Actinobacillus seminis Secretes a Metalloprotease That Degrades Bovine Fibrinogen and IgG

De la Cruz Montoya Aldo Hugo, Ramírez-Paz Y Puente Gerardo A, Vazquez-Cruz Candelario, Montes-García J, Negrete Y Abascal Erasmo

Actinobacillus seminis is a Gram-negative bacterium member of the Pasteurellaceae family. Even though it is part of the ovine microbiota it is also the causal agent of genital organs affections, particularly epididymitis and orchitis. Infections caused by this microorganism generate economic losses to the ovine industry due to impaired fertility and sterility in affected animals. Knowledge about virulence factors expressed by A. seminis is scarce. In the present work, we describe the expression of a metalloprotease secreted by A. seminis that can degrade bovine fibrinogen and immunoglobulin G and present homology with a carboxy-terminal protease from A. seminis. This metalloprotease presents an optimal activity at a pH between 6 and 7, is stable up to 60°C, inactive at higher temperatures, and completely inhibited by 30 mM EDTA. The expression of this proteolytic activity is controlled by temperature, calcium, and iron. Proteases degrading extracellular matrix components and molecules involved in the immune response could facilitate and improve host colonization and invasion by A. seminis.

INTRODUCTION

Ovine epididymitis is a transmissible disease commonly caused by Brucella ovis, Actinobacillus seminis, or Histophilus somni; of them, B. ovis is the microorganism most frequently isolated from infected animals. Although A. seminis has been considered part of the microbiota of the genital tract, it could be a primary pathogen as demonstrated by its solo inoculation in rams (Moustacas et al., 2014). Ovine epididymitis is considered one of the major causes of economic losses to the ovine industry due to impaired fertility and sterility in rams. Because young animals with epididymitis and orchitis caused by A. seminis are commonly asymptomatic in the early stages of illness, it is common that affected animals are detected until the disease has already progressed (Al-Katib and Dennis, 2009). This microorganism has also been isolated from the bovine semen of animals with testicular lesions (Santos et al., 2019) and uterine discharges from aboritions in cows. A. seminis can attach to bovine kidney epithelial cells and cultured human bladder cells (Healey et al., 1991; Montes-García et al., 2018), agglutinate fixed sheep red blood cells (Montes-García et al., 2019) and form in vitro biofilms (García et al., 2020). The capability of A. seminis to express RTX proteins by immune cross-reactivity with anti-Apx sera (Schaller et al., 2000) and to release outer membrane vesicles (Núñez-del Arco et al., 2006) has been suggested.

Mucosal pathogens can produce and excrete different kinds of precise nonspecific and specific proteases that degrade substrates involved in many functions, including the immune response, where they let them hide from the host defense mechanisms and take advantage of them (Potempa and Pike, 2009). A. seminis has been described as a habitant of the prepuce that ascends to the epididymis to express RTX proteins by immune cross-reactivity with anti-Apx sera (Schaller et al., 2000) and to release outer membrane vesicles (Núñez-del Arco et al., 2006) has been suggested. Mucosal pathogens can produce and excrete different kinds of precise nonspecific and specific proteases that degrade substrates involved in many functions, including the immune response, where they let them hide from the host defense mechanisms and take advantage of them (Potempa and Pike, 2009). A. seminis has been described as a habitant of the prepuce that ascends to the epididymis when hormonal changes occur during the ram’s sexual maturity (Al-Katib and Dennis, 2009), where it is subjected to different clearance mechanisms that it has to evade, including the immune response.

Different members of the Pasteurellaceae family, to which A. seminis belongs, express and secrete proteases as a mechanism to evade the clearance strategies of the different hosts these microorganisms colonize (Negrete-Abascal et al., 1998, 2004), as a means to get nutrients, expose putative receptors, and as an invasive strategy. Despite this knowledge, the virulence factors that participate in the potential pathogenesis of this microorganism are not completely understood. In the...
present work, we describe the expression of metalloproteases by *A. seminis*. These proteins can degrade bovine IgG and fibrinogen and their expression is controlled by temperature, the presence of calcium, and the iron concentration. These metalloproteases could be a mechanism to evade the immune response mediated by IgG and induce changes in the ram’s physiology during the *A. seminis* pathogenesis.

**MATERIALS AND METHODS**

**Bacterial strains:** *A. seminis* ATCC 15768 reference strain was cultured on red blood agar sheep plates to reactivates and determine the purity of trypsin soy broth (TSB) cultures. Plates were incubated at 37°C in candle jars with humidity for 24 h.

**Growth conditions:** To obtain the putative proteolytic activities, *A. seminis* strains were grown in TSB medium in agitation at 37°C overnight. This culture was used to inoculate TSB media incubated at 37°C or 39–40°C. Cultures were maintained in agitation until an OD600 nm 0.1 was obtained, and 10 mM CaCl₂ or 0.25 mM 2,2’ dipyridyl were added and incubated overnight. Media without CaCl₂ or 2,2’dipyridyl were used as controls. The purity of broth cultures was determined by growth on blood agar sheep plates.

**Secreted proteins:** Broth cultures were centrifuged at 10500g at 8°C for 25 minutes. Cultured supernatants without bacterial cells were precipitated overnight with ammonium sulfate at 60% saturation. Precipitated proteins were recovered by centrifugation at the same conditions and resuspended with PBS. Samples were frozen until their use.

**Zymograms:** The presence of proteolytic activity in the secreted proteins was determined through zymograms with porcine gelatin as previously described (Negrete-Abascal et al., 2004). To determine the optimal pH of the proteolytic activity, 10 µg of protein were loaded per well and, after electrophoretic separation, gels were incubated in three different conditions: i) 50 mM acetate buffer, pH 4 to 6; ii) 50 mM Tris-HCl, pH 7 or 8; and iii) 50 mM glycine-NaOH, pH 9 and 10. The effect of temperature on the proteolytic activity was determined at 4°C, room temperature, 37, 40, 50, 60, or 70°C for 10 minutes before electrophoresis. The effect of different protease inhibitors (10mM p-methylmercuric chloride [PMSF], 5 mM phenyl methyl sulphonyl fluoride [PMSF], or 10-40mM ethylenediaminetetraacetic acid [EDTA]) was determined by previous incubation with each inhibitor and their addition to the activation buffer at the same concentrations.

**Degradation of other substrates:** To establish the capability to degrade molecules of physiological importance, 20 µg of secreted proteins were mixed with 20 µg of bovine IgG or fibrinogen and incubated at 37°C overnight. Then, samples were separated by electrophoresis in a 10% SDS-PAGE. Bovine IgG or fibrinogen without secreted proteins were used as negative controls.

**Proteolytic activity quantification:** To know if the differences in proteolytic activity observed in zymograms were significant, we used azocasein as substrate (Sigma-Aldrich, St. Louis, MO, US) as described (Silva et al., 2015) and modified it with a 1% azocasein solution and 100 µg of sample in a final volume of 1 mL with PBS. As positive control, 10µg proteinase K (Sigma-Aldrich) was used. To quantify protease activity, one unit of activity was defined as an increase of 0.01 in absorbance units at 405 nm; results are expressed as specific activity units.

**Separation of proteases and immune recognition:** To separate proteolytic activity at the base of their molecular weight, we separated proteases using centrifugal filter devices (Centriprep®, Millipore, Burlington, MA, US) of 50 kDa or 100 kDa of the nominal molecular weight limit, according to the manufacturer. The samples recovered from Centriprep® devices were separated by electrophoresis, transferred to a nitrocellulose membrane, and incubated with serum against the *Actinobacillus pleuropneumoniae*-secreted metalloprotease (Negrete-Abascal et al., 1998) or the serum from sheep infected with *A. seminis* (Montes-García et al., 2018).

**Mass spectrometry analysis and phylogenetic analysis of the protease sequence:** A 50-kDa protein band was cut from the gel and analyzed for peptide mass fingerprints as previously described (Figueroa-Valenzuela et al., 2022). The trypptic peptide data were analyzed using Proteome Discoverer 1.4.1.14 (Thermo-Fisher Co). The protease's amino acid sequence was analyzed by BLASTP (https://blast.ncbi.nlm.nih.gov) to know its phylogenetic relationship with other proteases. One hundred seventy-two proteases' sequences were recovered from the GenBank and were aligned using Clustal Omega software (https://www.ebi.ac.uk/Tools/msa/clustalo/). The phylogenetic tree was obtained with the online version of Itol (https://itol.embl.de). Identity groups were indicated with color circles.

**RESULTS**

**Detection of secreted proteases by *A. seminis:**** *Actinobacillus seminis* secreted proteases of 100 kDa or higher molecular weight to the culture supernatant at 37°C in the presence of calcium or at 39 to 40°C in its absence, after 24 h of incubation (Fig. 1A), as can be observed on zymograms with porcine gelatin.

**Protease activity assay:** The proteolytic activities were active mainly at a pH of 6 to 7. At a pH of 8, the proteolytic activity diminished by approximately 50%, and no activity was detected at a pH lower than 4 or higher than 9 (data not shown). Proteases were active in the range of temperatures from 4 to 60°C. Higher temperatures inactivated them (Fig. 1B). Proteolytic activity was not affected by 10 mM p-hydroxymercury benzoate [PHMB], 5 mM phenyl methyl sulphonyl fluoride [PMSF], or 10-40mM ethylenediaminetetraacetic acid [EDTA]) was determined by previous incubation with each inhibitor and their addition to the activation buffer at the same concentrations.

**Degradation of other substrates:** The capability to degrade molecules of physiological importance, 20 µg of secreted proteins were mixed with 20 µg of bovine IgG or fibrinogen and incubated at 37°C overnight. Then, samples were separated by electrophoresis in a 10% SDS-PAGE. Bovine IgG or fibrinogen without secreted proteins were used as negative controls.

**Proteolytic activity quantification:** To know if the differences in proteolytic activity observed in zymograms were significant, we used azocasein as substrate (Sigma-Aldrich, St. Louis, MO, US) as described (Silva et al., 2015) and modified it with a 1% azocasein solution and 100 µg of sample in a final volume of 1 mL with PBS. As positive control, 10µg proteinase K (Sigma-Aldrich) was used. To quantify protease activity, one unit of activity was defined as an increase of 0.01 in absorbance units at 405 nm; results are expressed as specific activity units.
presence of calcium, at 37°C, increased the proteolytic activity in 10% (Fig. 3). However, a diminishing proteolytic activity, at a similar percent, was observed at 39°C.

Electrophoresis separation and immune recognition:

When proteases with proteolytic activities were separated by molecular weight, high molecular weight proteolytic activities were missing, but proteolytic activity was mainly observed at around 100, 75, and 50 kDa (Fig. 4), suggesting that higher molecular weight proteolytic activities are aggregates of this last band. Bands of approximately 50 kDa and higher were immune recognized by a polyclonal serum against a secreted metalloprotease from *A. pleuropneumoniae* and by a pool of serum from sheep infected with *A. seminis*.

Protein identity: The mass spectrometry analysis indicates that this protein presented identity with a carboxy-terminal protease from *A. seminis* (access number AOA263HFT9) (Fig. 5). However, it also presented identity with proteases DegP (access number A0A263HCG5), SobH (access number A0A263HAJ22), and Lon (access number A0A263HCR7), all from the same microorganism. The *A. seminis* protease with access number AOA263HFT9 is...
Homologous to WP_094945459.1 sequence of the GenBank. For this reason, this last sequence was used for the analysis. This sequence presented structural identity with protein structural models 4C2D (CBP protease) and 5WQL (AAA+ tail-protease) in the Protein Data Bank (PDB) (Supplemental Fig. X) reported as monomers or dimers subunits, indicating polymerizing properties. Comparison among A. seminis protease and B. subtilis 4C2D_1_ChainsABC presented a 136 score, with 44% coverage, 33% identity, and E value of 6e-39; comparison with E. coli 5WQL_2_ChainsC exhibited a 676 score with 92% coverage, 54.71% identity, and E value of 0.0. Both proteases, 4C2D and 5WQL, present a common amino acid sequence located in the central domain of the proteins. The phylogenetic relationship of the A. seminis protease clade includes P. maini, P. aerogenes, Necropsobacter massiliensis and Cavibacterium pharyngcola; access numbers of these microorganisms are WP_094945459, MCJ7480587, SUB33706, WP_040976124, WP_100296855.

Supplemental Fig. X: A. seminis protease (WP_094945459.1) structural homology using PDB. Identity of Swj.2.B, a PDZ protease with WP_094945459.1, was of 50.89% with QMEAN global of 0.79 ± 0.05; identity of 4C2D and 4C2E with WP_094945459.1 was of 36.04% with QMEAN global of 0.65 ± 0.05. Overlap of 4C2D and 4C2E 3D structures and Swj.2.B, 4C2D, and 4C2E, showing the multidomain spatial distribution corresponding to alignment of Supplemental figure Z.

Coagulation of blood is a process that prevents the loss of body fluids after an injury. During this event, soluble fibrinogen is transformed into insoluble fibrin (Dubin et al., 2013). Coagulation is not only a physiological process; it is also an important part of the innate immune response that allows higher organisms to contain and kill invading microorganisms. However, diverse microbial proteases can degrade fibrinogen molecules and enable pathogens to escape from these host immune responses. Also, other microorganisms can degrade host fibrinogen and cover themselves with it, creating a pseudo capsule and, thereby, being unchecked by the host's defensive mechanisms (Guggenberger et al., 2012).

A. seminis ram’s infection can be subacute, acute, or chronic and lesions can be purulent or necrotizing with fibrinous inflammation of the tunica vaginalis (Al-Katib et al., 2009; Moustacas et al., 2014).

We describe the expression of proteases secreted by A. seminis that can degrade bovine IgG and fibrinogen. This expression is controlled by temperature, the presence of calcium and iron concentration. Fibrinogen degradation by A. seminis proteases could contribute to the evasion of the
Supplemental Fig. Y: A. seminis protease (WP_094945459.1) analysis with MEROPS. Analysis of protein families with GENE3D, PANTHER, PFAM, PIRSF, PRINTS, PRODOM, PROFILE, PROSITE, SMART, SSF, TIGERFAMs and Unclassified shows possible homologues, in higher identity with Tail-specific proteases and next with carboxy terminal proteases (CTP). Common identity to both kinds of proteases is located among amino acids T238 to Q486 of WP_094945459.1 protease.

Domains in A. seminis protease CtpA-like

Supplemental Fig. Z: Search of domains in WP_094945459.1 sequence and its comparison with three multidomain PDZ type proteins. Alignment showing homologous domains present in CtpA proteases of the super family cl34043 (COG0793, in https://www.ncbi.nlm.nih.gov/Structure/cdd/cddsrv.cgi) present in pathogenic bacteria. Numbers in green indicate the position of amino acids containing the analyzed domains of three Gram-negative bacteria expressing CTP proteases.
host clearance mechanisms, inducing the observed lesions; these processes could also be promoted and enhanced by IgG degradation.

A. seminis proteases were active in a pH range of 6 to 7, and the proteolytic activity diminished considerably at a pH lower than 5 or higher than 8. Proteases secreted by A. pleuropneumoniae and Actinobacillus suis, which are swine pathogens and members of the Pasteurellaceae family, like A. seminis, have proteolytic activity at a wide range of pH, 4 to 8 and 5 to 10, respectively (Negrete-Abascal et al., 1998, 2004), suggesting a complex structure and high stability even with drastic pH changes. Previous reports on A. pleuropneumoniae-secreted proteases have demonstrated their activity in the presence of 0.25 M urea or after heating to 90°C for 5 min (Negrete-Abascal et al., 1998). A. seminis proteases diminish their proteolytic activity in the presence of 0.25 M urea (data not shown) and this activity was not observed at temperatures higher than 60°C.

The limited range of pH and temperature in which A. seminis proteases present activity suggests specific sites or tissues in which they could be acting. It has been described that the ductus epididymis has a pH of 7.2 and the region of sperm storage at the cauda has a pH of 6 (Rodriguez-Martinez et al., 1990). A. seminis secreted proteases were observed in zymograms at 40°C or 37°C in the presence of calcium. The normal temperature of goats and sheep is 38.5 to 40°C and the preputial temperature in bulls is considered to be 37.81°C ± 1.76, with an average pH of 8.45 (Koziol et al., 2017). This information supports why these proteases do not present proteolytic activity in cultures incubated at 37°C without calcium, and probably explains why they activate at higher temperatures.

The proteolytic activity of proteases secreted in A. seminis cultures is favored by temperature, the presence of calcium, and iron availability. The proteolytic activity A. seminis expression at 40°C could be the response of this microorganism to host defensive mechanisms through immunoglobulins when this microorganism is invading its host. Calcium could be a signal that indicates to this microorganism its ubiquation and protease expression could be needed by A. seminis for tissues colonization, evasion of the host defenses or to obtain nutrients when it is colonizing the epididymis. Calcium is present in the epididymis because it participates in spermatozoids maturation and activation of their motility. On the other hand, iron-restricted conditions have been described as a “nutritional immunity” that a host can use as a growth-restricting strategy. However, pathogens can take advantage of it by promoting the expression of different bacterial proteins to overcome this restriction. Bacillus anthracis over-expresses InhA1, a secreted metalloprotease that mediates the proteolysis of human hemoglobin to release iron and amino acids that enhance its growth (Terwilliger et al., 2015). Aeromonas hydrophila presented an increase in the expression of different virulence factors such as protease activity, hemolytic activity, lipase activity, and swimming ability when it is grown in iron-restricted conditions (Teng et al., 2018).

The separation of proteases with proteolytic activities in 75 kDa and 50 kDa by Centriprep® devices suggests that they can aggregate and give rise to proteolytic activities of higher molecular weights similarly to secreted proteases from other Pasteurellaceae members (Negrete-Abascal et al., 1998, 2004). The aggregation capability of the A. seminis protease to form high molecular oligomers could be due to the presence of other molecules, such as LPS. Association of LPS with a Mannheimia hemolytica leukotoxin allows this protein to associate with the membrane and form high molecular weight masses oligomers (Li and Chickenbeard, 1999). The presence of LPS could also explain how this protein resists denaturalization by temperature or urea. If this is the case in the A. seminis protease, it could be possible that its separation by Centriprep® devices removes LPS because the A. seminis protease is inactivated by temperature or urea. The possibility of the A. seminis protease to form higher molecular masses oligomers is supported by the immune recognition of bands of 50, 75 kDa, or higher molecular weight with polyclonal serum against the A. pleuropneumoniae secreted metalloprotease.

The identity of this A. seminis protease as carboxy-terminal protease (CTP) is not conclusive, due to the sharing homology with other proteases and that those are mainly serine proteases; the A. seminis protease identified here was identified as a metalloprotease. This conclusion will need more work; however, CTPs have been implicated in diverse processes such as regulation, stress, and virulence (Sommerfield and Darwin, 2022).

Proteases identified as related to the A. seminis protease were evaluated by sequence similarity according to the protease’s characteristics indicated in the MEROPS database that considers structural characteristics GEN3D, PANTHER, PFAM, PIRSF, PRINTS, PRODOM, PROFILE, PROSITE, SMART, SSF, TIGERFAMS, and Unclassified. Higher identity relates this A. seminis protease with AAA+ type proteases and next with carboxypeptidases. Structural search in the PDB indicates identity values of 50% with QMEAN Global of 0.79 ± 0.05 and 0.65 ± 0.05 with PDZ-protease, and Pre-protease, respectively, suggesting that the A. seminis protease is a PDZ protease or multidomain protein (Supplemental Fig. Z). These proteases form oligomers to process efficiently their substrates and can be compartmentalized in any of the cellular membranes. They seem to contain AAA+ domains necessary to use ATP to act on their substrates. Ex situ activity (on zymograms) suggests a dual effect; to function as an ATP-dependent protease in cells, processing the proteins needed for cell function and as an ATP-independent protease in direct contact with a partially degraded substrate (gelatin) with an unspecific effect.

The expression of proteases secreted by A. seminis that can degrade bovine IgG and fibrinogen is controlled by temperature, the presence of calcium, and the iron concentration. The latter could make these proteases a relevant virulence factor that can act in different adverse situations in response to the host immune system and explain part of the A. seminis pathogenesis.

Funding: This study was funded by a grant from UNAM, DGAPA-PAPIIT IN204122.

Authors contribution: CVC and ENA designed the experiments, AHDCM, GARPP, and FMG performed the experiments. AHDCM, ENA, and CVC wrote the draft of the article. AHDCM, ENA, FMG, and CVC revised this last version of the manuscript.
Availability of data and materials: In this study, all data generated or analyzed are included in the article. Code Availability. Not applicable.

Declarations: Conflict of interest. The authors declare that there is no conflict of interest.

REFERENCES


