

Determination of glycosidase activity in porcine oviductal fluid at the different phases of the estrous cycle

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Abstract

Sperm–oocyte binding and gamete–oviductal epithelium interactions are carbohydrate-mediated events occurring in the oviductal fluid (OF). Thus, knowledge about the activities of glycosidases (enzymes catalyzing hydrolytic cleavage of terminal sugar residues) in this milieu would help us understand the molecular mechanisms involved in these events. This work was carried out to investigate the glycosidase activity, protein content, and volume of OF collected from gilts and sows. Oviducts were classified into four phases of the estrous cycle (early follicular, late follicular, early luteal, and late luteal) based on the appearance of the ovaries. OF was aspirated, centrifuged, measured for volume, and frozen until assay. Substrates conjugated to 4-methylumbelliferyl were used to screen the activities of seven different glycosidases at physiological pH (7.2). α -L-Fucosidase and β -N-acetyl-glucosaminidase activities increased at the late follicular phase to decrease after ovulation. β -D-Galactosidase, α -D-mannosidase, and β -N-acetyl-galactosaminidase showed higher activities at the early follicular phase, which decreased after ovulation. N-Acetyl-neuraminidase and α -D-galactosidase did not show activity at any phase of estrous cycle neither in sows nor in gilts at pH 7.2, although it did at acidic pH (4.4) in the follicular and luteal phase samples. Total protein also changed during the cycle showing the maximum secretion at the late follicular phase (2118.6 ± 200.7 μ g/oviduct). The highest volumes of OF were collected from the oviducts at the late follicular phase (50.7 ± 1.3 μ l/oviduct). These results indicate that OF from sows and gilts shows glycosidase activity varying throughout the estrous cycle suggesting a role of these enzymes in carbohydrate-mediated events.

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Introduction

Sperm–egg interactions in numerous species including mammals are mediated by protein receptors on the sperm plasma membrane attaching to carbohydrate-containing molecules on the surface (i.e., zona pellucida) of oocytes reviewed by Benoff (1997). Binding of boar sperm to epithelial cells in the oviduct is a selective carbohydrate-mediated process (Talbot *et al.* 2003, Rath *et al.* 2006, Taylor *et al.* 2008). All these events take place in the oviduct where appropriate enzymes may change the structure of the interacting oligosaccharides. Glycosidases are enzymes, usually contained in the lysosomes that catalyze hydrolytic cleavage of terminal sugar residues from the glycan portion of glycoproteins and glycolipids. Although it was expected for glycosidases to be functional only in acidic environments, Tulsiani *et al.* (1995) demonstrated that this is not always the case. As an example, β -galactosidase optimally cleaves the synthetic substrate at acidic pH, but it shows maximum activity toward glycoprotein substrates in a neutral environment. This may explain how the

β -galactosidase or perhaps other ‘acidic’ glycosidases could be functional in the different extracellular sites where they have been detected, such as blood (Tulsiani & Touster 1981), sperm membranes (Tulsiani *et al.* 1989, Cornwall *et al.* 1991), epididymal luminal fluid (Skudlarek *et al.* 1993), and fluids from the female reproductive tract (Roberts *et al.* 1975, 1976, Tulsiani *et al.* 1996).

Oviductal fluid (OF) is the physiological milieu where fertilization occurs. However, precise studies about glycosidase activity in the OF during the estrous cycle in mammalian species such as the pig or cow, where reproductive technologies are usually and widely performed, are scarce (Roberts *et al.* 1975, 1976, Tulsiani *et al.* 1996). On the contrary, studies on several glycosidase activities in sperm membranes (Tulsiani *et al.* 1989, Cornwall *et al.* 1991) and epididymal fluid (Skudlarek *et al.* 1993) are available.

Although most glycosidases are most active at acidic pH (Tulsiani *et al.* 1995), they can show activity at higher pH. The pH profile in the pig oviduct is not static, but differs considerably with region of the oviduct and with

stage of cycle (Nichol *et al.* 1997). In cannulated animals, it was 7.9 (Engle *et al.* 1968) and 8.1 just before ovulation on the second day of estrus (Hunter 1988). A wider study showed that fluid collected from the ampullae of cannulated animals was 7.9 and 7.3 in preovulatory and postovulatory phases (Nichol *et al.* 1997) respectively, and we have observed that porcine fluid obtained after oviduct aspiration is around 6.6–7.3 (unpublished data). Therefore, the presence of active glycosidases at a neutral pH in the OF could have potential roles in different events related to sperm reservoir formation, sperm capacitation, final oocyte maturation, gamete interaction, and early embryo development. It has already been proposed that glycosidases could be involved in the control of polyspermy (Miller *et al.* 1993, Velasquez *et al.* 2007), sperm–oviductal epithelial cells interaction (Lefebvre *et al.* 1997), sperm–zona pellucida binding (Miller *et al.* 1993, Matsumoto *et al.* 2002, Venditti *et al.* 2007), sperm capacitation (Taitzoglou *et al.* 2007), or dispersion of cumulus cells (Takada *et al.* 1994). All these events take place in the oviduct and have been the aim of studies to increase our knowledge about the molecular basis of reproductive functions, but, surprisingly, we lack of precise studies about the activities of the different glycosidases in the OF. The exception could be found in some early reports from Roberts *et al.* (1975, 1976) showing low activities for α -L-fucosidase, β -D-fucosidase, α -D-galactosidase, β -D-galactosidase, α -D-glucosidase, β -D-glucosidase, β -N-acetyl-galactosaminidase, β -N-acetyl-glucosaminidase, α -D-mannosidase, and β -D-mannosidase in the OF from cattle and sheep, with a significant increase during diestrus and pregnancy for the activities of β -N-acetyl-galactosaminidase and β -N-acetyl-glucosaminidase. Apart from these studies, performed at acidic pH, there is no further available information.

The main objective of the present study was to determine the activities of seven exoglycosidases, whose roles in different reproductive events have been speculatively proposed, in the OF of gilts and sows at the follicular or luteal phase of the estrous cycle. The assayed glycosidases were α -L-fucosidase, β -N-acetyl-glucosaminidase, β -D-galactosidase, α -D-mannosidase, β -N-acetyl-galactosaminidase, α -D-galactosidase, and N-acetyl-neuraminidase.

Results

Glycosidase activity

Sow and gilt OF displayed α -L-fucosidase, β -N-acetyl-glucosaminidase, β -D-galactosidase, α -D-mannosidase, and β -N-acetyl-galactosaminidase activities at all phases of the cycle when assessed at pH 7.2. OF from sows showed statistical differences among follicular and luteal phases for α -L-fucosidase ($P < 0.01$; Fig. 1A),

β -N-acetyl-glucosaminidase ($P < 0.01$; Fig. 1B), β -D-galactosidase ($P < 0.01$; Fig. 1C), α -D-mannosidase ($P < 0.01$; Fig. 1D), and β -N-acetyl-galactosaminidase ($P < 0.01$; Fig. 1E) activities. The significances were maintained when the data were transformed into specific enzymatic activity (SEA; Fig. 1). Some patterns could be observed in the activity of the enzymes. On the one hand, α -L-fucosidase and β -N-acetyl-glucosaminidase showed maximum activity at the late follicular phase (28.4 and 36.2 U respectively) with a decrease of 50% in the activity after ovulation and then steadily increased their activity at the late luteal and early follicular phases. On the other hand, β -D-galactosidase, α -D-mannosidase, and β -N-acetyl-galactosaminidase showed higher activity at the early follicular phase (34.3, 51.0, and 34.1 U respectively) decreasing 65–70% of their activity close to ovulation and maintaining these low levels during the luteal phase.

Glycosidase activity at pH 7.2 in OF from gilts at the early follicular phase is shown in Fig. 2. Comparison between EA of OF from sows and gilts at the same cycle phase (early follicular phase) is shown in Fig. 3. Comparison of EA did not reach statistical significance but α -L-fucosidase had a tendency to be more active in OF from gilts ($P = 0.06$). When SEA data were compared, it was observed that β -D-galactosidase had a higher activity in OF from sows ($P = 0.04$) being the SEA of the remaining glycosidases similar between prepubertal and pubertal animals.

All fluorescence data (arbitrary fluorescence units) obtained for α -L-fucosidase, β -D-glucosaminidase, β -D-galactosidase, α -D-mannosidase, and β -D-galactosaminidase were higher in the OF than in the positive controls run in human seminal plasma (data not shown).

Neither OF from sows nor from gilts showed α -D-galactosidase or N-acetyl-neuraminidase activity in any sample at any phase of the estrous cycle at pH 7.2. Positive controls reached 26 453 and 13 438 fluorescence units for α -D-galactosidase and N-acetyl-neuraminidase respectively. However, both enzymes showed activity at pH 4.4. EA of α -D-galactosidase was 97.27 ± 34.79 and 116.20 ± 68.09 U respectively for follicular and luteal phases. Neuraminidase reached a discrete activity with 4.48 ± 1.60 and 5.36 ± 3.14 U respectively for follicular and luteal phases.

Protein content and volume of OF

Concentration of protein ($\mu\text{g}/\mu\text{l}$ OF) in OF from sows is shown in Table 1. A lower concentration of protein was observed at the late follicular phase. However, the volume of OF reached its maximum at the late follicular phase ($50.7 \mu\text{l}/\text{oviduct}$; Table 1) to decrease progressively after ovulation (early luteal phase). Therefore, when the total amount of protein ($\mu\text{g}/\text{oviduct}$) was calculated, significant differences were found with the highest secretion of protein close to the moment of ovulation ($2118.6 \pm 200.7 \mu\text{g}/\text{oviduct}$) decreasing at the early luteal phase (after ovulation).

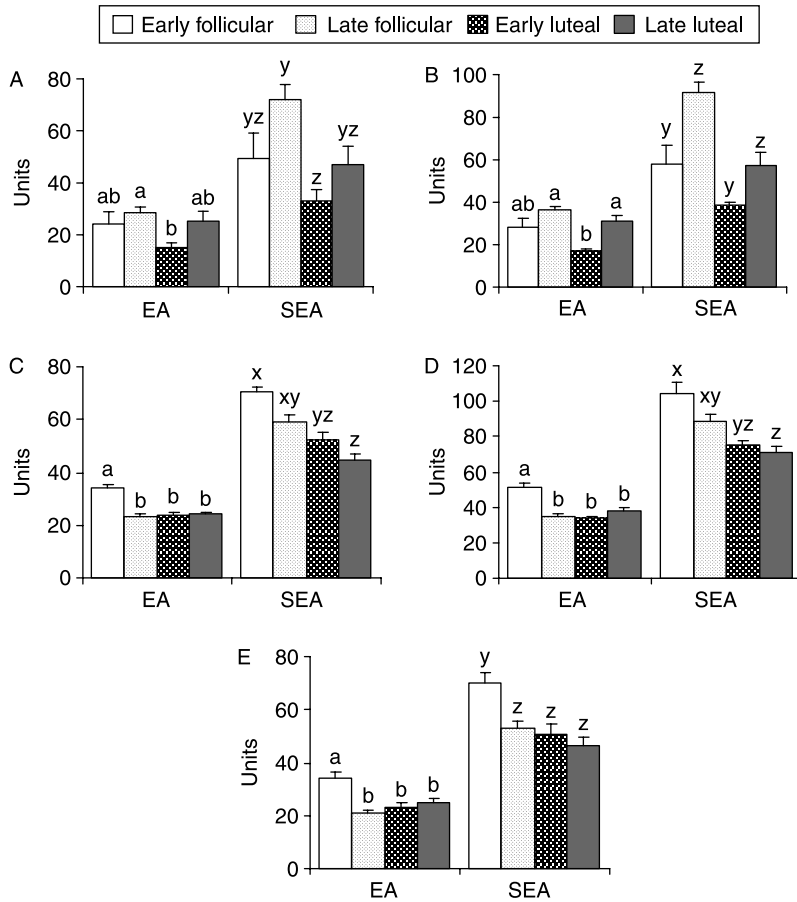


Figure 1 Units for enzymatic activity (EA) and specific enzymatic activity (SEA) for several glycosidases assessed at pH 7.2 in oviductal fluid collected from sows at different stages of the estrous cycle. (A) α -L-fucosidase, (B) β -N-acetyl-glucosaminidase, (C) β -D-galactosidase, (D) α -D-mannosidase and (E) N-acetyl-galactosaminidase. Different letters in each enzyme and in the same activity denote statistical significance ($P < 0.01$).

Data from OF from gilts are presented in Table 2. Comparison of the total amount of protein ($\mu\text{g}/\text{oviduct}$) between prepubertal and pubertal animals at the early follicular phase did not show any difference.

Discussion

Oviduct-sperm interaction and fertilization in mammals are carbohydrate-mediated processes that take place in the oviduct (reviewed by Talbot *et al.* (2003)). It is obvious then to suppose that oviductal glycosidases, if present and active at a physiological pH, may have a role in fertilization, as has been reported for other glycosidases in sperm membranes (Tulsiani *et al.* 1989, Cornwall *et al.* 1991) or epididymal fluid (Skudlarek *et al.* 1993). However, and surprisingly, no studies about enzymatic composition in such an important biological fluid as the OF have been carried out in mammals, except some old studies in cows and sheep (Roberts *et al.* 1975, 1976) or more recently in the hamster (Tulsiani *et al.* 1996). The results from this study show detailed and novel data about the glycosidase activity at physiological pH and protein concentration in the

porcine OF. Due to the lack of previous reports demonstrating such an activity and the lack of knowledge about the specific molecules involved in the porcine gametes interaction, all our interpretations for their presence in this milieu are only speculative.

Porcine OF from gilts and sows showed α -L-fucosidase, β -N-acetyl-glucosaminidase, β -D-galactosidase, α -D-mannosidase, and β -N-acetyl-galactosaminidase activities when assessed at neutral pH with variations during the estrous cycle. All these glycosidases showed maximum activity at the follicular phase, decreasing after ovulation. We have to bear in mind that in our study fluid from whole oviducts was pooled. It has been shown that the pH profile in the pig oviduct is not static and differs considerably with region of the oviduct and stage of cycle (Nichol *et al.* 1997), so *in vivo* oviductal EA could be slightly different in the specific regions of the oviduct. Given that glycosidases are glycoproteins, and the oviductal protein secretion is importantly regulated by ovarian steroids, mainly estrogen (Buhi *et al.* 2000), it could be inferred that the changes in glycosidase activity during the estrous cycle might be hormonally regulated.

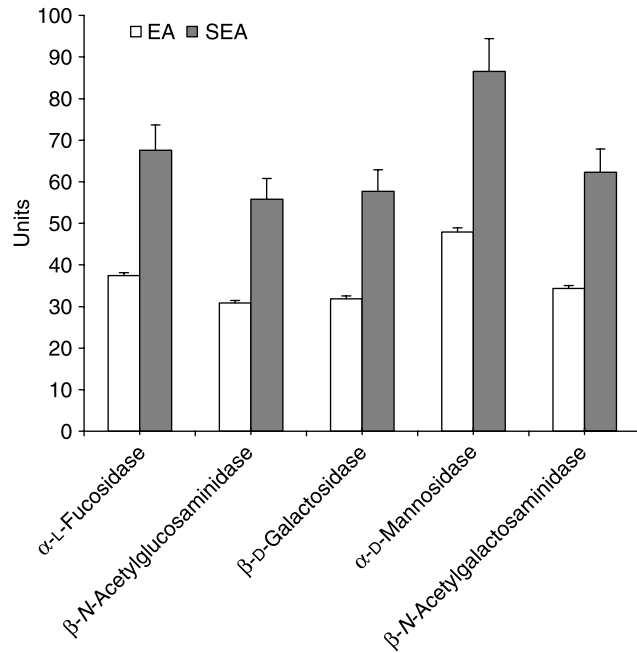


Figure 2 Units of enzymatic activity (EA) and specific enzymatic activity (SEA) for several glycosidases assessed at neutral pH in porcine oviductal fluid collected from gilts at early follicular phase.

In this study, we show that porcine OF has α -L-fucosidase activity that is coincident with previous studies in hamster OF (Tulsiani *et al.* 1996). In pigs, experiments with lectins show that carbohydrates are differently expressed among regions of the oviduct during the different phases of the estrous cycle (Raychoudhury *et al.* 1993, Walter & Bavdek 1997, Sant'ana *et al.* 2005). *Lotus tetragonolobus* lectin mainly recognizes α -L-fucose residues, and during the follicular phase its binding sites are only present on epithelial cells of the isthmic segment, the ampulla and infundibulum being unreactive (Walter & Bavdek 1997). Besides, there is evidence for a fucose-binding protein in boar (Topfer-Petersen *et al.* 1985) and, also, bovine (Ignatz *et al.* 2007) spermatozoa. In theory, the release of spermatozoa from the sperm reservoir could either be a consequence of a loss of binding sites or due to alterations in the spermatozoa themselves (reviewed by Rodriguez-Martinez (2007)). Thus, the active oviductal α -L-fucosidase could remove the α -L-fucose residues and likely take part in sperm release from the oviduct since it is known that the isthmus is the functional sperm reservoir in pigs (Hunter 2005). Other mechanisms already described that such activation of sperm motility by sperm bicarbonate uptake might also contribute to this release, together with α -L-fucosidase (Brandt *et al.* 2006). This hypothesis needs to be confirmed but it is supported by our observation of maximum α -L-fucosidase activity close to the moment of ovulation.

According to our results, OF shows hexosaminidase activity (β -N-acetylglucosaminidase and β -N-acetyl-

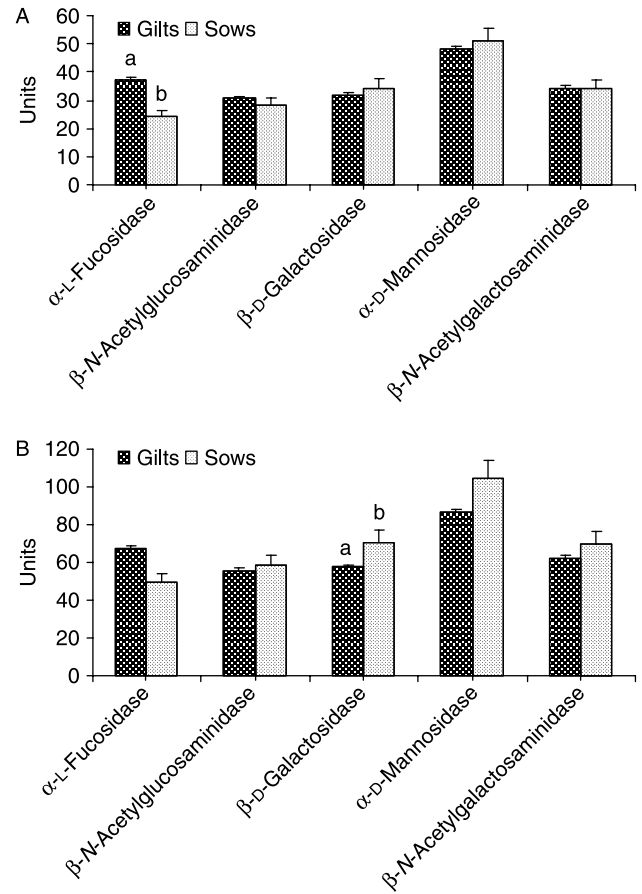


Figure 3 Comparison of enzymatic activity for several glycosidases assessed at pH 7.2 in oviductal fluid collected from sows and gilts at early follicular phase. (A) Units of enzymatic activity (EA). Different letters in each enzyme and in the same activity denote statistical significance ($P=0.06$). (B) Units of specific enzymatic activity (SEA). Different letters in each enzyme and in the same activity denote statistical differences ($P<0.05$).

galactosaminidase). β -N-Acetylglucosaminidase activity has been already described in the oviduct from other species (Roberts *et al.* 1975, 1976, Tulsiani *et al.* 1996, Droba *et al.* 2005), and in our study the highest activity was at the late follicular phase. Regarding oviductal β -N-acetyl-galactosaminidase activity, no studies are available and we observed its maximum activity at the early follicular phase. These results are in concordance with lectin studies in the porcine oviduct. On the one hand, it has been shown that *Triticum vulgare*, specific against D-N-acetylglucosamine sugar residues, reacts strongly with the oviduct epithelium during the follicular phase (Walter & Bavdek 1997). On the other hand, studies with *Helix pomatia* (Walter & Bavdek 1997) and *Dolichos biflorus* lectins, which recognize α -GalNAc residues, have shown that the porcine oviduct expresses this sugar especially during the follicular phase. Therefore, oviductal hexosaminidase might have a role in remodeling the oviduct

Table 1 Protein content, volume, and total amount of protein in oviductal fluid from sows at different phases of estrous cycle.

OF from sows	Protein (µg/µl OF)	Volume (µl OF/oviduct)	Total protein (µg/oviduct)
Early follicular	48.9 ± 3.6 ^{a,b} (Ns = 25)	27.9 ± 0.3 ^a (No = 49)	1354.8 ± 172.8 ^a
Late follicular	39.5 ± 1.4 ^a (Ns = 57)	50.7 ± 1.3 ^b (No = 48)	2118.6 ± 200.7 ^b
Early luteal	45.3 ± 2.5 ^{a,b} (Ns = 53)	40.2 ± 1.3 ^a (No = 60)	1680.5 ± 122.8 ^a
Late luteal	53.6 ± 4.0 ^b (Ns = 33)	30.0 ± 0.5 ^a (No = 69)	1563.2 ± 149.8 ^a
<i>P</i> value	<0.01	<0.01	0.02

^{a,b}Different letters in the same column represent statistically significant differences. Ns, number of samples analyzed; No, number of oviducts aspirated.

surface, thus affecting gamete interactions with oviductal cells. Although specific GlcNAc and GalNAc lectins in boar sperm have not been described yet, a lectin binding GalNAc in rat spermatozoa has been described (Abdullah & Kierszenbaum 1989). Another role that could be hypothesized for the oviductal hexosaminidase concerns the dispersion of cumulus cells from ovulated oocytes. This role has been attributed to the boar acrosomal β-N-acetyl-glucosaminidase that has been proposed to facilitate the passing by sperm through cumulus cells before they bind to the zona pellucida (Takada *et al.* 1994). However, the acrosomal content of the fertilizing spermatozoa is mainly released after sperm binding to the zona pellucida (ZP), making it difficult for the acrosomal β-N-acetyl-glucosaminidase to participate in the cumulus dispersion (Fazeli *et al.* 1997, Topper *et al.* 1999). We hypothesize that it might be the oviductal β-N-acetyl-glucosaminidase that is responsible for such an effect. Besides, in our study, maximum activity of oviductal β-N-acetyl-glucosaminidase was observed around fertilization time. Finally, a last role for the oviductal hexosaminidase might be related to the sperm–oocyte interaction, hydrolyzing the β-N-acetyl-glucosamine moieties present at the porcine ZP and thus affecting the sperm binding to the zona pellucida.

Porcine OF showed β-D-galactosidase activity as previously found in OF from several species (Roberts *et al.* 1975, 1976, Tulsiani *et al.* 1996, Droba *et al.* 2005). A feasible role for oviductal β-D-galactosidase would concern remodeling the ZP since β-galactosyl residues in the ZP oligosaccharides are involved in porcine sperm–egg binding (Yonezawa *et al.* 2005). These authors showed that treatment with β-D-galactosidase and *endo*-β-D-galactosidase of isolated porcine ZPs reduced the number of sperm bound and inhibited the sperm penetration in treated oocytes. These data

imply that *in vivo*, β-D-galactosidase could regulate the sperm binding sites present in the zona pellucida, reducing the sperm that could fertilize the oocyte and consequently reducing polyspermy. This could be one of the reasons for the different rate of polyspermy observed under *in vivo* and *in vitro* fertilizations.

In our study, α-D-mannosidase activity was maximal during the follicular phase of the cycle, similar to the observations in the rat (Pizarro *et al.* 1984). Oviductal α-D-mannosidase activity has also been described in ruminants and hamster (Roberts *et al.* 1975, 1976, Tulsiani *et al.* 1996). The role of oviductal α-D-mannosidase might be mainly modulating the sperm–oviduct interaction. Porcine oviduct shows reactivity toward concanavalin A lectin that binds to α-D-mannose residues (Walter & Bavdek 1997), and it is known that mannose acts as a sperm receptor in the porcine oviductal cells (Wagner *et al.* 2002). Therefore, α-mannose residues could be eliminated by oviductal α-D-mannosidase, thus regulating the population of sperm attaching to the oviduct. Once attached, their release could be regulated by means of the α-mannosidase anchored in the plasma membrane of the boar acrosomal region (Kuno *et al.* 2000) or even by the oviductal one. Boar spermadhesin AWN-1 is a sperm surface-associated lectin and a major protein of porcine seminal plasma (Calvete *et al.* 1997). Recent studies have shown that boar spermadhesins AWN and AQN1 are the dominant carbohydrate-binding sperm proteins and AQN1 has been proposed as the molecule involved in the formation of the oviductal sperm reservoir (Ekhlasi-Hundrieser *et al.* 2005). Binding of boar sperm to oviductal epithelium was inhibited by the addition of AQN1 but not by AWN (Ekhlasi-Hundrieser *et al.* 2005). AQN1 recognizes both α- and β-linked galactose as well as Manα1-3 (Manα1-6)Man structures. Recently, it has been shown that an oviductal protein, sperm binding glycoprotein, binds to boar sperm and exposes carbohydrate groups that can be later recognized by AQN1 (Pérez *et al.* 2006), and that AQN3 in porcine sperm apical plasma membrane firmly binds to ZP (van Gestel *et al.* 2007). Therefore, both oviductal α-D-mannosidase and β-D-galactosidase might remove these residues affecting sperm interaction with oviductal cells and/or ZP binding. In fact, we have observed that both enzymes have its maximum activity at the early follicular phase, but it significantly decreases at the late follicular and luteal phases. This activity decrease

Table 2 Protein content, volume, and total amount of protein in oviductal fluid from gilts at the early follicular phase of estrous cycle.

OF from gilts	Protein (µg/µl OF)	Volume (µl OF/oviduct)	Total protein (µg/oviduct)
Early follicular	55.3 ± 4.3 (Ns = 27)	23.5 ± 0.6 (No = 43)	1367.8 ± 86.1

Ns, number of samples analyzed; No, number of oviducts aspirated.

might help to maintain boar spermatozoa in the isthmus and to create the sperm reservoir, but at the same time their moderate activity might steadily release the sperm facilitating the fertilization of ovulated oocytes, although this hypothesis needs to be confirmed.

N-acetyl-neuraminidase and α -D-galactosidase did not show activity in the OF from sows or gilts at any stage of the cycle when assessed at neutral pH, but they displayed activity at acidic pH. These results suggest that the role of these enzymes at the fertilization process, if any, might be acting as lectins rather than as catalysts. It has been shown that boar spermadhesin AWN-1 binds to proteins containing *O*-linked NeuAc α (2–3/6)-Gal/3(1–3)-GalNAc and NeuAc α (2–3/6)-Gal/3(1–4)-GlcNAc sequences in *N*-linked triantennary structures (Dostalova *et al.* 1995). Besides, the absence of terminal sialic acid decreased fivefold the binding affinity. Maybe the presence of an active sialidase in the OF would strongly hamper the porcine sperm–ZP interaction. Regarding α -D-galactosidase, spermadhesin AWN binds only the galactose residues (Gal α 1,3GalNAc and Gal β 1,3GalNAc) and its addition to oviductal epithelium did not affect boar sperm binding (Ekhlesi-Hundrieser *et al.* 2005). It might be that α -D-Gal residues do not function as sperm receptors in the porcine species as in the mouse, where the Gal α 1,3Gal oocyte epitopes implicated in sperm adhesion to the ZP3 glycoprotein are not required for fertilization (Thall *et al.* 1995). This would help to explain the lack of an active oviductal α -D-galactosidase at physiological pH, although this hypothesis needs to be confirmed.

Protein content in OF was higher at estrus as was OF volume. In sows, Iritani *et al.* (1974) described no changes in protein concentration during the estrous cycle. A higher secretion rate of proteins has been described at proestrus and estrus when events such as fertilization and early cleavage stages of embryos occur (Buhi *et al.* 1989). This would be more in agreement with our results showing a higher total amount of protein at estrus. Regarding the volume of fluid, there are numerous studies indicating that OF secretion increases during the estrus and decrease during the diestrus (Iritani *et al.* 1974, Wiseman *et al.* 1992). This is coincident with our results since we obtained the maximum volume of OF from the oviducts at the late follicular phase and a decrease as the estrous cycle progressed.

In conclusion, we demonstrate that porcine OF from prepubertal and pubertal animals has α -L-fucosidase, β -*N*-acetyl-glucosaminidase, β -D-galactosidase, α -D-mannosidase, and β -*N*-acetyl-galactosaminidase activities with variations during the estrous cycle, but it did not show *N*-acetyl-neuraminidase or α -D-galactosidase activity. This suggests a role of the active glycosidases in reproductive events. Further experiments are necessary to find out the specific functions and role in porcine fertilization for each of the active enzymes.

Materials and Methods

Unless otherwise indicated, all chemicals and reagents were purchased from Sigma–Aldrich Química S A (Madrid, Spain).

Oviducts classification and collection of OF

Genital tracts from gilts and sows (Landrace \times Large White) were obtained at the abattoir and transported to the laboratory on ice. The cyclic stage of animals was assessed once in the laboratory, on the basis of ovarian morphology on both ovaries from the same female. Oviducts from sows were classified as early follicular, late follicular, early luteal, or late luteal phase, according to the criteria defined by Hafez & Hafez (2000; Table 3; Fig. 4). In the tracts from gilts, only ovaries at the early follicular phase were observed (Fig. 4). Both oviducts coming from the same genital tract were classified as in the same stage of the cycle. Tracts with ovaries not clearly matching these criteria, polycystic, and genitalis from pregnant animals were discarded from the study.

Once classified, a total of 43 gilt oviducts and 226 sow oviducts were separated from the tracts and quickly washed once with 0.4% v/v cetrimide (alkyltrimethylammonium bromide) solution and two times with Dulbecco's PBS and transferred to Petri dishes on ice and dissected. Once dissected, the oviducts were not opened lengthwise but the porcine OF from whole oviduct was collected by aspiration with an automatic pipette by introducing the tip into the ampulla for a maximum 200 μ l volume and making a manual ascendent pressure from the isthmus to the ampulla as described previously (Carrasco *et al.* 2008). Once aspirated, the OF was centrifuged at 7000 *g* for 10 min at 4 $^{\circ}$ C to remove cellular debris. Then the supernatant was immediately stored at -80° C until use for glycosidase and protein determinations. After centrifugation, the number of oviducts dissected and OF volume obtained per sample were recorded. Because preliminary experiments showed variations in EA, all samples were analyzed within 2 weeks of freezing.

Assays for glycosidases

Seven glycosidases were assayed at pH 7.2 in each sample: *N*-acetyl-neuraminidase (EC 3.2.1.18), α -D-galactosidase (EC 3.2.1.22), α -L-fucosidase (EC 3.2.1.51), β -*N*-acetyl-glucosaminidase (EC 3.2.1.52), β -D-galactosidase (EC 3.2.1.23), α -D-mannosidase (EC 3.2.1.24), and β -*N*-acetyl-galactosaminidase (EC 3.2.1.53). Glycosidase activities were assayed, as we have previously described (Aviles *et al.* 1996, Abascal *et al.* 1998), using 4-methylumbelliferyl-glucopyranosides as substrates. Briefly, stock solutions for substrates (0.1 M) were prepared in purified water and kept at -80° C until use. The day of the assay, OF samples were thawed and working solutions for all substrates prepared (1 mM) by dilution in assay buffer (pH 7.2; 137.1 mM NaCl, 2.7 mM KCl, 8.4 mM Na₂HPO₄, 1.46 mM KH₂PO₄, 0.32 mM Na pyruvate, 11.0 mM glucose, and 0.007 g/l kanamycin). In an ice bath, 40 μ l assay buffer, 20 μ l working solution substrate, and 10 μ l OF were added in a microtube. Duplicates were prepared for each OF sample. The blank in each sample consisted of 60 μ l assay buffer and 10 μ l OF. Substrate blanks for each enzyme were prepared with 50 μ l assay buffer and 20 μ l

Table 3 Classification of porcine oviducts according to ovarian morphology.

	Estrous cycle phase	Ovary morphology
Gilts (prepubertal)	Early follicular	Growing follicles (2–5 mm diameter); absence of signals from previous cycles (no CH, CL, or CA)
Sows (pubertal)	Early follicular	Growing follicles (2–5 mm diameter), CL under regression without surface vascularization or CA from previous cycle
	Late follicular	Periovarian follicles (8–11 mm diameter) or close to ovulate (> 11 mm diameter) and CA from previous cycle
	Early luteal	Recent CH or CL very vascularized in the periphery and purple stigma; small growing follicles and presence/absence of CA
	Late luteal	Developed CL with some surface vascularization or CA from previous cycle; small growing follicles (<2 mm)

CH, corpora hemorrhagic; CL, corpora lutea; CA, corpora albicans. Adapted from Hafez Hafez & Hafez (2000).

working solution substrate. Human seminal plasma (10 µl) was used as a positive control since it has shown activity for α-L-fucosidase (Alhadeff *et al.* 1999), β-N-acetyl-glucosaminidase (Yoshida *et al.* 1987), β-D-galactosidase (Corrales *et al.* 2002), α-D-galactosidase (Spiessens *et al.* 1998), α-D-mannosidase (Corrales *et al.* 2002), and β-N-acetyl-galactosaminidase (Kapur & Gupta 1985). Human seminal plasma was obtained from clinic IVI-Murcia (Murcia, Spain). Semen samples were centrifuged at 500 g for 10 min and the supernatant aliquoted and stored at

–80 °C until assay. The use of the samples in the present investigation was approved by the local ethical committee and consent was obtained from patients. Positive control for N-acetylneuraminidase was made with 10 µl (0.05 IU) of the commercial enzyme from *Clostridium perfringens* (*C. welchii*) since its presence has not been described in seminal plasma. All positive controls were run at pH 7.2. Incubation of samples, blanks, and controls was for 240 min at 37 °C and the reaction was stopped by adding to each microtube 0.5 ml glycine buffer containing

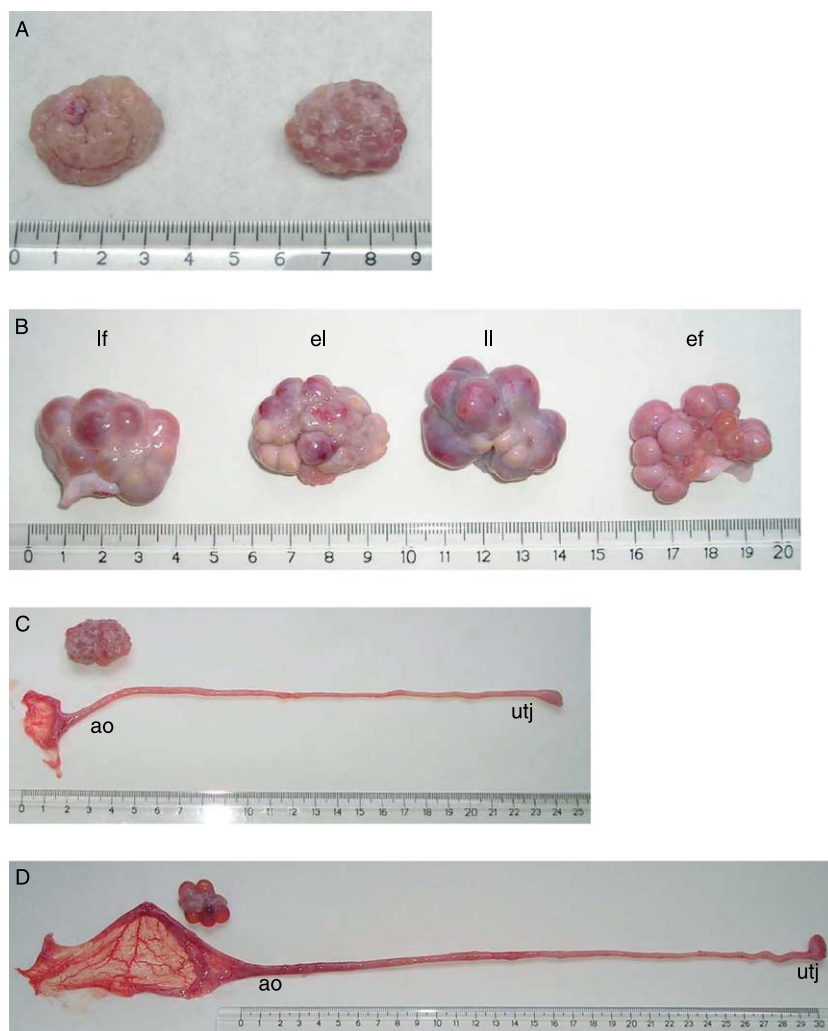


Figure 4 (A) Ovaries from gilts at the early follicular phase. (B) Ovaries from sows at the early follicular (ef), late follicular (lf), early luteal (el), and late luteal (ll) phases. (C) Dissected oviducts from a gilt at the early follicular phase with its corresponding ovary. (D) Dissected oviduct from a sow at the late follicular phase with its corresponding ovary. oa, oviductal ampulla; utj, utero tubal junction. Scale in cm.

0.0085 M glycine–CaCO₃, adjusted to pH 10.0 with 1 M NaOH. Samples, blanks, and controls were run concurrently and fluorescences were read on a spectrofluorimeter (FLUOstar Galaxy; BMG Lab. Technologies, Durham, NC, USA) using wavelengths of 340 and 450 nm for excitation and emission respectively, and corrected by subtracting both blanks.

Because OF did not show *N*-acetyl-neuraminidase and α -D-galactosidase activities at pH 7.2, a small trial was run to assay these two enzymes at acidic pH. Acidic neuraminidase and α -D-galactosidase of lysosomal origin have an optimum pH of 4.6–4.8 (Samollow *et al.* 1990) and 4.4 (Ohshima *et al.* 1997) respectively. Therefore, the enzymatic assays for samples, blanks, and controls were done, as described previously, but using a sodium acetate–acetic acid (0.2 M), adjusted to pH 4.4 as the assay buffer.

One unit (U) of glycosidase activity was defined in all cases as the amount of enzyme necessary to hydrolyze 1 nmol substrate/min at 37 °C under the above-defined conditions. One unit of glycosidase specific activity was the activity of an enzyme per milligram of total protein.

Protein determination

Protein concentration in OF samples was determined by the bicinchoninic acid assay (BCA method; Smith *et al.* 1985; Pierce, Rockford, IL, USA). Following the manufacturer's instructions, incubation of samples with the provided BCA reactive was performed at 37 °C for 30 min followed by 15 min at room temperature and reading of absorbances at 560 nm on a spectrofluorimeter (FLUOstar Galaxy; BMG Lab. Technologies, Durham, NC, USA). Bovine serum albumin was used as the standard for the protein assays. Three measurements of protein were run in each OF sample with 12, 8, and 4 μ l OF. Assays for glycosidases and proteins were run in the same OF sample. For each sample, the concentration of protein was the mean among these three measurements. To calculate the SEA, the mean protein value in each phase was used.

Statistical analysis

Data are presented as the mean \pm S.E.M. The variable EA, SEA, protein concentration, and OF volume were analyzed by one-way ANOVA with cycle stage (follicular versus luteal) or age of animal (gilts versus sows) as a fixed factor. When ANOVA revealed a significant effect, the values were compared by the Tukey test. $P < 0.05$ was considered to be statistically significant.

Declaration of interest

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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