Autolytic Activity and Plasma Binding Study of Aap, a Novel Minor Autolysin of *Streptococcus pneumoniae*

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Abstract- Pneumococcal autolysins are enzymes involved in cell wall turnover and cellular division physiologically. They have been found to be involved in the pneumococcus pathogenesis. The aim of this study was to identify the autolytic activity of Spr1754 as a novel protein of *Streptococcus pneumoniae*. Moreover, the binding of the recombinant protein to plasma proteins was also determined. The *spr1754* gene was amplified by PCR and cloned into the *p*ET21a(+) prokaryotic expression vector. The constructed *p*ET21a(+)/*spr1754* recombinant plasmid was transformed into *E. coli* Origami (DE3) and induced using IPTG. The recombinant protein of Spr1754 was purified by Ni-NTA affinity chromatography and confirmed by SDS-PAGE and Western blot analysis using anti-His tag monoclonal antibody. Autolytic activity and the ability of the recombinant protein in binding to plasma proteins were performed using zymogram analysis and western blot, respectively. The *spr1754* with expected size was cloned and overexpressed in *Escherichia coli* Origami (DE3), successfully. After purification of the Spr1754 recombinant protein, the autolytic activity was observed by zymography. Of the four plasma proteins used in this study, binding of lactoferrin to Spr1754 recombinant protein was shown. The Spr1754 recombinant protein has a bifunctional activity, i.e., as being autolysin and lactoferrin binding and designated as Aap (autolytic/ adhesion/ pneumococcus). Nevertheless, characterization of the Aap needs to be followed using gene inactivation and cell wall localization.
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Introduction

Streptococcus pneumoniae is a major pathogen in a family of streptococcaceae causing severe infectious diseases such as meningitis, pneumonia and otitis media (1). The pathogen expresses a wide variety of virulence factors that play important roles in its pathogenicity i.e. autolysins.

The role of autolysins in pneumococcal pathogenicity has been shown in animal models. Pneumococcal endophthalmitis in animal models shows that the autolysin-deficient pneumococci in comparison with wild-type strains have a low virulence potential. Furthermore, autolysins are involved in the pathogenesis of pneumococcal meningitis (2,3). The autolysins cause inflammation by releasing cell wall components that stimulate inflammatory intermediates (4). Nevertheless, autolysins are related with numerous biological functions, such as cell wall turnover, cell separation, cell division and antibiotic-induced autolysis (5,6). The enzymes include N-acetylmuramidase, N-acetylmuramyl-L-alanine amidase, N-acetyl glucosaminidase and endopeptidase that cleave the bonds within the peptidoglycan (7-9). Structurally, the pneumococcal autolysins have two domains including N-terminal domain with catalytic function, following C-terminal domain with six repeats domain and a short tail which is responsible for recognition of choline residues of pneumococcal cell walls (10). A number of pneumococcal autolysin-encoding genes have been characterised. LytA, a mainly studied gene, is conserved and encodes major autolysin in *Streptococcus pneumoniae* localized on the cell surface (11). Although this gene is conserved in all pneumococcal strains, nevertheless blasting the sequence show that it has high similarity with related sequences of mitis group streptococci (12). Despite that, a recent study showed that lytA PCR is specific for detection of S. pneumoniae

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in plasma samples (13). Binding of plasma proteins to autolysins was also reported in other streptococci. The *S. mutans* autolysin (AtlA) is a fibronectin binding protein and may play a role in bacterial resistance to phagocytosis (14). These data also indicate that the autolysins may be multifunctional/bifunctional proteins (15).

In the present study, using bioinformatic analysis *spr1754* gene was identified, and an amidase domain with N-acetylmuramyl-L-alanine amidase activity is showed. The aim of this study was a characterization of Spr1754 autolytic activity by zymography. Moreover, binding of the recombinant protein to plasma proteins was also studied.

Materials and Methods

Cloning and Expression

A conventional cloning protocol was used to the cloning of spr1754 gene. Briefly, *Streptococcus pneumoniae* (ATCC 49619) was cultured on blood agar medium and incubated at 37°C for overnight. Genomic DNA was extracted using QIAamp DNA Mini Kit (Qiagen, Germany) according to the manufacturer's instruction. The coding sequence *spr*1754 gene was amplified by PCR directly from the genome of the *Streptococcus pneumoniae* strain (ATCC 49619) by using specific forward and reverse primers(F-5′-GCGCGCCATATGGAAATTAATGTGAGTAAATTAAGAA-3′ and R:5′-GCGCGCCTCGAGTTTTACTGTAATCAAGCCATCTG-3′) carrying NdeI and Xho-I restriction sites, respectively. The resulting PCR product was digested with Nde1 and Xho-1 restriction enzymes (Fermentas, Germany) and ligated into a *p*ET21a vector (Novagen, USA), which was pre-digested with the same enzymes. Recombinant vector was transformed into competent *E. coli* DH5α cells and finally sequenced to confirm the correct gene composition.

Expression of recombinant protein carried out in Origami™ Host strain. For this means, a colony of *E. coli* (Origami DE3) harboring *p*ET21a was cultured in LB broth containing ampicillin (1 µg/ml) and tetracycline (1/5 µg/ml) and incubated at 37°C for 24 hours while shaking. After this step, 200 µl of overnight culture were transferred into 5 mL of LB broth containing ampicillin (1 µg/ml) at 37°C, when the OD600 reached 0.6, IPTG was added to a final concentration of 0.1mM and the cells were grown for 4 hours at 37°C with continuous shaking. Parallel cultures, without the addition of IPTG, were used as controls. The bacterial cultures were transferred to centrifuge bottles and centrifuged at 5,000 rpm for 5 minutes. The pellet and the supernatant were transferred to separate fresh tubes, and the cell pellets were resuspended in 100 µl SDS-PAGE sample buffer and were studied by 12% SDS-PAGE (16). Protein extraction was carried out using protein extraction kit (Qiagen, Germany) according to the manufacturer's instruction.

Western blot analysis

The purified recombinant protein was electrophoresed on a 12% polyacrylamide SDS gel and subsequently transferred to nitrocellulose membrane. Following stages carried out as described by Mahmood and Yang (17).

Zymogram

The autolytic activity of the recombinant protein was carried out by SDS-polyacrylamide gel electrophoresis. In this procedure, purified peptidoglycan (Sigma-Aldrich) was resuspended in dH2O (10µg/ml), and the suspension was added to a polyacrylamide gel at a final concentration of 0/5%. Following stages were conducted as described by Liu *et al*., (18). The SDS-PAGE gel was run at 150 V until the bromophenol blue markers were separated.

Electrophoresed gel were rinsed in dH2O to remove SDS and then in 200 ml of renaturing solution(5ml 1M MgCl2, 12.5ml 1M Tris HCL(pH7.5), 0.5 ml Triton X-100 and 482 ml dH2O) at RT for 30 minutes, followed by incubation overnight at 37°C in another 200 ml of renaturing solution. The gel was then rinsed in dH2O and incubated in 1x renaturing stain (2gr Methylene blue, 1.79 ml 2M potassium hydroxide and dH2O to 200 ml) for about 3h at RT. After destaining in dH2O, peptidoglycan hydrolase activity was visualised as a zone of clearing in the blue opaque background. Molecular masses were determined by comparison to protein standard with known size that runs on the same gel, cut off and Coomassie blue stained.

Binding assay

Binding assay, using lactoferrin, fibronectin, mucin and fibrinogen (Sigma-Aldrich) carried out as described by Hammerschmidt and other researchers (19-21).

Results

Cloning and expression of spr1754

The PCR product amplified from genomic DNA of pneumococcus is shown in Figure 1. As far as shown in Figure 1, the expected fragment size (957-bp) was

obtained. After digestion with *Nde*-1 and *Xho*-1 restriction enzymes, the digested fragment was ligated into corresponding sites of the *p*ET-21a (+) vector.

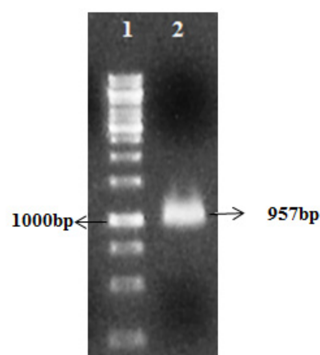

Figure 1. Gel electrophoresis of amplified *spr1754* on an agarose gel (1%). Lane 1, 100 bp DNA size marker. lane2 amplified *spr*1754.

The recombinant plasmids pET-21a(+)-spr1754 were digested by *Nde*-1 and *Xho*-1 and analyzed on gel agarose electrophoresis as shown in Figure 2. Sequence accuracy was confirmed by two-directional sequencing (GenBank accession number KJ094563).

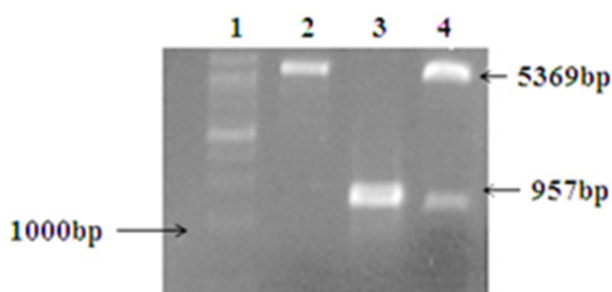

Figure 2. Gel electrophoresis of digested recombinant vector on an agarose gel (0/8%). lane 1 DNA Marker (1Kb), lane 2 digested vector with a Xho-1 restriction enzyme, lane 3 PCR product and lane4 double digest of the recombinant vector.

SDS-PAGE and Western blot analysis

The SDS-PAGE assay showed that the recombinant Spr1754 protein has approximately 36-kDa molecular weight (Figure 3).

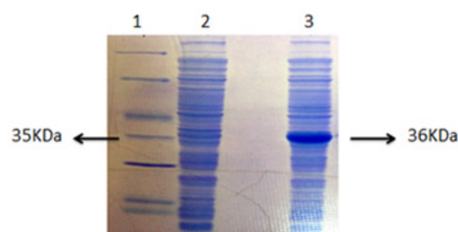

Figure 3. Analysis of recombinant Spr1754 protein expression by SDS-PAGE. Lane 1: Molecular mass marker, lane2 non-induced whole cell lysate (T0) and line3 induced host with 0.4 mM IPTG (T4)

The best expression conditions were 4 h at 37°C after IPTG addition. The His-tagged recombinant Spr 1754 protein was confirmed using Anti-His-antibody (Figure 4).

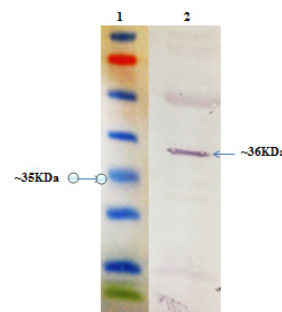

Figure 4.Western is blotting analysis of recombinant protein Spr1754. Lane 1: Protein size marker, lane 2. Recombinant protein western blotting with His-Tag monoclonal antibody.

Zymogram

Cell wall lytic activity was obtained with zymogram. The zymogram made with Bacillus cereus purified peptidoglycan as the substrate. The gel was stained with 0.1% (w/v) methylene blue for 2h and washed until clear band became visible. As far as shown in Figure 2, one translucent zone was revealed.

Binding

Binding of lactoferrin, fibronectin, mucin, and fibrinogen to a recombinant protein of Aap was tested. A positive interaction was only observed between lactoferrin and recombinant protein. The ScaA protein as control positive was bind to all four proteins used in the study (Table 1).

Table 1. Interactions between lactoferrin, fibronectin, mucin, and fibrinogen and Aap recombinant protein. ScaA used as control positive protein.

	Fibronectin	Fibrinogen	Musin	Lactoferrin
Spr1754	-	-	-	+
ScaA	+	+	+	+

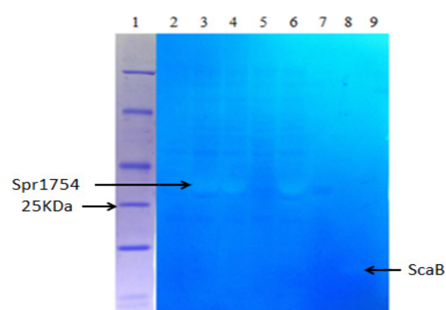

Figure 5. Zymogram analysis of recombinant protein Spr 1754, lane 1 molecular mass marker, lane 2, 3 and 6 protein Spr1754 and lane 8 ScaB as a control.

Discussion

Autolysins are enzymes that digest the peptidoglycan of the bacteria and play a role in producing them. It appears that the autolysins to be universal among all bacteria in their cell wall peptidoglycan (22). These enzymes are not only associated with many functions i.e. cell growth, cell-wall turnover, peptidoglycan maturation, cell division, and separation (23) but adhesive function has also been reported (24). As well as the pneumococcal autolysins have a role in its tissues damage. However, the intact molecular mechanisms of this process have not been understood (25). Several autolysin-encoded genes of *Streptococcus pneumoniae* have been studied bioinformatically, and experimental analysis has been conducted. In this study, binding to plasma proteins and autolytic activity of *Spr1754*, a hypothetical autolysin gene, was demonstrated. To our knowledge, this gene has not been studied yet. However, there are several studies about other pneumococcal and non-pneumococcal autolysins. The *spr1754* gene encodes a protein of 319 amino acids with an assumed molecular mass of ~36 kDa. In our study, lactoferrin binding property has also been shown. Therefore, the Spr1754 protein is predicted to have at least two or more-domain structure. It is considered that this feature is to be a common feature in autolysins (26). Past studies also indicate that the autolysins of *Streptococcus pneumoniae* are composed of two domains (27). According to our experiment, the Spr1754 protein designated as Aap (for autolysin/adherence protein).

The BLAST results showed that the *spr1754* gene is conserved, and therefore, it may be used in detective targets. Some researchers also believe that the autolysins loci are a useful tool for the sensitive diagnosis of pneumococcal disease (28,29).

In conclusion, although in this study the autolytic activity of the Spr1754 was demonstrated but it may have other functions. However, further research is required.

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References

1. Domingues S, Aires AC, Mohedano ML, et al. A new tool for cloning and gene expression in *Streptococcus pneumoniae*. Plasmid 2013 Sep;70(2):247-53.
2. Ng EW, Costa JR, Samiy N, et al. Contribution of pneumolysin and autolysin to the pathogenesis of experimental pneumococcal endophthalmitis. Retina 2002 Oct;22(5):622-32.
3. Hirst RA, Gosai B, Rutman A, al. Streptococcus pneumoniae deficient in pneumolysin or autolysin has reduced virulence in meningitis. J Infect Dis 2008;197(5):744-51.
4. Tuomanen E, Liu H, Hengstler B, et al. The induction of meningeal inflammation by components of the pneumococcal cell wall. J Infect Dis 1985;151(5):859-68.
5. Whatmore AM, Dowson CG. The autolysin-encoding gene (lytA) of *Streptococcus pneumoniae* displays restricted allelic variation despite localized recombination events with genes of pneumococcal bacteriophage encoding cell wall lytic enzymes. Infect Immun 1999;67(9):4551-6.
6. Ju CX, Gu HW, Lu CP. Characterization and functional analysis of atl, a novel gene encoding autolysin in *Streptococcus suis*. J Bacteriol 2012;194(6):1464-73.
7. Ingavale SS, van WW, Cheung AL. Characterization of RAT, an autolysis regulator in *Staphylococcus aureus*. Mol Microbiol 2003;48(6):1451-66.
8. Ramadurai L, Lockwood KJ, Nadakavukaren MJ, et al. Characterization of a chromosomally encoded glycylglycine endopeptidase of *Staphylococcus aureus*. Microbiology 1999;145(Pt 4):801-8.
9. Ramadurai L, Jayaswal RK. Molecular cloning, sequencing, and expression of lytM, a unique autolytic gene of *Staphylococcus aureus*. J Bacteriol 1997;179(11):3625-31.
10. Diaz E, Lopez R, Garcia JL. Chimeric phage-bacterial enzymes: a clue to the modular evolution of genes. Proc Natl Acad Sci U S A 1990;87(20):8125-9.
11. Mellroth P, Daniels R, Eberhardt A, et al. LytA, major

autolysin of *Streptococcus pneumoniae*, requires access to nascent peptidoglycan. J Biol Chem 2012;287(14):11018-29.
12. Greve T, Moller JK. The accuracy of using the lytA gene to distinguish *Streptococcus pneumoniae* from related species. J Med Microbiol 2012;61(Pt 4):478-82.
13. Abdeldaim G, Herrmann B, Molling P, et al. Usefulness of real-time PCR for lytA, ply, and Spn9802 on plasma samples for the diagnosis of *pneumococcal pneumonia*. Clin Microbiol Infect 2010;16(8):1135-41.
14. Jung CJ, Zheng QH, Shieh YH, et al. *Streptococcus mutans* autolysin AtlA is a fibronectin-binding protein and contributes to bacterial survival in the bloodstream and virulence for infective endocarditis. Mol Microbiol 2009;74(4):888-902.
15. Bourgeois I, Camiade E, Biswas R, et al. Characterization of AtlL, a bifunctional autolysin of *Staphylococcus lugdunensis* with N-acetylglucosaminidase and N-acetylmuramoyl-l-alanine amidase activities. FEMS Microbiol Lett 2009;290(1):105-13.
16. Kurien BT, Scofield RH. A brief review of other notable protein detection methods on acrylamide gels. Methods Mol Biol 2012;869:617-20.
17. Mahmood T, Yang PC. Western blot: technique, theory, and trouble shooting. N Am J Med Sci 2012;4(9):429-34.
18. Ju CX, Gu HW, Lu CP. Characterization and functional analysis of atl, a novel gene encoding autolysin in *Streptococcus suis*. J Bacteriol 2012 Mar;194(6):1464-73.
19. Hakansson A, Roche H, Mirza S, et al. Characterization of binding of human lactoferrin to pneumococcal surface protein A. Infect Immun 2001;69(5):3372-81.
20. Chia JS, Yeh CY, Chen JY. Identification of a fibronectin binding protein from *Streptococcus mutans*. Infect Immun 2000;68(4):1864-70.
21. Hammerschmidt S, Bethe G, Remane PH, et al. Identification of pneumococcal surface protein A as a lactoferrin-binding protein of *Streptococcus pneumoniae*. Infect Immun 1999;67(4):1683-7.
22. Shockman GD, Daneo-Moore L, Kariyama R, et al. Bacterial walls, peptidoglycan hydrolases, autolysins, and autolysis. Microb Drug Resist 1996;2(1):95-8.
23. Foster SJ. The role and regulation of cell wall structural dynamics during differentiation of endospore-forming bacteria. Soc Appl Bacteriol Symp Ser 1994;23:25S-39S.
24. Heilmann C, Hartleib J, Hussain MS, Peters G. The multifunctional *Staphylococcus aureus* autolysin aaa mediates adherence to immobilized fibrinogen and fibronectin. Infect Immun 2005;73(8):4793-802.
25. Sourav S, Patricia A, Sharma S, et al. Detection of pneumolysin and autolysin genes among antibiotic resistant *Streptococcus pneumoniae* in invasive infections. Indian J Med Microbiol 2010;28(1):34-9.
26. McLaughlan AM, Foster SJ. Molecular characterization of an autolytic amidase of *Listeria monocytogenes* EGD. Microbiology 1998;144 (Pt 5):1359-67.
27. Garcia E, Garcia JL, Garcia P, et al. Molecular evolution of lytic enzymes of *Streptococcus pneumoniae* and its bacteriophages. Proc Natl Acad Sci U S A 1988;85(3):914-8.
28. Lorente ML, Falguera M, Nogues A, et al. Diagnosis of pneumococcal pneumonia by polymerase chain reaction (PCR) in whole blood: a prospective clinical study. Thorax 2000;55(2):133-7.
29. Sheppard CL, Harrison TG, Morris R, et al. Autolysin-targeted LightCycler assay including internal process control for detection of *Streptococcus pneumoniae* DNA in clinical samples. J Med Microbiol 2004;53(Pt 3):189-95.