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Assessment of the potential utility of different regions of *Streptococcus uberis* adhesion molecule (SUAM) for mastitis subunit vaccine development

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ABSTRACT

Streptococcus uberis is one of the most prevalent pathogens causing clinical and subclinical mastitis worldwide. Among bacterial factors involved in intramammary infections caused by this organism, *S. uberis* adhesion molecule (SUAM) is one of the main virulence factors identified. This molecule is involved in *S. uberis* internalization to mammary epithelial cells through lactoferrin (Lf) binding. The objective of this study was to evaluate SUAM properties as a potential subunit vaccine component for prevention of *S. uberis* mastitis. B epitope prediction analysis of SUAM sequence was used to identify potentially immunogenic regions. Since these regions were detected all along the gene, this criterion did not allow selecting a specific region as a potential immunogen. Hence, four fractions of SUAM (-1fr, 2fr, 3fr and 4fr), comprising most of the protein, were cloned and expressed. Every fraction elicited a humoral immune response in mice as predicted by bioinformatics analysis. SUAM-1fr generated antibodies with the highest recognition ability towards SUAM native protein. Moreover, antibodies against SUAM-1fr produced the highest proportion of internalization inhibition of *S. uberis* to mammary epithelial cells. In conclusion, SUAM immunogenic and functionally relevant regions were identified and allowed to propose SUAM-1fr as a potential candidate for a subunit vaccine for *S. uberis* mastitis prevention.

1. Introduction

Bovine mastitis is the most significant limiting factor for profitable dairying worldwide. Total costs of mastitis in Argentina, including direct production losses and disease control expenses, have recently been estimated to be ≥ 1 US$/milking cow/day [1]. In addition to decreased milk production and quality, extensive antibiotic usage for treatment and prevention of intramammary infections (IMI) is a serious concern for dairy industry and public health [2].

Several streptococcal species, including *Streptococcus uberis*, are capable of causing IMI. This organism, ubiquitous in the cow's environment, causes IMI both during lactation and the nonlactating period and accounts for the majority of environmental streptococcal mastitis cases in heifers within the first week of lactation [3]. Continuous mammary gland exposure to *S. uberis* and poor control of IMI caused by this organism through recommended control procedures, has led to a search for enhancing cow's resistance to infection through vaccination [4].

To develop an effective vaccine for the control of *S. uberis* mastitis, knowledge of its virulence factors and their capability to generate a protective immune response is a critical step. Several virulence factors that contribute to *S. uberis* pathogenesis have been described. Among these, *S. uberis* adhesion molecule (SUAM) is involved in bacterial adherence, internalization and persistence in bovine mammary epithelial cells [5]. The extraordinary diversity of *S. uberis* strains [3] has also been a great difficulty in designing a

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vaccine that could confer protection against field strains [6]. Recent research conducted at our laboratory determined that 97.8% of *S. uberis* isolated from bovine IMI in Argentina harbored the *sua* gene [7]. In addition, we found that SUAM nucleotide and amino acid sequences showed an identity between 95% and 100% with respect to all reference sequences registered in GenBank [7]. The high prevalence of SUAM among *S. uberis* isolated from bovine IMI as well as its conservation among isolates makes it an attractive candidate for development of a subunit recombinant vaccine for mastitis prevention. However, production of recombinant proteins with coding sequences >1000 bp poses technical difficulties [8]. Considering that SUAM molecular weight is 112 KDa with a nucleotide sequence of approximately 2700 bp, the objectives of this study were to examine by in silico and experimental criteria SUAM regions rich in B epitopes in order to adjust the recombinant protein fractions size containing these regions, to produce selected molecule fractions and to characterize their immunogenicity.

2. Methods

2.1. Bacterial strains and growth conditions

Two strains of *S. uberis* designated SU05 and SU42 isolated from cows with mastitis were used. These strains were characterized previously in our laboratory [7]. These strains are not epidemiologically related and were isolated from different types of mastitis, SU42 from subclinical mastitis and SU05 clinical mastitis [7]. The isolates were initially identified using standard conventional biochemical tests [9] and further confirmed using restriction fragment length polymorphism analysis of 16S rDNA as previously described [10]. Streptococcal stocks were stored at −80 °C in Todd−Hewitt broth (THB) (Sigma-Aldrich Co., St. Louis, MO) with 20% glycerol (Promega, Madison, USA) until further use. *Escherichia coli* BL21 (DE3) strain (Novagen, Madison, WI, USA) was grown in Luria-Bertani (LB) medium (Britania) supplemented with ampicillin (Amp) (100 μg/mL) (Sigma Chemical Co., St.Louis, USA) as needed for plasmid maintenance.

2.2. Epitope prediction

The amino acid sequence of SUAM from *S. uberis* UT888 (GenBank accession number, ABB52003.1), a strain originally isolated from a cow with chronic mastitis, was used for all analyses [11,12]. The epitope prediction study was carried out using two online prediction methods, ABCpred and AAPPred. The first (http://www.imtech.res.in/raghava/abcpred/), allows to predict B epitopes, of defined length (10, 12, 14, 16° 20 aa) and the antigenicity analysis is based on an artificial neural network, using a database of known epitopes [13]. In this work a length defined between 10 and 20 aa was used. The second method, AAPPred (http://www.bioinf.ru/aappred/), determines the antigenicity of amino acid pairs (AAP), evaluating the frequency at which they appear next to a scale of hydrophobicity propensity, flexibility, accessibility within a protein and polarity [14].

2.3. Design, expression and purification of recombinant proteins

To obtain the recombinant SUAM fractions, five pairs of primers were designed (Table 1) using the Primer Select (DNAstart®) software. This software selects primers with the highest score, resulting in some cases the superposition of the template sequences. The oligonucleotide sequences included sites for restriction enzymes *Eco*RI and *Hind*III. PCRs were performed, from SU05 of *S. uberis* strain bacterial genome, by running 35 cycles with a temperature profile of 1 min at 94 °C, 1 min at 57 °C, and 1 min at 68 °C. The purified PCR products were digested with *Eco*RI and *Hind*III, and ligated into the pET-32a plasmid (Novagen, Madison, WI, USA) at the corresponding restriction sites. The resultant recombinant vectors were referred to as SUAM-1fr/pET-32a, SUAM-2fr/pET-32a, SUAM-3fr/pET-32a, SUAM-4fr/pET-32a and SUAM-5fr/pET-32a, respectively for each fraction. The identity of the cloned fragments was confirmed by sequencing, yielding a percentage higher than 98% homology to the sequence available in GenBank DQ232760.1. The pET-32a constructions were transformed into the *E. coli* strain BL21 (DE3) for protein expression. *E. coli* BL21 (DE3) cells were transformed with the different construction plasmids and grown in LB medium supplemented with Amp at 37 °C. When the culture optical density $(OD)_{600}$ reached 0.6, protein expression was initiated by adding isopropyl-β-D-thiogalactopyranoside (IPTG) (0.5 mM) (Promega). After additional 3 h cultivation, cells were harvested by centrifugation, resuspended in buffer (50 mM Na_2HPO4 pH 8, 300 mM NaCl, 10 mM Imidazol, 1 mM phenyl methyl sulfonyl fluoride) and sonicated with 50% pulses for 2 min at 600 W. The whole cell lysate was then centrifuged at 13,000 rpm for 10 min to separate soluble and insoluble portions and the supernatant was recovered. Proteins were purified from supernatant with a nickel pseudo-affinity IDA-Sepharose column (Novagen, Madison, WI, USA). Purity and concentration of the proteins were evaluated with 15% polyacrylamide gel electrophoresis under denaturing conditions (SDS−PAGE) [15] and subsequent Coomassie Brilliant Blue (Sigma) staining [16]. Quantification was performed by densitometry using Qubit (Invitrogen®) fluorometer.

2.4. Native SUAM extraction

Native SUAM (SUAM*wt*) was extracted as described previously [5]. Briefly, SU42 was grown in THB overnight at 37 °C, washed with sterile PBS (pH 7.4) and resuspended in 0.2% sodium dodecyl sulfate (Bio-Rad, Hercules, CA, USA) for 1 h at 37 °C with intermittent mixing. After incubation, bacterial suspensions were aliquoted, microfuged for 5 min and stored at −70 °C.

2.5. Production of SUAMwt, SUAM-1fr, SUAM-2fr, SUAM-3fr and SUAM-4fr antibodies

For production of antibodies against SUAM*wt* and recombinant fractions, 6 weeks-old CF-1 female mice were immunized by intraperitoneal route. Groups of 6 mice were inoculated with 0.2 mg of the corresponding purified recombinant SUAM fraction and SUAM*wt* using complete Freund's adjuvant for the initial injection and incomplete Freund's adjuvant for the following doses. After the first doses, mice were boosted two times every two weeks. Control group was inoculated with PBS. Serum samples were obtained before initial inoculation and one week after each additional injection. Serum was separated by centrifugation and stored at −20 °C. All procedures used in this study were approved by the Institutional Ethic Committee of the School of Biochemistry and Biological Sciences (Universidad Nacional del Litoral) being performed according to the recommendations of the Guide for the Care and Use of Laboratory Animals [17].

2.6. Measurement of antibodies

Antibody levels (total IgG) in all serum samples, were measured by indirect ELISA. Briefly, 96-well polystyrene EIA microtiter flat bottom plates (Greiner Bio-One, Frickenhausen, Germany) were coated with the corresponding purified protein at 0.5 μg/well. Purified proteins were diluted in carbonate/bicarbonate buffer at pH 9.6. Plates were blocked for 1 h with PBS-5% skimmed milk. Mice sera were diluted in PBS-1% skimmed milk and assayed in triplicate.