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—Research Note—

A 32-kDa Tyrosine-phosphorylated Protein Shows a Protease-dependent Increase in Dead Boar Spermatozoa

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Abstract. Boar sperm TyrP32 is a 32-kDa tyrosine-phosphorylated protein that increases during the capacitation and acrosome reaction and during cryocapacitation. However, it is still unclear whether the increase in TyrP32 is an event that is limited to the process of sperm fertilization, including cryocapacitation. The aims of the present study were to demonstrate that TyrP32 is increased in dead spermatozoa after freeze-thawing without a cryoprotectant and to find the causal factors for this increase. Washed spermatozoa were resuspended in a salt solution and then frozen. The frozen samples were rapidly thawed in a warm water bath and then used for sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE)/Western blotting to detect TyrP32, SDS-PAGE/silver staining of sperm proteins and staining of acrosomal contents with fluorescein isothiocyanate (FITC)-conjugated peanut agglutinin (PNA). In the samples before freezing, TyrP32 was barely detectable, and the distribution of the acrosomal contents was normal in most spermatozoa. One cycle of freeze-thawing induced an increase in TyrP32, a decrease in major sperm proteins and disorder in the acrosomal contents. However, the addition of a protease inhibitor (APMSF, 1 mM) suppressed the increase in TyrP32 and the decrease in the major sperm proteins, although it did not have any influence on the disorder in the acrosomal contents. Additionally, the spermatozoa did not exhibit any flagellar movement after freeze-thawing, which showed that almost all of them were dead. These results indicate that TyrP32 can show a protease-dependent increase in dead spermatozoa after freeze-thawing without a cryoprotectant even though the dead spermatozoa do not undergo cryocapacitation.

Key words: Acrosome, Boar, Peanut agglutinin (PNA), Protease, Sperm, Tyrosine

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In mammalian spermatozoa, a variety of tyrosine-phosphorylated proteins increase during incubation under capacitation-supporting conditions. These proteins have been considered to be involved in regulation of fertilization-related events [1, 2]. It is generally believed that the increase in tyrosine-phosphorylated proteins results from activation of protein tyrosine kinases and inactivation of protein tyrosine phosphatases that are controlled by cyclic adenosine 3′,5′-monophosphate (cAMP)/protein kinase A (PKA) signaling [1, 3–5]. However, a cAMP-dependent increase in 32-kDa tyrosine-phosphorylated protein (called TyrP32 or p32) in boar spermatozoa is exceptionally suppressed by inhibition of tyrosine phosphatases and barely affected by inhibition of protein tyrosine kinases [6]. This tyrosine-phosphorylated protein is primarily found as a capacitation-related protein [7–9] and has recently been identified as a (pro)acrosin-binding protein [10, 11]. We have previously shown that the cAMP-dependent increase in TyrP32 is greater in samples containing many spermatozoa that undergo calcium-dependent changes in acrosomal morphology (acrosome reaction) [6]. Bailey *et al*. [11, 12] have also reported that calcium deficiency suppresses both increase in TyrP32 and progression of capacitation in boar spermatozoa incubated in a capacitation-supporting medium. Moreover, treatment with calcium ionophore Br-A23187 accelerates the increase in TyrP32 [12]. These results indicate possible roles of TyrP32 in calcium-dependent events including capacitation and the acrosome reaction.

The increase in TyrP32 is not limited to the process leading to capacitation and the acrosome reaction. This increase in TyrP32 can also be observed in boar spermatozoa after cooling treatments. Moreover, some cooled spermatozoa exhibit capacitation-like molecular alterations in the sperm head [9, 13]. Similarly, frozen-thawed bull spermatozoa are likely to undergo premature capacitation (cryocapacitation) [14], which is a causal factor for low pregnancy rates in artificial insemination with frozen-thawed spermatozoa [15]. In cooled or frozen-thawed spermatozoa, the plasma membrane becomes unstable and partially damaged, and acrosomal contents leak onto the surface. These changes might mimic molecular alterations of the sperm membranes that occur during the capacitation and subsequent acrosome reaction [14, 16]. However, it is still unclear whether the increase in TyrP32 is an event that is limited to the process of sperm fertilization including cryocapacitation. In this study, we first showed that freeze-thawing without a cryoprotectant could increase TyrP32 in dead spermatozoa from boars with a deficiency in external calcium. We then examined the effects of inhibitors for tyrosine kinases or proteases on the increase in TyrP32, the composition of sperm proteins and the distribution of acrosomal contents in frozen-thawed dead spermatozoa.

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Materials and Methods

An animal use ethics statement

This study was approved by the Institutional Animal Care and Use Committee of Kobe University (Permission number: 16-04-08) and carried out in accordance with the Kobe University Animal Experimentation Regulations.

Preparation of sperm samples

Sperm-rich fractions from ejaculates were obtained from four mature boars by a manual method. The spermatozoa were washed in isotonic Percoll (GE Healthcare UK, Buckinghamshire, UK) and then in phosphate-buffered saline (PBS: 136.9 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4 and 1.5 mM KH_2PO_4; pH 7.4) containing 0.1% (w/v); polyvinyl alcohol (PVA; Sigma-Aldrich, St. Louis, MO, USA) by centrifugation as described previously [6, 17]. The resultant sperm pellets were resuspended in a modified Krebs-Ringer Hepes medium containing 0.1% (w/v) PVA (mKRH-PVA) [5, 6] or PBS to adjust the final sperm concentration to 1.0×10^8 cells/ml and were then transferred into a freezer (at –70 or –80 C). The frozen sperm samples were thawed rapidly in a warm water bath (at 38.5 C) or slowly in wet ice and were then used for assessment of acrosomal contents by fluorescein isothiocyanate (FITC)-conjugated peanut agglutinin (PNA) staining, detection of TyrP32 by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE)/Western blotting and detection of sperm proteins by SDS-PAGE/silver staining. In some experiments, herbimycin A (an inhibitor for tyrosine kinases; Sigma-Aldrich), *O,O′*-bis(2-aminoethyl) ethyleneglycol-*N,N,N′,N′*-tetraacetic acid [(EGTA), an inhibitor for metal proteases and a calcium chelator; Dojindo Corporate, Kumamoto, Japan and (*p*-amidinophenyl) methanesulfonyl fluoride hydrochloride [(APMSF), an inhibitor for trypsin-like serine proteases; Wako Pure Chemical, Osaka, Japan] were dissolved in dimethyl sulfoxide (DMSO; Wako, for herbimycin A) or PBS (for EGTA and APMSF) and then either of them was added to the sperm suspension. The DMSO or PBS was also added to the control samples without the inhibitors to equalize the concentration of solvent among the samples.

Assessment of acrosomal contents by FITC-PNA staining

FITC-PNA staining was performed as described previously [18] with some modifications. In brief, frozen-thawed spermatozoa were diluted with a five-fold volume of PBS, recovered by centrifugation (700 g, 5 min) and then fixed with 3% (w/v) paraformaldehyde (Nacalai Tesque, Kyoto, Japan) in PBS for 30 min. After being washed three times with 1% (w/v) bovine serum albumin (BSA; Wako) and 100 mM glycine (Gly; Wako) in PBS [(BSA/Gly-PBS), 1000 g, 1 min each], the spermatozoa were treated with 1% Triton X-100 (Nacalai) in PBS for 5 min and then washed three times with BSA/Gly-PBS. They were then stained with FITC-PNA (20 μg/ml, Sigma-Aldrich) in PBS for 30 min and subsequently washed with BSA/Gly-PBS three times. Finally, sperm nuclei were counterstained with 400 μg/ml propidium iodide (Invitrogen, Carlsbad, CA, USA) in PBS for 10 min. After being washed three times, the sperm samples were put onto glass slides and then covered with 0.22 M 1,4-diazabicyclo [2,2,2] octane (Sigma-Aldrich) dissolved in a glycerol-PBS mixture (9:1) and coverslips. The preparations were examined under a differential interference microscope equipped with epifluorescence (mirror unit U-MWB2: excitation filter BP460–490, dichroic mirror DM500 and emission filter BA520IF; Olympus, Tokyo, Japan). One hundred spermatozoa from each preparation were classified into six categories according to the fluorescent intensity of FITC-PNA in the acrosomes (Fig. 1). In Fig. 1, pattern A represents normal distribution of acrosomal contents, pattern B represents disorder of acrosomal contents with highly bright fluorescence, pattern C represents moderately-decreased acrosomal contents in the whole acrosome, pattern D represents moderately-decreased acrosomal contents in the apical region of the acrosome, pattern E represents moderately-decreased acrosomal contents in the whole acrosome, which was outlined with weak fluorescence, and pattern F represents almost no acrosomal contents.

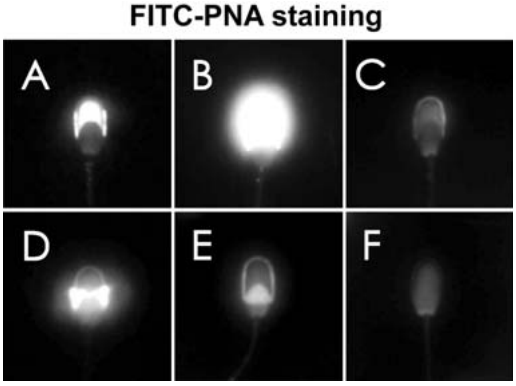

Fig. 1. Classification of fluorescein isothiocyanate-conjugated peanut agglutinin (FITC-PNA) staining patterns of boar sperm acrosomal contents. Spermatozoa were classified into six categories (patterns A–F) according to the fluorescence intensity of FITC-PNA in the acrosomes. Pattern A represents normal distribution of acrosomal contents, pattern B represents disorder of acrosomal contents with highly bright fluorescence, pattern C represents moderately-decreased acrosomal contents in the whole acrosome, pattern D represents moderately-decreased acrosomal contents in the apical region of the acrosome, pattern E represents moderately-decreased acrosomal contents in the whole acrosome, which was outlined with weak fluorescence, and pattern F represents almost no acrosomal contents.

Detection of TyrP32 by Western blotting

The procedures for Western blotting are described in our previous reports [4–6]. The antibodies used in the present study were mouse anti-phosphotyrosine monoclonal antibody (clone 4G10, anti-pY; Upstate Cell Signaling Solutions, Charlottesville, VA, USA, 1:10,000) and horseradish peroxidase-conjugated goat anti-mouse immunoglobulins (1:10,000; Dako Cytomation Denmark A/S, Glostrup, Denmark).

Table 1. Effects of freezing-rapid thawing on the fluorescein isothiocyanate-conjugated peanut agglutinin (FITC-PNA) staining patterns of boar sperm acrosomal contents (n=3)

Cycles of freezing-rapid thawing	FITC-PNA staining patterns (%)			
	A	B	C	D–F
0 (control)	96 ± 3[a]	2 ± 2[b]	1 ± 1	1 ± 1[c]
1	1 ± 1[b]	76 ± 9[a]	7 ± 6	16 ± 5[b]
3	0 ± 0*	68 ± 9[a]	1 ± 1	31 ± 8[ab]
5	0 ± 0*	55 ± 16[a]	0 ± 0*	45 ± 16[a]

Freeze-thawing (one to five cycles) was performed for boar spermatozoa resuspended in a modified Krebs-Ringer Hepes medium containing 0.1% polyvinyl alcohol. Frozen samples were rapidly thawed in a warm water bath (at 38.5 C). Values are means ± standard deviations (SD). [a–c]Values with different superscripts within the same column differ significantly ($P<0.05$). Values with asterisks were excluded from the statistical analyses because their SDs were zero.

Statistical analysis

The obtained data were subjected to one-way analysis of variance (ANOVA) or two-tailed paired *t*-test after arcsine transformation. When F-test results were significant in ANOVA, individual means were further tested by Tukey's multiple range test [19].

Results

Effects of freezing-rapid thawing on the FITC-PNA staining patterns of acrosomal contents, the increase in TyrP32 and viability

As shown in Table 1 and Fig. 2 panel A, almost all spermatozoa before freeze-thawing in mKRH-PVA were classified into pattern A, which was characterized by a normal distribution of acrosomal contents. After one cycle of freezing-rapid thawing, however, many spermatozoa (76%) exhibited pattern B, and most of the rest (23%) were classified into patterns C, D, E or F. Moreover, 5 cycles of freezing-rapid thawing significantly increased the number of spermatozoa in patterns D-F. Fig. 2 panel B shows representative Western blots of frozen-thawed spermatozoa which were detected with the anti-pY. In the sperm samples before freeze-thawing, several tyrosine-phosphorylated proteins were detected with molecular masses between 45 and 30 kDa, but TyrP32 (a 32-kDa band) was barely or slightly detected. However, the detection level of TyrP32 was dramatically enhanced in the sperm samples after one cycle of freezing-rapid thawing, although additional cycles of the treatment had only a slight influence on the detection pattern of TyrP32. In addition, the assays of sperm motility and viability using SYBR14/PI staining, the methods of which have been described previously [5, 6, 20], revealed that all of the frozen-thawed spermatozoa were dead (data not shown).

Effects of different thawing methods on the FITC-PNA staining patterns of acrosomal contents and the increase in TyrP32

Most of the spermatozoa after one cycle of freeze-thawing in PBS were classified into pattern B irrespective of the thawing method (Table 2). In the sperm samples after one cycle of freezing-slow thawing, however, the detection level of TyrP32 was not enhanced in the Western blots (Fig. 3 left panel). These results suggest that rapid thawing in 38.5 C water bath (*i.e.*, warming) is effective for increasing TyrP32. Possibly, temperature-dependent sperm components (*e.g.*, enzymes) are involved in this event.

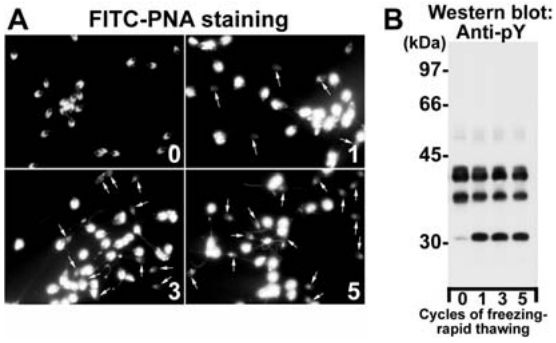

Fig. 2. Effects of freezing-rapid thawing on the FITC-PNA staining patterns of acrosomal contents and the increase in TyrP32 in boar spermatozoa. Freezing-rapid thawing (one to five cycles) was performed for boar spermatozoa resuspended in a modified Krebs-Ringer Hepes medium containing 0.1% polyvinyl alcohol. Frozen samples were rapidly thawed in a warm water bath (at 38.5 C). Panel A shows spermatozoa stained with FITC-PNA. The numbers on the photographs indicate the number of cycles of freezing and rapid-thawing. Arrows indicate spermatozoa classified into patterns D–F. Panel B shows a Western blot of boar spermatozoa which was detected with mouse anti-phosphotyrosine monoclonal antibody (clone 4G10, anti-pY) and horseradish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulins. An extract from 1×10^6 spermatozoa was loaded on each lane of the gel. The images of FITC-PNA staining and Western blot are representative of three replicates.

Effects of inhibitors for protein tyrosine kinases or proteases on the FITC-PNA staining patterns of acrosomal contents and the increase in TyrP32

In order to determine the temperature-dependent sperm components involved in the increase in TyrP32 after freeze-thawing, we examined the effects of herbimycin A (5 μM), EGTA (1 mM) and APMSF (1 mM) on the increase in TyrP32 in spermatozoa after one cycle of freezing-rapid thawing in PBS. As shown in the central and right panels of Fig. 3, APMSF apparently suppressed the increase in TyrP32, although the other reagents had no influence on the detection level of TyrP32. In addition, we found no effects of

Table 2. Effects of different thawing methods on the FITC-PNA staining patterns of boar sperm acrosomal contents after one cycle of freeze-thawing (n=3)

Samples	FITC-PNA staining patterns (%)			
	A	B	C	D–F
Before freezing (control)	92 ± 8*	3 ± 2[e]	3 ± 4*	2 ± 3
After rapid thawing[a] (control)	0 ± 0*	87 ± 6[d]	0 ± 0*	13 ± 6
After slow thawing[b]	0 ± 0*	98 ± 2[c]	0 ± 0*	2 ± 2

One cycle of freeze-thawing was performed for boar spermatozoa resuspended in phosphate-buffered saline (PBS). [a]Frozen samples were rapidly thawed in a warm water bath (at 38.5 C). [b]Frozen samples were slowly thawed in wet ice. Values are means ± SD. [c–e]Values with different superscripts within the same column differ significantly (P<0.05). Values with asterisks were excluded from the statistical analyses.

Table 3. Effects of protease inhibitor on the FITC-PNA staining patterns of boar sperm acrosomal contents after one cycle of freezing-rapid thawing (n=3)

Protease inhibitor	FITC-PNA staining patterns (%)			
	A	B	C	D–F
Control	0 ± 1	86 ± 8	1 ± 1	13 ± 9
APMSF (1 mM)	0 ± 1	88 ± 6	1 ± 1	11 ± 6

One cycle of freeze-thawing was performed for boar spermatozoa resuspended in PBS. Frozen samples were rapidly thawed in a warm water bath (at 38.5 C). Values are means ± SD.

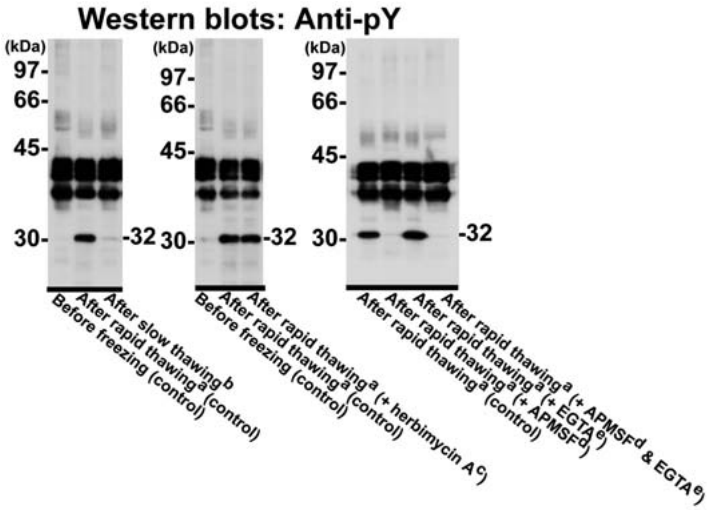

Fig. 3. Effects of different thawing methods and addition of inhibitors for protein tyrosine kinases or proteases on the increase in TyrP32 in boar spermatozoa. Panels: Western blots of boar spermatozoa which were detected with anti-pY and HRP-conjugated anti-mouse immunoglobulins. Each Western blot is representative of three replicates. One cycle of freeze-thawing was performed for boar spermatozoa resuspended in phosphate-buffered saline (PBS) containing herbimycin A[c] (5 μM), (*p*-amidinophenyl) methanesulfonyl fluoride hydrochloride (APMSF[d], 1 mM) or *O,O′*-bis(2-aminoethyl) ethyleneglycol-*N,N,N′,N′*-tetraacetic acid (EGTA[e], 1 mM). [a] Frozen samples were rapidly thawed in a warm water bath (at 38.5 C). [b] Frozen samples were slowly thawed in wet ice. An extract from 1×10^6 spermatozoa was loaded onto each lane of the gel.

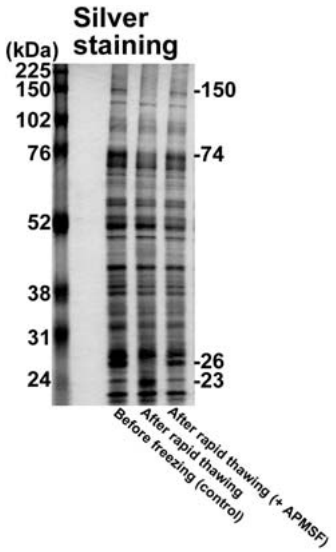

Fig. 4. Effects of freezing-rapid thawing and addition of protease inhibitor on the protein composition of boar spermatozoa. SDS-PAGE and silver-staining patterns of boar spermatozoa. This electrophoretogram is representative of three replicates. One cycle of freezing-rapid thawing was performed for boar spermatozoa resuspended in PBS with or without APMSF (1 mM). An extract from 5×10^5 spermatozoa was loaded onto each lane of the gel.

APMSF on the FITC-PNA staining patterns after one cycle of freezing-rapid thawing (Table 3).

Effects of freezing-rapid thawing treatment on the composition of sperm proteins

We examined the effects of freezing-rapid thawing in PBS on the composition of sperm proteins by SDS-PAGE and silver staining. Fig. 4 shows a decrease in at least three major proteins (150, 74 and 26 kDa) and an increase in one protein with a lower molecular mass (23 kDa) in the spermatozoa after freezing-rapid thawing. However, addition of APMSF suppressed the above-mentioned changes. These results indicate that APMSF-sensitive proteases digested sperm proteins during freezing-rapid thawing.

Discussion

Relationship between acrosomal condition and the increase in TyrP32

Boar sperm TyrP32 is a tyrosine-phosphorylated form of the 32-kDa (pro)acrosin-binding protein that is localized in the acrosome [10, 11]. In the present study, the FITC-PNA staining patterns of most spermatozoa changed from A to B after one cycle of freezing-rapid thawing. Coincidently, an increase in TyrP32 was observed. Moreover, additional cycles of the treatments changed the FITC-PNA staining patterns from B to D-F, although they had only a slight influence on the detection pattern of TyrP32 (Table 1 and Fig. 2). These results suggest that the increase in TyrP32 is linked to changes in acrosomal contents, which are indicated by the changes in the FITC-PNA patterns from A to B, and that it is barely associated with the increase in D-F pattern spermatozoa.

Mechanism for increase of TyrP32

When boar spermatozoa were incubated in mKRH-PVA (which did not contain BSA and $NaHCO_3$, but did contain 1.71 mM $CaCl_2$), TyrP32 increased within 30 min and then showed only minor changes thereafter. Moreover, addition of the cell-permeable cAMP analog cBiMPS induced a further increase in TyrP32 in the spermatozoa after incubation for 120–180 min. This cAMP-dependent second increase seemed to be regulated by a calcium-dependent mechanism that was not mediated by protein tyrosine kinases and protein tyrosine phosphatases [6, 20]. In addition, cBiMPS effectively promoted capacitation and the subsequent acrosome reaction in boar spermatozoa incubated in mKRH-PVA [6, 21]. Bailey *et al*. [11] reported that appearance of TyrP32 (which they called p32) was dependent on $CaCl_2$, but not on BSA and $NaHCO_3$, in boar spermatozoa incubated in Krebs Ringer-based solutions for 4.5 h, although they did not detect a second increase. They [11] also found a 32-kDa protein tyrosine kinase (TK-32) in capacitated boar spermatozoa using an assay for in-gel ^{32}P-labeled tyrosine kinase activity. This kinase is likely different from TyrP32 and is speculated to be a MAPK-like kinase that phosphorylates serine/threonine and tyrosine residues. However, the substrates for TK-32 remain to be determined in capacitating spermatozoa, although it is expected that TK-32 might phosphorylate TyrP32.

In the present study, external calcium was not required to increase TyrP32 in dead spermatozoa from boars after freezing-rapid thawing in PBS or PBS containing EGTA (Fig. 3 right panel). Moreover, addition of herbimycin A had no affect on the increase in TyrP32 (Fig. 3 central panel), indicating that herbimycin A-sensitive tyrosine kinases are not involved in this event. These results suggest that there are different mechanisms for the increase in TyrP32 between dead and capacitated/acrosome-reacted spermatozoa. Rapid thawing in a warm water bath (*i.e.*, warming) was important for the increase in TyrP32 (Fig. 3 left panel). Similarly, Bravo *et al*. [22] pointed out the importance of warming (at 39 C) for the increase in TyrP32 in cooled spermatozoa. Moreover, inhibition of sperm proteases with APMSF dramatically blocked the increase in TyrP32 in the spermatozoa after freezing-rapid thawing (Fig. 3 right panel), although it did not have any effects on changes in the distribution of acrosomal contents (Table 3). As described above, changes in the distribution of the acrosomal contents were observed in the spermatozoa after freezing-rapid thawing (Table 1 and Fig. 2 panel A). Acrosomal contents include a variety of proteases that can digest zona pellucida proteins after exocytosis [23, 24], and the predominant enzyme is acrosin [25]. Indeed, some major sperm proteins were digested by APMSF-sensitive proteases during freezing-rapid thawing (Fig. 4). Interestingly, TyrP32 is a (pro)acrosin-binding protein, and this indicates direct interaction between TyrP32 and acrosin. Thus, we conclude that TyrP32 can show a protease-dependent increase in dead spermatozoa after freeze-thawing without a cryoprotectant even though the dead spermatozoa do not undergo cryocapacitation.

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