



# Activation of proacrosin accompanies upregulation of sp32 protein tyrosine phosphorylation in pig sperm

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**ABSTRACT.** This study investigated the relationship between acrosin activation and pig sperm proacrosin binding protein (sp32) phosphorylation levels. Differently processed pig spermatozoa (fresh semen sperm, capacitation sperm, acrosome reaction sperm, capacitation-like sperm, and thawed sperm) were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis and Western blot analysis. The fresh semen and capacitation sperm groups both produced proacrosin protein bands of 55 kDa; however, the result of the fresh semen sperm group was clearer than that of the capacitation sperm group. The thawed sperm group showed a shallow strip at 55 kDa. The capacitation and acrosome reaction sperm groups produced obvious proacrosin protein bands at 35 kDa, and the strips of the capacitation sperm group were again clearer. A faint band was visible at 32 kDa in the acrosome reaction sperm group. The capacitation, thawed, and acrosome reaction sperm groups showed significant strips in sp32, and the bands of the acrosome reaction sperm group were shallower than those of the 2 other groups. The capacitation and thawed sperm groups produced significant strips at 40 kDa, and the capacitation sperm group produced an additional strip at 55 kDa. In conclusion,

sp32 phosphorylation levels can promote proacrosin activation into the active acrosin.

**Key words:** Pig sperm; Proacrosin; Acrosin; sp32; Phosphorylation

## INTRODUCTION

Many similarities exist between *in vitro* capacitated sperm and cooling-induced capacitation-like sperm. Sperm surviving after cold shock treatments suffers damage due to capacitation-like changes, which are the main causes of low fertilization rates after cooling, freezing, and thawing.

The sperm acrosome contains 24 types of enzymes, including the proteolytic enzyme and phosphatase, among others. Among all 24 enzymes, acrosin has been most closely associated with fertilization, and 85-90% of acrosome proteins have been found in proacrosin form in original semen. The pig sperm proacrosin has a molecular weight of 55 and 53 kDa. When automatically activated, it first converts into 49 kDa intermediates, and after a series of changes finally forms a mature top body protein with a molecular weight of 35 kDa. Although the natural substrate of sperm acrosin has been shown to be the mature oocyte zona pellucida, purified acrosin can also digest the mammalian zona. In a study of the conversion process of proacrosin to acrosin within sperm, Töpfer-Petersen et al. (2008) demonstrated that its regulatory mechanism is controlled by contact of the sperm surface with the zona pellucida. Acrosin is not only able to identify oocyte zona, but is also able to penetrate it. When the sperm acrosin is lost, sperm can neither identify oocytes nor penetrate the zona pellucida, so that the sperm can no longer bind with the oocyte (Barros et al., 1996; Bailey et al., 2000). Our previous studies have shown that under the sperm capacitation state, most of the pig sperm proacrosin/acrosin system exists in the acrosome protein form, and the sperm could only be activated to acrosin when combined with transparent tape. However, when capacitation-like sperm is activated into acrosin, they lose fertilization opportunities if not promptly combined with oocytes (Sillerico et al., 1996). The molecular mechanisms involved in the activation of capacitation and capacitation-like pig sperm proacrosin/acrosin kinetics are not currently clear (Cortes et al., 2006).

In its excited state, the 32-kDa proacrosin-specific binding sperm protein (sp32) automatically activates the proacrosin protein and converts it into 49 kDa intermediates, subsequently forming a mature acrosin of 35 kDa (Baba et al., 1994). In addition, Dubé et al. (2003, 2005) confirmed that sp32 combines with proacrosin to participate in acrosin maturation. However, very little is known about differences in the phosphorylation of sp32 between capacitation and capacitation-like sperm. Therefore, the aim of this study was to explore the early activation mechanism of cold shocked sperm by comparing capacitation and capacitation-like sperm states. This data will be informative for effective measures to prevent or reverse these changes, or to delay the process.

## MATERIAL AND METHODS

### Experimental animals

Semen was collected from healthy adult Landrace boars obtained from Yanji City

Hanji Mu Co., Ltd. Semen samples were sealed in dark mugs and transported to the laboratory within 2 h while maintained at  $24 \pm 2^\circ\text{C}$ . The semen used in the study complied with the following conditions: 1) milky-white color; 2) vitality  $>80\%$ ; 3) acrosome integrity  $>75\%$ .

## Main drugs

In this study, all drugs were purchased from Sigma. For the acrosin (proacrosin) activation pathway experiments with pre-stained protein markers, the polyvinylidene fluoride (PVDF) membrane, DAB color reagent, Western blot primary antibodies (rabbit anti-porcine proacrosin antibody, rabbit anti-porcine acrosin antibody, and rabbit anti-porcine sp32 antibody), and secondary antibodies [goat anti-rabbit IgG antibody HRP, goat anti-rabbit IgG antibody fluorescein isothiocyanate (FITC)] were all purchased from Beijing Huaxia Ocean Biological Technology Co., Ltd. For the protein tyrosine phosphorylation experiments with pre-stained protein markers, the PVDF membrane, immunoblotting primary antibodies (mouse anti-phosphotyrosine rabbit anti-porcine p32 antibody), and secondary antibodies [goat anti-mouse IgG (H+L) HRP and goat anti-mouse IgG (H+L), FITC] were all purchased from Hangzhou MultiSciences Biotech Co., Ltd. (Beijing).

## Antibody dilution

Antibody dilutions were prepared by adding 50  $\mu\text{L}$  Tween 20 to 100 mL phosphate-buffered solution (PBS), uniformly mixed with a stirrer, filtered, and stored at  $4^\circ\text{C}$ .

Primary antibodies (anti-top body protein antibody, anti-acrosome antibody) were diluted with antibody dilution solution (at 1:50), and prepared for use in sperm immunofluorescence staining. The solution was further diluted in the dark at a 1:6 ratio for the Western blotting analysis and maintained at  $4^\circ\text{C}$  until use.

Secondary antibodies [HRP goat anti-rabbit IgG (H+L)] were diluted with antibody dilution (at 1:1000), and maintained in dark conditions.

## Protamine separation

Pig sperm protein separation was performed essentially as described previously (Dubé et al., 2003). Briefly,  $5 \times 10^6$  sperm/mL aliquots of differently processed noncapacitated and capacitated sperm were obtained. After the addition of 0.2 mM sodium orthovanadate, samples were centrifuged (4 min, 16,060 g, at ambient temperature), the obtained sperm particles were suspended in non-2-mercaptoethanol sample buffer, and then heated at  $95^\circ\text{C}$  for 1 min. The sperm dilution was centrifuged again (4 min, 16,060 g), and 5% 2-mercaptoethanol was added to the supernatant. The protamine was then prepared for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis.

## SDS-PAGE

The Laemmli electrophoresis buffer system was used for SDS-PAGE analysis (Raftogianni et al., 2012). Twenty-microliter protein samples were analyzed on a U2800 UV spectrophotometer, and the protein concentration was adjusted to be the same in all tubes.

Samples were denatured by boiling, immediately cooled, high speed centrifuged for 1 min, and the supernatant was removed by pipette. Twenty microliter samples were loaded on 8% separating gel and 5% stacking gel, and then the power supply was connected with the anode below and the cathode above. Electrophoresis was run at constant pressure (80 V stacking gel, 120 V separating gel) for 2 h, and stopped when bromophenol blue reached to about 1 cm under the mouth of the gel. After electrophoresis, the gel was removed, and part of the film was stained with Brilliant Blue solution for decolorization. Photographs were obtained for visualization once the protein bands were clear.

### **Western blotting**

After SDS-PAGE, the transfer protein gel was cut, along with 6 equal-sized pieces of filter paper, and the PVDF membrane was soaked in transfer buffer for 30 min until no bubbles were present. After sequentially stacking and aligning 3 layers of filter paper, gel, and PVDF membrane, and another 3 layers of filter paper, ensuring no air bubbles were present between the layers, the positive electrode was connected to the PVDF membrane, the negative electrode was connected to the gel, and electric transfer was run at 0.8 mA/cm<sup>2</sup> for 1 h. After the transfer, the PVDF membrane was placed on a Petri dish, and blocking solution was added to the plates [5% skim milk dissolved in Tris-buffered saline (TBS)] and blocked overnight at 4°C. Meanwhile, the primary antibody was diluted at 1:200 using blocking solution while proteins on the gel were checked for complete transfer. After sealing, the blocking solution was discarded, the PVDF membrane was rinsed with TBS 3-4 times for 5 min each, and primary antibodies were added to react at 37°C for 1 h. The PVDF membrane was washed again with TBS solution for 5 min 3-4 times, and then again with the washing buffer (containing 5% skim milk), and the secondary antibody (goat anti-rabbit IgG antibody, HRP-labeled) was added, diluted at 1:1000, to make sure the transfer film surface made full contact with the antibody solution in 37°C under the reaction conditions for 1 h. The PVDF membrane was washed again for 5 min 3-4 times, and finally, color developed in the DAB color liquid. The PVDF membrane was immersed in the color-developing solution and gently shaken at room temperature. When a clear purpose belt was evident, the membrane was immediately washed with water, and then the film was transferred to TBS for preservation and photography.

### **FITC immunofluorescence**

FITC immunofluorescence staining was used in each semen experimental group at room temperature with 4% paraformaldehyde/PBS fixed sperm for 30 min. PBS was washed 2 times, smeared, and sealed in 10% bovine serum albumin for 30 min at room temperature. Fifty microliters of primary antibody (mouse anti-phosphotyrosine, 1:50 dilution), or Antibody Diluent (negative control) was added dropwise and incubated overnight at 4°C. The solution was then washed with PBS 3 times, and 50 µL (1:100 dilution) secondary antibody [goat anti-mouse IgG (H+L) FITC] was added, and incubated at 37°C in the dark for 40 min. Samples were washed 3 more times with PBS, sealed at the edge with nail polish, and observed by fluorescent microscope.

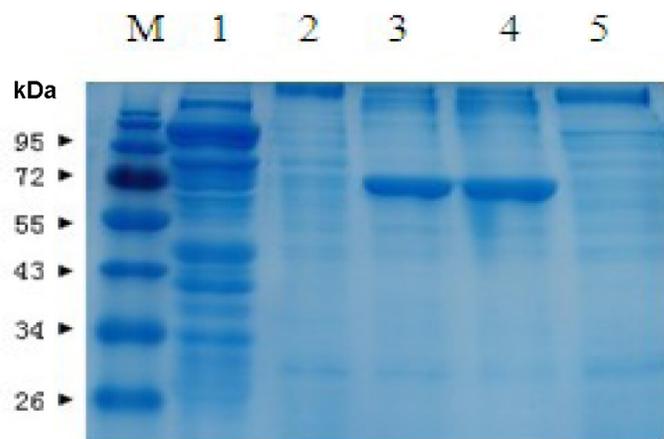
### **Statistical analysis**

The data obtained were analyzed using the SPSS v.11.4 software, and differences at  $P < 0.05$  were considered to be statistically significant.

## RESULTS

### Protamine SDS-PAGE analysis of different treatment groups

Protein contents of the control group (fresh semen) and treatment groups (capacitation, acrosome reaction group, capacitation-like group, and freeze-thaw group) were compared by SDS-PAGE. Figure 1 shows that proteins of the samples from each treatment group were separated, with molecular weights of 55-95 kDa evident in the fresh semen group. The capacitation group protein species showed uniform distribution in which new strips were generated and some bands were lost. A clear band emerged in the acrosome reaction and capacitation-like groups at 70 kDa. Total protein concentrations were higher in the capacitation-like group compared to the acrosome reaction group. The total protein content of the freeze-thaw group was significantly lower than those of the other groups. These results demonstrated greater changes in protein species and expression levels of the treatment groups compared to the control group.

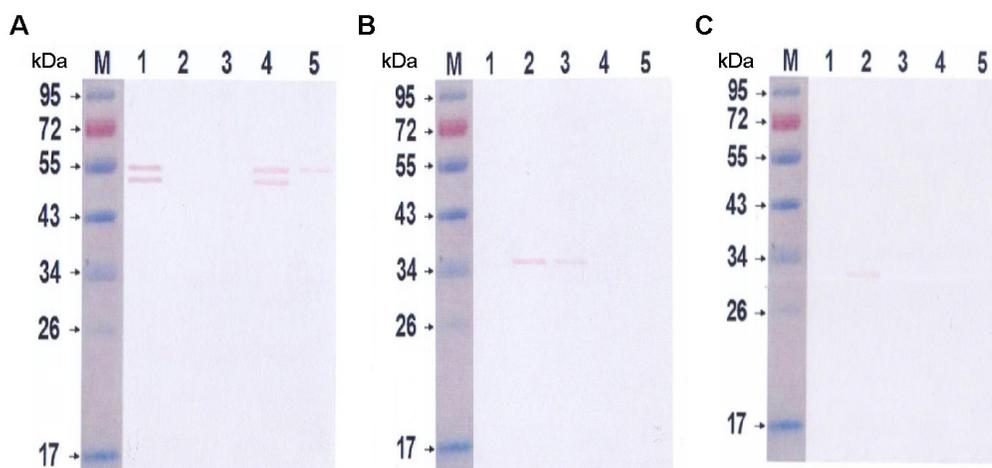


**Figure 1.** SDS-PAGE analysis of sperm proteins of different treatment groups. Lane M = molecular marker; lane 1 = fresh semen group; lane 2 = capacitation group; lane 3 = acrosome reaction group; lane 4 = “capacitation-like” group; lane 5 = freeze-thaw group.

### Western blotting results

After SDS-PAGE analysis, samples of the control group (fresh semen) and treatment groups (capacitation group, acrosome reaction group, capacitation-like group, and freeze-thaw group) were treated with acrosome and HRP secondary antibodies. Western blot analysis (Figure 2A) revealed proacrosin protein bands at 55 kDa in the fresh semen and capacitation-like groups, although bands were clearer in the fresh semen group. This result demonstrated that the fresh sperm and capacitation-like groups were in the proacrosin state, and proacrosin failed to activate into active acrosin. The proacrosin partially activated into active acrosin in the freeze-thaw group and completely activated into active acrosin in the capacitation and acrosome reaction groups. As shown in Figure 2B, clear bands appeared in the capacitation and acrosome reaction groups at 35 kDa, although bands of the capacitation group were clearer.

Figure 2A and B show that the proacrosin of the capacitation and acrosome reaction groups almost fully activated into the active acrosin, and part of the acrosin in the acrosome reaction group discharged into the semen via exocytosis.



**Figure 2.** Western blotting analysis in different treatment groups. **A.** Proacrosin. **B.** Acrosin. **C.** sp32. Lane M = molecular marker; lane 1 = fresh semen; lane 2 = capacitation; lane 3 = acrosome reaction; lane 4 = “capacitation-like”; lane 5 = freeze-thaw.

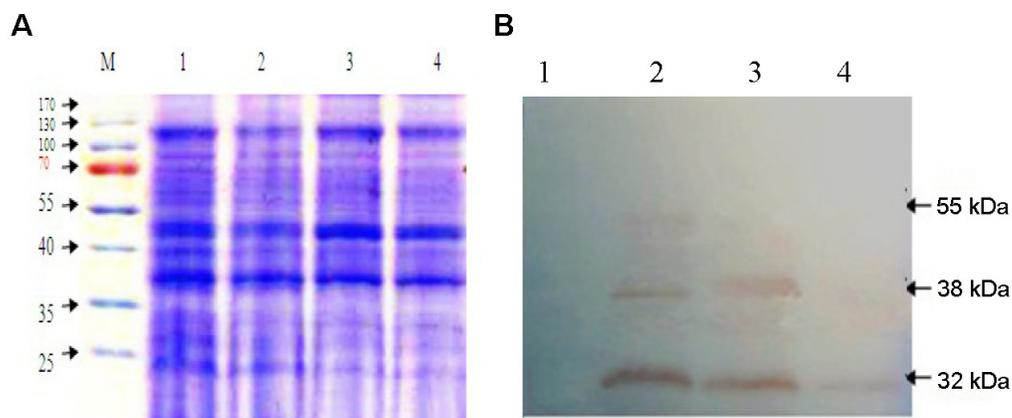
After SDS-PAGE analysis, the above samples were treated with sp32 protein antibody and HRP secondary antibody. Western blot analysis showed that only a faint band appeared from the capacitation group (Figure 2C). These results indicated that the sp32 protein began to express when the sperm was capacitated.

### The role of p32

To further investigate the role of sp32, SDS-PAGE was used to analyze the control group (fresh semen) and the treatment groups (capacitation, freeze-thaw, and acrosome reaction). Figure 3A shows that proteins of the samples were separated in each treatment group, with molecular weights between 35-135 kDa in the fresh semen group. The protein species distribution of the capacitation group was uniform, with new strips generated and some bands lost. Clear bands emerged in the acrosome reaction and freeze-thaw groups at 70 kDa. The total protein content of the capacitation-like group was higher than that of the acrosome reaction group, whereas it was significantly lower in the freeze-thaw group compared to all other groups. These results indicated that protein species and expression levels changed more in the treatment groups compared with the control group.

Western blot analysis (Figure 3B) showed the presence of clear bands in the capacitation, freeze-thaw, and acrosome reaction groups at sp32, although the bands of the acrosome reaction group were less clear than those of the capacitation and freeze-thaw groups. Clear bands emerged in the capacitation and freeze-thaw groups at 70 kDa. In addition, another band appeared in the capacitation group at 55 kDa. These results demonstrated that the capacita-

tion and freeze-thawed sperm had similar levels of protein phosphorylation. While the sp32 phosphorylation level can reach its threshold when sperm is capacitated, during the acrosomal reaction, this level decreased gradually and ultimately disappeared.



**Figure 3.** sp32 in different treatment groups. **A.** SDS-PAGE analysis. *Lane M* = molecular marker; *lane 1* = room temperature; *lane 2* = capacitation sperm; *lane 3* = freezing and thawing; *lane 4* = acrosome reaction. **B.** Western blotting analysis; *lane 1* = room temperature; *lane 2* = capacitation sperm; *lane 3* = freeze-thaw sperm; *lane 4* = acrosome reaction sperm.

## DISCUSSION

Results of this study revealed that pig sperm proacrosin is automatically activated and converted from 55 and 53 kDa molecular weight proteins into 49 kDa intermediates, eventually forming a mature acrosomal protein weighing 35 kDa after a series of changes. Although capacitation-like and capacitation sperm have many similarities, an improvement of the Kennedy et al. (1989) method to detect sperm acrosome protein levels revealed that the level of acrosomal protein was significantly different among diluted semen, cooled semen, and freeze-thawed semen samples, and acrosome protein activity positively correlated with the *in vitro* fertilization rate. In the present study, SDS-PAGE was used to analyze differently treated pig sperm samples (fresh, capacitation, acrosome reaction, cooling, freeze-thaw), and then pre-acrosomal antibodies, acrosomal protein antibodies, and HRP secondary antibodies were applied. Western blot analysis showed that the acrosomal protein band appeared in the fresh semen and cooling groups at 55 and 53 kDa, while only dimly visible protein bands appeared in the freeze-thaw group at about 55 kDa. No acrosome protein bands appeared in any of these 3 groups. The acrosome protein band was clearer in the fresh semen group than in the cooling and freeze-thaw groups. Bands appeared at 35 kDa in the capacitation and acrosome reaction groups, and the band was clearer in the former group. Previous studies have shown that low temperatures tend to promote pre-acrosome protein activation; however, in the present study, pre-acrosome sperm protein did not transform into acrosome sperm protein in the cooling or freeze-thaw groups, possibly due to a small transformation amount or loss of the acrosome protein in the capacitation sperm. This may also be related to capacitation injury of the survived sperm that experienced cooling or freeze-thaw (Watson, 1995). The capacitation

and acrosome reaction groups showed the presence of bands at 35 kDa, which were clearer in the capacitation group. This band corresponded to the acrosome protein, which not only recognizes, but can also penetrate, the oocyte transparent zone. If the acrosome protein of sperm is damaged, it can neither identify oocytes nor penetrate the zona pellucida, resulting in the loss of fertilization capacity, which contributes to the low fertilization rates observed in cooling and freeze-thawed sperm.

Protein phosphorylation is a process of post-translational processing and modification. In eukaryotic cells, protein phosphorylation/dephosphorylation is one of the most common ways of regulating protein biological activities, and regulates physiological activities of several cell types. Mature sperm is a highly differentiated specific cell that cannot translate or synthesize new proteins. Therefore, post-translational modifications, for example, protein phosphorylation/dephosphorylation of sperm, is very important to its capacitation, super activating motion maintenance, and acrosome reaction. sp32 can combine with acrosin at capacitation at the early or end stages of the acrosome reaction, and is not detected in sperm that have undergone acrosome reaction (Baba et al., 1989). This is consistent with results of the present study, which suggest that sp32 is released along with the acrosome reaction or is hydrolyzed by protease. In addition, our results demonstrated that clear bands appeared in capacitation and freeze-thaw sperm at 38-40 kDa, which appears to be closely related to sperm capacitation.

In conclusion, our results showed that the fresh semen and capacitation-like treated groups were in the proacrosin forms, and proacrosin failed to activate into active acrosin overall, although a small amount of proacrosin could be activated in the freeze-thaw group. Most proacrosin of the capacitation and acrosome reaction groups could be completely activated into acrosin, whereas acrosin of the acrosome reaction group was partially discharged into semen by exocytosis. The sp32 protein began to express when sperm was capacitating. Capacitation and freeze thawing sperm have similar levels of protein phosphorylation. While the sp32 phosphorylation level can reach its threshold when capacitated, it decreases gradually when processed to the acrosomal reaction until finally disappearing. In conclusion, pig sperm sp32 phosphorylation levels can promote proacrosin activation into active acrosin.

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