Characterization of Sperm Agglutinating Factor Isolated from *Staphylococcus Aureus* and its Corresponding Receptor from Spermatozoa

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Abstract

Spermagglutination factor isolated from *Staphylococcus aureus* showed spermagglutinating and spermicidal properties in vitro. In an attempt to characterize SAF, it was tested for the various factors produced by *S. aureus* viz. catalase, coagulase, hemolysins, protease, lipase, phospholipase C, nuclease and protein A. As, SAF did not show any of these activities, therefore to identify it, peptide mass fingerprinting using Matrix Assisted Laser Desorption Ionization-Time of flight (MALDI-TOF) was done. Matching of the mass spectrum in NCBI database showed sequence homology with hypothetical protein BACPEC_00178 of *Bacteroides pectinophilus* ATCC 43243. SAF conjugated with FITC was able to bind to spermatozoa indicating the presence of receptor on sperm surface. Calorimetric analysis of SAF and this purified receptor interaction showed the binding constant, enthalpy of binding ΔH° as 1130/M and -11.6kJ/mole while the values of free energy and entropy were -18.1kJ/mole and 20.9J/moleK, respectively. The receptor showed sequence similarities with chain A crystal structure of Ga module complexed with human serum albumin when subjected to MALDI-TOF.

Keywords: *Staphylococcus aureus*, spermagglutinating factor, calorimetry, MALDI-TOF

Staphylococcus aureus is known to elaborate a plethora of toxins and enzymes. Nearly all strains secrete a group of exoproteins such as nucleases, proteases, lipases, hyaluronidase, and collagenase as well as several cytolytic toxins including α , β and γ -hemolysin, leukocidin, Panton-Valentine leukocidin (PVL), toxic shock syndrome toxin-1 (TSST-1), the staphylococcal enterotoxins and the exfoliative toxins (Dinges *et al.*, 2000; Kaneko and Kamio 2004; Lina *et al.*, 2004; Holtfreter and Bröker, 2005). The attachment of *S. aureus* to the host cell surface initiating the colonization process is mediated by several adhesins comprising proteins covalently anchored to cell peptidoglycan which specifically attach to the plasma or extracellular matrix (ECM) components and collectively are termed the microbial surface component recognizing adhesive matrix molecules (MSCRAMMs). These include staphylococcal protein A (SpA), fibronectin-binding proteins A and B (FnbpA and FnbpB), collagen-binding protein, and clumping factor (Clf) A and B proteins (Speziale *et al.*, 2009; Marraffini *et al.*, 2006; Maresso and Schneewind, 2008). All these proteins facilitate in the pathogenesis of *S. aureus*. In earlier work carried out in laboratory, we isolated a strain of *S. aureus* from the cervix of a woman suffering from unexplained infertility. This strain showed spermagglutinating property and could also adhere to human spermatozoa in vitro (Ohri and Prabha, 2006). Further studies led to isolation and purification of a spermagglutinating factor from this strain which could inflict morphological damages on human spermatozoa and exhibited spermicidal property in vitro (Ohri and Prabha, 2006; Kaur *et al.*, 2010). The corresponding receptor was isolated from human spermatozoa and purified using salt extraction and column chromatography. This receptor could efficiently block SAF mediated changes on spermatozoa. Though both SAF and receptor had been isolated and purified, but their identity remained elusive. Therefore, the present study was carried out with an aim to characterize both these proteins.

Semen samples were obtained from normozoospermic males undergoing evaluation of fertility by masturbation into sterile wide-mouth containers, following a 24h abstinence period. The ejaculates were collected from Government Multispeciality Hospital, Sector 16 Chandigarh, India, after informed consent. Only ejaculates showing normal semen parameters according to World Health Organization (2010) criteria were used. Samples were allowed to liquefy at room temperature for 30min.

Spermagglutinating factor (SAF) used in the present work was isolated from spermagglutinating isolate of *S. aureus* and this factor also showed this spermagglutination and spermicidal property. It had been earlier suggested that the negative effect of *S. aureus* on sperm motility may be a result of various toxins and enzymes produced by it, however this is a subject of further investigation (Jiang and Lu, 1996). Therefore, SAF was checked for various activities such as catalase, coagulase, hemolysin, lipase, phospholipase c, protease, deoxyribonuclease and protein A, so as to ascertain its nature.

For detection of catalase activity, SAF was mixed with 3% (v/v) H₂O₂ on a clean

glass slide and observed for effervescence. Positive control comprised of catalase positive bacterial culture. The absence of effervescence indicated the lack of catalase activity of SAF. Most *S. aureus* strains form both free and bound coagulase and a few strains form only one or the other kind. Therefore, both tube and slide coagulase tests were performed in order to check for the free and bound coagulase activity respectively. For tube coagulase, two fold dilutions of purified SAF were prepared in PBS (20mM, pH 7.2) in 3ml test tubes. To each tube, 500 μ l of (1:1000 diluted) human plasma was added. The tubes were incubated in water bath at 37°C and observed at hourly intervals for 3h for presence of clot formation. In case of slide coagulase test, a drop of SAF was mixed with a drop of undiluted human plasma on a clean glass slide and observed for the appearance of coarse clumping within 5-10s. Positive control was made by mixing a coagulase positive culture with plasma. The absence of clumping within 10s was read as negative result. The results showed that SAF did not exhibit either slide or tube coagulase activity.

SAF was also checked for α , β and δ hemolysin activity for which rabbit, sheep and human sRBCs were used, respectively. The blood samples from rabbit, sheep and human (10ml each) were collected in sterile flasks containing Alsever's anticoagulant. The samples were centrifuged and a 2% RBC suspension was prepared for each. Two fold serial dilutions of purified SAF were prepared in PBS in a microtitre plate. To all the dilutions equal volume of 2% suspension of either rabbit, sheep or human RBCs was added. The plate was incubated at 37°C for 2h and hemolysin activity was determined by the presence of lysed RBCs while the presence of button formation was regarded as negative. Controls consisted of RBCs incubated with PBS without SAF. The presence of button formation in all the cases showed that SAF lacked α , β and δ hemolysin activity. Lipase, phospholipase C, protease and deoxyribonuclease (DNase) activities were determined by respective plate assays on 1% tributyrin, 10% egg yolk, 5% skimmed milk and DNase agar. Steel wells of 8mm diameter were placed onto these plates. The wells were filled with SAF (100 μ l) and incubated for 24-48h at 37°C. As control, purified lipase, protease, lecithinase and DNase were used. The absence of zone of clearance around the wells containing SAF on tributyrin, skimmed milk and DNase test agar indicated that SAF lacks lipase (Figure 1a), protease (Figure 1b) and DNase (Figure 1d) activities, respectively and the absence of zone of opalescence on egg yolk agar indicated that SAF also lacked phospholipase C activity (Figure 1c). Protein A activity was checked using tube agglutination test with sheep RBCs (SRBC) (Maxim *et al.*, 1976). Sheep erythrocytes were obtained in sterile flasks containing Alsevers solution. A 1% suspension was prepared in PBS and sensitized with rabbit anti-SRBC hemolysin. The cells were washed with PBS and adjusted as 2% suspension. 50 μ l of SAF was incubated with 50 μ l of sensitized SRBC. In parallel, SRBC with PBS alone and SRBC incubated with purified protein A were set up as negative and positive controls, respectively. The results were read from 2-5h after addition of SRBCs. Absence of hemagglutination in wells containing SAF indicated lack of protein A activity.

These results in general showed that SAF did not possess catalase, coagulase, hemolysin, lipase, phospholipase C, protease, deoxyribonuclease and protein A

activity. As, SAF lacked any of these activities therefore it was subjected to peptide mass fingerprinting by Matrix Assisted Laser Desorption and Ionization-Time of Flight (MALDI-TOF) to ascertain the identity of the protein. The services for MALDI-TOF were outsourced to The Centre for Genomic Applications (TCGA), New Delhi, India. For this, the purified SAF was run on a 10% Polyacrylamide gel and stained with Coomassie Brilliant Blue. Entire gel was rinsed in ultrapure water for 1-2h. The gel piece containing protein band was excised further to 1-1.5mm cubes and transferred to 1.5ml microfuge tubes. Gel pieces were washed once with 100 μ l HPLC grade water for 10min followed by destaining using 50% 100mM ammonium bicarbonate in 50% acetonitrile (ACN) at 37°C for 30min. Dehydration was done using ACN followed by reduction and alkylation using 10mM of dithiothritol/100mM of ammonium bicarbonate and 50mM iodoacetamide/100mM ammonium bicarbonate, respectively. The gel piece was washed again with destaining solution followed by dehydration. Gel pieces were rehydrated in ~5 μ l trypsin solution (20ng/ μ l) and incubated at 37°C for 16h. Peptide extraction solution (1% Trifluoroacetic acid) was added to extract the peptides which were used for subsequent MS analysis. The resulting spectrum was searched for matching proteins in the NCBI database using the MASCOT search engine (Matrix Science). SAF showed sequence homology to hypothetical protein BACPEC_00178 of *Bacteroides pectinophilus* ATCC 43243 with a score of 82.7 (protein scores greater than 61 are significant; $P < 0.05$) (S1).

SAF (1mg/ml) was conjugated with Fluorescein isothiocyanate (FITC) using a FITC Protein Labeling Kit (Bangalore Genei Pvt. Ltd., India) and incubated with spermatozoa to observe binding of SAF. Fluorescence microscopy showed that SAF could bind to complete spermatozoa as observed by uniform fluorescence on spermatozoa (Figure 2). This pointed towards the presence of receptor on the surface. The corresponding receptor has already been isolated and purified in our laboratory and the receptor could block SAF induced changes on spermatozoa [9]. This pointed towards a receptor-ligand (SAF) interaction between the two purified proteins which was evaluated in the present study using micro reaction calorimeter in isothermal titration mode. Microcalorimetry is a label-free method that is useful in the characterization of biomolecular interactions of membrane proteins (Salemme *et al.*, 1997). The enthalpy of binding of SAF with the receptor was determined in PBS using a microreaction calorimeter. Two experimental vials (reference and test) filled with 1.5ml of receptor dissolved in PBS were placed in each of the calorimetric blocks maintained at 37°C. A 250 μ l syringe was loaded with the buffer in case of reference and with SAF in case of test. The experiment was conducted using titration mode of addition of SAF solution. An initial period of 200s was allowed for baseline measurement with an injection interval of 800s. The binding constant, K and enthalpy of binding ΔH_0 were computed by using iterative non-linear least square regression method. The values for Gibbs free energy and entropy were calculated from the equations: $\Delta G^\circ = -RT \ln K$ and $\Delta S^\circ = (\Delta H^\circ - \Delta G^\circ)/\Delta T$.

When two components bind, heat is either generated or absorbed. Measurement of this heat allows accurate determination of binding constants (K_b), reaction stoichiometry (n), enthalpy (ΔH) and entropy (ΔS) (Heerklotz 2004; Wiseman *et al.*,

1989; Leavitt and Freire, 2001; Weber *et al.*, 2003; Ladbury 2004). Calorimetric analysis of SAF-receptor binding indicated the specificity of the interaction as the binding was enthalpically (-11.4kJ mole⁻¹) as well as entropically (20.9J/moleK) favoured resulting in the Gibb's free energy of -18.1kJ/mole (Table 1). Further, characterization of receptor using MALDI-TOF showed its sequence similarities with chain A crystal structure of Ga module, complexed with human serum albumin from *Homo sapiens* as determined by matching peptide mass fingerprints in NCBI database with a score of 247 (protein scores greater than 61 are significant; $P < 0.05$) (S2).

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Figure 1: Evaluation of SAF for various activities (A) Lipase, (B) Protease, (C) Phospholipase C, and (D) Deoxyribonuclease. Arrows indicate positive controls.

Figure 2: Fluorescein isothiocyanate (FITC)-sperm agglutinating factor (SAF)-labeled spermatozoa. Spermatozoa were prepared as described in the text and were allowed to dry on slides. The slides were examined at X1000 magnification under a fluorescent microscope.

Table

Table 1: Calorimetric assessment of heat released during receptor-ligand (SAF) binding.

$\mu\text{moles of SAF}$	$\mu\text{moles of receptor}$	Enthalpy (mJ)
2.31×10^{-4}	1.071×10^{-3}	83.25
4.61×10^{-4}	1.071×10^{-3}	83.15
6.92×10^{-4}	1.071×10^{-3}	81.15
9.23×10^{-4}	1.071×10^{-3}	80.95
3.81×10^{-4}	1.071×10^{-3}	76.25
7.69×10^{-4}	1.071×10^{-3}	73.65
11.53×10^{-4}	1.071×10^{-3}	63.25

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