

ORIGINAL ARTICLE

Stabilization of membrane-associated α -L-fucosidase by the human sperm equatorial segment

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Summary

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Previous reports from this laboratory documented the existence of two novel isoforms of α -L-fucosidase in human semen and showed that membrane-associated α -L-fucosidase is cryptically held within the acrosomal compartment and enriched within the sperm equatorial segment. The occurrence of these novel isoforms is provocative. Sperm proteins potentially involved in sperm-egg interactions must maintain their functional integrity as they travel through the female reproductive tract. The goal of this project was to investigate the stability of membrane-associated α -L-fucosidase in human sperm. Whole seminal plasma and Percoll[®] purified sperm cell populations were incubated for 72 h at 37 °C, with 5% CO₂ or ambient air. At various times during prolonged incubation, sperm cells were permeabilized with 0.1% Triton[®]X-100 and enzyme assays using the fluorogenic substrate 4-MU-fuc were performed to evaluate the stability of both the seminal plasma and membrane-associated α -L-fucosidase. Here, we report seminal plasma α -L-fucosidase activity rapidly decreased within 24 h. Conversely, α -L-fucosidase activity from Percoll[®] purified sperm cell populations persisted up to 72 h. Data from these experiments support the notions that (i) membrane-associated α -L-fucosidase is stable for extended periods of time, consistent with a possible role in sperm-egg interaction and (ii) membrane domains and compartmentalization within the human sperm are key to preserving protein integrity.

Introduction

Compartmentalization of the mammalian sperm cell is key to maintaining its functional capacity. The progress of fertilization requires an orchestrated, sequential exposure or activation of sperm surfaces, structures and functions. Unique division of the sperm head into compartments provides a mechanism by which the cell can sequester proteins until they are needed. The life history of the sperm cell is complex and different proteins become exposed/available as the membranes change during capacitation and the acrosomal compartment is opened. Three distinct regions of the sperm head, the acrosome (Ac), equatorial segment (EqS) and post-acrosome (Pac), each have exclusive subcellular structures and proteins. Of these regions, significant interest revolves around the EqS because it seems crucial during fertilization.

Although the EqS has been described for decades, new details continue to emerge as novel techniques are used to

explore this region. It has been well established that the EqS is a critical part of the sperm acrosome during fertilization. As early as 1970, it was reported that the EqS was of great functional importance because it (i) remained intact after the acrosome reaction, (ii) lies beneath the plasma membrane domain involved in egg membrane fusion and (iii) is the site for sperm nuclear envelope break down (Yanagimachi & Noda, 1970). Surface replicas of eight mammalian species revealed structural evidence that the EqS was composed of hexagonally packed particles, spaced 170 Å apart (Phillips, 1977); an observation consistent with previous cytochemical studies (Yanagimachi & Teichman, 1972). More recently, Ellis *et al.* (2002) employed atomic force microscopy to reveal topographical differences between the sperm plasma membrane regions. Their results indicate the EqS has a relatively smooth surface covering the width of the sperm head, compared to the rough, uneven surface of the Pac (Ellis *et al.*, 2002). A subcompartment, the equatorial subsegment (EqSS) was also observed as a

semicircular structure within the EqS with a coarse, uneven surface. The EqSS has been described for the Artiodactyla order and appears to change topographically following acrosome reaction (Ellis *et al.*, 2002; Jones *et al.*, 2007).

Specialized diffusion properties exist in the EqS. During capacitation, the sperm plasma membrane changes as it prepares to undergo the acrosome reaction. Unlike the plasma membrane, the EqS does not vesiculate during this stage and becomes fusogenic only after completion of the acrosome reaction (Allen & Green, 1995; Takano *et al.*, 1993; Yanagimachi, 1994). Utilization of fluorescence loss in photobleaching (FLIP) and fluorescence recovery after photobleaching (FRAP) techniques revealed the presence of diffusion barriers or molecular filters, between the EqS and the PAc compartments. FLIP analysis using 1,1'-dihexadecyl-3,3,3',3'-tetramethylindocarbocyanine (DiI_{C16}) showed single lipid molecules freely moving throughout the plasma membrane domains; however, large molecular complexes were unable to cross the boundary between the PAc and the EqS (James *et al.*, 2004).

The list of specialized proteins localized in the sperm EqS continues to grow. Previous work in our lab has shown localization of membrane-associated α -L-fucosidase to the EqS of human sperm cells (Venditti *et al.*, 2007). Here, we report the in situ enzyme stability for both seminal plasma and membrane-associated isoforms. Direct enzyme assays were conducted using seminal plasma and Percoll[®] washed human sperm to evaluate enzyme activity over 72 h. Results from all experiments support the conclusion that the functional integrity of membrane-associated α -L-fucosidase is stabilized by its association with the EqS, making this enzyme available for sperm-egg interactions and perhaps post-fusion events.

Materials and methods

Semen specimens

Human semen specimens were obtained from healthy volunteers, at least 18 years of age, in accord with approved human subjects protocols. Except as specified for frozen-thawed semen, all data reported here were obtained using fresh, individual semen samples collected on separate occasions for each experiment from four different semen donors. Each experiment was performed on a single semen specimen and no semen samples were pooled. All semen samples were characterized and assessed according to World Health Organization criteria (WHO, 1999).

Enzyme assays and stability conditions

α -L-Fucosidase activity was quantified as previously described (Venditti *et al.*, 2007). Briefly, each assay mixture contained 40 μ L 4-methyl-umbelliferyl- α -L-fucopyrano-

side (4-MU-fuc) (Sigma M8527), 10 μ L Human Sperm Medium (HSM) (Suarez *et al.*, 1986) and 10 μ L enzyme source. Briefly, HSM (pH 7.4 and 280 milliosmolar) contained 117 mM NaCl, 0.3 mM NaPO₄, 8.6 mM KCl, 2.5 mM CaCl₂, 0.49 mM MgCl₂, 2 mM glucose, 19 mM sodium lactate, 25 mM NaHCO₃, 0.25 mM sodium pyruvate along with penicillin (5000 IU/mL) and streptomycin (5000 μ g/mL) to inhibit bacterial growth. Enzyme activity was quantified by fluorescence of 4-methylumbelliferone (4-MU) at discrete time-points without the addition of stop reagents. All assays were conducted in duplicate for 10 min at pH = 7.0. Fluorescence was quantified using a TBS 380 fluorometer with minicell adaptor (Turner BioSystems, Sunnydale, CA, USA). For stability studies, samples of seminal plasma or Percoll[®] purified sperm (see below) were taken at incubation times 0, 24, 48 and 72 h, and subjected to 10 min assays for α -L-fucosidase. Stability of α -L-fucosidase was determined under two conditions: (i) 37 °C, 5% CO₂, high humidity and (ii) 37 °C, ambient air. All enzyme assays were conducted at the corresponding stability condition.

Preparation of Percoll[®] purified sperm cells

Human sperm cells were washed using a Percoll[®] gradient as described by Suarez *et al.* (1986). Two additional 10-min washes in 10 mL of HSM were done to remove remaining seminal plasma α -L-fucosidase. To assess enzyme stability, a 750 μ L stock solution of washed cells was incubated in an open microfuge tube under both conditions described above. At $t = 0, 24, 48$ and 72 h, the stock suspension was gently mixed and 45 μ L was removed and added to 5 μ L of 1% Triton[®]X-100 (0.1% final) and incubated 5 min at the respective incubation condition. Following permeabilization, enzyme activity was measured, with readings recorded at $t = 0$ and 10 min. Assay mixtures were incubated at their initial incubation condition during the 10-min time course. Enzyme assays were performed in duplicate at each time-point for each specimen tested. Throughout the time course of the incubation, sterile, neutralized, distilled water pre-equilibrated to each incubation condition was added to each stock tube to replace water lost as a result of evaporation. The beginning 750 μ L volume remained constant throughout the entire experiment. In addition, pH of the reaction mixture was recorded following completion of each assay using a colorfast pH strip. To evaluate sperm viability for each incubation condition, 10 μ L of sperm cells were removed at 0, 24, 48 and 72 h, mixed with propidium iodide (PI) (12 μ M final concentration) and evaluated using a Zeiss LSM 510 Meta confocal microscope (Carl Zeiss, Inc., Jena, Germany). Viability was quantified by calculating the percentage of PI-stained

sperm cells for each time-point. Data are reported here as average % PI-stained sperm cells at $t = 0, 24, 48$ and 72 h.

Preparation of seminal plasma

For seminal plasma α -L-fucosidase preparation, 500 μ L of whole semen was centrifuged at 10 000 g for 10 min. The supernatant was removed, microscopically inspected to confirm the absence of cells and transferred to a new microfuge tube containing 500 μ L of HSM. The sample was centrifuged for 10 min at 10 000 g and the top 250 μ L of supernatant was removed and mixed with 500 μ L of HSM in a fresh microfuge tube. Because α -L-fucosidase activity is substantially high in the seminal plasma, the sample was pre-diluted 1 : 100 by mixing 20 μ L of the seminal plasma stock and 1980 μ L of HSM. The final dilution was aliquoted into two microfuge tubes. This solution served as the seminal plasma α -L-fucosidase stock for the 72 h incubation. Throughout the course of the experiment, sterile, neutralized, distilled water pre-equilibrated to each incubation condition was added to each tube to replace water lost as a result of evaporation. Enzyme activity was measured at $t = 0, 24, 48$ and 72 h. Assay mixtures were incubated at their initial temperature conditions and fluorescence measurements recorded at $t = 0$ and 10 min. For each time-point, the seminal plasma stock was gently mixed and assays performed in duplicate.

Preparation of liquid nitrogen stored whole semen

Whole semen stored in 10% glycerol in liquid nitrogen was defrosted by removing the tube from liquid nitrogen and placing it in a -20 °C freezer for 20 min. The sample was then moved to 4 °C for 45 min. Once thawed, the sample was placed in a 15-mL conical vial and HSM was added to bring the volume to 10 mL. The sample was centrifuged for 10 min at 500 g . Following centrifugation, the supernatant was removed and HSM was added to bring the volume to 5 mL. The sample was centrifuged a final time for 5 min at 500 g . The pellet was resuspended in 1 mL HSM and α -L-fucosidase activity was measured over 72 h as previously described for Percoll[®] purified sperm cells.

Stability controls and calculations

Control α -L-fucosidase assays consisted of 40 μ L 4-MU-fuc, 10 μ L HSM and 10 μ L of either HSM, 1% Triton[®]X-100 or seminal plasma that had been previously boiled 10 min at 100 °C. Inclusion of these controls confirmed that enzyme assays measured α -L-fucosidase activity. No α -L-fucosidase activity was detected in any of these conditions. For 3-day stability studies, the enzymatic activity value for time zero was defined as the baseline

(100%) of α -L-fucosidase activity present for that particular sample and used to calculate percent α -L-fucosidase remaining at each subsequent time-point. α -L-fucosidase activity from Percoll[®] purified and liquid nitrogen-stored sperm preparations was calculated similarly, except fluorescence values were converted to average fluorescence per 100 000 cells. Again, average fluorescence at each discrete time-point was compared to $t = 0$ to determine % enzyme activity present. Data were plotted as time vs. average % relative α -L-fucosidase activity.

Immunolocalization of α -L-fucosidase

Immunolocalization studies were performed as previously described (Venditti *et al.*, 2007) for the Percoll[®] purified sperm cells prepared as described above, except sperm were not doubly labelled with fluorescein-conjugated *Pisum sativum* agglutinin (FITC-PSA). Briefly, an 8 μ L aliquot was removed at $t = 0, 24, 48$ and 72 h for each incubation condition. Samples were fixed onto Esco fluoro slides with 100% methanol and stained with polyclonal goat-anti-human liver α -L-fucosidase antibodies followed by a rhodamine-conjugated secondary rabbit-anti-goat-IgG antibody (KPL, Inc., Gaithersburg, MD, USA). Slides were evaluated using a Zeiss LSM 510 Meta confocal microscope. Images were reconfigured with Adobe Photoshop CS2 and presented here as confocal maximum projections.

Results

Stability of seminal plasma α -L-fucosidase

Previous biochemical analyses of seminal plasma α -L-fucosidase indicated a single subunit 56 kDa in size present in high concentration in human seminal plasma (Khunsook *et al.*, 2002). In this study, its stability in situ was investigated to further elucidate its possible roles during fertilization. Figure 1 shows the 72 h stability of seminal plasma α -L-fucosidase (cell free sample) graphically represented as time vs. average % relative α -L-fucosidase activity. After 24 h, less than 10% of the original enzyme activity was detectable, corresponding to a loss of 90% enzyme activity. By 72 h, no activity was detected. Although present in high quantities, the seminal plasma isoform is highly unstable under both assay conditions. Given this instability, seminal plasma α -L-fucosidase may function prior to fertilization; possibly during penetration of the cervical mucus or the cumulus mass.

Stability of membrane-associated α -L-fucosidase

Membrane-associated α -L-fucosidase is a single pass transmembrane protein 51 kDa in size distributed over the

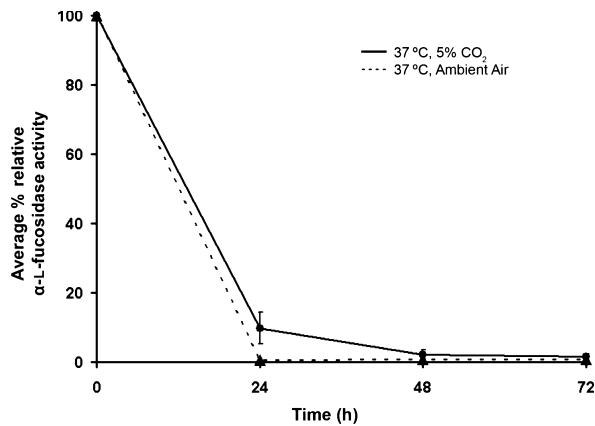


Figure 1 Stability of seminal plasma α -L-fucosidase at 37 °C, 5% CO₂, high humidity and 37 °C, ambient air. Data represent averages from four independent experiments. Error bars represent mean standard error.

entire sperm plasma membrane system with enrichment in the EqS (Khunsook *et al.*, 2003; Venditti *et al.*, 2007). Biochemically, the two semen isoforms of α -L-fucosidase are different, and here we report significant differences in their stabilities. As shown in Fig. 2, more than 80% of the original α -L-fucosidase activity was detectable at 24 h when incubated at 37 °C, 5% CO₂, high humidity and more than 70% was present after 72 h. After 3 days of incubation, only 30% of the original enzyme activity is lost, indicative of enzyme stabilization. For both incubation conditions, initial pH at $t = 0$ was 7.0. Following 72 h of incubation at 37 °C, 5% CO₂, the pH remained constant. By contrast, at 37 °C, ambient air, the pH increased from the initial 7.0 to 9.0 after 72 h.

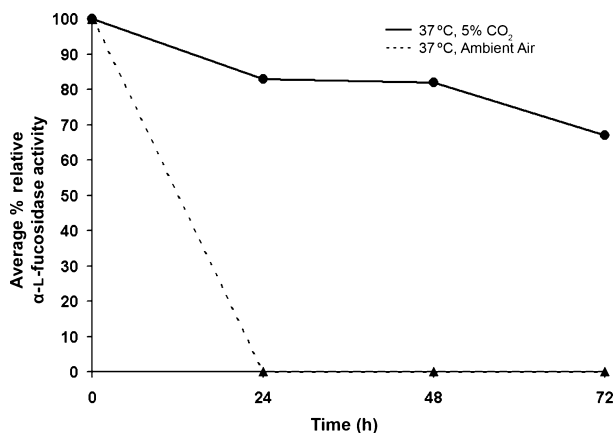


Figure 2 Stability of membrane-associated α -L-fucosidase at 37 °C, 5% CO₂, high humidity and 37 °C, ambient air. Data represent averages from nine independent experiments. Mean standard errors were less than 0.2 (not included on graph).

Table 1 Sperm cell viability quantified using propidium iodide (PI)

Time (h)	Average % PI-stained sperm
A	
0	71.2
24	88.6
48	100
72	100
B	
0	71.2
24	98
48	100
72	100

Human sperm cells were labelled with 12 μ M PI at 0, 24, 48 and 72 h post-incubation at 37 °C, 5% CO₂ (A) and 37 °C, ambient air (B). For each specimen, five different microscopic fields were evaluated at each time-point and the numbers of stained and unstained sperm were recorded. The % PI was calculated by the number of stained sperm divided by the total number of sperm evaluated, multiplied by 100.

Experiments evaluating sperm cell viability were performed by staining whole semen and Percoll[®] purified sperm preparations with 12 μ M PI. Results are reported here in Table 1 as average % PI-stained sperm cells for each time-point. On average, whole semen contained 18% PI-stained sperm cells. Following manipulation of sperm cells in the laboratory, namely Percoll[®] purification and washing, the average % PI-stained sperm cells increased to 71.2%. As shown in Table 1, after 24 h of incubation at 37 °C, 5% CO₂ and 37 °C, ambient air, the average % PI-stained cells increased to 88.6 and 98%, respectively. By 48 h for both conditions, all sperm cells were stained with PI. The increased PI staining at $t = 0$ compared to the whole semen sample was likely due to the manipulation of sperm cells required to enrich for sperm and remove seminal plasma. Despite this apparent cell death, sperm membrane-associated α -L-fucosidase retains its activity for up to 72 h.

Immunolocalization studies were performed to evaluate the association of α -L-fucosidase with the sperm membrane over the course of the 72 h incubation period. Figure 3 shows confocal maximum projections of Percoll[®] purified sperm cells at 24 and 72 h post-incubation at 37 °C, 5% CO₂ and 37 °C, ambient air. Sperm were labelled with anti-fucosidase antibodies (red). As seen in Fig. 3, α -L-fucosidase was associated with the sperm membrane throughout the entire length of the 72 h incubation. These results confirm that enzyme assays indeed measured the stability of membrane-associated α -L-fucosidase.

Membrane-associated α -L-fucosidase activity can be recovered from whole semen samples stored with 10%

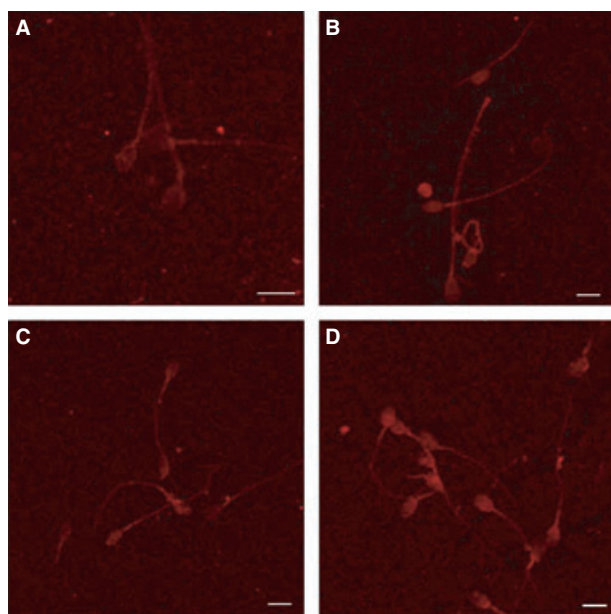


Figure 3 Immunolocalization of α -L-fucosidase in human sperm cells. Confocal maximum projections of sperm labelled with anti-fucosidase antibodies following 24 h of incubation at 37 °C, 5% CO₂ (A), 37 °C, ambient air (C) and following 72 h incubation at 37 °C, 5% CO₂ (B), 37 °C, ambient air (D). Scale bar represents 10 μ m.

glycerol in liquid nitrogen as shown in Fig. 4. Aliquots of semen samples from two different donors previously used for membrane-associated stability tests were frozen in liquid nitrogen and subsequently thawed to measure α -L-fucosidase stability. Although the α -L-fucosidase present was not as stable as the fresh membrane-associated isoform, it is clearly more stable than seminal plasma enzyme. Stability of α -L-fucosidase from liquid nitrogen stored semen samples was evaluated only at 37 °C, 5%

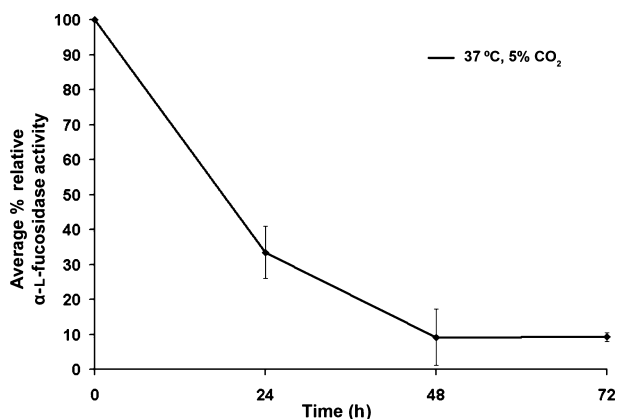


Figure 4 Stability of α -L-fucosidase from liquid nitrogen stored sperm at 37 °C, 5% CO₂, high humidity. Data represent averages from three independent experiments. Error bars represent mean standard error.

CO₂, because these laboratory conditions more closely resemble the physiological environment that a sperm would experience during an insemination procedure. Despite variation in stability of α -L-fucosidase from liquid nitrogen stored semen samples, ample enzyme activity is present to complete a fertilization event within the normal timeline of donor insemination.

Discussion

Sperm-associated α -L-fucosidases have been reported for humans (Alhadeff *et al.*, 1999; Khunsook *et al.*, 2002, 2003), rats (Hancock *et al.*, 1993; Aviles *et al.*, 1996; Abascal *et al.*, 1998), *Drosophila* (Intra *et al.*, 2006), ascidians (Matsumoto *et al.*, 2002), certain fishes of the family Percidae (Venditti, Mendelson and Bean, in preparation), bulls (Jauhainen & Vanha-Perttula, 1986), chimpanzees (Srivastava *et al.*, 1981), *Unio elongatulus* (Focarelli *et al.*, 1997) and Syrian hamsters (J. J. Venditti and B. S. Bean, unpublished observations). The presence of these isoforms in diverse organisms is consistent with the importance of carbohydrates during fertilization. Several studies provide direct evidence supporting roles for fucose-containing polysaccharides and/or fucosidases during fertilization. In the ascidian *Halocynthia roretzi*, sperm-egg binding is mediated by sperm α -L-fucosidase and complementary L-fucose residues found on glycoproteins of the vitelline envelope (Matsumoto *et al.*, 2002). The sperm-associated α -L-fucosidase stability data reported here extends previous arguments placing α -L-fucosidase on the list of proteins required during fertilization.

Results reported here document a striking difference between the seminal plasma and membrane-associated isoforms of α -L-fucosidase. As evident in Fig. 1, seminal plasma α -L-fucosidase is highly unstable at 37 °C, 5% CO₂ and under ambient air conditions. The variation in the stability of seminal plasma α -L-fucosidase after 24 h of incubation at 37 °C, 5% CO₂ is not unexpected. One possible explanation is the inconsistency between semen samples from different donors. Human semen parameters are not homeostatically regulated. For example, protein content and pH vary between donors such that no two semen samples are identical. Enzyme assays performed in these studies apparently reflect the natural decay of α -L-fucosidase activity over time. Previous studies in our laboratory have shown that purified seminal plasma α -L-fucosidase is labile unless frozen following purification (Khunsook *et al.*, 2002).

Unlike the seminal plasma isoform, membrane-associated α -L-fucosidase retains substantial enzyme activity for 72 h during incubation at 37 °C, 5% CO₂, high humidity. In sharp contrast, during and following purification to homogeneity, the human sperm membrane-associated

α -L-fucosidase is highly labile (Khunsook *et al.*, 2003). Its stability in situ seems attributable to its integration into the EqS. α -L-Fucosidase preparations similar to those reported here have been previously shown to be inhibitable by deoxyfuconjirimycin (DFJ), a specific, known potent inhibitor of α -L-fucosidase activity (Winchester *et al.*, 1990; Venditti *et al.*, 2007), confirming these assays indeed measured α -L-fucosidase activity. Although the membrane-associated α -L-fucosidase appears unstable at 37 °C, ambient air, this decrease in enzyme activity is most likely attributable to a basic shift in pH. Previous biochemical studies using purified human membrane-associated α -L-fucosidase revealed optimal activity at acidic pH (4.0–6.0) with 80% of the maximal activity at pH 7.0 (Khunsook *et al.*, 2003).

We have previously demonstrated that α -L-fucosidase activity is cryptically stored within the EqS (Venditti *et al.*, 2007). Prior to measuring enzyme activity at each time-point, sperm cells were permeabilized with 0.1% Triton[®]X-100, exposing the cryptic stores of α -L-fucosidase activity. This procedure renders the remaining total active α -L-fucosidase accessible to substrate, regardless of the physiological condition of individual cells. Thus, the assay focused on stabilized α -L-fucosidase activity, and not the live or dead status attributable to populations. In addition, data from the immunolocalization studies presented here reinforce the notion of the EqS as a stabilizing feature of the sperm cell, as α -L-fucosidase remained associated with the sperm membrane throughout the 72 h incubation. Studies on the immunolocalization of fucosidase protein, following various incubation and preparation treatments, showed that the protein remained associated with the membranous structures of human sperm (Fig. 3). As expected, subcellular immunolocalization patterns reveal enrichment of the protein in the equatorial and posterior head regions of the sperm. These patterns appear to persist in cells that may have undergone acrosome reaction or death prior to sampling for immunolocalization.

Data from these stability studies reinforce the notion that membrane-associated α -L-fucosidase is likely to have a role in sperm–egg interaction. Under physiologically relevant conditions, 37 °C, 5% CO₂, membrane-associated α -L-fucosidase is 8.5 times more stable than the soluble seminal plasma isoform after 24 h of incubation. As evident by the striking contrast in enzyme stability, association of α -L-fucosidase with the sperm membranes, and especially the EqS, not only sequesters this enzyme but stabilizes its function beyond the timeframe expected for a successful fertilization event. Exposure of the EqS occurs after release of the acrosomal compartment, consistent with expected roles during fertilization. Localization to the EqS and stability up to 72 h delineates a timeline for

possible action of α -L-fucosidase during sperm–egg interaction.

Our findings on the stability and functional distribution of the human sperm membrane-associated α -L-fucosidase are provocative and invite further research. All lines of evidence continue to support the hypothesis that membrane-associated α -L-fucosidase is required during sperm–egg interactions. Specific roles of α -L-fucosidase during and following sperm–egg interaction in mammals remain to be identified. Understanding the role of this enzyme during fertilization could advance the development of improved diagnosis and/or treatment of infertility.

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