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ORIGINAL ARTICLE

Characterization of Afb, a novel bifunctional protein in *Streptococcus agalactiae*

Sanaz Dehbashi[1], Mohammad Reza Pourmand[2*], Rahil Mashhadi[2]

[1]Department of Pathobiology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran
[2]Urology Research Center, Tehran University of Medical Sciences, Tehran, Iran

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ABSTRACT

Background and Objectives: *Streptococcus agalactiae* is the leading cause of bacterial sepsis and meningitis in newborns and results in pneumonia and bacteremia in adults. A number of *S. agalactiae* components are involved in colonization of target cells. Destruction of peptidoglycan and division of covalently linked daughter cells is mediated by autolysins. In this study, autolytic activity and plasma binding ability of AFb novel recombinant protein of *S. agalactiae* was investigated.
Materials and Methods: The *gbs1805* gene was cloned and expressed. *E. coli* strains DH5α and BL21 were used as cloning and expression hosts, respectively. After purification, antigenicity and binding ability to plasma proteins of the recombinant protein was evaluated.
Results: AFb, the 18KDa protein was purified successfully. The insoluble mature protein revealed the ability to bind to fibrinogen and fibronectin. This insoluble mature protein revealed that it has the ability to bind to fibrinogen and fibronectin plasma proteins. Furthermore, in *silico* analysis demonstrated the AFb has an autolytic activity.
Conclusions: AFb is a novel protein capable of binding to fibrinogen and fibronectin. This findings lay a ground work for further investigation of the role of the bacteria in adhesion and colonization to the host.

Keywords: *Streptococcus agalactiae,* Fibrinogen, Fibronectin, Autolysin

INTRODUCTION

Streptococcus agalactiae is the principal cause of bacterial sepsis, neonatal meningitis and endocarditis in parturient women. It is also a cause of pneumonia particularly in the immunocompromised elderly (1). *S. agalactiae* commonly colonizes the gastrointestinal and urogenital tracts of humans (2) and the proteins enabling the bacteria for colonization, invasion and evasion are poorly defined (3).

The interaction between *S. agalactiae* and the epithelial cells is mediated by a heterogeneous system known as extracellular matrix (ECM). This system includes the components both fibronectin and fibrinogen (2). The fibronectin functions as a binding site for *S. agalactiae* through an insoluble phase (4, 5). A sequence of arginine, glycine and aspartic acid (RGD) in fibronectin is also involved in binding to proteins (6). Furthermore, C5a peptidase has a high affinity to the RGD sequence of the fibronectin and plays a significant role in adhesion and invasion (4). However, the fibrinogen in *S. agalactiae* performs key role in

*Corresponding author: Mohammad Reza Pourmand
Address: Department of Pathobiology, School of Public Health and Biotechnology Research Center, Tehran University of Medical Sciences, Tehran, Iran.
Tel: +98-21- 88954910
E-mail: mpourmand@tums.ac.ir

attachment (7) and FbsA and FbsB are recognized fibrinogen-binding proteins (2). The FbsA binds to cell wall covalently because of LPXTG motif while the FbsB seems to be secreted due to lack of such motif (8). Both proteins promote the entry of the *S. agalactiae* into the epithelial cells (9).

S. agalactiae also possess a dynamic structure called peptidoglycan. This structure like other Gram-positive bacterial cell wall contains peptidoglycan hydrolases. These enzymes play an important role in remodeling, turnover, division and separation of the cell wall (10). In critical situations, some of the peptidoglycan hydrolases(autolysin) facilitate self-disintegration(autolysis) of the peptidoglycan (11, 12). *S. agalactiae* and *Staphylococcus aureus* are both Gram positive cocci from different genus. While there is no clear evidence about the details of autolysin in *S. agalactiae, S. aureus* (Atl) contains two major domains of autolysin: amidase and glucosaminidase (11). The glucosaminidase domain ionically binds to fibronectin (13). Another autolysin of *S. aureus* (Aaa) is bifunctional, and it also mediates adherence to immobilized fibrinogen and fibronectin (14). Similar roles were found in other *Streptococcus* species such as *Streptococcus pneumoniae* (15). The *S. agalactiae* contains proteins which are not fully understood of their roles, like Gbs1805.

In this study, AFb, which is a novel protein encoded by *gbs1805* gene in *S. agalactiae* was cloned, over expressed and purified. *In silico* analysis of AFb indicates amidase activity and great homology to other *Streptococci* genus. Moreover, the binding ability of AFb to plasma proteins was examined.

MATERIALS AND METHODS

This experimental study was performed on functional analysis of *gbs1805* gene of *S. agalactiae.*

Bacterial strains, plasmids and culture conditions. The strains and plasmids used in this study are presented in Table 1. The *S. agalactiae* isolate were grown on blood agar enrichedby 5% sheep blood. Also, *Escherichia coli* strains were cultured overnight on Luria Bertani (LB) (Merck, Germany) broth at 37 ˚C. Ampicillin (0.1mg/ml) was added to the cultures when required.

PCR amplification of the *gbs1805* gene. Chromosomal DNA of *S. agalactiae* was extracted using DNA Extraction Kit (Promega, USA) based on the manufacturer's instructions. The upstream (GCGCGCCATATGATACATATAACTATGCAGTAGATGTA) and the downstream (GCGCGCCTCGAGATTCGGATAAATGTAGCTAACTAC) primers (20pmol/µl) with the underlined restriction sites were used to amplify the *gbs1805* gene at: 95 ˚C for 5 min, 95 ˚C for 1 min, 59 ˚C for 45 seconds (30 cycles), 72 ˚C for 45 seconds and 72 ˚C for 10 seconds.

Cloning and Overexpression. The amplified gene and *p*ET21a were double digested by *Nde*I and *Xho*I enzymes (Takara, Japan) and ligated using T4 ligase (Takara, Japan). Then, the recombinant vector was transformed to *Escherichia coli* DH5α. Then positive clones were chosen fromthe ampicillin-supplemented LB agar plates and were confirmed by colony PCR and sequencing of plasmid. The positive clones then were transformed to the expression host, *E. coli* BL21.The overexpression was optimized at 37 ˚C, 1mM/ml IPTG (Fermentas, USA) for 4 hours oncethe culture has reached OD_{600} of 0.6.

Strains and plasmids	Genotype or Description	Source
E. coli strains DH5α	dlacZ Delta M15 Delta(lacZYA-argF) U169 recA1 endA1 hsdR17(rK-mK+) supE44 thi-1 gyrA96 relA1	Novagen, UK
BL21	F_ *ompT gal (dcm) (lon) hsdS*B (rB_ mB_)	
Streptococcus agalactiae	Clinical isolate	This Study
*p*ET21a	His6-tagged vector Amp^r	Stratagen, UK

Table1. Bacterial strains and plasmids

Protein purification. The recombinant insoluble protein was purified by Qiagen purification kit (Qiagen, Netherland) based on the manufacturer's instructions. The purification process included 4 steps: cell lysis, binding, washing and elution.The procedure was performed using urea buffers under PH reducing conditions.

SDS-PAGE, Western blotting. The purified protein was indicated by SDS-PAGE. 12.5% SDS-PAGE gel was applied to analyze the purified recombinant protein by Coomassie brilliant blue staining. Western blot was used to confirm the His-tagged recombinant protein. Briefly, the recombinant protein was size-separated on SDS-PAGE gel. Then it was electroblotted to nitrocellulose membrane. The membrane was blocked overnight (4 ˚C) with 3% skimmed milk in PBS. Subsequently, it was washed three times in a washing buffer (Tween 20 and PBS) and incubated in His-tag antibody (Sigma, USA) diluted in1:1000 in PBS for 1h at room temperature. Afterwards, the membrane was washed three times and DAB substrate (3, 3'-Diaminobenzidine) (Sigma, USA)was added to detect the recombinant protein.

Western blotting was used to assess binding to biotinylated plasma proteins (fibrinogen and fibronectin). Ant II was used as a positive control. The recombinant protein was electroblotted on nitrocellulose membrane as previously mentioned. After blocking and washing the membrane, it was incubated in plasma protein (fibrinogen and fibronectin) diluted in1:1000 for 3h at room temperature. Then the membrane was washed as before and incubated in Streptavidin (Roche, Germany) diluted washing buffer in 1:30000 at room temperature for 3h. Subsequently, the membrane was washed and incubated in alkaline phosphatase buffer for 5 min. Then the membrane was immersedin NBT/BCIP (Roche, Germany) solution and kept in dark condition until the protein band appears.

RESULTS

The *gbs1805* gene was amplified by PCR (Fig. 1) and cloned into *p*ET21a in *E. coli* DH5α as cloning host. Then, the constructed plasmid was transformed into BL21 and expressed as a hexahistidyl-tagged protein. The recombinant protein was successfully purified using Ni-NTA (Qiagen, Netherland), as predicted by bioinformatics analysis in JCVI. A full sized 18 KDa mature protein was indicated by the SDS- PAGE (Fig. 2). Then, the purified His6-tagged recombinant protein was confirmed by anti his-tag

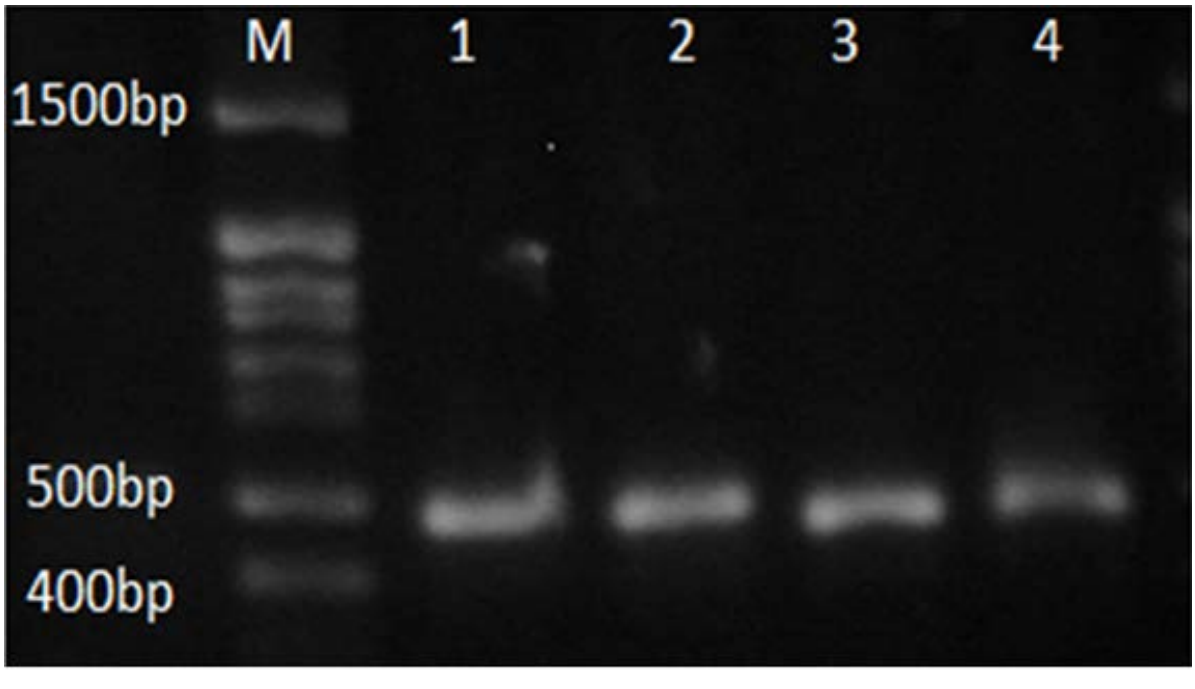

Fig. 1. Amplification of *gbs1805* gene (432bp). (M)Marker 100bp (GeneOn, USA), (1) Positive Control *Streptococcus agalactiae* NEM316, (2-4) Clinical isolates.

western blot experiment (Fig. 3). The binding ability of the bacterium to the fibrinogen and fibronectin are shown Fig. 4.

DISCUSSION

Attachment to specific receptors is the initial step in the pathogenesis of Gram-positive bacteria (16). The most common receptors mediating adherence to epithelial cells including fibrinogen and fibronectin are parts of the extra cellular matrix (ECM) (2, 17) and fibrinogen and fibronectin are known to be the major

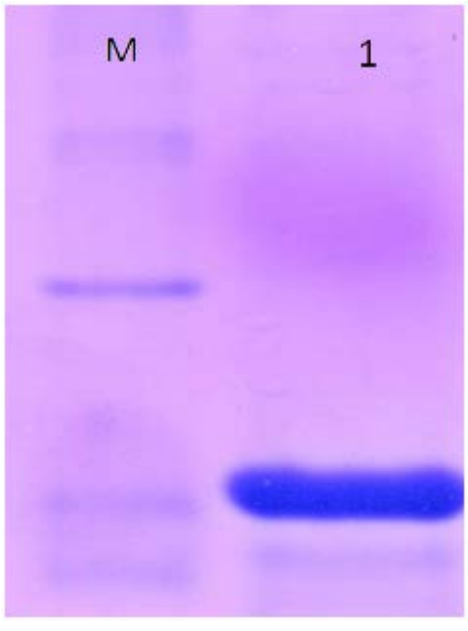

Fig 2. Confirmation of protein purification by SDS PAGE. (M): Unstained protein marker (Fermentas, USA), Lane1: Purified Recombinant protein (18 KDa).

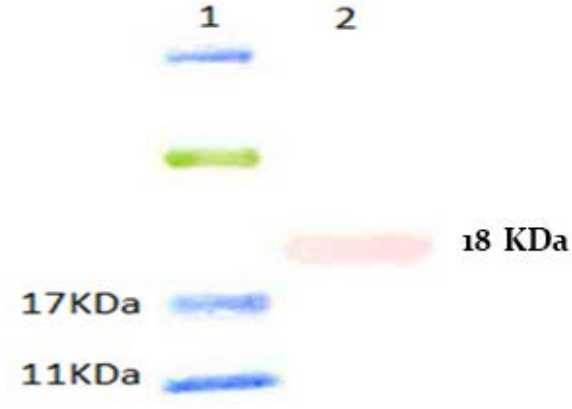

Fig 3. Anti His6-tagged recombinant protein. Lane1: Prestained Marker (Sinaclon, Iran), Lane2: Recombinant protein (18 KDa).

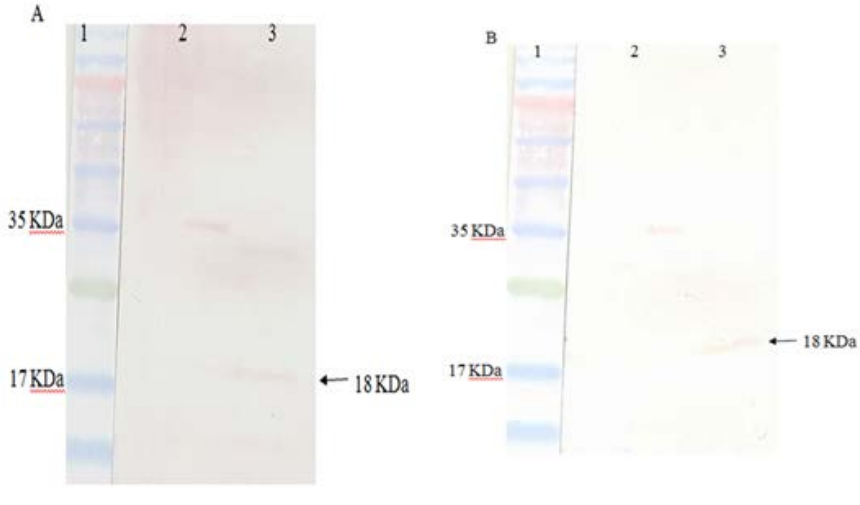

Fig 4. (A) Fibrinogen protein binding assay. Lane 1: Prestained Marker (Sinaclon, Iran), Lane 2: Positive control (Ant II), Lane 3: Recombinant protein (18 KDa). (B) Fibronectin protein binding assay. Lane 1: Prestained Marker (Sinaclon, Iran), Lane 2: Positive control (Ant II), Lane 3: Recombinant protein (18 KDa).

components of the ECM in a number of Gram-positive bacteria (18, 19). In this study, AFb (Autolysin, Fibrinogen/Fibronectin binding), is identified to be a novel protein of Group B Streptococcus capable of bindingto fibrinogen and fibronectin. *S. agalactiae* interacts with fibrinogen via a variety of proteins including FbsA, FbsB, FbsC, Srr1 and Srr2 (2, 8, 20, 21). Fibrinogen is expressed on epithelial and endothelial cell surfaces and interferes complement activation (22), phagocytosis and polymorphonuclear activation (23). Since FbsA protects *S. agalactiae* against opsonophagocytosis (9), deletion of *fbsA* leads to reduced proliferation of the bacterium in blood (7). The interaction of Srr1 and fibrinogen contribute to pathogenesis and plays key role in attachment to brain microvascular endothelial cells (24). The binding ability to epithelial and endothelial cells, biofilm formation and invasion decreases significantly in *fbsC*-negative mutants (20). Furthermore, FbsA and FbsB also participate in evasion of host defense (23).

Fibronectin, another component of ECM, producesA549 on cell surfaces in the respiratory tract and on vaginal and cervical cells (6, 25). The well-defined fibronectin binding protein of Group B Streptococcus encoded by *scpB* gene, known asC5a peptidase, adheres to epithelial cells and involved in evasion (4). *S. agalactiae* binds to immobilized fibronectin (5). However, the *scpB* defective mutant *S. agalactiae's* attachment to fibronectin significantly decreaseswhen compared to the wild strain (6). This suggeststhe involvement ofmultiple factors in the fibronectin binding process. In addition to the binding activity of AFb, it seems to be a peptidoglycan hydrolase. The possible role of the *AFb* in the attachment process and its substantial pathogenicity is not well recognized due to the lack of afb-defective mutant *S. agalactiae.*

Commonly, peptidoglycan hydrolases deal with cell separation, turn over, remodeling, and degradation of cell wall. Evidence shows that peptidoglycan hydrolases, autolysins, are involved in the pathogenesis and virulence of Gram-positive bacteria by mediating bacterial adherence (26, 27). To date numerous autolysin/adhesion proteins including Aaa, Atl, Aas, Aae, LytM, AmiC, etc which confirmed binding to ECM are described undergram-positive bacteria (11, 13-15, 28). In vivo studies demonstrated that autolysin-defective mutants to be less virulent than wild type strains of *Listeria monocytogenes, S. aureus, S. pneumonia,* and *S. epidermidis* (29-31). *In silico* studies of AFb demonstrated high homology to amidase, and zymography confirmed the activity in vitro (Fig 5). Therefore, AFb can be a bifunctional protein, based on its binding affinity to two plasma proteins and amidase like activity. Using patients' sera, we did not get any

Fig 5. (A). Sequence of the *gbs1805* gene. Red: Nucleic acid sequence of signal peptide. (B). Amino acid sequence of the AFb (the recombinant protein). (C). Multiple alignment of AFb to other protein of *Streptococcus agalactiae*

evidence showing antigenic activity.

CONCLUSION

The AFb is a novel protein encoded by *gbs1805* in *S. agalactiae* and is capable to bind with the plasma proteins - fibrinogen and fibronectin. The role of the protein in adhesion and colonization of the bacterium is unknown and needs further investigation.

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