ORIGINAL ARTICLE

Detection of Biofilm Proteins from *Aggregatibacter actinomycetemcomitans* Induced by Glucose, Lactose, Soy Protein, and Iron Along with Protein Density Analysis

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ABSTRACT

Introduction: *Aggregatibacter actinomycetemcomitans* (*A.a*) is the main bacterium that causes aggressive periodontitis which is capable of forming biofilms on the surface of periodontal tissues. The objective of the study of the bacterial biofilm proteins is to provide an alternative to early prevention of oral infection of Periodontitis caused by *A.a*. Biofilms formed characterized by its molecular weight will then be used for the purpose of making Periodontal Disease Detection Kits. This project is aimed to select the molecular weight of biofilm protein of *A.a* exposed to 5% glucose, lactose, soy protein, and iron. **Methods:** Exposure to 5% glucose, lactose, soy protein, and iron is necessary to form biofilm of *A.a*. Then, the biofilm is characterized by SDS-PAGE electrophoresis to measure the molecular weight. **Results:** From this study *A.a* biofilm bands were formed with the number of protein bands that varied depending on the induction used. *A.a* induced by 5% glucose had one protein band (37.5 kDa), *A.a* induced by 5% lactose had five protein bands (77.9 kDa, 52.6 kDa, 46.8 kDa, 36.6 kDa, and 28.5 kDa), *A.a* induced by Soy Protein had seven protein bands (77.9 kDa, 71.3 kDa, 47.4 kDa, 40.4 kDa, 37.2 kDa, 28.8 kDa and 11.8 kDa) and *A.a* induced by 5% iron did not form protein band. **Conclusion:** Exposure to 5% glucose, lactose, soy protein, and iron to *A.a* resulted in band of biofilm protein. However, *A.a* biofilm induced by 5% iron has no protein bands.

Keywords: *Aggregatibacter actinomycetemcomitans*, nutrient intake, biofilm, biofilm protein

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INTRODUCTION

Periodontitis is an infectious disease caused by a group of certain microorganisms resulting in damages to the periodontal ligament and alveolar bone, with the formation of pocket and gingival recession or both. It begins with the invasion of microbe pathogens (*Aggregatibacter actinomycetemcomitans* = *A.a*) that colonize biofilms on the root surface of the tooth. Microorganisms that cause periodontitis occur a lot in patients with poor oral hygiene. *A.a* is the most bacteria with a frequency around 90% in agrresive periodontitis, 21% in chronic periodontitis, and 17% in healthy individuals (1). *A.a* are opportunistic pathogens and are part of the normal flora that colonizes the oral mucosa, teeth, and oropharynx (2).

Biofilm is an aggregation of microorganisms that attach to the surface and are enveloped by an extracellular matrix as a defense mechanism of external factors such as antimicrobial material (3). Biofilm formation in periodontal tissue is a gradual and continuous process. Clinically, the process of periodontitis causes an increase in sub gingival inflammation and formation of periodontal pockets (4). As Hajishengallis, et al. (5) has stated, pathogenic bacteria in periodontal infections may not really be the dominant species in biofilms, but can cause changes in other constituent species. That means the regulation of proteins in biofilms will be very important in understanding the role of individual microbe in function and integrity of biofilm. Most studies have discussed the role of parts of proteins, than all protein profile in biofilms. To reveal the relationship among the species, it is necessary to understand the role of biofilm in oral cavity. There are many interactions in various microbial species of the human oral cavity (6).

Food ingredients that are often consumed daily can induce *A.a* to form biofilms, including: glucose, lactose, iron, and also protein. These materials have their respective roles in the biofilm of *A.a* bacteria,

such as glucose for the biofilm defense system itself. Biofilms formed due to induction by these materials have different specific protein characteristics compared to their planktonic biofilms. The inducer material can express the same or different biofilm proteins from one ingredient to another. Biofilm proteins originating from daily food intake will be used as a detection test for the severity of periodontal disease caused by *A.a*. Therefore, candidates for *A.a* biofilm protein induced from several food ingredients variants need to be studied to determine their molecular weight and density.

In previous studies, variations in gene expression were related to biofilm formation as influenced by environmental conditions (7). Therefore, it is necessary to do research to determine the strength of each biofilm protein by the *A.a* bacteria induced by a variety of ingredients. The present study is expected to determine the strength of protein expression through the density of protein bands with the Gel Doc ™EZ Imager software for each of these inducers.

MATERIALS AND METHODS

In this study there was treatment, but replication had not been done. This study was carried out at Microbiology Laboratory of Faculty of Medicine and Biomedical Laboratory of Brawijaya University, Malang. This research procedures are divided into two major groups, the first one is the procedure *A.a* bacterial biofilm growth, the second is the procedure electrophoresis biofilm proteins using SDS-PAGE.

The first procedure is the growth of *A.a* bacterial biofilm including: the production of bacterial growth medium (BHIB and TSB), bacterial culture, and bacterial biofilm growth. Making BHIB medium is carried out by weighing 11.1 grams of BHIB powder/medium powder and put it into a 500 ml erlenmeyer flask, then adding the distilled water gradually while stirring up to 300 ml. The erlenmeyer flask is placed over the hotplate stirrer for 15 minutes until it is homogeneous. Pour 50ml of BHIB media on four 100 ml erlenmeyer flask containing 2.5 gr of inducer. The four tubes are labeled according to the inducer (glucose, lactose, iron, and control without inducers). Making TSB medium is done by weighing 9 grams of powder and put in a 100 ml erlenmeyer flask. Add some aquades little by little while shaking up to 50 ml. The erlenmeyer flask is placed over the hotplate stirrer for 15 minutes until it is homogeneous. Medium BHIB and TSB which already contain a 5% inducer are ready to be sterilized in an autoclave with a temperature of 121oC pressure of 2 atm for 15 minutes. After passing the sterility test, the medium is ready for use. Induction of soy protein is obtained from TSB media which is a special medium for growing biofilms with soy protein content.

For bacterial culture and growth of bacterial biofilms carried out by means of bacterial stocks that will be used to be isolated in new mediums to rejuvenate the colony at the same time to ensure bacterial purity. By using a sterile curved ose, take one pure colony and put it in 10 ml of liquid culture media. Media that already contains bacteria is incubated 37oC for 24 hours. The bacteria that growth equivalent to turbidity Mc Farland 8 standard done with gram staining and then observed in a microscope to ensure the culture is not contaminated. Then take the culture with a micropipette and put it into a medium containing inducer (1 ml each). All erlenmeyer flask are inserted into the incubator and incubated at 37oC for 2x24 hours to grow bacterial biofilms from each inducer.

The second procedure was electrophoresis including: protein isolation and electrophoresis preparation with SDS-PAGE. Pellet protein isolation was carried out by transferring the results of bacterial culture from the erlenmeyer flask to a 50ml falcon tube without shaking, and centrifuge at 6000 rpm fpr 15 minutes was done and the supernatant was discarded. Washing the centrifuged sediment with PBS and centrifuged at 6000 rpm for 15 minutes to remove the remaining medium. Add PBS + Tween 0.05% to the pellets. Perform sonication with an amplitude of 40 Hz [(on 1 minute, off 20 seconds) x 2] to break down pellet protein. The sonication results were centrifuged again at a speed of 12,000 rpm for10 minutes. Protein is obtained in the supernatant and the sediment contains debris. Take the supernatant and precipitation with 1: 1 alcohol and incubate for 1 night. Centrifuge again at 12,000 rpm for 10 minutes. Dissolve precipitate containing protein with PBS buffer solution. Calculates protein concentration using Nanodrop. Biofilm protein was isolated by scraping biofilms attaching at the base of the erlenmeyer flask, then added with PBS + Tween 0.05%, and transferred to eppendorf. After that it is centrifuged at 12,000 rpm for 10 minutes. Then supernatant was transferred to eppendorf and feed it with alcohol 1:1, and then incubated for overnight. To calculates the protein concentration, Nanodrop was applied.

Electrophoresis preparation procedure is carried out by making a gel with a 12% separating gel mixture inserted into a cassette using a micropipette. Allow 10-30 minutes to harden and form a gel. Next 4% stacking gel is poured on top of the separating gel and added with H_2O to adjust the volume while combing it into the gel cassette and waiting until the stacking gel hardens. Then prepare the sample to be used by adding RSB (Reducing Sample Buffer) to the sample with a ratio of 1: 1 and heated for approximately five minutes to denaturation the protein.

Conducting electrophoresis running process by entering 10 µl marker of protein into the well. Then enter the sample into each printed well (15-20 µl / well). Running gel for 100 minutes with a voltage of 200 V. Noting the

movement of marker protein and tracking dye (blue), if the tracking dye has reached the green line from the gel cassette, the running process can be stopped. Then release the gel from the cassette slowly and put it in the staining box. Then pour the commasie blue buffer dye until the gel is submerged perfectly. Incubate for ± four hours-overnight in the shaker incubator. Replace the staining buffer solution with a de-staining buffer solution and incubate in a shaker incubator until the protein bands are clearly visible. Gel electrogram from SDS-PAGE was used to analyzed the molecular weight and protein band density using the Gel Doc ™EZ Imager software.

RESULTS

Results of protein isolation planktonic, pellet induced, and induced biofilm to be used for running the SDS-PAGE electrophoresis carried Nanodrop procedure prior to measuring the protein concentration. The concentration of this protein is important to know before proceeding to the electrophoresis stage (Table I).

Table I: Results of Nanodrop to look at protein concentrations

SAMPLE	CONCENTRATION(mg/ml)
Planktonic	24.58
Glucose-induced pellet	14.07
Glucose-induced biofilm	2.22
Lactose-induced pellet	9.60
Lactose-induced biofilm	1.99
Soy Protein-induced pellet	58.86
Soy Protein-induced biofilm	3.07
Iron-induced pellet	1.25
Iron-induced Biofilm	5.64

Running electrophoresis SDS-PAGE procedure used the composition of stacking gel 4% and separating gel 12%. Marker protein used Jena Bioscience Blueray containing 10 standard proteins ranging from 11-180 kDa. And then the gel was removed and stained with commasie blue so that the image of protein bands could be seen. The gel is scanned to read the density of protein fractions using Gel Doc ™EZ Imager software.

From reading the results using Gel Doc ™ EZ Imager software, protein bands were obtained in the biofilm of *A.a* bacteria with kDa (Kilo Dalton) and also Intensity units respectively (Figure 1). We can know the molecular weight as follows:

a. In *A.a* biofilm that has been induced with 5% glucose there is one band of biofilm protein that appears, which is 37.5 kDa.

b. In *A.a* biofilm that has been induced with 5% lactose contained five protein bands biofilm that appears, namely 77.9 kDa, 52.6 kDa, 46.8 kDa, 36.6 kDa, and 28.5 kDa.

c. In *A.a* biofilm that has been induced by soy protein contained seven protein bands appear, namely 77.9 kDa, 71.3 kDa, 47.4 kDa, 40.4 kDa, 37.2 kDa, 28.8 kDa, and 11.8 kDa.

d. In *A.a* biofilm that has been induced with 5% iron contained no protein bands appear.

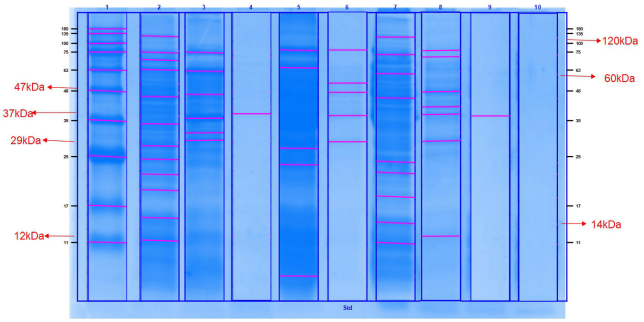

Figure 1: Results of SDS-PAGE electrophoresis. in kilo Dalton (kDa), tracks 1-10, are marker, standard (planktonic), glucose-induced pellet, glucose-induced biofilm, lactose-induced pellet, lactose-induced biofilm, soy protein-induced pellet, soy protein-induced biofilm, liron-induced pellet, lane iron-induced biofilm respectively.

In the procedure for calculating protein density using the Gel Doc ™EZ Imager software it is known that protein bands that appear have different densities (Table II).

Table II. Density of biofilm proteins from each inducer

INDUCER	BAND NO.	MOL. WT. (KDa)	DENSITY (Int)
Glucose	1	37,5	48.709
Lactose	1	77,9	46.565
	2	52,6	155.775
	3	46,8	154.971
	4	36,6	189.409
	5	28,5	134.33
Soy Protein	1	77,9	95.274
	2	71,3	36.180
	3	47,4	348.333
	4	40,4	109.478
	5	37,2	291.316
	6	28,8	366.088
	7	11,8	342.705
Iron	-	-	-

DISCUSSION

Biofilm protein analysis in this study aims to determine the profile of biofilm proteins based on the molecular weight that appears in the analysis of proteins by SDS-PAGE electrophoresis. The standard marker protein used is Jena Bioscience Blueray. Gel electrophoresis results of SDS-PAGE by staining commasie own blue color levels are different. The thickness of the staining results that appear on a gel containing the sample can be due to differences in the concentration of protein in the sample. Therefore, it is necessary to calculate protein

concentration before SDS-PAGE electrophoresis is performed using the Nanodrop method. It is intended that the sample concentration is not less than the detection limit of the dye used (detection limit commassie brilliant blue = 0.1µg) (8).

Biofilms that have been carried out by electrophoresis give rise to protein bands at each inducer. Protein bands that are examined for their molecular weight in each inducer are expressions of proteins that play a role in biofilm formation and can be targeted in determining specific biofilm proteins, which will later be used as biomarker candidates for an oral cavity infection. These protein bands are suspected biofilm specific proteins that need to be tested for further specificity to determine which proteins are truly specific from the biofilm inducer.

Protein bands produced from electrophoresis were then analyzed for each density using the Gel Doc ™EZ Imager software, resulting in results such as in Table II. From the measurement of protein band density the amount of density of each protein can be determined. Density measurement in this study aims to determine the amount of protein that will be used on the next test, namely Western Blotting.

The results of running SDS-PAGE electrophoresis on 5% glucose-induced biofilms, it is known that the pellet consists of 6 protein bands ranging from 29-75 kDa. While on biofilm it appears only protein band, with 37.5 kDa and a density of 48.709 Int. In planktonic lane, there was no protein band with 37.5 kDa was found, so that it can be said that the protein band that appeared in this glucose-induced biofilm is a suspected specific protein from glucose-induced biofilms.

In biofilm-induced lactose, there was seen five protein bands with 77.9 kDa, 52.6 kDa, 46.8 kDa, 36.6 kDa, and 28.5 kDa. If these five protein bands appear compared to planktonic protein bands, only one protein band has the same molecular weight as planktonic, which is 28.5 kDa. Each of these protein bands has different densities.

One protein band that appears in soy protein-induced biofilms, 11.8 kDa, is predicted to have the name ComE1, which according to Henderson et al. (9), the protein (11 kDa) all functions to bind a special bond to the fibronectin - FnIII9-10 domain. The bond in fibronectin above was also used to identify the combination of peptide synthesis and mutagenesis cutting.

In addition to the soy protein-induced biofilms contained protein bands with molecular weight of 40.4 kDa. According to Nagata et al., (10), the *A.a* bacterium which has a protein with a molecular weight of 40 kDa has a function as a hemoglobin binder as a source of iron for *A.a*. Because of the low source of pure iron in the host, the *A.a* bacteria must meet the iron requirements of the iron-carrying protein from the host, for example hemoglobin, myoglobin, lactoferrin, and transferrin. Only two proteins with molecular weights of 40 kDa and 65 kDa can bind to the hemoglobin protein that carries iron.

From the data above, protein bands that emerged from the planktonic and biofilm-induced glucose has a number of different proteins, the glucose-induced biofilms have a number of protein bands were less than planktonic. This is in accordance with the statement of Llama-Palacios, et al. (11) which states that there are often significant differences in *A.a* biofilm growth characterized by down-regulation in protein expression. Based on the research on protein function, the expression of protein had a tendeto go down influenced by stages of metabolic regulation, biosynthesis (such as amino acid biosynthesis, coenzymes, cofactors, and fatty acids), and also low transportation, which causes decreased metabolic activity that occurs in biofilm. So that the protein band that appears on the biofilm is less than the planktonic.

Not every induction component can form a biofilm. This is evident in iron-induced biofilms. In the results of a study of the detection of *A.a* biofilm proteins by various types of inducers, there was no protein band found in iron-induced biofilms. Iron can form biofilms at certain doses as biofilm forming activators by the bacteria *A.a*, but if iron exposure in the *A.a* bacteria is excessive, iron can cause toxicity. Where this will damage the toxic properties of proteins that do not form protein fractions and does not raise protein bands on electrophoresis results. According to previous research (12), excessive iron intake can reduce biofilm resistance to the presence of macrophages and will destroy biofilms previously formed by *A.a*.

CONCLUSION

Glucose induced 5% *Aggregatibacter actinomcetemcomitans* biofilm has a protein band candidate of 37.5 kDa, 5% lactose has a candidate protein band: 77.9 kDa; 52.6 kDa; 46 kDa; 36.6 kDa; 28.5 kDa, and soy protein has a candidate protein band: 77.9 kDa; 71.3 kDa; 47.4 kDa; 40.4 kDa; 37.2 kDa; 28.8 kDa; and 11.8 kDa, these can be used as an ingredient for the detection of Periodontal Disease.

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