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Cloning, expression, purification and ligand binding studies of novel fibrinogen-binding protein FbsB of *Streptococcus agalactiae*

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ABSTRACT

Fibrinogen (Fg) is often a common site for bacterial recognition. In *Streptococcus agalactiae*, two surface proteins that recognize Fg are FbsA and FbsB. FbsA and the N-terminal region of FbsB have been shown to bind to human Fg, while the C-terminal region of FbsB [FbsB(C)] has been speculated to bind to bovine Fg. This C-terminal region which is conserved in many of the *S. agalactiae* strains was tested for binding to bovine Fg. For this, FbsB(C) was cloned, expressed and purified. Dot blot, Western blot and ELISA experiments carried out with the purified protein showed that FbsB(C) has the ability to bind to bovine Fg. It was also observed that other than binding to the native form of Fg, FbsB(C) also has the ability to bind to the Fg subunits when reduced. On studying the influence of Ca^{2+} on the FbsB(C)–bovine Fg binding it was observed that the addition of Ca^{2+} in the assay experiment greatly stimulated the binding. When the primary structure of FbsB(C) was analyzed, it was seen that other than similarities with strains of the same organism, it does not have any similarity with any protein characterized so far. In addition to this, its secondary structure component analysis by circular dichroism revealed that it is composed mainly of alpha helices and random coils unlike other Fg-binding surface proteins where beta sheets are dominant. FbsB(C) indeed is a novel protein and understanding the mechanism of its interaction with Fg would be useful in developing strategies to fight against infections by *Streptococcus*.

Introduction

With the emerging bacterial strains resistant to antimicrobial drugs, a major aim in the current field of medicine is to develop strategies to fight against bacterial infections. Attempts are therefore being made to study the diverse population of bacteria that cause diseases as a result of which genome sequencing and protein characterization for a number of bacteria have been done. One among them is of a Group B Streptococcus (GBS), *Streptococcus agalactiae*. This Gram-positive cocci is a commensal organism which is best known for postpartum infection and neonatal sepsis [1]. Common manifestations of it are urinary tract infections, pneumonia, skin and soft tissue infections, septic arthritis, osteomilitis, meningitis, peritonitis and endo-opthalmitis [1,2]. In addition, it also colonizes the mammary glands of ruminants causing mastitis leading to major financial losses in the dairy industry [1,3]. Pathogenesis in Gram-positive bacteria is normally initiated by the adherence of the bacteria to the host via its surface adhesins. A subfamily of adhesins known as MSCRAMMs (*M*icrobial *S*urface *C*omponent *R*ecognizing *A*dhesive *M*atrix *M*olecules) binds specifically to extracellular matrix molecules (ECM) like fibrinogen, fibronectin, collagen, laminin etc. [4]. The MSCRAMM–ECM interaction is one of the good targets for developing new therapeutic agents.

Fibrinogen (Fg)[1], an important component of the blood plasma, is often a site of binding among Gram-positive bacteria. Among the well characterized Fg-binding adhesins from Gram-positive bacteria are the ClfA and ClfB from *Staphylococcus aureus* [5,6], Fbe from *Staphylococcus epidermidis* [7] and FgBP from *Streptococcus equi* subsp. *equi*, a Group C Streptococcus [8]. In *S. agalactiae*, two surface proteins that bind to Fg have been characterized. They are Fg-binding surface protein A (FbsA) [9] and Fg-binding surface protein B (FbsB) [10]. FbsA has the LPXTG motif, a specific signal for cell wall anchoring, which is generally seen in the structural organization of MSCRAMMs, however, FbsB is devoid of such a motif [10,11] suggesting that it is a secreted protein rather than a covalently surface-exposed protein. Although both FbsA and FbsB bind to Fg, there is no sequence homology between them. While FbsA is

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[1] *Abbreviations used:* Fg, fibrinogen; GBS, Group B Streptococcus; MSCRAMMs, *M*icrobial *S*urface *C*omponent *R*ecognizing *A*dhesive *M*atrix *M*olecules; ECM, extracellular matrix molecules; FbsA, Fg-binding surface protein A; FbsB, Fg-binding surface protein B; ATCC, American Type Culture Collection; THB, Todd-Hewitt broth; LB, Luria Bertani; IPTG, isopropyl β-D-1-thiogalactopyranoside; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; NBT, nitro blue tetrazolium chloride; *p*Npp, *para* nitrophenyl phosphate; ELISA, enzyme-linked immunosorbent assay; CD, circular dichroism.

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a repeat protein composed of several repetitive units, each 16 amino acids in length [9], FbsB is a non-repetitive protein. The common feature in both FbsA and FbsB is the number of amino acids that constitute the protein which varies from one strain to another.

FbsB [10] from the human strain *S. agalactiae* NEM316, a type III GBS, is a 635 amino acid (73 kDa) protein. Its homologous protein in a bovine strain of *S. agalactiae* was termed as Fgag [3]. The sequence comparison of FbsB/Fgag using MultAlin [12] in various *S. agalactiae* strains is shown in Fig. 1. FbsB has a conserved N-terminal signal peptide and a C-terminal region of 223 residues. The N-terminal region between the signal peptide and the C-terminal region varies in size from one strain to another and also not conserved. However, this region has been shown to bind to human Fg [10]. In contrast to this observation it has been shown that the conserved C-terminal region in Fgag binds to bovine Fg and not to human Fg [3].

The protein sequence analysis of the C-terminal region of FbsB [FbsB(C), corresponding to 413–635 residues] from *S. agalactiae* NEM316 using BLAST indicates that other than similarities with strains of the same organism, there is no similarity with any characterized protein. This shows that FbsB(C) is a novel protein. Here we report the cloning, expression, purification, secondary structural analysis and binding studies of FbsB(C) with bovine Fg.

Materials and methods

Bacterial strains, plasmids and culture conditions

Freeze-dried culture of Type III *S. agalactiae* NEM 316 strain was obtained from American Type Culture Collection (ATCC). *Escherichia coli* DH5α was used for plasmid cloning and Rosetta-gami(-DE3)pLysS for protein expression. pET20b(+) from Novagen was used to make the vector-DNA construct. The streptococcal strain was grown in Todd-Hewitt broth (THB) supplemented with 0.1% yeast extract and all other *E. coli* strains were grown in Luria Bertani (LB) broth and when required 100 μg/ml of ampicillin was supplemented. All cultures were grown at 37 °C. Cloning and expression hosts were made competent by the $CaCl_2$ method.

PCR Amplification of fbsB(C) gene and preparation of fusion protein

Chromosomal DNA from *S. agalactiae* NEM316 was prepared by following the protocol given by Cheng [13]. It was used as a template for PCR amplification. The upstream (TGCCTTGCCATGGGTTG AATGGCTCCCAAAAAC) and downstream (GCGGACAGCTCGAGC TCTTTTATACGCGATGAG) primers with the restriction sites NcoI and XhoI (underlined), respectively, as reported by Gutekunst [10] were used to amplify the *fbsB(C)* gene. The amplified gene corresponds to amino acid residues 408–635 which is the C-terminal region of FbsB. Amplification was done at 98 °C for 3 min and then at 98 °C for 10 s, 52 °C for 30 s, and 72 °C for 30 s for a total of 30 cycles, followed by 72 °C for 4 min.

The amplified product was double digested with the restriction enzymes NcoI and XhoI and ligated with pET20b(+) which had been digested with the same set of enzymes. This vector places a hexahistidyl tag (6xHis) at the C-terminal region of the protein which will aid in the purification process. The ligated product was transformed into competent DH5α cells and selection of positive clones was done on ampicillin-supplemented agar plates. Colonies obtained were screened using colony PCR and plasmid mini preps followed by gene sequencing. The positive clones were transformed into various expression hosts of *E. coli* such as BL21(DE3), BL21(DE3)pLysS, C43(DE3), Rosetta(DE3)pLysS and Rosetta-gami-(DE3)pLysS and expression condition was optimized.

Optimization of the protein expression was done at different temperature, induction period and IPTG (Isopropyl β-D-1-thiogalactopyranoside) concentration conditions. The protein expression was best in Rosetta-gami(DE3)pLysS with 1 mM IPTG induction at 37 °C overnight after the culture reached an OD_{600} of 0.6.

Purification of the 6xHis-tagged rFbsB(C)$_{408–635}$ protein

The cells from the induced culture were harvested by centrifugation and resuspended in 50 mM phosphate buffer, pH 7.0. The cells were disrupted using a sonicator and centrifuged at 10,000 rpm at 4 °C for 30 min. Most of the rFbsB(C)$_{408–635}$ protein was found in the soluble cytoplasmic fraction. This was dialyzed overnight against 20 mM Tris–HCl (pH 7.0), 300 mM NaCl, 5 mM βME (β-mercaptoethanol) and 5% glycerol at 4 °C. The dialyzed sample was loaded on a nickel–nitrilotriacetic acid (Ni–NTA) column and bound proteins were eluted with increasing concentrations of imidazole (50, 100, 200 and 300 mM). Eluted sample was subsequently diluted tenfold with Tris–HCl buffer without NaCl and loaded onto the Q-Sepharose anion exchange column (GE Health Sciences). Elution was done with a stepwise increase in NaCl concentration. Final purification was done by using Sephacryl 200 size-exclusion chromatography column. Peak fractions were pooled and concentrated. A final yield of 7 mg protein per 1L culture was obtained.

SDS–PAGE, Western blot, Dot blot and native PAGE analysis

Sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) and Western blotting were used to confirm the expression of the recombinant protein. Fifteen percent SDS–PAGE gels were used for the analysis and staining was done using Coomassie brilliant blue. For Western blot experiment to confirm the expression of the 6xHis-tagged protein, rFbsB(C)$_{408–635}$ was size-separated on a 15% SDS–PAGE gel and electroblotted onto nitrocellulose membrane. The membrane was blocked overnight with 3% BSA (bovine-serum albumin) in PBST (phosphate buffered saline with 0.1% Tween 20) incubated with purified rFbsB(C)$_{408–635}$ for 1 h at room temperature. Subsequently, the membrane was washed three times with PBST and incubated with His-tag monoclonal antibody (Novagen), diluted 1:1000 in PBS, for 1 h at room temperature. Membrane was again washed three times with PBST and incubated for 1 h with goat anti-mouse IgG alkaline phosphatase conjugate (Calbiochem), diluted 1:10,000 in PBS. Finally, alkaline phosphatase conjugated antibodies were detected by adding the substrates 5-bromo-4-chloro-3-indoyl phosphate disodium salt (BCIP; 1 ml of 1% BCIP in 100% dimethylformamide) and nitro blue tetrazolium chloride (NBT; 1 ml of 1.5% NBT in 70% dimethylformamide) in 20 ml PBST. Visualizing the bound protein was done by incubating the membrane in a developing solution (100 mM Tris–HCl, pH 9.5, with 1 mM $MgCl_2$) for about 30 min in the dark. When the bands are intense enough, the membrane was washed with water and air dried.

For Western blot to identify the subunit of Fg to which FbsB(C) binds to, bovine Fg was separated into its three subunits on a 12.5% SDS–PAGE gel, electroblotted onto nitrocellulose membrane and blocked overnight with 3% BSA in PBST. The membrane was then incubated with purified rFbsB(C)$_{408–635}$ for 1 h at room temperature. Following this, incubation of the membrane with primary and secondary antibodies and detection of the bound protein were carried out as above.

For the Dot blot experiment, 2 μl and 1 μl of 100 μg/ml of native bovine Fg were spotted on a piece of nitrocellulose membrane and allowed to air dry at room temperature. The membrane was blocked with 3% BSA in PBST overnight at 4 °C followed by washing it once with 1× PBS for 5 min and incubating for 1 h at room temperature with rFbsB(C)$_{408–635}$. At the end of the incubation period, the membrane was washed with 1× PBS and incubated with