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Detection of the Matrix Metalloproteinases MMP-2 and MMP-9 and Tissue Inhibitors of Metalloproteinases TIMP-1 and TIMP-2 in Llama (*Lama glama*) Oviduct

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Contents

Matrix metalloproteinases (MMPs) and their inhibitors (TIMPs) are involved in several reproductive events like oocyte–spermatozoa interaction and semen liquefaction. In order to study their role in the llama oviductal reproductive process, MMP activity in oviductal fluid (OF) was assayed. Considering that llama genome sequences are partially known, a strategy to procure cDNA sequences of MMP-2, MMP-9, TIMP-1 and TIMP-2 was designed. Afterwards, their expression patterns in the different llama oviductal segments were assayed. Gelatine zymograms detected 62 and 94 kDa protease activities that matched MMP-2 and pro-MMP-9, respectively. Expression pattern analysis showed that MMP and TIMP mRNAs were present in ampulla, isthmus, utero-tubal junction (UTJ) and papilla. Altogether, these findings support the argument that MMPs/TIMPs are produced in the oviduct and secreted into the oviductal lumen. Our results encourage further studies to elucidate the role of these proteins in reproductive oviductal events.

Introduction

Applied and basic research on the South American camelid (SAC) has gained considerable interest in the last decades because the animal has become important mainly as a result of the excellent quality of its fibre and meat. The development and improvement of reproductive biotechnology would allow the propagation of genetically superior individuals, providing alternative methods to breeding and selection within the species. It would also provide a solution for wild/endangered camelids, whose embryos could be transferred to domestic SACs. However, the techniques and protocols successfully applied in other mammals cannot be easily extrapolated to SACs due to their reproductive peculiarities. SACs are induced ovulators with an extended interval between mating and ovulation (Ratto et al. 2006), and they present ovarian activity in waves of follicular growth and regression (Chaves et al. 2002). Besides, once spermatozoa are ejaculated, they are entrapped in thick viscid seminal plasma (Giuliano et al. 2010) that is even present in the oviductal sperm reservoirs (Apichela et al. 2009, 2014).

It has been suggested that oviductal secretions could be involved in the control of gametes and embryo functions (Hunter 2005; Rodriguez-Martinez 2007). The oviduct provides an environment not only suitable for pickingup, transport and maturation of the oocyte, fertilization and early embryo development, but also for transport, storage and capacitation of sperm (Hunter 2005). A comprehensive understanding of the oviductal environment will allow better *in vitro* fertilization (IVF) and embryo culturing success rates. Different members of proteolytic systems such as matrix metalloproteinases and tissue inhibitors of metalloproteinases (MMPs/TIMPs) have been detected in the mammalian oviduct and oviductal fluid (OF) (Satoh et al. 1994; Buhi et al. 1997; Gabler et al. 2001; Kim et al. 2001, 2003). MMP and TIMP oviductal functions remain elusive although it is well known that this system is involved in several aspects of the ovarian and uterine function (Curry and Osteen 2001). MMPs are a large family of proteolytic enzymes able to degrade components of the extracellular matrix (ECM) or activate growth factors and cytokines (Fowlkes and Winkler 2002) through a zinc-catalysed mechanism. They are classified into different subfamilies according to their substrate specificity and molecular structure (collagenases, gelatinases, stromelysins, matrilysins and membrane-type MMPs among others), and they are synthesized as inactive proenzymes (zymogens), which are activated through the cleavage of pro-peptides by various enzymes, including MMPs themselves (previously activated). MMP activity is regulated mainly by tissue inhibitors of metalloproteinases (TIMPs). There are four TIMPs (TIMP-1, TIMP-2, TIMP-3 and TIMP-4) that are able to inhibit MMPs by forming non-covalent 1 : 1 stoichiometric complexes (Visse and Nagase 2003).

Regarding oviductal MMPs/TIMPs, it is known that TIMP-1 is synthesized in a bovine oviductal epithelial cell culture system (Satoh et al. 1994), and it is present in the pig oviduct (Buhi et al. 1997). In addition, Gabler et al. (2001) detected MMP-1, MMP-2 and TIMP-1 expression in bovine oviductal epithelial cells during the oestrous cycle.

Currently, the role of the oviduct and its secretions during the SAC reproductive process are poorly understood. Besides, availability of genomic sequences of these species is still limited, restricting genetic and molecular biology analyses. It is obvious that knowledge of basic reproductive biology is necessary in order to adapt reproductive technologies to SAC species. Therefore, the current study analysed the MMP/TIMP proteolytic system in the oviduct of a domestic SAC, the llama (*Lama glama*). The objectives were to detect metalloproteinases with gelatinolytic activity in llama OF, to clone and sequence MMPs previously detected in the OF and their corresponding inhibitors (TIMP-1 and TIMP-2) in order to detect their expression in the different oviductal segments.

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Materials and Methods

Animals and samples

Non-lactating and non-pregnant fertile female llamas (*Lama glama*) were used in this study (n = 6). The llamas belonged to Campo Experimental de Altura, Instituto Nacional de Tecnología Agropecuaria (INTA) located in Abra Pampa (22°S 65°O, Jujuy, Argentina) at 3484 m altitude.

Llama reproductive tracts were obtained immediately after animals were slaughtered. Oviducts were separated, and OF was obtained by perfusion of the oviducts (right and left, separately) with 100 μl of 10 mM Tris–HCl, 150 mM NaCl (pH 7.4) at 4°C, and the liquid was subsequently centrifuged to remove cellular debris. Then, oviducts were sliced into ampulla, isthmus, utero-tubal junction (UTJ) and papilla segments and placed in RNAlater solution (Ambion, Austin, TX, USA), according to the manufacturer's instructions. Afterwards, oviductal tissue segments and OF were transported to our laboratory in liquid nitrogen and stored at −70°C until use.

Gelatine zymography

Gelatine zymography was used to assess the presence of matrix metalloproteinases with gelatinase activity in OF. Total protein amount was determined using a Micro BCA kit (Thermo Fisher Scientific, Rockford, IL, USA). For zymography, 30 μg of total proteins was separated under non-reducing conditions on 8% polyacrylamide gels containing 1 mg/ml gelatine. Five μl of a PageRuler Unstained Broad Range Protein Ladder (Thermo Fisher Scientific) was loaded onto the gel in separate wells. Gels were run at 150 V at room temperature. After electrophoresis, gels were washed 6 times for 15 min with enzyme renaturing buffer (200 mM NaCl, 5 mM $CaCl_2$, 5 μM $ZnCl_2$, 2.5% Triton X-100, 0.02% NaN_3, 50 mM Tris–HCl, pH 7.5) to remove SDS and incubated in developing buffer (200 mM NaCl, 5 mM $CaCl_2$, 5 μM $ZnCl_2$, 0.02% NaN_3, 50 mM Tris–HCl, pH 7.5) for 42 h at 37°C. Gels were then stained for 40 min with 0.125% Coomassie Brilliant Blue R-250 in 50% methanol and 20% acetic acid with gentle agitation and destained in 30% methanol and 10% acetic acid until clear bands were visible against a dark blue background.

To confirm that the proteases observed were metalloproteinases, 10 mM EDTA (a metalloproteinase inhibitor) or 10 mM benzamidine-HCl (a serine protease inhibitor) was added to renaturing and developing buffer before gel incubation.

The molecular weight of the bands was calculated using GelAnalyzer 2010a freeware software (Copyright 2010 by Istvan Lazar and Dr. Istvan Lazar, Hungary).

RNA isolation and cDNA synthesis

Total RNA from oviductal tissue segments (ampulla, isthmus, UTJ and papilla) was isolated using the SV total RNA isolation system (Promega, Madison, WI, USA) according to the manufacturer's instructions. RNA was quantified spectrophotometrically at 260 nm, and RNA integrity was examined by electrophoresis on 1.5% agarose gels.

Total oviduct RNA (1 μg) was reverse-transcribed with M-MLV reverse transcriptase (Promega) and oligo-dT primer in a 25 μl volume reaction mixture according to the manufacturer's instructions.

Identification of MMP and TIMP sequences in llama

PCR

Considering that the llama genome is not yet sequenced, MMP-2, MMP-9, TIMP-1 and TIMP-2 primers were designed using known mRNA sequences of other mammals (porcine, bovine, equine and human mRNA) (Table 1). Briefly, each MMP or TIMP sequence of the four species mentioned above was aligned using MULTALIN (http://multalin.toulouse.inra.fr/multalin/). Once gene-conserved regions were determined, primers were designed with the Primer3 program (http://frodo.wi.mit.edu/) to amplify these regions. Table 2 shows primer sequences used to clone MMPs and TIMPs from llama oviduct.

PCR assays were carried out in a total volume of 20 μl, containing 10 mM Tris–HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 1 U of *Taq* polymerase (Invitrogen, Carlsbad, CA, USA), 1 μM of each primer and 1 μl of oviduct cDNA. PCR conditions were as follows: 94°C for 5 min; 30 cycles of 94°C for 15 s, 58°C for 30 s and 72°C for 30 s; followed by a final extension at 72°C for 5 min.

Cloning and sequencing

PCR products were separated on 1.5% agarose gels containing SYBR Safe (10 000 X; Invitrogen). Amplicons of the expected size were purified using a PureLink Quick Gel Extraction kit (Invitrogen) and then cloned into the pGEM-T easy vector (Promega). The plasmids

Table 1. Accession numbers of nucleotide (bold type) and amino acid sequences of mammalian MMPs and TIMPs used for primer design and alignments

Species	MMP-2	MMP-9	TIMP-1	TIMP-2
Sus scrofa (porcine)	**NM_214192.1**	**NM_001038004.1**	**NM_213857.1**	**NM_001145985.1**
	NP_999357.1	NP_001033093.1	NP_999022.1	NP_001139457.1
Bos taurus (bovine)	**NM_174745.2**	**NM_174744.2**	**NM_174471.3**	**NM_174472.4**
	NP_777170.1	NP_777169.1	NP_776896.1	NP_776897.2
Equus caballus (equine)	**XM_001493281.1**	**NM_001111302.1**	**NM_001082515.1**	**XM_001916682.2**
	XP_001493331.1	NP_001104772.1	NP_001075984.1	XP_001916717.2
Homo sapiens (humans)	**NM_001127891.1**	**NM_004994.2**	**NM_003254.2**	**NM_003255.4**
	NP_004521.1	NP_004985.2	NP_003245.1	NP_003246.1

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Table 2. Primer sequences used to clone MMP and TIMP cDNA from llama

	Primer sequences (5′-3′)	Expected size (bp)
mmp2 forward	GATGTGACTCCGCTACGGTT	286
mmp2 reverse	TGCAGCTGGTGTACTCCTTG	
mmp9 forward	GAGTTGTGGTCTCTGGGCAA	543
mmp9 reverse	GCAGAAGCCCCACTTCTTGT	
timp1 forward	CCTGGTCATCAGGGCCAAGT	437
timp1 reverse	GGCTCTGGAAGCCCTTGTCA	
timp2 forward	ACGACATCTACGGCAACCC	577
timp2 reverse	TCATGCTGTTTCCAGGGAGG	

were used to transform *E. coli* DH5α. The recombined colonies were identified and the plasmids were extracted using a PureLink Quick Plasmid Miniprep kit (Invitrogen). The amplicons, contained in the plasmids, were sequenced by Macrogen (Applied Biosystems 3730xl, Grand Island, NY, USA). The nucleotide sequences and the deduced amino acid sequences were analysed using the BLAST program and the Conserved Domains Architecture Retrieval tool (NCBI). Once identified, the sequences were submitted to GenBank (http://www.ncbi.nlm.nih.gov/genbank/).

Expression of MMPs and TIMPs in oviductal segments

Specific primers based on llama MMP and TIMP sequences previously identified were designed with Primer3 software (Table 3) and used to analyse expression in oviductal segments (ampulla, isthmus, UTJ and papilla). PCR assays were performed as described above, using the following conditions: 94°C for 5 min; followed by 35 cycles of 94°C for 15 s, 60°C for 20 s, 72°C for 10 s; and a final extension at 72°C for 5 min. The *β*-actin gene was used as internal control, and samples were analysed using 1.5% SYBR Safe-stained agarose gel electrophoresis.

Results

Gelatinase activity in llama oviductal fluid

Gelatine zymography was performed to detect gelatinases in llama OF. Samples typically showed two gelatinolytic bands corresponding to 62 and 94 kDa. The 62 kDa band was most prominent, whereas the 94 kDa band showed weak activity and was absent in some samples. Particularly, OF samples belonging to the same animal displayed the same pattern of gelatinolytic bands, indicating no difference between the right and left oviduct (Fig. 1a). When the gels were washed and incubated in the presence of EDTA, all gelatinolytic bands were inhibited (Fig. 1b). In contrast, treatment with benzamidine-HCl (a serine protease inhibitor) showed no inhibitory effect on gelatinase activity in OF (Fig. 1c), which is consistent with the metalloproteinase character of these bands.

Considering the molecular masses, the 62 and 94 kDa bands likely represent the active form of MMP-2 and the latent form of MMP-9, respectively.

Identification of nucleotide sequences of MMP-2, MMP-9, TIMP-1 and TIMP-2 in llama

Based on the zymography results, the nucleotide sequences of the gelatinases present in the OF and their main inhibitors were determined. Four single amplicons of 286, 543, 437 and 577 bp were obtained. The size of these bands corresponds to the expected molecular weight for the amplification products of MMP-2, MMP-9, TIMP-1 and TIMP-2, respectively (Fig. 2). The amplicons were excised from the gel, cloned and sequenced and then compared with the known sequences available in databases. Bioinformatics analysis confirmed that the 286, 543, 437 and 577 bp amplicons correspond to MMP-2, MMP-9, TIMP-1 and TIMP-2 sequences, respectively. These novel sequences showed a high percentage of identity with porcine, bovine, equine and human orthologous sequences (Table 4). The MMP-2 nucleotide sequence encodes an 81-amino acid peptide, corresponding to a region of the catalytic (amino acids 1–57) and fibronectin (amino acids 67–81) domains (Fig. 3a), while the MMP-9 nucleotide sequence encodes a fragment of 167 amino acids, comprising the three fibronectin domains (amino acids 10–57, 68–115 and 127–167), characteristic of gelatinases (Fig. 3b). The partial nucleotide sequences of TIMP-1 and TIMP-2 encode 131 and 155 amino acids, respectively, corresponding in both cases to a region of the amino- and carboxy-terminal domains (Fig. 4a,b). Like the nucleotide sequences mentioned before, the deduced partial amino acid sequences for MMP-2 and MMP-9 showed a high percentage of identity with porcine, bovine, equine and human sequences (Table 5). The deduced amino acid sequences for TIMP-1 and TIMP-2 also showed a high percentage of identity ranging between 85 and 98% when compared with their homologous porcine, bovine, equine and human sequences (Table 5).

Table 3. Specific primer sequences used to detect gene expression in llama oviductal segments (ampulla, isthmus, UTJ and papilla)

	Primer sequences (5′-3′)	Amplicon size (bp)	Accession number
MMP-2 forward	ACCCCTTTGATGGCAAAGAT	174	GQ244429.1
MMP-2 reverse	TGAACAGGAAGGGGAACTTG		
MMP-9 forward	GTTCGATGTGAAGACGCAGA	175	GU207475.1
MMP-9 reverse	GTCCACCTGGTTCACCTCAT		
TIMP-1 forward	GTGGCTCCCTGGAACAGTC	143	KC425456
TIMP-1 reverse	TCGGTCCACAAGCAATGAGT		
TIMP-2 forward	GCACCACCCAGAAGAAGAGC	117	KC425455
TIMP-2 reverse	CCATCCAGAGGCACTCATCC		
β-actin forward	GCGGGACCACCATGTACC	183	XM_003357928.1
β-actin reverse	ACTCCTGCTTGCTGATCCAC		

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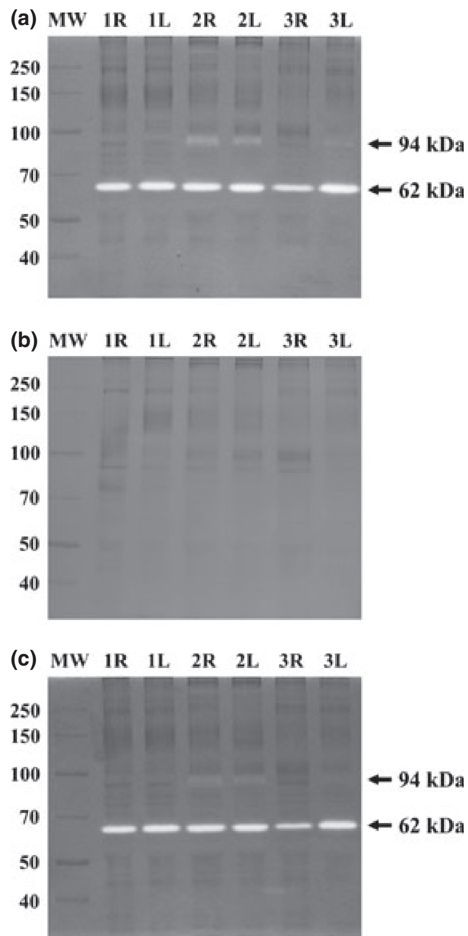

Fig. 1. Gelatine zymography of llama OF obtained from the right (R) and left (L) oviduct from three animals (1, 2 and 3). (a) Arrows indicate the position of bands with gelatinase activities and their calculated molecular weight (MW). (b) Effect of EDTA and (c) benzamidine on gelatinase activities of llama OF

The novel llama nucleotide sequences have been deposited in GenBank: Lama glama matrix metalloproteinase 2 mRNA, partial CDS, under accession number GQ244429; Lama glama matrix metalloproteinase 9 mRNA, partial CDS, accession number GU207475; Lama glama tissue inhibitor metalloprotease 1 (TIMP1) mRNA, partial CDS, accession number KC425456; and Lama glama tissue inhibitor metalloprotease 2 (TIMP2) mRNA, partial CDS, under accession number KC425455.

Expression of MMP-2, MMP-9, TIMP-1 and TIMP-2 in ampulla, isthmus, UTJ and papilla

In order to examine whether the genes were expressed in ampulla, isthmus, UTJ and papilla oviductal segments, specific primers were designed based on the llama MMP-2, MMP-9, TIMP-1 and TIMP-2 sequences. Expression of MMP-2, MMP-9, TIMP-1 and TIMP-2 was observed in all four segments of the llama oviduct (Fig. 5).

Table 4. Percentage of identity between MMP-2, MMP-9, TIMP-1 and TIM-2 nucleotide sequences from llama and other mammals

	Sus scrofa	*Bos taurus*	*Equus caballus*	*Homo sapiens*
MMP-2	93	93	93	91
MMP-9	92	91	89	86
TIMP-1	95	92	90	90
TIMP-2	92	90	91	91

Discussion

The present study demonstrates the presence of gelatinolytic activity in llama OF. Two gelatinolytic bands were detected by gelatine zymography. Even though identification with zymograms is not definite, it can be affirmed that the 62 and 94 kDa gelatinase bands were indeed metalloproteinases because gelatinolytic activity was inhibited by EDTA, a metalloproteinase inhibitor, but not by benzamidine-HCl, a serine protease inhibitor, which is consistent with a metalloproteinase character.

Previous studies about gelatinolytic activity in OF were only carried out in bovine and human oviducts (Gabler et al. 2001; Kim et al. 2001, 2003). In agreement with our findings, Kim et al. (2001) detected two gelatinase bands of 97 and 62 kDa in bovine OF. The authors suggested that based on its molecular weight, the 62 kDa form could correspond to MMP-2. Different findings have been reported by Gabler et al. (2001) who detected a unique gelatinase band of 72 kDa, proposed as pro-MMP-2. In addition, Kim et al. (2003) observed two intense bands of 98 and 62 kDa in human OF. In llama, the 94 kDa band detected by zymography would agree with the 92 kDa pro-MMP-9 reported in bovine placental tissues (Maj and Kankofer 1997) and porcine uterine tissue/fluid (Lenhart et al. 2001). Minor differences in molecular weight of these gelatinases with other mammals may be attributed to species differences. Besides, the degree of glycosylation could determine the presence of MMP-9 isoforms with different molecular masses.

Once the gelatinase activity of the OF was proven to be specific for metalloproteases, initially identified as MMP-2 and MMP-9, interest grew to determine whether these molecules and their inhibitors are expressed by oviductal tissue. Taking into account that the llama genome is still unknown, the first step to study expression of the oviduct gene was to establish the transcript sequences of MMP-2, MMP-9, TIMP-1 and

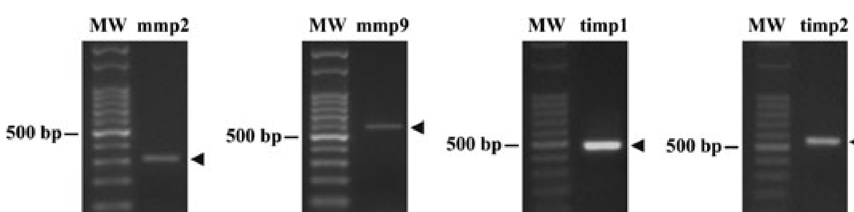

Fig. 2. RT-PCR products obtained for MMP-2, MMP-9, TIMP-1 and TIMP-2 in llama oviduct and separated with agarose gel electrophoresis. Arrowheads denote the positions of amplicons obtained. MW: molecular weight

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(a)

```
ttctcggatccatgatggagaggctgacatcatgatcaactttggccgctgggagcatgga
 S  R  I  H  D  G  E  A  D  I  M  I  N  F  G  R  W  E  H  G
 gatggatacccctttgatggcaaagatgggctcctggctcacgccttcgcccctggccct
 D  G  Y  P  F  D  G  K  D  G  L  L  A  H  A  F  A  P  G  P
 ggcgttggggagactcccactttgatgacgatgagctgtggaccctgggggaagggcaa
 G  V  G  G  D  S  H  F  D  D  D  E  L  W  T  L  G  E  G  Q
 gtggtccgcgtgaagtatgggaacgccgacggggagtattgcaagttccccttcctgttc
 V  V  R  V  K  Y  G  N  A  D  G  E  Y  C  K  F  P  F  L  F
 aacgg
 N
```

(b)

```
gggcgtcgtggttccgacctacttcggaaacgcaaatggcgccgcctgccacttccccttc
 G  V  V  V  P  T  Y  F  G  N  A  N  G  A  A  C  H  F  P  F
 acctttgaaggccgctcctactcggcctgcactacggacggccgctccgacgacatgctc
 T  F  E  G  R  S  Y  S  A  C  T  T  D  G  R  S  D  D  M  L
 tggtgcagcaccacggccgactatgacacagaccacaagttcggtttctgccccagcgag
 W  C  S  T  T  A  D  Y  D  T  D  H  K  F  G  F  C  P  S  E
 agactctacacccgggacggcaatgccgacggcaagccctgcgtgtttccgttcactttc
 R  L  Y  T  R  D  G  N  A  D  G  K  P  C  V  F  P  F  T  F
 gagggccgctcctactctgcctgcaccaccgacggtcgctcggacggctaccgctggtgc
 E  G  R  S  Y  S  A  C  T  T  D  G  R  S  D  G  Y  R  W  C
 gccaccaccgccaactacgatcaggacaagctctatggcttctgtcctacccgagccgac
 A  T  T  A  N  Y  D  Q  D  K  L  Y  G  F  C  P  T  R  A  D
 tcgacggtgactgggggcaactcggcgggagagctgtgcgtcttccccttcaccttcctg
 S  T  V  T  G  G  N  S  A  G  E  L  C  V  F  P  F  T  F  L
 ggcaaggagtactcgacctgcaccagagagggccgcaatgatgggcacctctggtgtgcc
 G  K  E  Y  S  T  C  T  R  E  G  R  N  D  G  H  L  W  C  A
 accacctctaacttcgacagag
 T  T  S  N  F  D  R
```

Fig. 3. Llama MMP nucleotide and amino acid sequences. The active domains are boxed, and the fibronectin domains are highlighted. Conserved cysteines are expressed in bold type and underlined. (a) MMP-2. (b) MMP-9

(a)

```
tcgtggggactgcagaagtcaaccagaccgccttataccagcgttatgagatcaagatgacc
  V  G  T  A  E  V  N  Q  T  A  L  Y  Q  R  Y  E  I  K  M  T
 aagatgttcaaagggttcaatgccttgggggatgcccccgacatccggtttatctacacc
  K  M  F  K  G  F  N  A  L  G  D  A  P  D  I  R  F  I  Y  T
 cccgccatggagagcgtctgcggatacttccacaggtcccagaaccgcagcgaggaattt
  P  A  M  E  S  V  C  G  Y  F  H  R  S  Q  N  R  S  E  E  F
 ctcatagctggacaactgtggaacgggcacctgcacatcaccacctgcagcttcgtggct
  L  I  A  G  Q  L  W  N  G  H  L  H  I  T  T  C  S  F  V  A
 ccctggaacagtctgagctctgctcaacgcaggggcttcaccaagacctacgctgctggc
  P  W  N  S  L  S  S  A  Q  R  R  G  F  T  K  T  Y  A  A  G
 tgtgaggaatgcacagtgtttccctgttcatccatcccctgcaaactgcagagtgacact
  C  E  E  C  T  V  F  P  C  S  S  I  P  C  K  L  Q  S  D  T
 cattgcttgtggaccgaccagctcctcacaggctc
  H  C  L  W  T  D  Q  L  L  T  G
```

(b)

```
catcaagcgcatccagtacgaggtcaagcagataaagatgttcaagggacccgacaaggac
 I  K  R  I  Q  Y  E  V  K  Q  I  K  M  F  K  G  P  D  K  D
 atcgagtttatctacacggcgccctcctctgccgtgtgcggggtctcgctggacgtcgga
 I  E  F  I  Y  T  A  P  S  S  A  V  C  G  V  S  L  D  V  G
 ggaaagaaggagtatctcattgcaggaaaggccgagggcaatggcaaaatgcacatcacc
 G  K  K  E  Y  L  I  A  G  K  A  E  G  N  G  K  M  H  I  T
 ctctgcgacttcatcgtgccctgggacaccctgagcaccacccagaagaagagcctgaac
 L  C  D  F  I  V  P  W  D  T  L  S  T  T  Q  K  K  S  L  N
 cacaggtaccagatgggctgcgagtgcaagatcacgcgctgccccatgatcccctgctac
 H  R  Y  Q  M  G  C  E  C  K  I  T  R  C  P  M  I  P  C  Y
 atctccgccccggatgagtgcctctggatggactgggtcacggagaagaacatcaatggg
 I  S  A  P  D  E  C  L  W  M  D  W  V  T  E  K  N  I  N  G
 caccaggccaagttcttctcctgcatcaagagaagcgacgggtcctgtgcgtggtaccgc
 H  Q  A  K  F  F  S  C  I  K  R  S  D  G  S  C  A  W  Y  R
 gggcggcacccccccaagcaggagtttctcgacatcgaggacccgtaagcgggccgccag
 G  A  A  P  P  K  Q  E  F  L  D  I  E  D  P  -
 caccccgcggcctgtcagaagcctcccagagtctagactggtccagctccgacatc
```

Fig. 4. Llama TIMP nucleotide and amino acid sequences. The N-terminal domains are highlighted, and the C-terminal domains are boxed. Conserved cysteines are expressed in bold type and underlined. (a) TIMP-1. (b) TIMP-2

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Table 5. Percentage of identity between MMP-2, MMP-9, TIMP-1 and TIMP-2 deduced amino acid sequences from llama and other mammals

	Sus scrofa	*Bos taurus*	*Equus caballus*	*Homo sapiens*
MMP-2	98	97	100	98
MMP-9	94	91	89	87
TIMP-1	92	95	85	89
TIMP-2	97	95	98	97

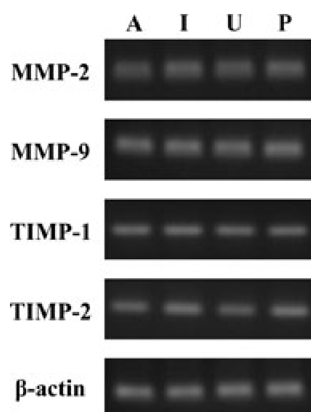

Fig. 5. RT-PCR products for MMP-2 (174 bp), MMP-9 (175 bp), TIMP-1 (143 bp), TIMP-2 (117 bp) and β-actin (183 bp) in llama oviductal segments. A: ampulla, I: isthmus, U: utero-tubal junction, P: papilla

TIMP-2. The nucleotide and deduced amino acid sequences obtained in llama showed a high degree of identity with other mammalian species (pig, cow, horse and humans). These findings suggest that MMP-2, MMP-9, TIMP-1 and TIMP-2 are highly conserved proteins.

Expression of MMP-2, MMP-9, TIMP-1 and TIMP-2 was detected in all examined oviductal segments, showing that the llama oviduct is a source of both proteases and their specific inhibitors. Our results might indicate that these MMPs play a biological role, probably participating in the reproductive events that occur in the oviduct. The organ probably also regulates MMP activity through TIMPs. MMP-2 has been proposed to be involved in ovum cumulus expansion and sperm–egg interaction as part of a successful fertilization (Gabler et al. 2001). In addition, presence of TIMP-1 could be interesting because of its embryotrophic activity; it has been identified as a stimulating factor of *in vitro* embryo development (Satoh et al. 1994).

These enzymes and their inhibitors might act on seminal plasma and sperm. SAC semen is characterized by its viscosity and mucous consistency, condition that impedes the homogenous mixing of semen with extender, thereby restricting contact between the sperm cell membrane and cryoprotective compounds during cryopreservation. Recent studies suggest that the glycosylated protein mucin 5B causes the mucous consistency in alpaca semen (Kershaw-Young and Maxwell 2012). This finding supports the fact that many proteases have proven to be effective in reducing the viscosity of camelid seminal plasma (Bravo et al. 2000; Kershaw-Young et al. 2013). The identity of the specific enzyme that is produced *in vivo* to liquefy camelid semen is still unknown. Interestingly, it has been reported that collagenase 0.1% (a bacterial metalloproteinase) treatment improves semen rheological properties and sperm progressive motility, while maintaining sperm membrane functionality and integrity (Giuliano et al. 2010). Moreover, collagenase has proven to be effective for improving sperm quality before IVF (Conde et al. 2008). On the other hand, previous studies demonstrated that seminal plasma is still present during sperm storage in the oviduct (Apichela et al. 2010, 2014). Necessarily, seminal plasma must be degraded into the oviduct by proteolytic action at the time of ovulation, allowing the sperm releasing in order to fecundation occur. Our results, together with those above mentioned, could lead to think that MMPs/TIMPs are involved in semen liquefaction. Nevertheless, this hypothesis has to be proven.

In conclusion, the current study has demonstrated the presence of metalloproteases with gelatinolytic activity in llama OF and detected the expression of MMP-2, MMP-9, TIMP-1 and TIMP-2 in the llama oviduct. Our results support the hypothesis that MMPs are produced in the oviduct and secreted into the oviductal lumen. Additionally, to the best of our knowledge, this is the first report that describes cloning of *Camelidae* (llama) MMPs/TIMPs. These data will allow us to perform further studies regarding the role of these proteins in the reproductive oviductal events above mentioned.

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Conflict of interest

The authors have declared no conflicts of interest.

Author contributions

R Zampini contributed with experimental design, experimental assays, data analysis and drafting the article. ME Argañaraz contributed with experimental design, analysis and interpretation of data and revising paper. DC Miceli contributed with experimental design and interpretation of data. SA Apichela contributed with experimental design, analysis and interpretation of data and revising paper.

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