CSIRO PUBLISHING

Reproduction, Fertility and Development, 2008, **20**, 808–817 www.publish.csiro.au/journals/rfd

Glycosidase determination in bovine oviducal fluid at the follicular and luteal phases of the oestrous cycle

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Abstract. Gamete recognition and binding of spermatozoa to the oviduct are carbohydrate-mediated processes in which several glycosidases are thought to have a role, although this has not been demonstrated unequivocally. Oviducal fluid is the biological milieu in which fertilisation and early embryo development take place, but the enzyme composition of oviducal fluid is largely unknown. The aim of the present study was to determine glycosidase activity and protein content in bovine oviducal fluid (bOF) and the volume of fluid collected per oviduct. Oviducts obtained from a slaughterhouse were classified as either in the follicular or luteal phase on the basis of ovarian luteal morphology. Oviducal fluid was aspirated, centrifuged and the volume determined. Samples were then frozen until assay. Substrates conjugated to 4-methylumbelliferyl were used to screen for the activity of seven glycosidases at pH 7.2. The results indicate that bOF has α-L-fucosidase, β-*N*-acetyl-glucosaminidase, β-D-galactosidase, α-D-mannosidase and β-*N*-acetyl-galactosaminidase activity during both phases of the cycle, with the specific activity of the latter two enzymes being higher during the follicular phase. There was no *N*-acetyl-neuraminidase or α-D-galactosidase activity detected in bOF at either phase of the oestrous cycle at pH 7.2, but activity for both glycosidases was detected at pH 4.4. There were no differences in protein concentration or the volume of bOF collected between the two phases of the cycle. These findings indicate that oviducal fluid exhibits glycosidase activity, with specific variations throughout the oestrous cycle, suggesting that these enzymes play a role in carbohydrate-mediated events.

Introduction

The oviduct is a key reproductive organ because it is the place where the capacitation of spermatozoa, fertilisation and early embryo development take place (for a review, see Hunter 2005*a*). The internal luminal fluid (i.e. the oviducal fluid), which has a neutral or slightly basic pH at the time of fertilisation (Nichol *et al*. 1997; Lane *et al*. 1999), is the physiological milieu in which these important events take place. The functioning of the oviduct is under hormonal control (mainly ovarian steroids) and it is therefore a dynamic environment, with changes in the composition of the oviducal fluid in different regions of the oviduct as well as during different stages of the oestrous cycle (for a review, see Hunter 2005*b*). Many studies have investigated the composition of oviducal fluid (for a review, see Leese *et al*. 2008), even using a proteomics approach (Georgiou *et al*. 2005). The knowledge gained from these studies has been used to formulate new culture media to mimic *in vivo* fertilisation under *in vitro* fertilisation (IVF) and early embryo culture conditions (Tervit *et al*. 1972; Walker *et al*. 1996; Li *et al*. 2006). However, the complex sequence of biological steps involved in *in vivo* reproduction is only partially reproduced in current IVF/*in vitro* embryo production (Talevi and Gualtieri 2004) and the precise composition of such a dynamic and complex milieu as the oviducal fluid remains unknown.

Fertilisation is a carbohydrate-mediated process, beginning with the adhesion of ovulated cumulus oocyte–complexes to the oviduct (oocyte captation) and the transport of gametes in the reproductive tract, ending with fusion of a spermatozoon with the oocyte membrane (for a review, see Talbot *et al*. 2003). Capacitated (acrosome-intact) spermatozoa loosely and reversibly adhere to zona-intact oocytes and, in a second stage, tight irreversible binding occurs. Both types of binding are attributed to the presence of glycan-binding proteins (receptors) on the plasma membrane of spermatozoa and complementary bioactive glycan units (ligands) on the surface of the zona pellucida (Tulsiani 2000). Taking all these facts into consideration, glycosidases are important components of the oviducal fluid as enzymes capable of modifying the carbohydrate moieties of

 10.1071/RD08113 1031-3613/08/070808

glycoproteins. These enzymes catalyse the hydrolytic cleavage of terminal sugar residues from the glycan portion of glycoproteins and glycolipids. So, oviducal glycosidases may have a role in modifying the oligosaccharides involved in the different steps of fertilisation. Usually glycosidases are present in lysosomes acting in an acidic environment (Tulsiani *et al*. 1995). However, β-galactosidase has been shown to have maximum activity in a neutral environment in rat epididymal luminal fluid (Tulsiani *et al*. 1995). Other 'acidic' glycosidases have been found to be active in different extracellular sites, such as the blood (Tulsiani and Touster 1981), spermatozoan 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). Therefore, active glycosidases present in a neutral environment, such as the oviducal fluid, could have potential roles in different events related to gamete interaction and early embryo development.

Although several studies have been performed investigating glycosidase activity in spermatozoan membranes and epididymal fluid, similar studies on oviducal fluid are scarce, despite the fact that it has been proposed that glycosidases could be involved in the control of polyspermy (Miller *et al*. 1993*a*; Velasquez *et al*. 2007), in the interaction between spermatozoa and oviducal epithelial cells (Lefebvre *et al*. 1997), in the binding of spermatozoa to the zona pellucida (Miller *et al*. 1993*b*; Matsumoto *et al*. 2002; Venditti *et al*. 2007), in capacitation of spermatozoa (Taitzoglou *et al*. 2007) and in the dispersion of cumulus cells (Takada *et al*. 1994). All these events take place in the oviduct and have been the focus of research aimed at increasing our knowledge of the molecular basis of reproductive function. However, and surprisingly, there have been no indepth studies investigating the activity of different glycosidases in the oviducal fluid throughout the oestrous cycle in mammalian species, such as the porcine or bovine, in which assisted reproduction techniques (ART) are routinely performed. The exceptions are some early reports of studies performed at an acidic pH that indicated low activity of 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 oviducal fluid from cows and sheep, with a significant increase the activity of β-*N*-acetyl-galactosaminidase and β-*N*-acetyl-glucosaminidase during dioestrus and pregnancy (Roberts *et al*. 1975, 1976). More recently, Tulsiani *et al*. (1996) described α-D-mannosidase, β-D-galactosidase, β-D-glucuronidase, *N*-acetyl-β-D-glucosaminidase and α-D-fucosidase activity in the oviducal fluid of hamsters, with no differences in activity throughout the oestrous cycle. Apart from these studies, there is no other information available regarding glycosidase activity in the oviducal fluid of mammals.

The main aim of the present study was to determine the activity of seven exoglycosidases, for which roles in different reproductive events have been postulated, in the oviducal fluid of heifers during the follicular or luteal phase of the oestrous cycle. The glycosidases assayed 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.

Material and methods

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

Oviduct classification and collection of oviducal fluid

Genital tracts from 14–20-month-old heifers (Charolais, Limousine and Simmental) were obtained at the abattoir and transported to the laboratory on ice. Once in the laboratory, the stage of the oestrous cycle (follicular or luteal) was assessed on the basis of ovarian luteal morphology, according to the criteria defined by Orsi *et al*. (2005), on both ovaries from the same female.

Briefly, follicular-stage oviducts were defined as those coming from ovaries showing at least one large dominant follicle (>12 mm diameter) or a follicle close to ovulation (approximately 15 mm diameter) with a regressed corpus luteum (CL), no surface vasculature and the absence of red/brown colouration (Fig. 1*a*, *c*). Individual follicles were graded on the basis of dominance and assessed as to whether they were the largest follicle or not because steroid profiles were not determined. Luteal-phase oviducts were designated as those coming from tracts showing ovaries with growing follicles (<4 mm diameter) and recently ovulated follicles, or a new CL with vasculature at its periphery. When bisected, the apex of the follicle was coloured red/brown, whereas the remainder was orange/yellow (Fig. 1*b*, *d*). Both oviducts coming from the same genital tract were classified as follicular or luteal phase. Tracts with ovaries not clearly matching these criteria, showing polycystic ovaries or from pregnant animals, were not included in the study.

Once classified, the oviducts ($n = 237$) were separated from the tracts and quickly washed once in 0.4% v/v cetrimide solution, twice in Dulbecco's phosphate-buffered saline (PBS) and then transferred to Petri dishes on ice before being dissected individually (Fig. 2*a*). The bovine oviducal fluid (bOF) was collected by aspiration with an automatic pipette using a tip for a maximum 200 μL volume (Fig. 2*b*, *c*, *d*) and centrifuged at 7000*g* for 10 min at 4°C to remove cellular debris. The number of oviducts dissected and the volume of bOF obtained per sample were recorded. The supernatant was stored immediately at −80°C until use for glycosidase and protein determinations. In order to avoid sample damage and changes in enzymatic activity, all samples were analysed within 2 weeks of freezing.

Glycosidase assays

Seven glycosidases were assayed at pH 7.2 in each sample, namely 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), β-*N*-acetyl-galactosaminidase (EC 3.2.1.53), α-D-galactosidase (EC 3.2.1.22) and *N*-acetyl-neuraminidase (EC 3.2.1.18). Glycosidase activity was assayed as described previously (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. On the day of the assay, bOF samples were thawed and working

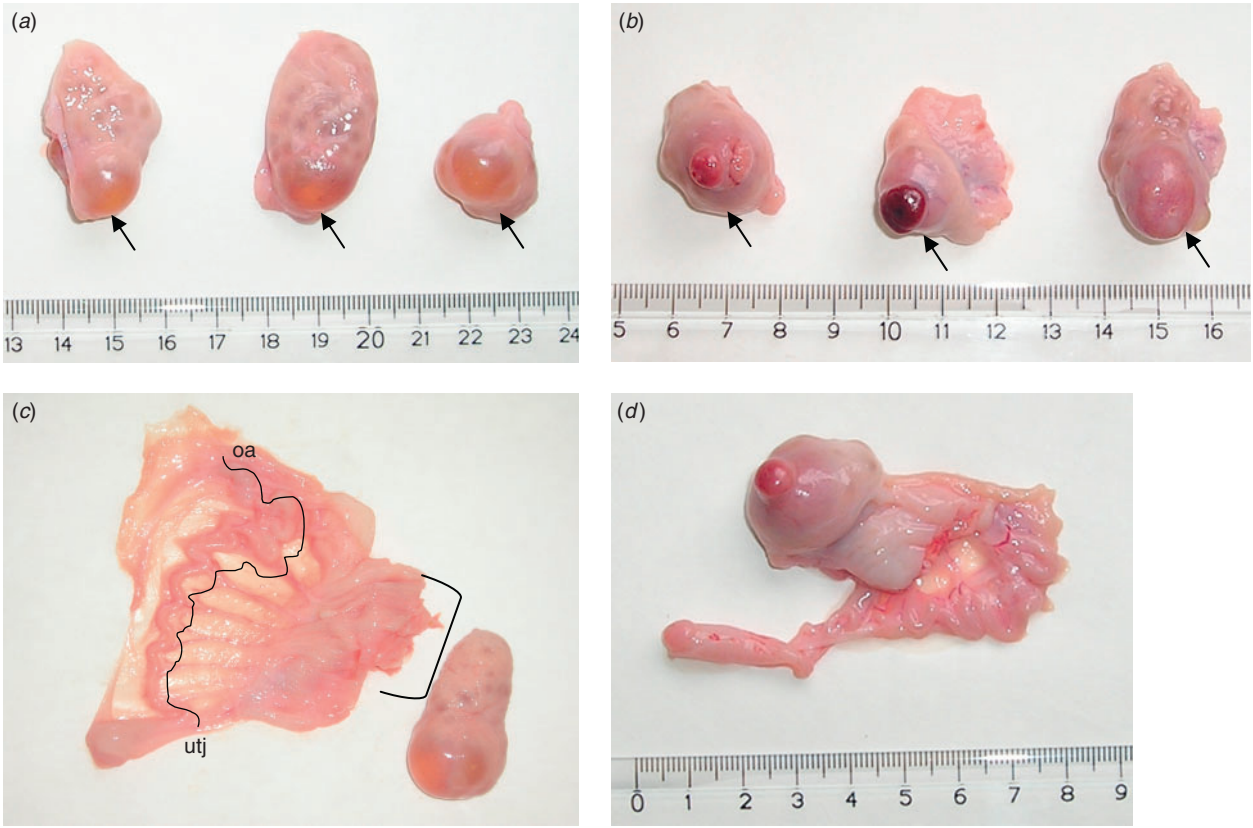

Fig. 1. Bovine ovaries classified as either from the (*a*) follicular stage, showing follicles over 12 mm in diameter (arrows), or as from the (*b*) luteal stage, showing recent ovulated follicles or corpora lutea (arrows). (*c*) An ovary at the follicular stage with its corresponding oviduct surrounded by mesosalpinx before dissection. The trajectory of the oviduct has been marked by a parallel line from the uterotubal junction (utj) to the oviducal ampulla (oa). Point of ovary–oviduct junction is remarked (hook). (*d*) An ovary at the luteal stage attached to its corresponding oviduct before dissection.

solutions for all substrates were prepared (1 mM) by dilution in assay buffer pH 7.2 (137.1 mM NaCl, 2.7 mM KCl, 8.4 mM Na_2HPO_4, 1.46 mM KH_2PO_4, 0.32 mM Na pyruvate, 11.0 mM glucose and 0.007 g L^{-1} kanamicine). In an ice bath, 40 μL assay buffer, 20 μL working solution substrate and 10 μL bOF were added to a microtube. Duplicates were prepared for each bOF sample. The blank in each sample consisted of 60 μL assay buffer and 10 μL bOF. Substrate blanks for each enzyme were prepared with 50 μL assay buffer and 20 μL working solution substrate. Human seminal plasma (10 μL) was used as a positive control because it has been demonstrated to have 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 and Gupta 1985) activity. The positive control for *N*-acetyl-neuraminidase consisted of 10 μL (0.05 IU) of the commercial enzyme from *Clostridium perfringens* (*C. welchii*) because this enzyme has not been described in seminal plasma. All positive controls were run at pH 7.2. The incubation of samples, blanks and controls was for 240 min at 37°C and reactions were stopped by the addition of 0.5 mL glycine buffer containing 0.0085 M glycine–$CaCO_3$, adjusted to pH 10.0 with 1 M NaOH, to each microtube. Samples, blanks and controls were run concurrently and fluorescence was read on an espectrofluorimeter (Fluostar Galaxy; BMG Laboratory Technologies, Durham, NC, USA) using excitation and emission wavelengths of 340 and 450 nm, respectively, and corrected by subtracting both blanks.

Because bOF did not show *N*-acetyl-neuraminidase and α-D-galactosidase activity at pH 7.2, a small trial was run to assay the activity of 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. So, the enzymatic assays for samples, blanks and controls were run as before, but using sodium acetate–acetic acid (0.2 M) adjusted to pH 4.4 as the assay buffer.

In all cases, 1 unit of glycosidase activity was defined as the amount of enzyme necessary to hydrolyse 1 nmol substrate per min at 37°C under the above-defined conditions. One unit of specific glycosidase enzyme activity was the activity of the enzyme per mg total protein. Total enzyme activity per oviduct

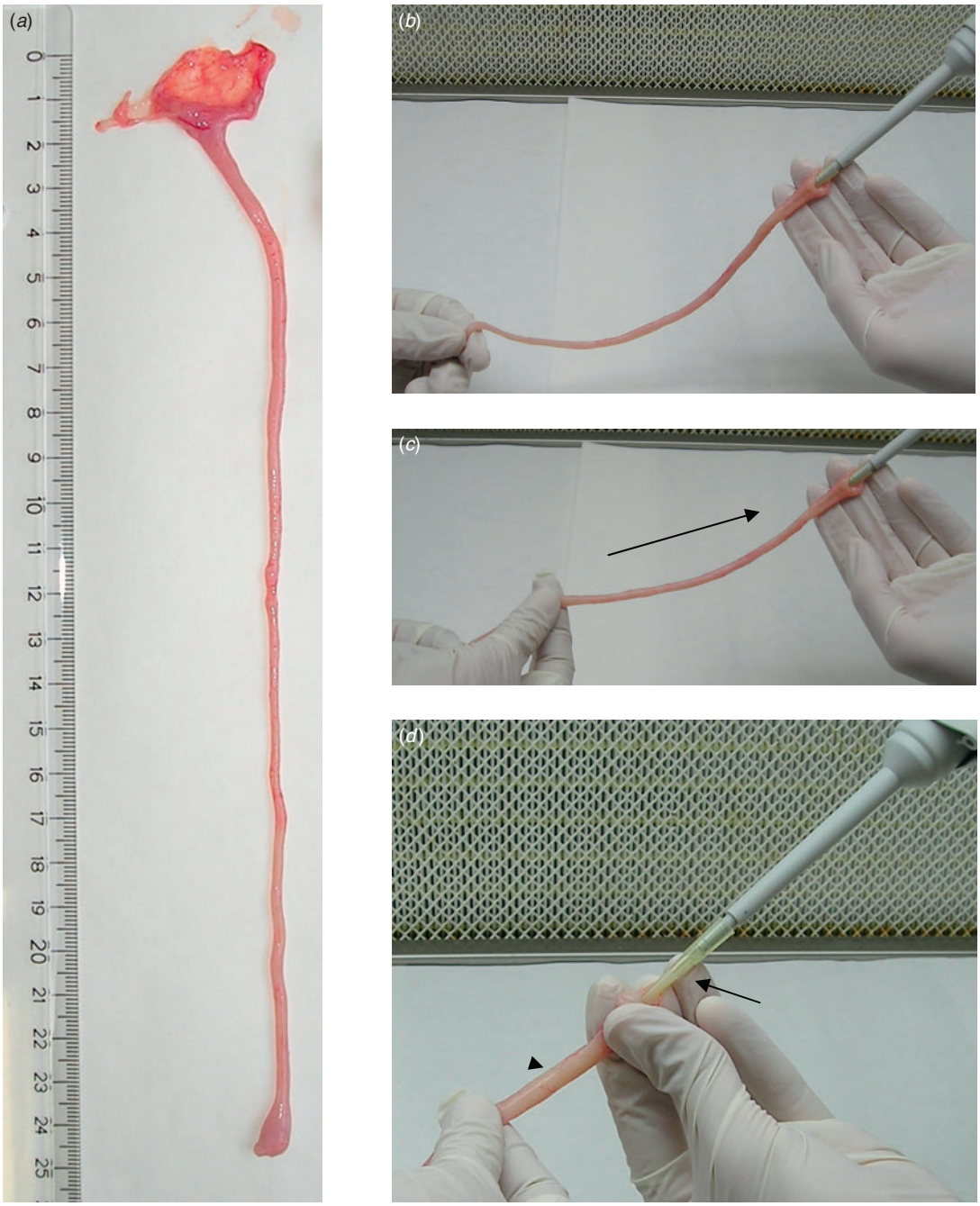

Fig. 2. (*a*) An oviduct after dissection (scale in cm). (*b*) Introduction of the tip pipette into the oviducal ampulla. (*c*) Ascendant mechanical pressure (from the uterotubal junction to the ampulla) to collect the oviducal fluid. (*d*) Oviducal fluid accumulates at the oviducal ampulla (arrowhead) and is aspirated with the pipette, where it can be observed inside the tip (arrow).

was defined as the product of enzyme activity and the volume of bOF per oviduct.

Protein determination

The protein concentration in oviducal fluid samples was determined by the bicinconinic acid assay (BCA method; Smith *et al*. 1985) accoding to the manufacturer's instructions (Pierce, Rockford, IL, USA). Samples were incubated with BCA at 37°C for 30 min, followed by 15 min incubation at room temperature. Absorbance was read at 560 nm on an espectrofluorimeter (Fluostar Galaxy; BMG Laboratory Technologies). Bovine serum albumin (BSA) was used as the standard for the protein assays.

Table 1. Protein concentration, volume of bovine oviducal fluid aspirated and total amount of protein per oviduct in samples obtained during the follicular and luteal stages of the oestrous cycle
Data are the mean ± s.e.m. *n*, number of samples analysed

	Protein ($\mu g\,\mu L^{-1}$)	Volume (μL per oviduct)	Total protein (μg per oviduct)
Follicular	54.28 ± 3.71 ($n = 34$)	39.09 ± 1.32 ($n = 34$)	2122.64 ± 163.91 ($n = 33$)
Luteal	55.89 ± 2.93 ($n = 78$)	43.45 ± 1.66 ($n = 87$)	2470.29 ± 177.63 ($n = 76$)
P	0.751	0.12	0.23

Three measurements of protein were run in each bOF sample with 12, 8 and 4 μL bOF. Assays for glycosidases and proteins were run in the same bOF sample. For each sample, the concentration of protein was taken as the mean of three measurements. Mean protein concentrations were used to calculate specific enzyme activity in each phase.

Statistical analysis

Data are presented as the mean ± s.e.m. Enzyme activity, specific enzyme activity, protein concentration and bOF volume, and the variables derived from these (i.e. total protein per oviduct, total enzyme activity per oviduct), were analysed by one-way ANOVA with cycle stage as a fixed factor. When the ANOVA indicated a significant effect, values were compared by Tukey's test. $P < 0.05$ was taken to denote statistical significance.

Results

Glycosidase activity

α-L-Fucosidase, β-*N*-acetyl-glucosaminidase, β-D-galactosidase, α-D-mannosidase and β-*N*-acetyl-galactosaminidase activity was found in the bOF, with no difference in activity between the follicular and luteal phases of the oestrous cycle ($P > 0.05$; Fig. 3). When data were transformed into specific enzyme activity (i.e. enzyme activity per mg protein), significantly higher α-D-mannosidase ($P = 0.033$) and β-*N*-acetyl-galactosaminidase ($P = 0.025$) activity was found in bOF collected from the follicular phase of the oestrous cycle; the activity of the other glycosidades remained similar in both phases (Fig. 3).

Results for total enzyme activity per oviduct are shown in Fig. 4. Higher α-L-fucosidase activity was found in oviducts in the luteal compared with the follicular phase (714.44 ± 124.69 *v.* 1051.92 ± 80.70 units, respectively; $P < 0.05$). There were no significant differences found for the remaining enzymes.

There was no α-D-galactosidase or *N*-acetyl-neuraminidase activity found in any sample of bOF at pH 7.2. However, when the assays were repeated at pH 4.4, α-D-galactosidase and *N*-acetyl-neuraminidase activity was found in bOF from both the follicular and luteal phases of the oestrous cycle. In the follicular and luteal phases, α-D-galactosidase activity in the bOF was determined to be 82.27 ± 44.37 and 49.34 ± 15.04 units, respectively; neuraminidase activity in bOF from the follicular and luteal phases was 3.79 ± 2.05 and 2.28 ± 0.69 units, respectively.

Protein concentration and volume of oviducal fluid

The protein concentration of the bOF was 54.28 ± 3.71 and 55.89 ± 2.93 $\mu g\,\mu L^{-1}$ during the follicular and luteal phases of the oestrous cycle, respectively, which was not significantly different. The volume of the bOF obtained per oviduct during the follicular and luteal phases was 39.09 ± 1.32 and 43.45 ± 1.66 μL, respectively ($P > 0.05$; Table 1).

There were no significant differences in the volume (μL per oviduct) and total protein concentration ($\mu g\,\mu L^{-1}$) per oviduct between the follicular and luteal phases of the oestrous cycle (Table 1).

Discussion

The oviduct provides an appropriate environment for fertilisation and early embryo development, but the composition of this important medium is not fully known. From spermatozoa–oviducal epithelium attachment to spermatozoa–zona pellucida binding and oocyte penetration, the entire process of mammalian fertilisation is a carbohydrate-mediated event (for a review, see Talbot *et al*. 2003). All these processes are modulated in the oviduct and the fact there are active enzymes present able to modify the structure of oligosaccharides involved (i.e. composition, sequence, linkages, etc.) is important. The results of the present study demonstrate α-L-fucosidase, β-*N*-acetyl-glucosaminidase, β-D-galactosidase, α-D-mannosidase and β-*N*-acetyl-galactosaminidase activity in oviducal fluid from heifers during the luteal and follicular phases of the oestrous cycle at neutral pH. Because there are few previous studies demonstrating this sort of enzyme activity, we can only speculate as to the role of these enzymes in the oviducal fluid.

α-L-Fucosidase activity has been detected previously in hamster oviducal fluid, but without variations in activity throughout the oestrous cycle (Tulsiani *et al*. 1996). Bull spermatozoa bind to an oligosaccharide ligand on the oviducal epithelium that resembles Lewis-a (α-L-Fuc[1–4]-[β-D-Gal-(1–3]-D-GlcNAc) oligosaccharide (Suarez *et al*. 1998) and the α-L-fucose present in annexins has been proposed to be the carbohydrate responsible for spermatozoa–oviducal epithelium binding (Ignotz *et al*. 2007). In fact, treatment of oviducal epithelium with α-L-fucosidase or the presence of fucose prevented spermatozoa from binding to oviducal cells (Lefebvre *et al*. 1997; Suarez *et al*. 1998). Consequently, the oviducal α-L-fucosidase activity we have found in the present study could be related to the release of spermatozoa from the isthmus reservoir to reach the oocyte in the ampullar–isthmic junction, especially considering that total α-L-fucosidase activity per oviduct increased significantly after ovulation. In addition, α-L-fucosidase may have a role in spermatozoon–oocyte fusion because the presence of

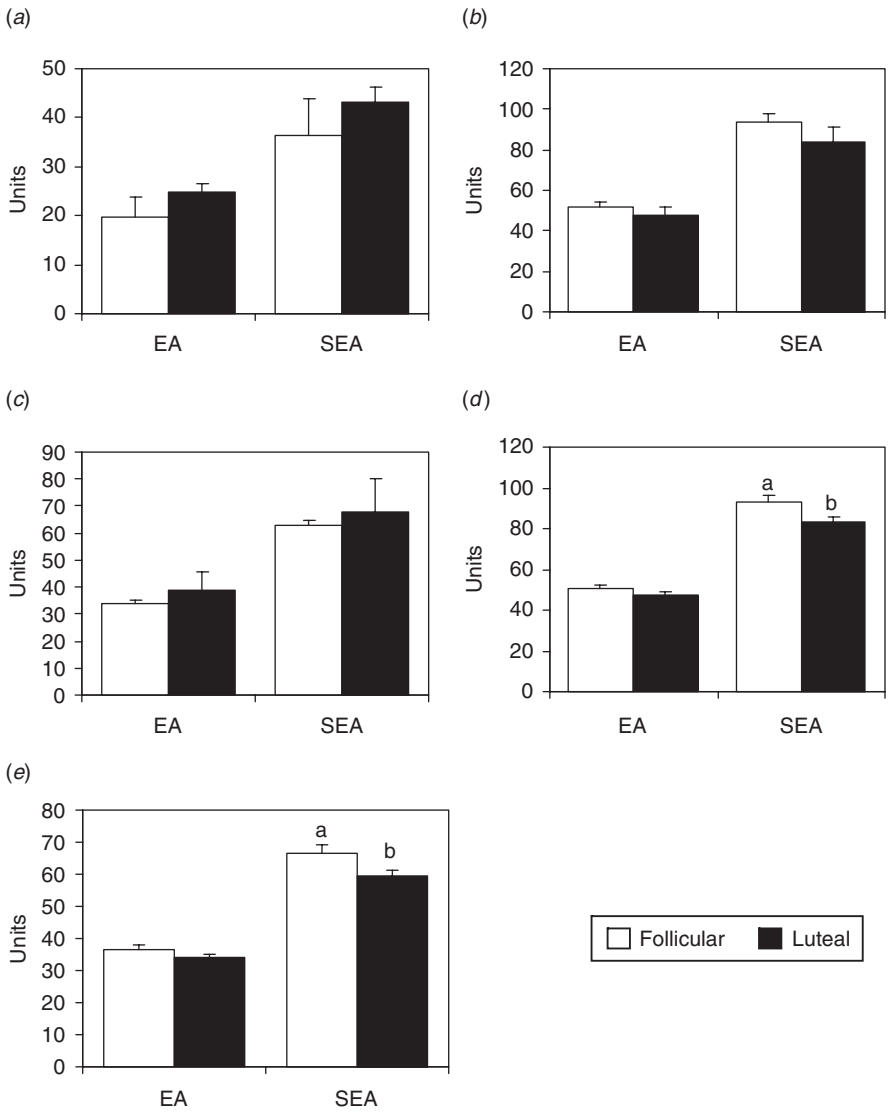

Fig. 3. Enzyme activity (EA) and specific enzyme activity (SEA) for (*a*) α-L-fucosidase, (*b*) β-*N*-acetyl-glucosaminidase, (*c*) β-D-galactosidase, (*d*) α-D-mannosidase and (*e*) β-*N*-acetyl-galactosaminidase assessed at neutral pH in bovine oviducal fluid collected from heifers during the follicular and luteal phases of the oestrous cycle. Data are the mean ± s.e.m. of 30 follicular samples and 75 luteal samples. Different letters denote statistical significance ($P < 0.05$).

L-fucose during bovine *in vitro* fertilisation produced a 50% reduction in penetration of spermatozoa (Tanghe *et al*. 2004*b*).

In the present study, hexosaminidase activity (β-*N*-acetyl-glucosaminidase and β-*N*-acetyl-galactosaminidase) was found in the bOF, which has also been demonstrated in hamster oviducal fluid (Tulsiani *et al*. 1996). β-*N*-Acetyl-glucosaminidase activity has also been described in hen (Droba and Droba 1987), sheep (Roberts *et al*. 1976) and cow (Roberts *et al*. 1975) oviducts. Tulsiani *et al*. (1996) did not observe significant differences in the activity of these enzymes throughout the oestrous cycle in hamsters, whereas Roberts *et al*. (1975, 1976) detected higher β-*N*-acetyl-glucosaminidase activity during dioestrus and pregnancy. The present study demonstrated hexosaminidase activity during the two phases of the oestrous cycle investigated, with significantly higher levels of β-*N*-acetyl-galactosaminidase activity during the follicular phase. There are several possible roles for oviducal hexosaminidase. First, these enzymes may be involved in cumulus dispersion of ovulated oocytes. According to Takada *et al*. (1994), β-*N*-acetyl-glucosaminidase in pig spermatozoa has cumulus dispersion activity at a physiological pH, suggesting that it plays a role in the passage of spermatozoa through cumulus cells before binding to the zona pellucida. Takada *et al*. (1994) attribute this role to the acrosomal enzyme, but we propose that the oviducal enzyme may also be involved

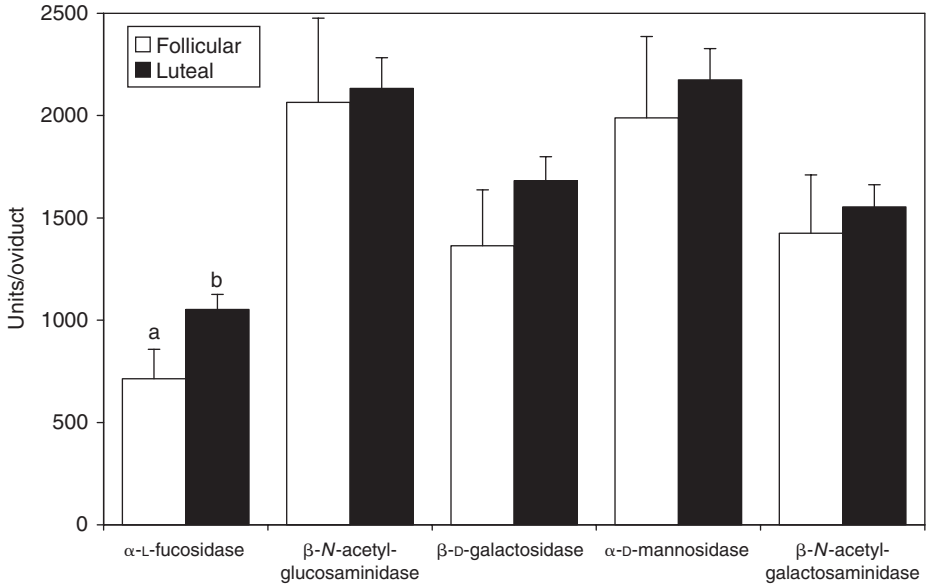

Fig. 4. Enzyme activity (units per oviduct) in bovine oviducal fluid collected from animals at different stages of the oestrous cycle and assessed at neutral pH. Data are the mean ± s.e.m. of 30 follicular samples and 75 luteal samples. Different letters denote statistical significance ($P < 0.05$).

because the acrosomal content of the fertilising spermatozoon is released after it binds to the zona pellucida, when it has already passed through the cumulus cells. This would make it difficult for the acrosomal β-*N*-acetyl-glucosaminidase to participate in cumulus dispersion (Fazeli *et al*. 1997; Topper *et al*. 1999), so oviducal β-*N*-acetyl-glucosaminidase, by dispersing cumulus cells, may facilitate contact of the spermatozoa with the oocyte.

Second, oviducal hexosaminidase may be related to the capacitation of spermatozoa. Bull spermatozoa incubated with bovine oviducal fluid showed a marked decrease in affinity for wheat germ agglutinin (WGA) and bandeiraea simplicifolia agglutinin I (BS-I) lectins in the spermatozoan membrane (Taitzoglou *et al*. 2007). WGA recognises both *N*-acetyl-glucosamine (GlcNAc) and sialic acid residues, whereas BS-I recognises both *N*-acetyl-galactosamine (GalNAc) and α-galactose (α-GAL) residues (Taitzoglou *et al*. 2007). In the present study, we did not detected sialidase or α-galactosidase activity, so we hypothesise that the decreased lectin affinity after capacitation is due mainly to hexosaminidase, although further studies are necessary to confirm this hypothesis.

As in the present study, β-D-galactosidase activity has been detected in oviducal fluid from hen (Droba and Droba 1987), hamster (Tulsiani *et al*. 1996), sheep and cow (Roberts *et al*. 1975, 1976). *N*-Acetyl-lactosamine residues have been demonstrated in oviducal epithelial cells in heifers (Cobo *et al*. 2004) and a galactose-binding protein has been identified in spermatozoa from stallion (Dobrinski *et al*. 1996) and rat (Abdullah and Kierszenbaum 1989). So, it is possible that oviducal β-D-galactosidase participates in the release of bull spermatozoa from the isthmus reservoir, as proposed for α-L-fucosidase, although this hypothesis needs further confirmation. Alternatively, β-D-galactosidase could also remove β-D-galactose residues from the oviducal epithelium, releasing this carbohydrate into the fluid and making it available for metabolism by the spermatozoa (Fleming *et al*. 2005). Galactose residues have been described previously in the bovine zona pellucida (Katsumata *et al*. 1996; Parillo *et al*. 2000; Ikeda *et al*. 2002), so the oviducal β-D-galactosidase may modify galactose residues contained in zona pellucida glycoproteins. The impact of this likely zona pellucida remodelling in spermatozoon–oocyte or oocyte–oviduct interaction is not known, but galactose has not been described as a spermatozoan receptor in bovine.

α-D-Mannosidase activity has been detected in hamster (Tulsiani *et al*. 1996), ovine and bovine (Roberts *et al*. 1975, 1976) oviducts. In the present study, the specific activity of this enzyme was significantly higher during the follicular than the luteal phase of the oestrous cycle, similar to observations made in the rat (Pizarro *et al*. 1984). It has been proposed that mannose residues contained in bovine zona pellucida glycoproteins are involved in fertilisation (Amari *et al*. 2001) and α-D-mannose affects the *in vitro* fusion between bovine gametes, because spermatozoa have receptors for mannose, which is necessary for fusion with the oolema (Tanghe *et al*. 2004*a*, 2004*b*). So, oviducal α-D-mannosidase, the activity of which decreases after ovulation, may act to eliminate some mannose residues from the zona pellucida, thereby helping control polyspermy. This hypothesis should be confirmed, because current opinion is that there are multiple ligands involved in spermatozoa–zona pellucida interaction (Lyng and Shur 2007) and that sialic acid may have a more essential role than α-D-mannose in bovine spermatozoon–oocyte interactions (Velasquez *et al*. 2007).

Regarding a possible role of oviducal α-D-mannose in spermatozoa capacitation, the incubation of bull spermatozoa in non-luteal oviducal fluid for 30 min produced a 76% decline for Concanavalin A (Con-A) lectin affinity (Taitzoglou *et al*. 2007). We suggest this decrease may be caused by oviducal α-D-mannosidase, the activity of which reaches a maximum during the non-luteal phase of the oestrous cycle. Mannose (binding to Con-A) has been also detected in the oviducal epithelium of heifers (Cobo *et al*. 2004), so the oviducal mannosidase may remodel the sugar components, thus affecting interactions with spermatozoa. Together, these observations suggest a possible role for this enzyme in spermatozoa capacitation inside the oviduct, although the mechanism remains unknown.

Neither *N*-acetyl-neuraminidase nor α-D-galactosidase activity was detected in bOF at any phase of the oestrous cycle at physiological pH. However, activity of both enzymes was found at acidic pH, the activity of the neuraminidase being less than that of the galactosidase. These results suggest that these enzymes may not have a crucial role in the fertilisation process as active enzymes but, because of their presence in the oviducal fluid, they may play a role as lectins. In a recent study, *N*-acetyl-neuraminidase was proposed to be present in the cortical granules of bovine oocytes (Velasquez *et al*. 2007). At fertilisation, and following cortical reaction, neuraminidase would cleave the terminal sialic acid from the zona pellucida, which would otherwise serve as a substrate for spermatozoa binding (Katsumata *et al*. 1996; Velasquez *et al*. 2007). This hypothesis is consistent with the reduced spermatozoa–zona pellucida binding and decreased fertilisation rates observed by Velasquez *et al*. (2007) when oocytes or isolated zona pellucida were incubated with neuraminidase. Thus, the presence of active oviducal *N*-acetyl-neuraminidase would hamper the spermatozoa–zona pellucida interaction and the absence of *N*-acetyl-neuraminidase activity was an expected result. Similarly, Roberts *et al*. (1975) showed that the activity of α-D-galactosidase in bOF was low and only slightly higher than the trace amounts of activity detected in serum. There could be several reasons for the differences between the present study and that Roberts *et al*. (1975), such as the different pH used for the assays, the small number of samples (three) used by Roberts *et al*. (1975) and the use of diluted samples of bOF. α-Galactose residues have been detected in bovine zona pellucida by means of BSA IB4 lectin (Parillo *et al*. 2000). It has been suggested that these galactose residues play a role in gamete interaction in the mouse (Bleil and Wassarman 1988; Litscher *et al*. 1995). However, there is no evidence supporting the involvement of this carbohydrate in gamete interaction in bovine, so our observation of a lack of α-D-galactosidase activity at physiological pH is not unexpected.

Protein content in oviducal fluid has been studied in numerous species. Proteins in the oviducal fluid come from mucosa transudation and direct secretion from oviducal cells, thus being regulated by steroid hormone levels. Some have observed a higher concentration of protein in bOF around oestrus (Ehrenwald *et al*. 1990), but results from different studies are contradictory mainly because of high individual variability (Carlson *et al*. 1970; Stanke *et al*. 1974; Roberts *et al*. 1975). Killian *et al*. (1989) described a higher secretion of proteins in bOF during the follicular phase of the oestrous cycle, despite a higher rate of protein secretion, due to the increase in oviducal fluid volume at this time. In the present study, we did not find any differences in protein concentration between the follicular and luteal phases of the oestrous cycle, which is in accordance with results from other studies (Carlson *et al*. 1970; Stanke *et al*. 1974; Roberts *et al*. 1975). In canulated animals, the volume of bOF secreted increases during the late follicular phase, decreasing during the early and late luteal phases of the oestrous cycle (Kavanaugh and Killian 1988). In addition, ampulla secretion is higher than isthmic secretion (1.04 *v.* 0.5 mL, respectively; Kavanaugh and Killian 1988). In the present study, we did not observe any differences between the follicular and luteal phases of the oestrous cycle probably because we grouped the oviducts only into these two groups and because we collected the fluid from the entire oviduct. In addition, because of the collection method used, we have to consider the possibility that some fluid was not aspirated, which may have impacted on the volume data, as well as on total enzyme activity per oviduct.

In conclusion, we have demonstrated that oviducal fluid has glycosidase activity, with variations in activity throughout the oestrous cycle, suggesting a role for these enzymes in reproductive events. The specific role of the glycosidases described herein in fertilisation is currently being investigated. This information will add to knowledge of the molecular mechanisms underlying the fertilisation process. The role of any of the glycosidases present in the oviduct can be now investigated by using similar levels of activity in culture media for IVF or gamete cocultures with oviducal epithelial cells.

Acknowledgements

The authors thank Dr Daulat Tulsiani for interesting comments and suggestions on the manuscript. The authors also thank the staff of the Orihuela and El Pozo slaughterhouse for supplying the genital tracts and Maria Roldan for help with the acidic enzyme assays. This work was supported by grants from the Spanish Ministry of Education and Science and FEDER (AGL2006–03495) and Murcia Regional Government through Seneca Foundation (Projects Seneca 03018/PI/05 and 04542/GERM/06).

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Manuscript received 20 May 2008, accepted 17 July 2008