolyze one micromole of N-p-Tosyl-Gly-Pro-Arg-p-nitroanilide per minute. Protein was estimated by the procedure of Bradford [52].

Purification of ALM polypeptides and peptide mapping

High pH extraction of ALM fraction in 0.1M CAPS (3-[cyclohexylamino]-1-propane sulfonic acid) buffer, pH 11.0, was performed following our published procedure [53]. ALM 29 and ALM 22 polypeptides were solubilized by high pH extraction of ALM. The supernatant fraction was dialyzed against water and lyophilized and subjected to a continuous-elution SDS-PAGE on 12% acrylamide gels using a Model 491 prep cell (Bio-Rad laboratories, Hercules, CA) to isolate homogeneous fractions of ALM 29 and ALM 22 polypeptides. Both polypeptides were then fractionated on 15% SDS-polyacrylamide gels in the presence of V8 protease following the method of Olson et al. [47]. Peptide fragments were electrophoretically transferred to PVDF membranes. Then they were stained with either Coomassie blue or with anti-ALM22 antibody. Other blots were subjected to blot overlay assay for the binding of acrosin to the V8- digested peptide fragments of both ALM 29 and ALM 22 polypeptides following the method of Nagdas et al. [21].

Dephosphorylation of ALM 29 and ALM 22 polypeptides

Dephosphorylation of ALM29 and ALM22 polypeptides in high-pH-soluble fraction were performed following the manufacturer's instructions using calf intestinal alkaline phosphatase (PromegaCorporation, Madison, WI). The dephosphorylation of ALM29 and ALM22 polypeptides was examined by immunoblot analyses using anti-ALM22 polyclonal antibody.

For the hydrolase binding experiments, the homogeneous fraction of ALM29 polypeptide was treated with calf intestinal alkaline phosphatase (PromegaCorporation, Madison, WI) following the manufacturer's instructions. After enzymatic digestion, the dephosphorylated ALM 29 polypeptide and native ALM 29 polypeptide were conjugated to AminoLink Plus coupling gel (Pierce Chemical Co., Rockford, IL) separately at pH 10.0 according to the manufacturer's instructions. As a control, the same units of alkaline phosphatase were conjugated to Amino Link Plus coupling gel. A centrifugation assay was performed to determine the binding efficacy of acrosin to the dephosphorylated ALM 29 polypeptide following the method of Nagdas et al. [20, 21].

Results

Biochemical characterization of the ALM complex

SDS-PAGE of the Percoll gradient-purified ALM complex revealed a spectrum of polypeptides (Figure 1A). The ALM complex contains two major polypeptides of Mr 29,000 (ALM29) and Mr 22,000 (ALM22) and several minor polypeptides (Figure 1A, lane 1). Both ALM29 and ALM22 polypeptides are selectively solubilized by extraction of ALM in 0.1M CAPS buffer (3-[cyclohexylamino]-1-propane sulfonic acid), pH 10.5 (Figure 1A, lane 3), while other minor polypeptides remain associated with the sedimentable "stripped" ALM (Figure 1A, lane 2). To examine charge variant isoforms of ALM29 and ALM22 polypeptides, the total ALM complex was separated using the two dimensional PAGE analysis. The gel was stained with Coomasie Brilliant blue displaying a profound separation between the two major acrosomal polypeptides (ALM29 and ALM22), exhibiting two distinct isoforms of pI ~ 5.8 and 6.0 (Figure 1B).

Immunoblot analysis

Western blot of ALM complex stained with polyclonal anti-ALM22 recognized both the 29kDa and 22kDa bands and a set of adjacent minor bands of total ALM fraction (Figure 1C, lane 1) and the in the high pH supernatant fraction of ALM (Figure 1C, lane 3). No immune reactive bands were present in the high pH extracted ALM pellet (Figure 1C, lane 2). Preimmune serum reacted with no polypeptides in the ALM fraction (data not shown). These results demonstrated that ALM29, ALM22, and the set of adjacent polypeptides are an antigenically related family.
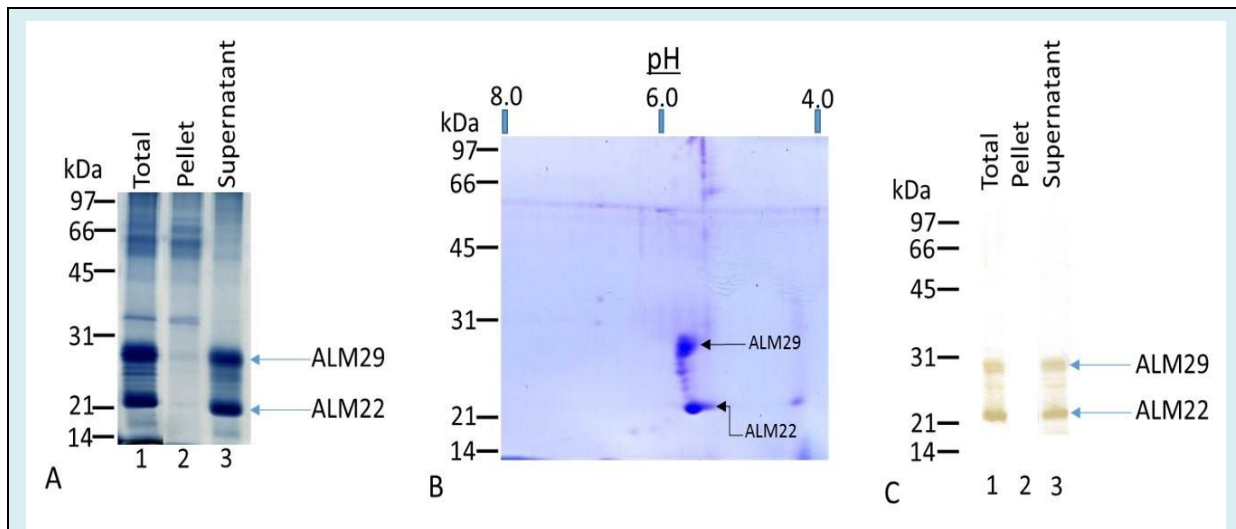
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Figure 1

A: Hamster Cauda sperm acrosomal matrix fraction analyzed by reducing SDS-PAGE on 12% gels and stained with Coomassie blue for total protein. Lane 1 shows the total acrosomal fraction; note that Coomassie blue staining reveals major acrosomal matrix polypeptides of 29 and 22kDa and several minor higher molecular weight polypeptides. Lane 2 shows the high-pH-insoluble acrosomal matrix fraction that contains several minor higher molecular weight polypeptides. Lane 3 shows the high pH soluble acrosomal fraction that contains the major acrosomal matrix polypeptides of 29 and 22 kDa. Each lane represents protein from 50µg of acrosomal fraction.

B: The two major polypeptides, ALM29 and ALM22, were separated using the two dimensional PAGE analysis. The gel was stained with Coomassie Brilliant blue displaying a profound separation between the two major acrosomal polypeptides, exhibiting two distinct isoforms of the ALM of pI ~ 5.8 and 6.0.

C: Immunoblot analysis of total acrosomal fraction (lane 1), the high-pH-insoluble acrosomal matrix fraction (lane 2), and the high pH soluble acrosomal fraction (lane 3) immunostained with anti-ALM 22 demonstrate the immunoreactivity to both ALM29 & ALM22 and a few minor polypeptide bands in total (lane 1) and the high pH soluble acrosomal fraction (lane 3). An identical blot stained with preimmune serum and shows no immunoreactivity (data not shown).

Diagonal gel

Diagonal gel electrophoresis was performed to examine whether both ALM29 and ALM22 polypeptides are cross-linked by disulfide bridge(s). ALM samples are sequentially electrophoresed in a cross-linked state and then a monomeric state [51]. For the first dimension separations cross-linked ALM fractions were solubilized in nonreducing sample buffer and fractionated on SDS-PAGE tube gels. The tube gels were incubated in reducing sample buffer containing DTT as a reducing agent to cleave disulfide-linked complexes into their monomeric components and then fractionated in the second dimension on SDS-polyacrylamide slab gels. Non cross-linked polypeptides migrate on a diagonal line with a comparable Rf in both dimensions; whereas, polypeptide members of cross-linked complexes are aligned in a vertical row below the diagonal [51]. Our data showed that both polypeptides migrate on a diagonal line with a comparable Rf under both non-reduced (Figure 2A) and reduced (Figure 2B) conditions, suggesting that both polypeptides are not joined by disulfide bridge(s).
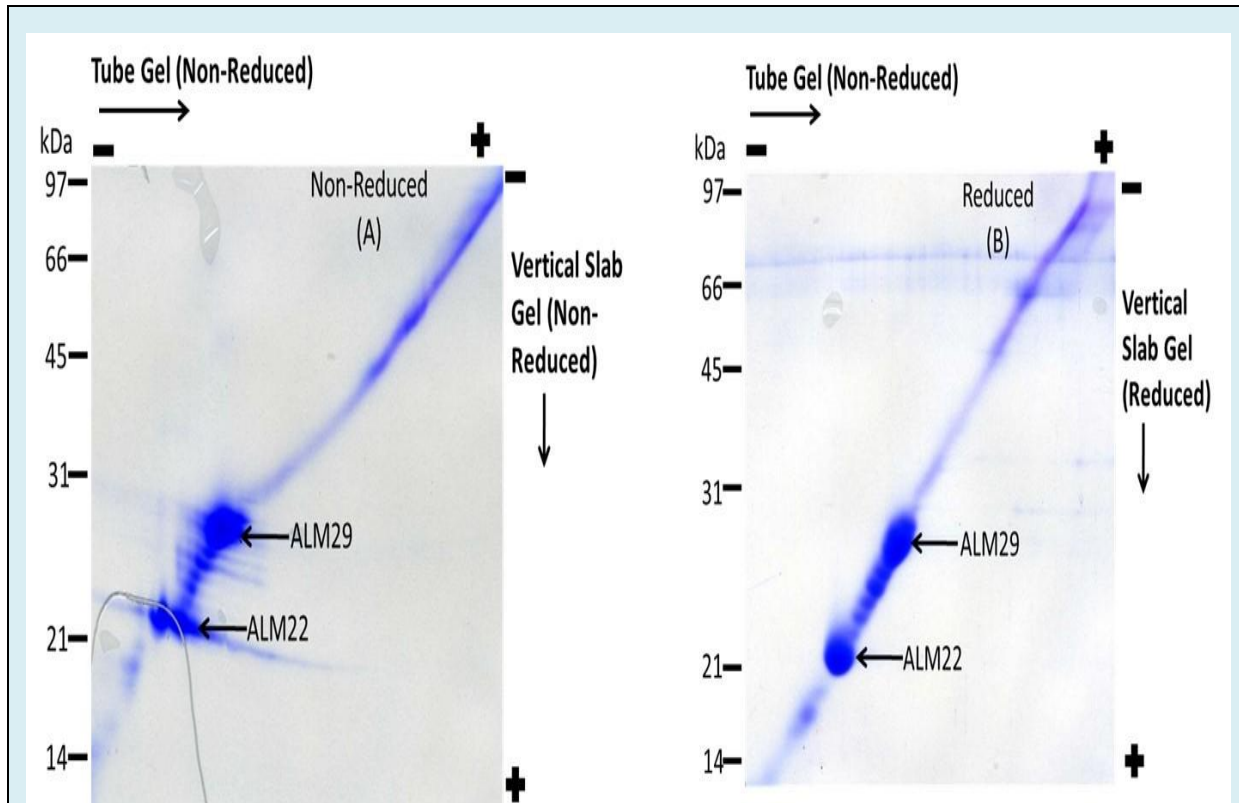
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Figure 2: Diagonal Gel Electrophoresis of ALM Complex (25μg protein) was performed to examine whether both ALM29 and ALM22 polypeptides are cross-linked by disulfide bridge(s). For the first dimension separations, cross-linked ALM fractions were solubilized in nonreducing sample buffer and fractionated on SDS-PAGE tube gels. The tube gels were incubated in reducing sample buffer containing DTT as a reducing agent to cleave disulfide-linked complexes into their monomeric components and then fractionated in the second dimension on SDS-polyacrylamide slab gels. A—Non-Reduced Conditions in both dimensions; B—Non-Reduced Conditions (1st Dimension) & Reduced Conditions (2nd Dimension).

Acrosin binding efficiency of V8-digested peptides of ALM29 and ALM22 polypeptides

Previously, we have shown that V8 digestion of ALM29 and ALM22 polypeptides generated an identical set of four polypeptide bands of 20kDa, 16kDa, 13kDa, and 12kDa suggesting a structural similarity of ALM29 and ALM22 polypeptides [47]. N-terminal amino acid sequence of the generated peptides demonstrated that ALM 29 and ALM22 polypeptides share sequence homology to each other [47]. In the current study, an identical pattern of V8-digested peptides of ALM 29 (Figure 3, lane 2) and ALM22 (Figure 3, lane 4) were obtained by Coomassie blue staining as we reported earlier [47]. To explore the antigenic relationship of V8-digested peptides of ALM29 and ALM22, an identical blot stained with anti-ALM22 polyclonal antibody exhibits that all V8-digested peptides of ALM29 (lane 6) and ALM22 (lane 8) showed immunoreactive bands suggesting that all peptides are antigenically related family. An identical blot was subjected to examine the acrosin binding efficiency by blot-overlay assay. Interestingly, only native ALM29 polypeptide binds acrosin (lane 9), neither V8-digested peptides (20kDa, 16kDa, 13kDa, and 12kDa) of ALM29 (lane 10), nor ALM22 polypeptide (lane 11) and V8-digested peptides of ALM22 (lane 12) bind acrosin. This study suggests that only the native ALM29 polypeptide possesses acrosin binding activity.
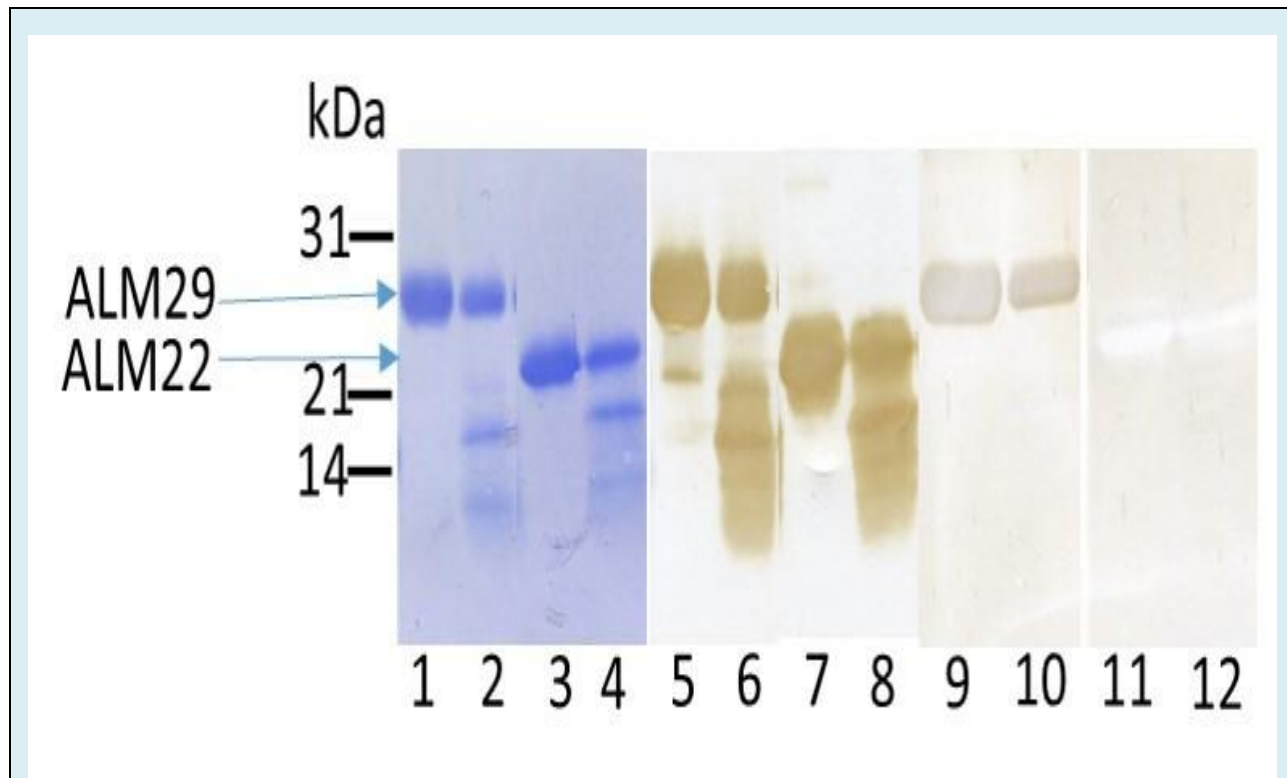
Figure 3: Peptide maps of ALM29 and ALM22 using V8 protease. SDS-PAGE purified ALM29 (lanes 1,2,5,6,9,10) and ALM22 (lanes 3,4,7,8,11,12) were incubated in the absence (lanes 1,3,5,7,9,11) or presence (lanes 2,4,6,8,10,12) of V8 protease and then fractionated by SDS-PAGE on 15% acrylamide gels, transferred to PVDF membranes.

Lanes 1-4 stained with Coomassie Blue Dye and lanes 5-8 stained with anti-ALM22 antibody. Lanes 9-12 was subjected to blot overlay assay to examine the binding efficiency of acrosin to the V8 protease-generated peptides of ALM29 and ALM22 polypeptides.

Alkaline phosphatase treatment of ALM29/ALM22 polypeptides

ALM 29 and ALM 22 polypeptides were solubilized by high pH extraction of ALM. High pH supernatant fraction was treated with alkaline phosphatase and then examined by Western Blot Analysis. Blots were stained with anti-ALM22. Immunoblot data (Figure 4, lane 1) shows the control experiment displaying two major polypeptides. Lane 2 reflects the alkaline phosphatase treated ALM29 and ALM22. Treated ALM29 and ALM22 were reduced in size by approximately 3 kDa (Figure. 4, lane 2). This study concluded that both ALM29 and ALM22 are phosphorylated.
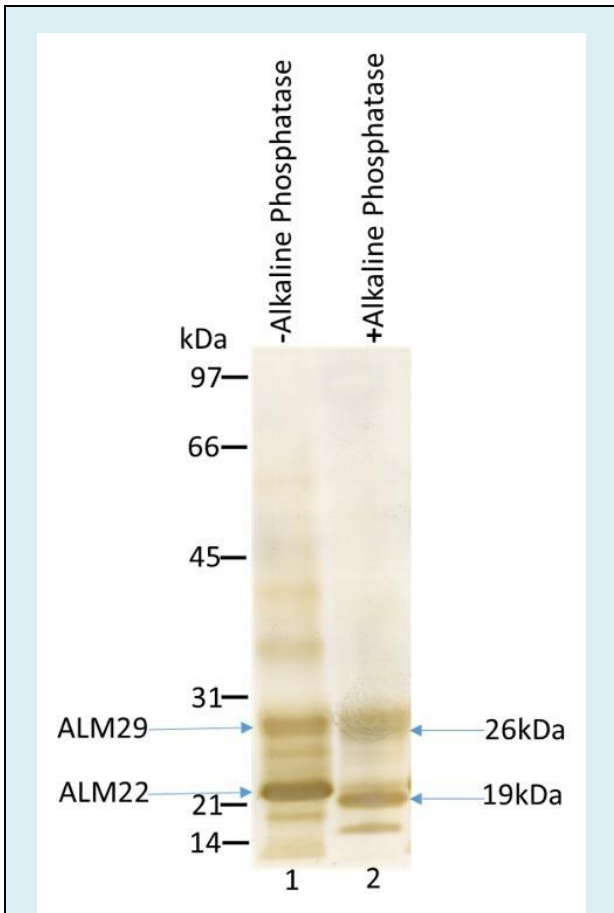
Figure 4: Both ALM29 and ALM22 polypeptides were stripped out of the ALM complex by high pH (pH-11) extraction. Both respective polypeptides were treated with alkaline phosphatase in 50mM Glycine-NaOH buffer, pH 11 for 4 hours at 37°C. A parallel ALM control was run without an enzyme. Both the control and enzyme-treated fractions were analyzed by Western blot analysis. Blots were immunostained with anti-ALM 22. Lane 1 shows the control experiment exhibiting the presence of two major polypeptides: ALM29 and ALM22. Lane 2 reveals the alkaline phosphatase treated ALM; there is a reduction in size (~3 kDa) of both the ALM29 & ALM22 polypeptides.

Demonstration of acrosin binding to dephosphorylated and native ALM29 polypeptide

A sedimentation assay was employed to determine the binding efficiency of dephosphorylated ALM29 to acrosin (Table-1). The dephosphorylated ALM29 polypeptide revealed a noticeable reduction in acrosin (~50% reduction) binding in comparison to the native ALM29 polypeptide binding to acrosin. In contrast, alkaline phosphatase conjugated beads, used as control for specificity, did not bind acrosin (data not shown) demonstrating the specificity of binding of acrosin to the native and dephosphorylated ALM29 polypeptide. Our results suggest that phosphate groups play an important role in acrosin binding.

	Native ALM29 Polypeptide	Dephosphorylated ALM29 olypeptide
Pellet	90±8	45±5
Supernatant	10±2	55±6

Table-1: Acrosin Binding to Native and Dephosphorylated ALM29 Polypeptide.

The distribution of across in between the pellet and supernatant fractions is shown in Table-1. Approximately, 50% reduction in acrosin binding to the dephosphorylated ALM29 polypeptide was noted. Values represent mean ± SD of three experiments.

Discussion

The ALM complex remains associated with the hybrid membrane complex after the acrosome reaction [47]. Utilizing both biochemical and immunological approaches, we have demonstrated that ALM29 and ALM22 polypeptides are structurally related and appear to arise from a common 40kDa precursor protein expressed in round spermatids [47]. SP10 related acrosomal proteins are reported in several species including human, baboon, fox, mouse, and bovine spermatozoa [54-59]. Both monoclonal and polyclonal antibodies to human SP-10 significantly lowered in vitro fertilization rates of bovine oocytes by bovine spermatozoa [59]. The potential of utilizing SP-10 antigen as targets for immunocontraceptive vaccines has previously been suggested [60]. Recently, Foster and Gerton [61] reported a list of mouse knockout genes of acrosomal matrix and associated proteins. Since a knockout mouse line of SP-10 has not been generated, the molecular mechanisms of acrosomal protein SP-10 in mammalian fertilization is yet to be elucidated. In the

present study, we address the biochemical characterization and assembly of ALM29 and ALM22 polypeptides. In addition, we also elucidate the mechanism of binding efficiency of acrosin to ALM29 polypeptide.

We have observed an identical architect of related proteins in the bovine sperm acrosomal matrix [20,62] reflecting the presence of a similar acrosomal distribution pattern of SP-10 related proteins in nonhuman spermatozoa. Recently, we have shown that proteomic identification of the OMC32 polypeptide (one of the bovine sperm acrosomal proteins), by MALDI-TOF-TOF analysis, yielded 2 peptides that matched the NCBI database sequence of acrosin-binding protein (Bos Taurus; GI: 194666681) [46]. 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 which are moderately acidic [46]. Human sperm SP-10 peptides from 24-34kDa have isoelectric points of approximately 4.9; whereas, the isoelectric point 17.5kDa peptide is basic [55]. Our results displayed the isoelectric points of both ALM29 and ALM22 polypeptides ranging between 5.8 and 6.0 which are slightly acidic. Results of several species strongly elucidate that the isoelectric points of acosomal matrix polypeptides are slightly acidic which could be one of the factors for the acrosomal hydrolases/proteins binding to the acrosmal matrix complex. By cross-linking experiments using the homobifunctional cleavable cross-linkers, dithiobis [sulfosuccinimidyl propionate] (DTSSP), we demonstrate that the ALM29 and ALM22 polypeptides are not cross-linked by disulfide bond. It is possible that both the ALM29 and ALM22 polypeptides interact with each other by other types of interaction. Future study will address this issue.

Our peptide mapping and blot overlay assay revealed that V8-digested peptides of the ALM29 and ALM22 polypeptides do not interact with acrosin; acrosin only binds to the intact ALM29 polypeptide-not the intact ALM22 polypeptide. N-terminal sequence of different forms of SP-10 (17.5-34kDa) by Edman degradation demonstrates that the processing of 34kDa to 17.5kDa occurs via N-terminal cleavage [63]. Since acrosin binds to intact ALM29 polypeptide only, it is suggested that the acrosin binding domain is present at the N-terminus end of ALM29 polypeptide probably at the 7kDa region of the N-terminus end of ALM29 polypeptide. When ALM29 is converted to the ALM22 polypeptide, due to the cleavage of peptide at the N-terminus, the acrosin binding domain is lost in ALM22 polypeptide.

Alkaline phosphatase treatment of both the ALM29 and ALM22 polypeptides exhibit ~3kDa reduction suggesting that both polypeptides are phosphorylated. Previously, Baker et al [64] reported sixty-eight rat sperm phosphopeptides during epididymal maturation. Out of sixty-eight, twenty-two polypeptides have been identified. Two of the proteins are acrosomal proteins; they are IZUMO1 and sperm acrosome membrane associated I proteins. By immunoblot analyses, they also showed the reduction of rat cauda sperm IZUMO1 by alkaline phosphatase treatment that also supports their phosphoproteomic analyses [64]. In the present study, we have also shown by sedimentation assay that there is a ~50% reduction of acrosin binding to the de-phosphorylated ALM29 polypeptide compared to the native ALM29 polypeptide. Our study suggests that the phosphate group of the ALM29 polypeptide is one of the components involved in acrosin binding. By peptide mapping study, we have also shown that the acrosin binding domain of the ALM29 polypeptide is present at 7kDa region of the peptide near the N-terminus end. It is assumed the phosphate group which is involved in the acrosin binding is present at the 7kDa region of ALM29 polypeptide near the N-terminus end.

In conclusion, we hypothesize that during the acrosome reaction the enzymatically inactive acrosomal phosphatase is converted to active phosphatase which cleaves the phosphate group of the ALM29 polypeptide leading to the dissociation of ALM29-acrosin interaction; consequently the release of acrosin occurs from the hamster sperm acrosomal matrix complex. How does the activation of phosphatase occur? It may be possible that the acrosomal phosphatase becomes enzymatically active by phosphorylation via a signal transduction pathway. Alternatively, the conversion of inactive to active phosphatase occurs by other biochemical modification(s). Future studies will elucidate all these possible pathways.

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