The PlySs9 endolysin and its amidase subdomain reveal potential roles in the treatment of Gram-positive bovine mastitis

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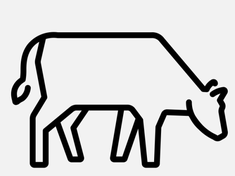
Gram-positive bovine mastitis.

Picture: Van Hoof M., Eksel, Belgium.

Introduction & Aim

Bovine mastitis, an infection of the cow's udder, causes major economic losses in the global dairy industry and remains one of the main reasons for antibiotic use in dairy cattle. The most prevalent Gram-positive causative agents of this disease are *Streptococcus uberis* (*S. uberis*) and *Staphylococcus aureus* (*S. aureus*). Bacteriophage-derived endolysins, such as LysK, B30 and λSA2 [1-2], are each known to lyse at least one of these pathogens both *in vitro* and in preclinical models. These endolysins typically consist of a cell wall-binding domain (CBD) and one or more enzymatically active domains (EADs), which allow them to either bind or cleave the bacterial peptidoglycan, respectively. Their known working spectrum can nevertheless be improved and/or expanded by engineering chimeric endolysins using platforms such as VersaTile shuffling [3] or non-covalent Barnase-Barstar fusion [4]. From this perspective, the in-depth functional characterization of different CBDs and EADs is mandatory before creating novel chimeric endolysins. Therefore, we here characterized the LysM-based CBD and N-terminal amidase EAD of PlySs9, an endolysin derived from a *Streptococcus suis* (*S. suis*) bacteriophage that also displays lytic activity against bovine *S. uberis* and *S. aureus*.



± $200 / Cow / Year [5]

Veterinary Treatment

Production Losses

Use of antibiotics and emerging resistance

Methods

Gene Cloning: The amidase EAD and CBD subdomains were amplified from the PlySs9 genome (the latter artificially synthesized and codon-optimised) through polymerase chain reactions (PCR) and Gibson assembled in a pBAD24 vector. After transformation in competent *E. coli* cells, colonies were picked from selective plates and PCR-screened for presence of the gene. Positive clones were subsequently sequenced. **Protein expression:** Transformed *E. coli BL21* were grown in 1,5L of selective Luria-Bertani broth until the OD_{600nm} reached 0,8 – 1,0. These cultures were subsequently induced with 0.25% L-arabinose and shaken overnight (18°C, 150 rotations per minute (RPM)). **Protein purification:** *E. coli BL21* were pelleted at 4000 RPM and resuspended in phosphate buffered saline (PBS) with 10 mM imidazole and 1 mM PMSF. Thereafter, cells were sonicated on ice for 15 minutes. Insoluble cellular debris was removed through centrifugation for 45 minutes at 13000 RPM and 4°C. His-tag/nickel chromatography further purified the protein from the centrifuged supernatant. Eluted fractions that contained the envisaged protein were combined and dialyzed for 4 hours at 4°C in 5,0L PBS to remove residual imidazole. The PlySs9 CBD needed an additional purification over an S-200 gel filtration column. Purified proteins were concentrated and stored in 20% glycerol at -80°C. **Turbidity reduction assays:** An overnight culture of each pathogen was washed twice and diluted with PBS until the desired optical density was reached. The assay was performed in a 96-well plate at 37°C with both 100µL of purified protein and pathogen. **Spot-on-plate assay:** Overnight cultures were washed with PBS and dissolved in 10mL of 0.7% liquid agarose. After solidification, 10µL was spotted on top of the agar and incubated overnight at 37°C. **Zymogram:** Overnight cultures were washed, dissolved in 2mL distilled water and embedded in an SDS-PAGE gel. Proteins were boiled for 2 minutes in an SDS buffer, ran on the gel, refolded with 5% Triton X-100, incubated in phosphate buffer at 37°C and stained with 0.1% crystalviolet. **Viable counts:** Overnight cultures were washed, resuspended in PBS and incubated for 2 hours with an equal volume of purified protein. Serial dilutions were made and 10µL was subsequently spotted on nutrient agar. **AlexaFluor 555 labeling:** Performed according to the manufacturer's protocol.

Results

1. Lytic activity of the PlySs9 parental endolysin

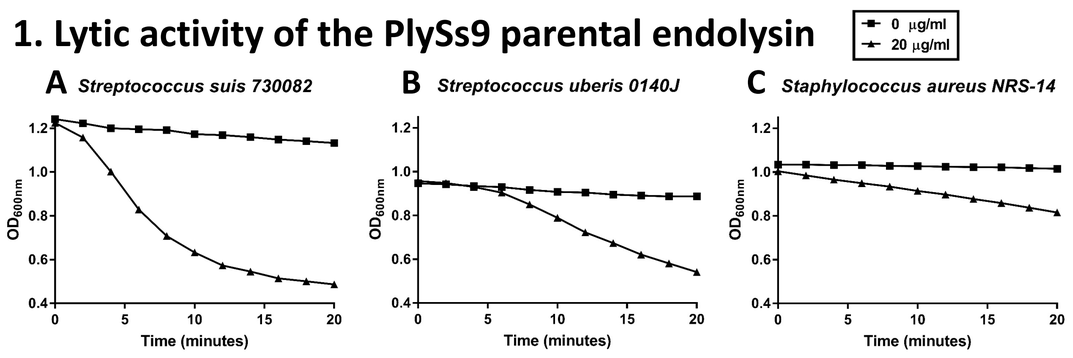


Figure 1. Turbidity reduction assays with the PlySs9 parental endolysin.

Optical density measured at 600nm during 20 minutes at 37°C with 0 and 20 µg/ml concentrations against (A) *S. suis 730082*, (B) *S. uberis 0140J* and (C) *S. aureus NRS-14.*

2. Lytic activity of the PlySs9 amidase EAD subdomain

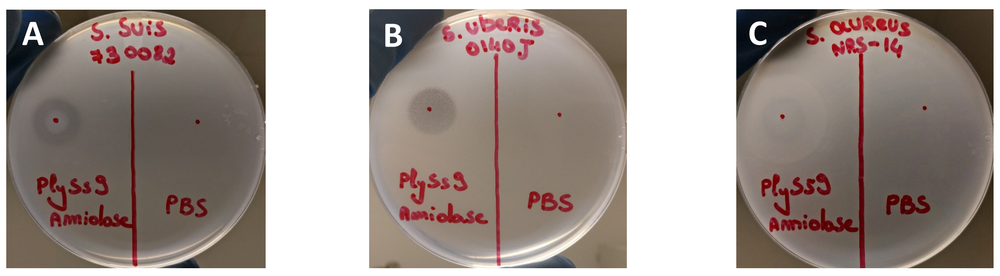


Figure 2. Lysis observed in a spot-on-plate assay with 3.0 mg/ml PlySs9 EAD amidase versus phoshate buffered saline (PBS) as a negative control after overnight incubation at 37°C.

(A) *S. suis 730082,* (B) *S. uberis 0140J* and (C) *S. aureus NRS-14.*

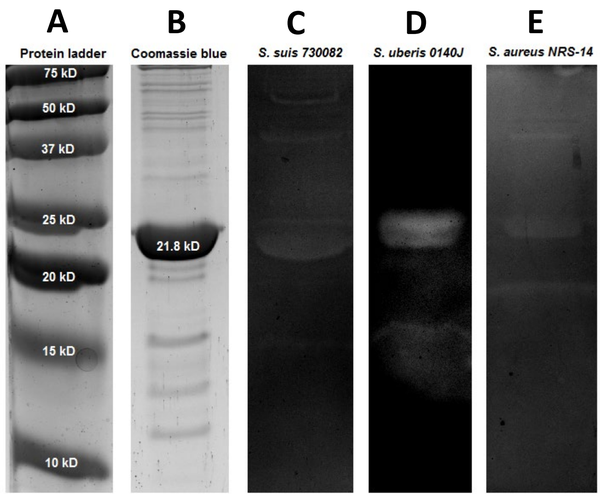


Figure 3. Lysis observed in a zymogram assay with 1.0 mg/ml PlySs9 EAD amidase.

(A) Prestained protein ladder.
(B) Coomassie blue staining of purified PlySs9 EAD Amidase.
(C) Zymogram *S. suis 730082.*
(D) Zymogram *S. uberis 0140J.*
(E) Zymogram *S. aureus NRS-14.*

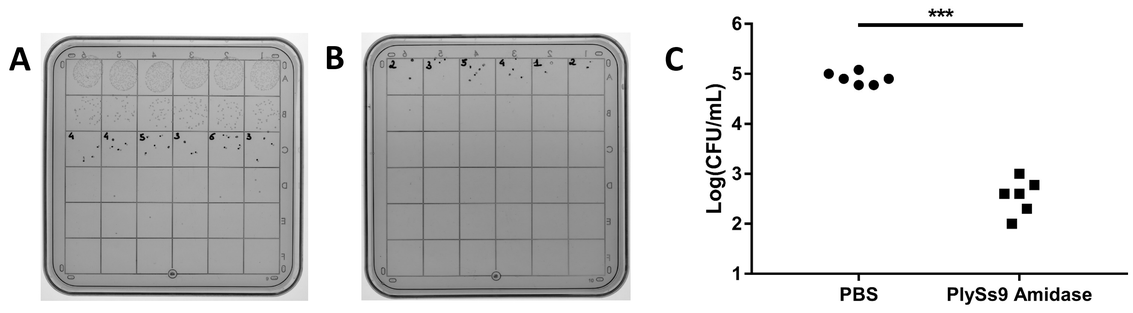


Figure 4. Plate count of *S. suis 730082* after a 2 hour incubation with either PBS or PlySs9 EAD amidase.

(A) Colonies counted in the 10^{-2} dilution of the PBS treated group.
(B) Idem Fig. 4A for the 10^{0} dilution of the PlySs9 EAD amidase treated group.
(C) Logaritmic killing of *S. suis 730082* by PlySs9 EAD amidase.
*** indicates P<0.001

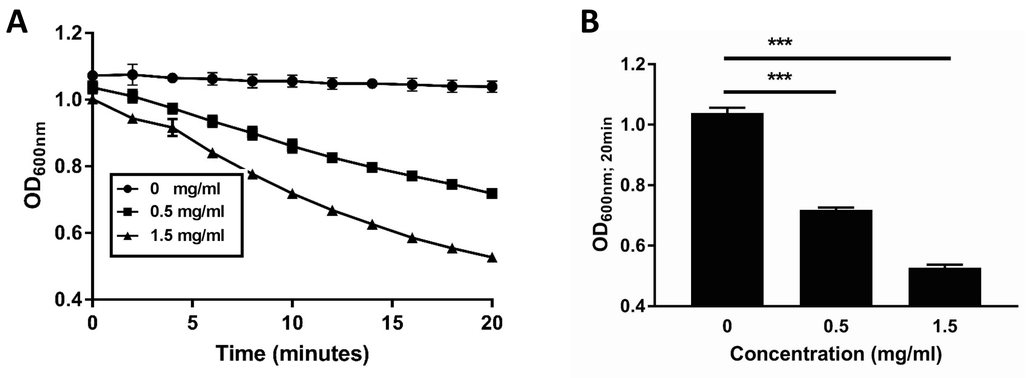


Figure 5. Turbidity reduction assay with PlySs9 EAD amidase on *S. suis 730082*.

(A) Optical density measured at 600nm during 20 minutes at 37°C with 0, 0.5 and 1.5 mg/ml concentrations.
(B) Difference in optical density after 20 minutes at 37°C with the same concentrations as in Fig. 5A.
*** indicates P<0.001

3. Binding of the AlexaFluor 555 labeled PlySs9 CBD subdomain

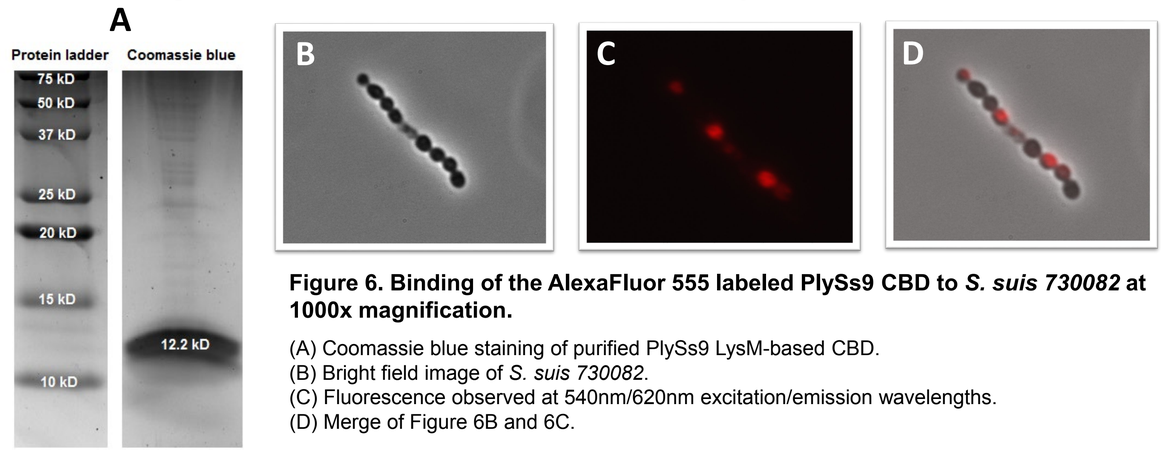


Figure 6. Binding of the AlexaFluor 555 labeled PlySs9 CBD to *S. suis 730082* at 1000x magnification.

(A) Coomassie blue staining of purified PlySs9 LysM-based CBD.
(B) Bright field image of *S. suis 730082*.
(C) Fluorescence observed at 540nm/620nm excitation/emission wavelengths.
(D) Merge of Figure 6B and 6C.

Conclusions

- The parental **PlySs9 endolysin** and its **amidase EAD subdomain expose lytic activity against *Streptococcus suis* and two major Gram-positive bovine mastitis pathogens**: *Streptococcus uberis and Staphylococcus aureus.*
- The **PlySs9 LysM-based CBD subdomain** is able to **bind *Streptococcus suis***. Binding to relevant mastitis-causing pathogens requires further in-depth analysis.

References

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