RESEARCH ARTICLE

SL15: a seminal plasma-derived lectin from the sperm of llama (*Lama glama*) †

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Abbreviations:

GalNAc [-polyacrylamide-FITC], *N*-acetylgalactosamine [and fluorescein isothiocyanate-conjugated polyacrylamide]; SAP, sperm-adsorbed proteins; SL, seminal lectin; SPP, seminal plasma proteins.

Summary

The oviductal sperm reservoir of South American camelids is formed when sperm bind to *N*-acetylgalactosamine (GalNAc) on the surface of oviductal epithelium. The aim of this study was to characterize the GalNAc-binding proteins on llama sperm, and to establish their origin. Sperm-adsorbed proteins were extracted with 0.5 M KCl in Hepes-balanced salts. Sperm-adsorbed and seminal plasma proteins were then subjected to ligand blotting for their GalNAc affinity, and the labeled bands were identified by mass spectrometry. Three proteins were identified in seminal plasma versus only one in the sperm-adsorbed population; SL15, a seminal lectin, was common to both. SL15 is a homologue of zymogen granule protein 16, homolog B-like, which belongs to the Jacalin-related lectin family. This lectin is likely presented to sperm via seminal plasma since epididymal sperm are not capable of binding GalNAc, whereas ejaculated sperm does, and its transcript was enriched predominantly in the prostate and bulbourethral glands. This is the first report of a seminal lectin in South American camelids that originates in the male reproductive tract, and is probably involved in sperm reservoir formation.

Keywords: Spermatozoa, sperm protein, N-acetylgalactosamine, sugar recognition, South American camelids

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Introduction

Protein composition of the seminal plasma varies widely among species (Druart et al., 2013). Many of the identified seminal plasma proteins (SPPs) from ungulates are predicted to function in fertility (bulls [Souza et al., 2008], boars [Caballero et al., 2009], stallions [Töpfer-Petersen et al., 2005], and buffalos [Harshan et al., 2009]); cryoprotection (bulls [Almaday et al., 2015] and rams [Pérez-Pé et al., 2001]); or sperm viability (bulls and rams [Barrios et al., 2000]). Thus, SPPs are thought to improve the function of semen extenders and, consequently, fertility. Despite the growing list of identified SPPs, the function of 21% of these proteins remains unknown (Druart et al., 2013).

Very little information is available about the composition and function of the seminal plasma of South American camelids (Ratto et al., 2011; Kershaw-Young and Maxwell, 2012; Apichela et al., 2014). One particular feature that could affect the application of reproductive technologies on these camelids is their lack of vesicular glands (Tibary and Anouassi, 1997): they only possess a prostate and two bulbourethral glands. In most mammals, secretions of the vesicular glands accounts for all the major SPPs (Bergeron et al., 2005), which adsorb to sperm and thus mediate sperm recognition and binding to the oviductal epithelium, allowing the formation of a reservoir that helps prolong sperm viability and fertilizing capacity – as documented in bovine (Gwathmey et al., 2003) and porcine (Maňásková et al., 2007). The absence of vesicular glands suggests that camelids have evolved a different method for sperm to attach to the oviduct in order to create a sperm reservoir.

We previously demonstrated that the llama sperm reservoir is formed at the utero-tubal junction by means of a lectin-like molecule that mediates sperm recognition of *N*-acetylgalactosamine (GalNAc) and galactose in the female oviduct (Apichela et al.,

2010). Under normal conditions, sperm remain attached to the llama utero-tubal junction at least 28 h after mating (Apichela et al., 2009); surgical removal of the bulbourethral glands diminished such sperm attachment (Apichela et al., 2014). The current study sought to isolate and identify the GalNAc-binding lectins present on llama sperm, and to test the hypothesis that they are provided by seminal plasma.

Results

Detection of N-*acetylgalactosamine-binding molecules on llama spermatozoa*

Fluorescein isothiocyanate-conjugated polyacrylamide substituted with *N*-acetylgalactosamine (GalNAc-polyacrylamide-FITC) was used to probe the proteins adsorbed on the surface of different llama spermatozoa populations (e.g. sperm-adsorbed proteins [SAPs]) to ask when their GalNAc-binding capacity was obtained. Whole, ejaculated spermatozoa revealed a strong fluorescent signal, whereas epididymal spermatozoa, obtained from the tail of the epididymis, did not (Figure 1). Very weak or no fluorescence was observed in untreated sperm.

Identification of N-*acetylgalactosamine-binding proteins in seminal plasma and adsorbed to sperm*

Ten protein bands, from 129 kDa to 13 kDa, were noted in seminal plasma (Figure 2a). Among them, four seminal lectins (SL) possessed the capacity to bind GalNAc (Figure 2b); the identity of three was determined by mass spectrometry: BPI fold-containing family B member 1 (SL54), prolactin-inducible protein homolog (SL16), and zymogen granule protein 16 homolog B-like (ZG16B) (SL15). The identity of the fourth, SL26, was undetermined due to the absence of a significant score and to a discrepancy between the experimental and theoretical molecular weight (Table 1).

Eleven protein bands, from 95 kDa to 13 kDa, were observed from a high-salt extraction of sperm-adsorbed proteins (Figure 2c). Only one of these proteins bound GalNAc (Figure 2d), and was identified as SL15 (Table 2).

SL15 ***sequence analysis***

The full-length llama *SL15* mRNA sequence matched the predicted *Vicugna pacos* zymogen granule protein 16, homolog B-like (LOC102538843) mRNA (XM_015239267.1) with 100% identity. The SL15 open reading frame consists of 459 base pairs (nucleotides 33 to 491), encoding a protein of 152 amino acids with a predicted 17-amino-acid signal peptide (Figure S1). The mature, deduced SL15 protein corresponds to a 135-amino-acid polypeptide chain with an estimated molecular weight of 15,237 Da and a calculated pI of 9.76. This protein has a predicted *N*-glycosylation site at N107, and three clusters predicted to bind *N*-acetylglucosamine, galactose, mannose, glucose, and GalNAc (Figure 3a): G25, G26, T27, F95, Y137, R138, L139, Y140, G141, and T143 (cluster 1); Y92, R93, K94, F95, I96, Q97, and G116 (cluster 2); G47, P48, V49, G50, L51, and K53 (cluster 3).

The llama SL15 amino acid sequence was queried against a database of proteins with experimentally determined three-dimensional structures, using the Phyre2 web portal (Kelley et al., 2015). This assessment returned two putative matches: 65% identity with the porcine seminal protein Chain B, crystal structure of a prostate-specific WGA16 glycoprotein lectin, Form 2 (PDB: 3WOC_B) and 46% identity with human protein Chain B, crystal structure of human Pancreatic Secretory Protein Zg16b (PDB: 3AQG_B). Both proteins belong to a large family of plant and animal lectins, the Jacalin-related lectins, which assume a β-prism fold consisting of three β-sheets (Figure

3b). Each β-sheet is made up of three to four β-strands, forming three Greek-key motif shavings (Raval et al., 2004).

SL15 expression in male reproductive organs

Gene expression was assessed by reverse-transcription PCR to identify the site of SL15 production within the llama male reproductive system. A 384-bp product was detected in the testicles, tail of the epididymis, prostate, and bulbourethral glands; no amplification was observed in the head or body of the epididymis. *SL15* expression was significantly higher in bulbourethral gland and prostate than in testis and epididymis tail (Figure 4).

Discussion

We previously demonstrated that ejaculated sperm are capable of adhering to GalNAc, and that excess GalNAc strongly inhibits sperm adhesion to oviductal epithelial cells in vitro (Apichela et al., 2010), indicating that sperm possess GalNAc-binding proteins. We hypothesized that these proteins are provided by seminal plasma, and found that ejaculated sperm, but not epididymal sperm, which have not been exposed to seminal plasma, were capable of binding GalNAc. Comparison of SPP and SAP profiles showed very different protein populations – although some bands seemed to be shared. The SAPs were easily removed by 0.5 M KCl, so we hypothesized that this protein population was derived from seminal plasma, which is consistent with descriptions for sperm-associated proteins in other livestock (e.g. bulls [Einspanier et al., 1994; Manjunath et al., 2007], rams [Pérez-Pe et al., 2001; Bergueron et al., 2005], and boars [Garénaux et al., 2015]).

Three GalNAc-binding protein unique to llama seminal plasma were identified. Fold-containing family B member 1 (BPI), an SPP that binds GalNAc, possesses antibacterial activity against Gram-negative bacteria (Schumann et al., 1990; Elsbach and Weiss, 1998) and was previously reported in ram seminal plasma (Martins et al., 2013). This molecule primarily associates with phospholipids and lipopolysaccharide (Hailman et al., 1996; Bruce et al., 1998), although it may also bind heparin (Martins et al., 2013) – which could be why we detected it with GalNAc. Prolactin-inducible Protein (PIP) was previously reported in human seminal plasma (Murphy et al., 1987), but its role is still undetermined. PIP is an IgG-binding protein, suggesting that it may protect sperm from the action of anti-sperm antibodies (Martínez-Heredia et al., 2008) and/or modulate the immune response during insemination (Caputo et al., 1999).

Llama SL15, the only lectin-type molecule identified from llama sperm, was likely adsorbed to sperm via the seminal plasma during ejaculation, given that it is mainly produced by prostate and bulbourethral glands. SL15 belongs to the Jacalin-related lectins, a large family of plant and animal lectins whose sugar-binding site is composed of three loops (GG loop, binding loop and recognition loop) (Raval et al., 2004); indeed, SL15 contains the signature motifs GG and GXXXD from the recognition loop (Meagher et al., 2005) as well as numerous predicted sugar-binding sites. A key residue present in most Jacalin-related lectins, first identified in human ZG16p (D151) (Kanagawa et al., 2011), is mutated in boar WGA16 (T143) as well as in llama SL15.

Boar WGA16, a lectin similar to SL15, was recently reported to bind to terminal β-*N*-acetylhexoseamine and *N*-acetylneuraminic acid residues (Garénaux et al., 2015). WGA16 is a prostate-derived seminal-plasma glycoprotein adsorbed to sperm that disappears from the sperm surface during capacitation, which was proposed to be related to its affinity towards heparin or sulfated glycosaminoglycans (Garénaux et al.,

2015). Site-directed mutagenesis defined the heparin-binding site of WGA16 in the basic cluster of K53 and K73 (Garénaux et al., 2015). Interestingly, these two amino acids are conserved in llama SL15, suggesting that SL15 also possesses heparin-binding capacity.

Seminal plasma-derived heparin-binding proteins that associate with ejaculated sperm are common in other species (Feng et al., 2006; Mader et al., 2006), including binder of sperm (BSP) proteins in bulls (Manjunath and Sairam, 1987), goats (Villemure et al., 2003), pigs (Lusignan et al., 2007), horses (Calvete et al., 1997), and bison (Boisvert et al., 2004). BSP interacts with heparin through its two fibronectin type II domains (Feng et al., 2006). The prediction that llama SL15 also binds heparin, based on sequence homology to boar WGA16, implies that heparin-binding activity is conserved and necessary for sperm physiology within the oviduct.

South American camelids are economically and socially essential for those who inhabit Andean plateaus, yet they are also endangered. The function of SL15 may prove to be beneficial for conservation and biotechnological applications – particularly in the development of semen extenders and protocols in vitro fertilization. Before this can be achieved, however, some basic questions must be addressed: Can SL15 prolong the lifespan of llama sperm by enabling binding to oviduct? Can SL15 modulate the capacitation process? If so, can SL15 improve epididymal sperm quality? Answers to these inquiries will also greatly enhance out understanding of GalNAc-binding proteins in the reproductive biology of these camelids.

Materials and Methods

Animals

Twenty-four fertile male llamas between 3 and 8 years old were used for seminal plasma and ejaculated sperm collection. The animals were kept at the experimental farm of Instituto Nacional de Tecnología Agropecuaria (INTA) in Abra Pampa (Jujuy, Argentina), located in the high Andean Plateau of northwest Argentina at 3484 m above sea level (22˚ 49'S latitude and 65˚ 47'W longitude). The animals were kept on natural pasture, and water was provided *ad libitum.* Semen was obtained from these animals (one ejaculate per animal) during the autumn of 2013-2014.

Three male llamas between 3 and 4 years old from a local abattoir (Bella Vista, Tucumán, Argentina) were used for reproductive organs and epididiymal sperm collection during the winter of 2016.

Seminal Plasma Preparation

Semen (n=10) was obtained by using a modified bovine artificial vagina – 20 cm in length and filled with water at 39˚C – after an abstinence period of five days, according to Giuliano et al., (2008). Semen was collected using a long plastic sleeve sealed at one end, and inserted within the latex inner lining of the artificial vagina. A teaser female was used for the collections. Duration of each collection was 20–25 min. Protease inhibitor cocktail (Sigma, St. Louis, MO, USA) was added to the semen to a final 1X concentration, and seminal plasma was recovered by centrifugation at 6000*g* for 20 min at 4˚C and filtered (0.2-µm cellulose acetate) to remove particulates. Protein content was determined using a Micro BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA) with a bovine serum albumin standard. Aliquots of clarified seminal plasma were stored at -70˚C until needed.

Sperm

Semen (n=5) was diluted fivefold with Hepes-balanced salt solution (HBSS) (25 mM Hepes, 130 mM NaCl, 5 mM KCl, 0.36 mM NaH_2PO_4, 0.49 mM $MgCl_2$, and 2.4 mM $CaCl_2$; pH 7.4, 290 mOsm/kg), and centrifuged at 1000*g* for 10 min at room temperature to remove the seminal plasma. Sperm were then dried on a positively-charged slide, and fixed with Carnoy´s solution (methanol/acetic acid 3:1) for 24 h at room temperature.

Sperm-adsorbed protein extraction

Three pools of three semen samples (n=9 total) from different males were used. Pools were diluted fivefold, and washed three times with HBSS to remove seminal plasma. Washed sperm were re-suspended in HBSS with 1X protease inhibitor cocktail (Sigma). An equal volume of 1 M KCl in HBSS was added, and sperm proteins were extracted by gentle mixing on a rotary shaker for 1 h at 4˚C. Spermatozoa were then removed by centrifugation at 6000*g* for 10 min, and discarded. Extracts were clarified by filtration (0.2-µm cellulose acetate). A series of centrifugations in 3-kDa cellulose filters (Amicon, Lexintong, MA, USA) were performed at 9000*g* for 5 min at 4˚C, re-diluting in HBSS every centrifugation to reduce the salts and to concentrate the sample. Protein content per sample was assessed before storage at -70˚C.

Reproductive organs

The reproductive organs of fertile male llamas (n=3) were obtained immediately after slaughtering, in accordance with protocols approved by local institutional animal care. The testis, epididymis (head, body, and tail), bulbourethral glands, and prostate were

dissected into small pieces (less than 0.5-cm thick), and stored in RNAlater solution (Ambion, Austin, TX, USA) at -70˚C until RNA isolation.

Epididymal sperm

Epididymides (n=3) were obtained *post mortem*. The caudal epididymis was excised to obtain sperm cells, which were suspended in 5 ml of HBSS and centrifuged at 1000*g* for 10 min at room temperature. The supernatant was removed, and the sperm were re-suspended in 200 µl of HBSS, dried, and fixed as described above.

Labeling of Spermatozoa

Slides containing the epididymal and ejaculated sperm were washed with phosphate-buffered saline (PBS), and then incubated for 1 h with 150 µg/ml of GalNAc-polyacrylamide-FITC conjugate (Glycotech, Rockville, MD, USA). The slides were then mounted with PBS-glycerol (9:1), and observed under an Olympus BX53 fluorescence microscope. The images were captured with an Olympus DP71 microscope digital camera. A negative control, consisting of untreated sperm samples, was also included.

Polyacrylamide Gel Electrophoresis

SPP and SAP samples were separated by denaturing polyacrylamide gel electrophoresis, according to Gevaert and Vandekerckhove (2000). Briefly, 40 µg of total protein was diluted (v/v) with sample buffer (0.1 M Tris–HCl, pH 6.8, 2% sodium dodecyl sulfate, 1% β–Mercaptoethanol, 30% glycerol, and 0.05% bromophenol blue), and loaded onto a 15% polyacrylamide resolving gel with a 4% stacker. Molecular masses were determined by running protein markers (PageRuler Unstained Broad Range Protein

Ladder and PageRuler Plus Prestained Protein Ladder) (Thermo Fisher Scientific) covering the range of 5–250 kDa. Gels were run in a PROTEAN II xi Cell (Bio-Rad, Hercules, CA, USA) at 150 V for 1 h at room temperature. The separated proteins were stained with colloidal Coomassie Blue G-250 (Sigma) (Neuhoff et al., 1990) or further processed for blotting (see Ligand Blot section). Coomassie Blue Gel images were obtained using a Pentax Optio M 90 camera (Pentax, Milan, Italy). GelAnalyzer, version 2010a, was used to determine the molecular weight of the detected bands on the digitized images.

Ligand Blot

Electrophoretically separated SPP and SAP samples were immobilized on a 0.45-µm nitrocellulose membrane (Sigma) using a Trans-Blot Semi-Dry Transfer Cell system (Bio-Rad). GalNAc-binding proteins were detected according to the methods of Ignotz et al. (2001), with modifications. Briefly, membranes were incubated for 1 h at room temperature in binding buffer (HBSS with 5% bovine serum albumin, 1 mM $NiCl_2$, and 0.05% [v/v] Tween20) to block any non-specific sites and to renature the blotted proteins. After three rinses in wash buffer (HBSS with 1 mM $NiCl_2$, 0.5% [v/v] Tween20), membranes were probed with 5 µg/ml GalNAc-polyacrylamide-FITC in binding buffer for 2 h at room temperature, followed by three 10-min washes. Membranes were then incubated with alkaline phosphatase-conjugated mouse monoclonal anti-FITC (Sigma) (1:10000 in binding buffer) for 1 h at room temperature, followed by additional washes. Visualization of GalNAc-binding proteins was achieved by incubating in 23.25 mg/ml SigmaFast chromogenic substrate (BCIP/NBT) (Sigma) until a violet precipitate developed.

Mass Spectrometry

Bands of interest were excised from a colloidal Coomassie Blue stained polyacrylamide gel for characterization using matrix-assisted laser desorption–ionization mass spectrometry (MALDI-MS), performed on an Ultraflex II TOF/TOF (Bruker Daltonics, Bremen, Germany) mass spectrometer at the CEQUIBIEM mass spectrometry facility (Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Argentina). Proteins were identified by peptide-mass fingerprinting with MASCOT v. 2.2.03. Fragmentation was carried out with the most intense peaks (MS/MS). When possible, MS and MS/MS data were combined for one or more peptide searches. De novo sequencing was inferred from BLAST results when peak fragmentation was allowed. The percentage of protein coverage was determined for each band using the MASCOT search.

RNA isolation and cDNA synthesis

mRNA from testis, epididymis (head, body and tail), bulbourethral gland, and prostate was isolated using the Genelute Direct mRNA Miniprep kit (Sigma), according to the manufacturer's instructions. Reverse transcription was performed using Moloney murine leukemia virus (M-MLV) reverse transcriptase (Promega, Madison, WI, USA) and oligo $(dT)_{15}$ primer. The reaction mixture (25 µl) consisted of 5.5 µl of mRNA, 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 0.5 mM of each dNTP, 25 pmol of oligo $(dT)_{15}$, 200 units of reverse transcriptase, and RNase-free water. Reactions were performed by incubating the mixture in a thermal cycler at 42˚C for 90 minutes, followed by enzyme inactivation at 94˚C for 5 minutes.

Sequencing of llama SL15 complete mRNA

The 5´ and 3´ ends of *SL15* transcript were amplified from prostate cDNA by PCR. Primers were designed using Primer3 (http://frodo.wi.mit.edu/), based on the predicted *Vicugna pacos* zymogen granule protein 16, homolog B-like (LOC102538843) mRNA sequence (XM_015239267.1), which was the predicted ortholog of SL15 by mass spectrometry. Primers were chosen to be approximately 20 bp in length and 60% GC content (Table 3). The absence of self-complementary sequences and primer-dimer interactions were checked.

Amplifications were carried out in a final volume of 10 µl containing 0.5 µl of prostate cDNA, 2 µl of 5X Green GoTaq Reaction Buffer (pH 8.5), 0.2 mM of each dNTP, 2.5 units of GoTaq DNA polymerase (Promega), and 1 µM of each primer pair. Different amplification conditions were assayed to determine the optimal PCR conditions: 94˚C for 5 min; 30 cycles of 94˚C for 15 sec, 60˚C for 15 sec, and 72˚C for 20 sec; followed by a final step at 72˚C for 5 min. PCR products were separated on 1.5% agarose gels containing SYBR Safe (Invitrogen, Carlsbad, CA, USA). Amplicons of the expected size were purified using a PureLink Quick Gel Extraction kit (Invitrogen), and then sequenced by CERELA Sequencing Service using an ABI/Hitachi Genetic Analyzer 3130.

SL15 Sequence Analysis

The full-length nucleotide sequence of llama *SL15* was obtained by aligning 5' and 3' partial sequences. A homology search was performed with the BLAST servers at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The inferred amino acid sequence was determined using Translate Tool of EXPASY (http://web.expasy.org/translate/), and the theoretical molecular weight of protein was estimated by Compute pI/Mw of EXPASY

(http://web.expasy.org/compute_pi/). The putative signal peptide sequence was determined using SignalP (http://www.cbs.dtu.dk/services/SignalP/). *N*-glycosylation sites were found using NetNGlyc from the Center for Biological Sequence Analysis (http://www.cbs.dtu.dk/services/). Phyre2 and 3DLigandSite were used to predict the three-dimensional structure of the protein amino acid sequence and sugar-binding sites, respectively.

Semi-quantitative PCR

SL15 gene expression in the testis, epididymis (head, body, and tail), bulbourethral gland, and prostate was analyzed by semi-quantitative PCR. PCR mixtures were performed as described above, using ZG16 pair A primers (Table 3). The reactions were carried out in a thermal cycler, following the same conditions described above. *ACTB* (β-actin) cDNA was amplified as an internal control (Table 3). The PCR products were analyzed on 1.5% agarose gels, and visualized with SYBR Safe DNA Gel Stain (Invitrogen).

For semi-quantitative expression analysis, gel images were captured with an Optio M 90 Pentax digital camera, and the optical densities of PCR products were quantified using ImageJ 1.42q software (NIH, Bethesda, Maryland, USA). The relative abundance of the *SL15* transcripts was normalized against that of *ACTB* (reference gene), and the transcript/*ACTB* ratio was calculated for each reproductive organ analyzed.

Statistical analysis

Statistical analysis was performed with InfoStat software (Di Rienzo et al., 2008). One-way Analysis of Variance (ANOVA) was used to analyze relative *SL15* mRNA expression. Fisher's LSD test was used to determine the level of significance when ANOVA showed differences. Results were considered statistically significant at $P<0.05$.

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Figure Legends

Figure 1. Ejaculated and epididymal llama sperm incubated with GalNAc-polyacrylamide-FITC. Ejaculated sperm with (a) or without (c) GalNAc. Epididymal sperm with (b) or without (d) GalNAc. Inset correspond to phase contrast images. Scale bar, 10 µm.

Figure 2. Electrophoretic profile and GalNAc-ligand blotting. (a-b) SPP samples or (c-d) sperm-adsorbed protein (SAP) samples were separated, and then stained with colloidal Coomassie Blue staining (a, c) or probed with GalNAc-polyacrylamide-FITC followed by alkaline phosphatase-conjugated anti-FITC for detection (b, d). The control blot was run without GalNAc. Arrows indicate bands that were characterized by mass spectrometry.

Figure 3. Scheme of SL15 protein structure (a). The signal peptide is shown in grey, and mature protein in violet. Numbers indicate amino acid positions. Secondary structure of llama SL15 is shown below the protein scheme. Predicted three-dimensional structure of SL15 is shown as a ribbon model (b). The β-sheet structures are highlighted in red, cyan, and green. Loops are colored in gray. N, amino-terminus; C, carboxyl-terminus.

Figure 4. Semi-quantitative analysis of *SL15* transcription in the testis (T), epididymis (head [HE], body [EB], and tail [ET]), bulbourethral gland (BG), and prostate (P). Relative mRNA expression, normalized to *ACTB* mRNA levels, is shown (mean ± standard error; n=3). Significant differences are indicated with different letters (P<0.05). NA, no amplification.

Supplemental Figure 1. Nucleotide (lowercase) and deduced amino acid sequence (uppercase) of SL15. In the nucleotide sequence, start and stop codons are in bold. In the amino acid sequence, the signal peptide is highlighted with a gray boxed, and green boxes indicate GG loop and GXXXD motifs that are characteristic of Jacalin-related lectins. Predicted *N*-glycosylation site at N107 is highlighted in magenta. Predicted amino acids involved in sugar binding are colored in red (cluster 1), orange (cluster 2), and blue (cluster 3). K53 and K73 heparin-binding sites are highlighted in yellow, and T143 in light blue. Peptides identified by mass spectrometry are underlined.

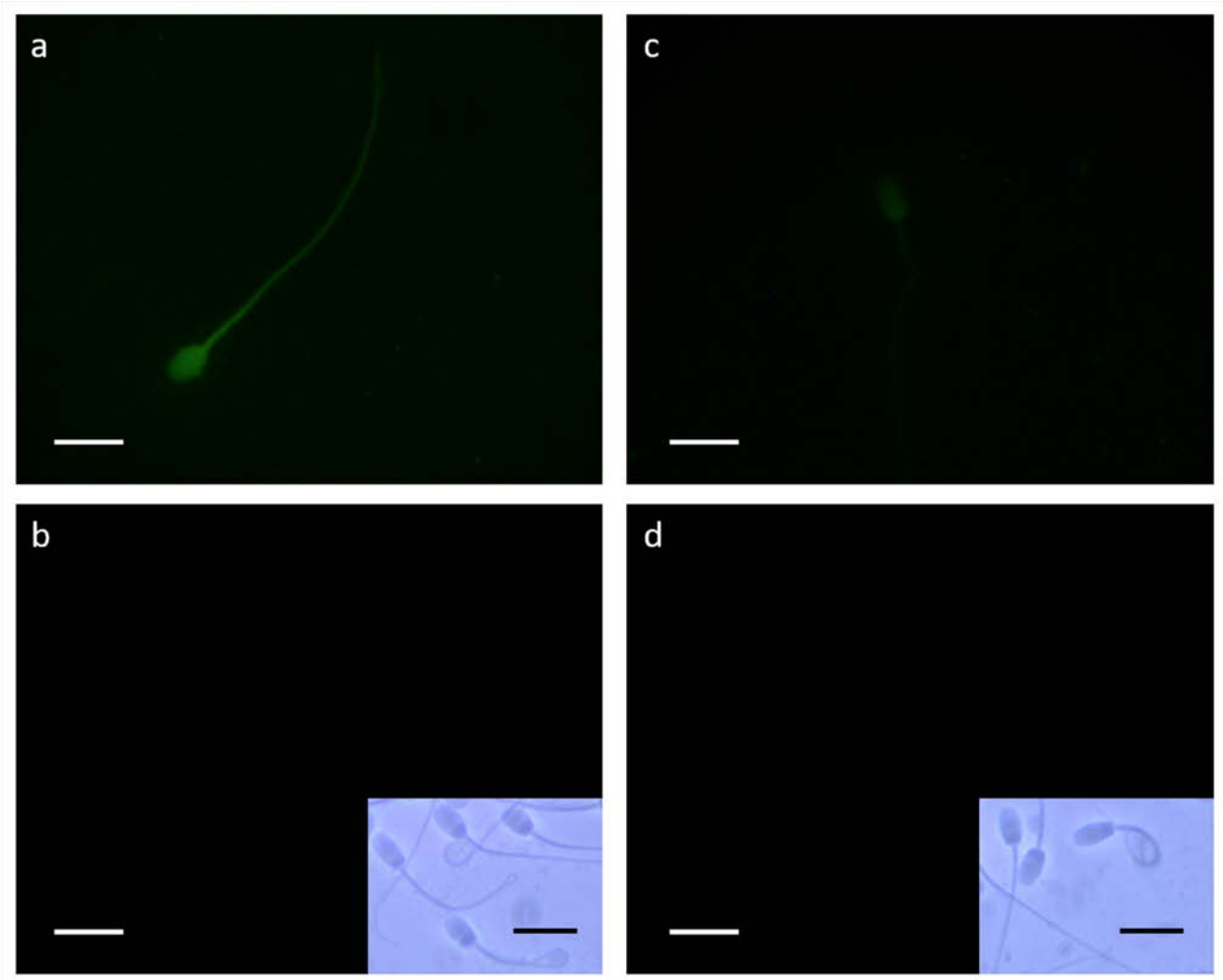

Figure 1

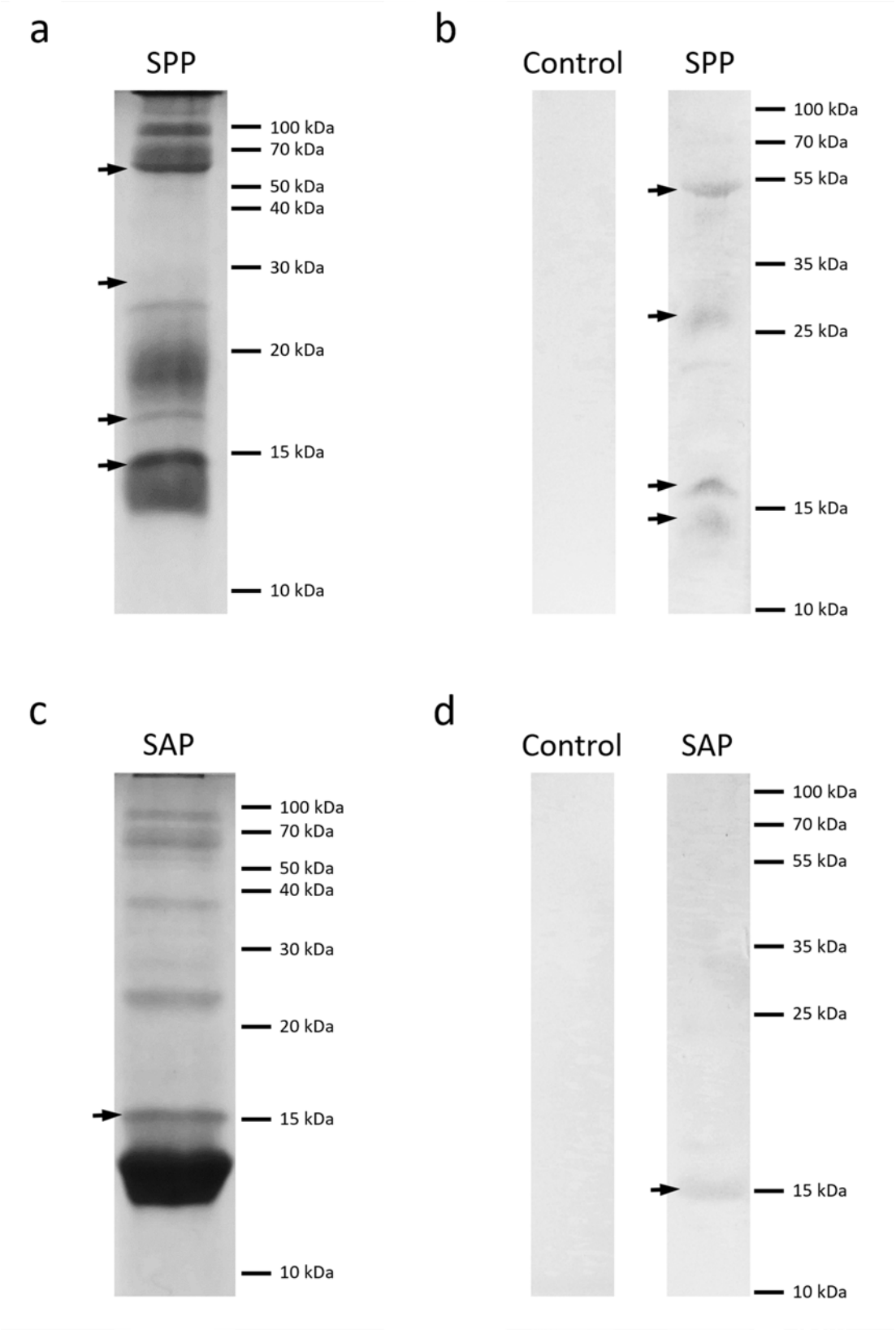

Figure 2

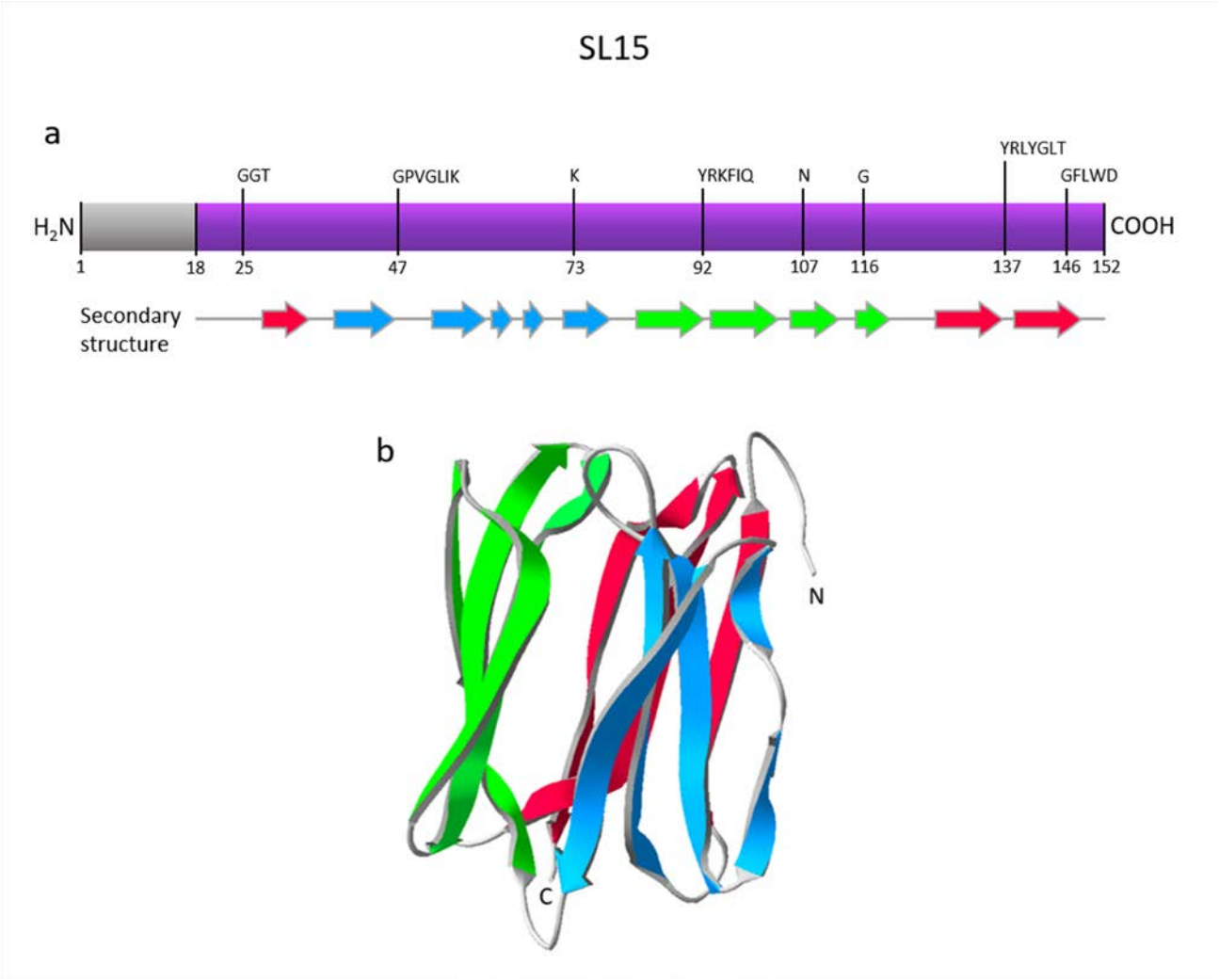

Figure 3

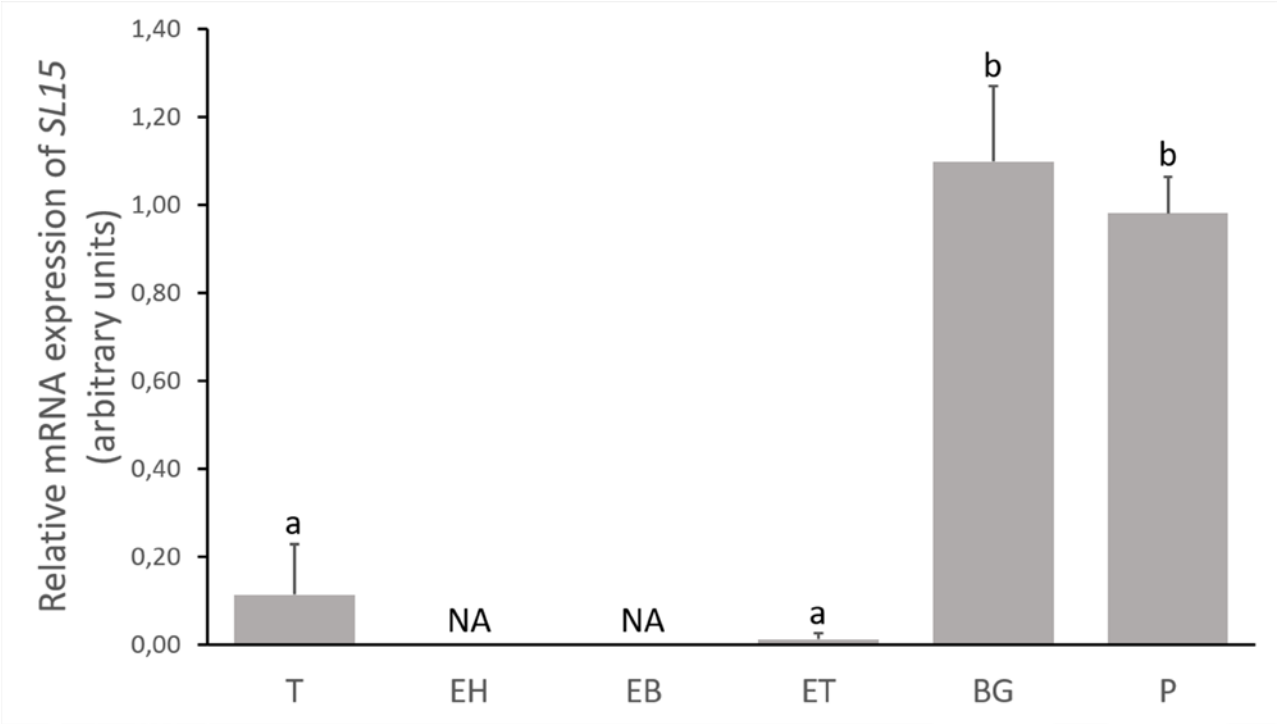

Figure 4

Table 1

Identity of seminal plasma bands that bind *N*-acetylgalactosamine.

Band	Identity	Accession Nº	Species	EPMW kDa	TPMW kDa	Coverage (%)	MP	S/SL	E
SL54	*PREDICTED: BPI fold-containing family B member 1*	XP_006202804.1	*Vicugna pacos*	54	52	30	18	318/76	3.8e-26
SL26	*Ig lambda chain C regions isoform 19-like protein*	EPY90124.1	*Camelus ferus*	26	15	22	2	74/76	0.092
SL16	*PREDICTED: prolactin-inducible protein homolog*	XP_015099709.1	*Vicugna pacos*	16	17	35	4	180/76	2.4e-12
SL15	*PREDICTED: zymogen granule protein 16 homolog B-like*	XP_015094753.1	*Vicugna pacos*	15	15	18	2	103/74	0.00012

EPMW, experimental protein molecular weight; TPMW, theoretical protein molecular weight; MP, matched peptides; S/SL, score/significance level; E, expectation value.

Table 2

Identity of the SAP band that recognize *N*-acetylgalactosamine.

Band	Identity	Accession Nº	Species	EPMW	TPMW	Coverage (%)	MP	S/SL	E
SL15	*PREDICTED: zymogen granule protein 16 homolog B-like*	XP_015094753.1	*Vicugna pacos*	15	15	31	4	125/74	4.5e-07

EPMW, experimental protein molecular weight; TPMW, theoretical protein molecular weight; MP, matched peptides; S/SL, score/significance level; E, expectation value.

Table 3

Primers sequences.

Primer name	Primer sequences	GenBank accession number	Amplicon size (bp)
ZG16 forward A	5'-GCCAGGGGCTTTTGACTCC	XM_015239267.1	384
ZG16 reverse A	5'-CCTTGCCAACTTCCTTTCCA		
ZG16 forward B	5'-AGATGAGACGTTTGGTCCCG	XM_015239267.1	431
ZG16 reverse B	5'-GTTGAGCAGAGGTCATCCCAC		
ACTB forward	5'-GCGGGACCACCATGTACC	XM_006210388.1	183
ACTB reverse	5'-ACTCCTGCTTGCTGATCCAC		