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Research Report

Density of *Streptococcus mutans* biofilm protein induced by glucose, lactose, soy protein and iron

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ABSTRACT

Background: *Caries constitute an infectious disease that result from the interaction of bacteria with the host and the oral environment. Streptococcus mutans (S. mutans) represents the main bacterium that causes caries. The ability of S. mutans to form biofilms in the oral cavity is influenced by daily nutrient intake. This study of bacterial biofilm proteins can be used in the manufacture of kits for the detection of infectious diseases such as caries in the oral cavity. A biomarker is required for the manufacture of the detection kit. Consequently, research must first be conducted to determine the molecular weight and density of S. mutans biofilm proteins induced by several different daily nutrients, namely; 5% glucose, 5% lactose, soy protein and 5% iron.* ***Purpose:*** *This study aimed to analyse the density of S. mutans biofilm protein induced by 5% glucose, 5% lactose, soy protein, and 5% iron.* ***Methods:*** *The density of the S. mutans biofilm protein bands induced were measured using EZ Imager Gel Doc™ software.* ***Results:*** *A band of biofilm protein (61.7 kDa) was obtained from S. mutans induced by 5% glucose, four bands of biofilm protein (180 kDa; 153,9 kDa; 43,9 kDa; 37,5 kDa) from 5% lactose induction and seven bands of biofilm protein (157,9 kDa; 86,6 kDa; 66,5 kDa; 50,1 kDa; 37,9 kDa; 32,3 kDa; 29,4 kDa) from soy protein induction. In contrast, S. mutans induced by 5% iron did not show any protein bands. The proteins that result from each inducer are of differing densities.* ***Conclusion:*** *The protein bands from each inducer are of different densities which can be used in the further test to make a biomarker for dental caries detection kits.*

Keywords: *caries; nutrient intake; protein biofilm; protein density; Streptococcus mutans*

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INTRODUCTION

Caries constitute an infectious disease that results from the interaction of bacteria with the host and the oral environment. Dental caries form when food waste adheres to the teeth producing a demineralization process involving bacterial interactions on the tooth surface. The etiologic factors of caries include: plaque microorganisms, diet and time.[1] The predominant microorganisms playing a role in the occurrence of caries are *Streptococcus mutans* (*S. mutans*) which demonstrate the ability to attach to enamel surfaces, produce acid metabolites and form damaging biofilms.[2]

Biofilms are layers formed by colonies of microbial cells, slimy in texture, which attach themselves to the surface and are difficult to remove. Plaque constitutes a biofilm that forms in the oral cavity[3] and whose subsequent development is influenced by changes in the prevailing environmental conditions. One such change results from exposure to chemical nutrients present in food. Examples of daily consumed food include glucose and lactose as sources of carbohydrate and soy protein, in addition to iron which is one of the minerals required by the body. Various kinds of food can induce *S. mutans* biofilm formation in the oral cavity, the most basic example being glucose. A high concentration of glucose modulated by hydrogen ions can increase the metabolism of *S. mutans* resulting in the formation of extracellular polymeric substances (EPS). These EPS will help *S. mutans* adhere to the tooth surface and form a matrix as a means of self-defense.[4]

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S. mutans in a microenvironment will form biofilms in individual cells whose character is influenced by the nutrients around them[5] and which will express the same or different proteins from the planktonic cell depending on the inducer. Biofilms formed by individual S. *mutans* cells are controlled by specific genes that express biofilm formation.[5] Because the biofilm proteins formed from daily food intake have different characteristics, they can be used as a reference in the manufacture of detection kits which measure the severity of *S. mutans*-induced dental caries. For the manufacture of disease detection devices, an accurate biomarker is required. This renders necessary calculating both the molecular weight and expression strength (density) of each specific protein. Therefore, research must be conducted on *S. mutans* biofilm protein candidates which have been induced by several variant nutrients in order to determine their density.

The aim of this study was to analyse the expression strength of *S. mutans* biofilm proteins by measuring the density of protein bands using the Gel Doc ™ EZ Imager software after induction by 5% glucose, 5% lactose, soy protein and 5% iron. These inducing agents represent food nutrients which form part of a daily diet, are consumed during metabolism and contain microbes.[6]

This method of measuring protein density is intended to determine the volume of biofilm proteins produced after the inducing of specific materials characterized by their greater density. Such materials are used to meet the minimum requirements of follow-up work to a Western Blot and also for manufacturing vaccines.

MATERIALS AND METHODS

This research was conducted at the Microbiology Laboratory, Faculty of Medical and the Biomedical Laboratory at Universitas Brawijaya, Malang. Procedures for measuring the protein density strength of biofilm include: bacterial culture, biofilm growth, isolation of biofilm proteins, sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) and protein density analysis using Gel Doc ™ EZ Imager software.

S. mutans were cultured anaerobically and replicated in Luria Bertani (LB) medium to ensure that bacterial growth occurred with an indication of turbidity equivalent to McFarland 8 standard. Gram staining was subsequently conducted and observed through a microscope to ensure that the culture results were not contaminated. Following biofilm growth, *S. mutans* was inserted into 50 ml brain heart infusion (BHI) in an Erlenmeyer tube supplemented with 5% glucose, 5% lactose, 5% iron (FeCl2) in each tube and *S. mutans* inserted into 50 ml BHI in an Erlenmeyer tube without an inducer (as a control group). For soy protein, *S. mutans* was inserted into a 50 ml tube with trypticase soy broth (TSB) before being incubated anaerobically overnight at 37°C in order to grow *S. mutans* biofilm.

Isolation of biofilm protein was performed by scraping biofilms formed from the base of each Erlenmeyer tube, adding phosphate buffer saline (PBS) + Tween 0.05% and transferring it to an Eppendorf tube prior to centrifuging it at 12,000 rpm for 10 minutes. The supernatant was transferred to an Eppendorf tube before being precipitated with alcohol at a ratio of 1:1, and incubated for one night. The concentrations of the proteins obtained were measured by means of nanodrop.[7]

The method of SDS-PAGE electrophoresis began with preparation of the gel. After the gel had been formed from a mixture of 12% separating gel and 4% stacking gel, the plate was mounted on an electrophoresis device and the buffer was poured into the electrophoresis vessel. Injection of samples into the gel made by inserting 10 µl of isolate protein into each *S. mutans* biofilm in TSB medium, glucose-induced *S. mutans* biofilm in BHI media, lactose-induced *S. mutans* biofilm in BHI media, and iron-induced *S. mutans* biofilm in BHI media was added to 10 µl Tris-Cl + 20 µl reducing sample buffer (RSB). It was then inserted into the micro tube, before being heated in a water heater at 100°C for five minutes. After being cooled, 20 µl of the sample was inserted into each of the gel wells. An electric current of 30 mA and 100 V for electrophoresis was activated. After the gel had been removed from the plate, staining and washing the gel was completed. Staining was performed by soaking the gel in a staining solution for ± 4 hours–overnight in a shaker incubator.[8]

The SDS-PAGE electrophoresis gel was documented in the form of images. The results in the form of this image were analyzed to measure the protein molecular weight and the strength of the expression (density) of the protein bands using Gel Doc ™ EZ Imager software. The program read the thickness of the band per column selected as a fluctuating curve.

RESULTS

From the SDS-PAGE procedure that used 4% stacking gel and 12% separating gel, each protein fraction that appeared could be seen in its molecular weight expressed in units of kDa and its density in units of Int. The results produced using EZ Imager Gel Doc ™ software indicated the presence of several *S. mutans* protein bands that appeared after being induced with 5% glucose, 5% lactose and soy protein, while *S. mutans* induced with 5% iron did not produce any protein band. The marker protein used was Jena Bioscience Blueray which contained ten standard proteins ranging from 11-180 kDa (Figure 1).

As shown from the contents of Table 1, within the procedure for calculating protein density using EZ Imager Gel Doc ™ software each visible biofilm protein band possessed a different density. The density of each protein band was expressed in units of intensity (Int). The manner in which the EZ Imager Gel Doc ™ software functions

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is simply to compare the peak of the protein band graphs which appear on the monitor screen. The thickness (density) of the protein band is illustrated by the graph above. The density of each protein band with a different inducer can be read in the data above (Bio-Rad Laboratories, Inc., 2014).

From the table above, it can be seen that biofilms induced by 5% glucose contain one protein band of 61.7 kDa with a density of 184.21 Int. Whereas biofilms induced by 5% lactose contain four protein bands, there are seven protein bands in biofilms induced by soy protein that appear with their respective molecular weight and density in Table 1. Each of these protein molecular weights can be said to be the biofilm protein candidates which require further tests to enable its use as a marker in detecting the severity of caries disease caused by *S. mutans*. In iron-induced biofilms, no protein bands appear which could be due to several factors preventing iron from forming protein bands.

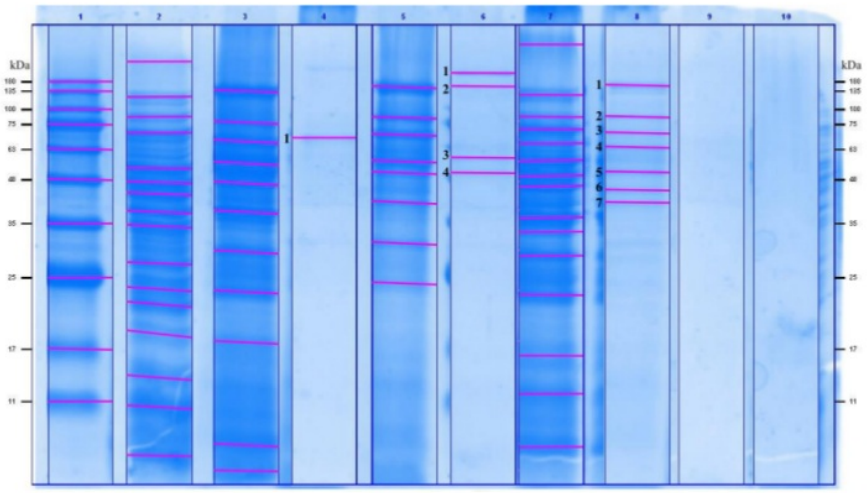

Figure 1. The result of electrophoresis. KDa = molecular weight in units of daltonkilo; lane 1 = marker; lane 2 = standard (planktonic); lane 3 = glucose-induced whole cell; lane 4 = glucose-induced biofilm; lane 5 = lactose-induced whole cell; lane 6 = lactose-induced biofilm; lane 7 = soy protein-induced whole cell; lane 8 = soy protein-induced biofilm; lane 9 = iron-induced whole cell; lane 10 = iron-induced biofilm.

Table 1. The molecular weight and density of *S. mutans* biofilm protein corresponding to each inducer

Inducer	Band no.	Mol. Wt. (kDa)	Volume (Int)
Planktonic (Control)	1	180.0	339.388
	2	122.8	401.676
	3	86.6	340.612
	4	66.5	702.712
	5	39.4	434.180
	6	34.2	356.184
	7	31.4	155.244
	8	27.3	138.108
	9	24.5	188.156
	10	18.8	137.904
	11	16.3	475.728
	12	15.5	380.392
	13	14.0	276.488
	14	12.0	767.992
	15	11.0	1.548.972
Glucose	1	61.7	184.21
Lactose	1	180.0	174.76
	2	153.9	76.64
	3	43.9	143.62
	4	37.5	155.72
Soy Protein	1	157.9	260.24
	2	86.6	152.86
	3	66.5	169.66
	4	50.1	175.17
	5	37.9	157.96
	6	32.3	102.54
	7	29.4	115.06
Iron	-	-	-

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DISCUSSION

The analysis in this study of *S. mutans* biofilm protein with SDS-PAGE electrophoresis aimed to determine *S. mutans* biofilm proteins that had been induced by 5% glucose, 5% lactose, soy protein and 5% iron. The density of the protein bands was subsequently analyzed using Gel Doc ™ EZ Imager software, the results of which are shown in Table 1. The density of each protein can be determined from the measurements taken in units of Int. The density measurement in this study was taken to determine the amount of protein to be used in subsequent Western blotting tests.

S. mutans induced by 5% glucose had one band of biofilm protein of 61.7 kDa and with a density of 184.21 Int. Because only one protein band appears, the protein can be considered to be a 5% glucose-induced *S. mutans* biofilm specific protein. Further tests such as Western blotting are required until the protein can finally be used as a marker in the identifiy of caries which result from glucose consumption.

S. mutans induced by 5% lactose contains four bands of biofilm proteins ranging from 37.5 kDa to180 kDa, each of which is of a different density. Protein with a molecular weight of 37.5 kDa has a density of 155.72 Int which is the highest protein density compared to that of the others. That means that protein content is dominant in lactose-induced biofilms.

S. mutans induced by soy protein contains seven biofilm protein bands which appear ranging from 29.4 kDa to 157.9 kDa. Each protein band is of a different density, but there is one protein with a more dominant protein content than the others. This is a protein whose molecular weight is 157.9 kDa and which has a density of 260.24 Int. According to Svensäter *et al*.[9] the names of proteins in *S. mutans*, it can be said that those protein bands with a molecular weight of 29.4 kDa are thought to be protein phosphoglycerate mutase. Proteins with a molecular weight of 32.3 kDa are regarded as GrpE proteins since the gene that becomes the protein is the *GrpE* gene. A protein with a molecular weight of 37.9 kDa is considered to be a protein exopolyphosphatase.[9]

S. mutans induced with 5% iron did not demonstrate the development of any protein band. This is probably due to excessive iron concentration which inhibits biofilm formation. Iron is a micronutrient crucial to the optimal growth of *S. mutans*. However, too high a concentration of this element can kill *S. mutans* bacteria. Ribeiro *et al*.[10] in their in situ study, stated that iron (Fe) at a concentration of 100µg/ml was able to reduce the number of *S. mutans* cells present in dental biofilms.[10] Fe has an antibacterial effect not only in terms of killing *S. mutans* cells, but also by disrupting the ability of these bacteria to form biofilms. It has been shown in situ that dental biofilms formed in humans exposed to Fe contain a lower number of *S. mutans*. In terms of its working mechanism, Fe possesses the ability to inhibit F-ATPase contained in *S. mutans*. As a result, Fe can affect the acidogenicity and aciduricity of *S. mutans*. On the other hand, by interfering with sucrose metabolism, Fe can reduce the production of EPS.[10]

Protein expression in biofilm formation inside the oral cavity is influenced by daily nutrient intake from the food consumed. The analysis above suggests that each inducer produces protein bands of differing molecular weights and densities which differ from those of plankton and from other inducers. Protein bands that result from 5% glucose induction, 5% lactose, soy protein and 5% iron are ones that play a role in biofilm formation. High or low protein density affects the calculation of the amount of protein that will be used in subsequent Western blot tests. The protein band with the highest density indicates the dominant protein content of each inducer. However, those proteins that have a dominant protein content are not necessarily a specific protein from each inducer. Rather, all protein bands that appear after being induced by each inducer are only candidate proteins that can be further tested to identify *S. mutans* biofilm specific proteins. Further analysis to determine the function of these specific biofilm proteins is a prerequisite to the protein being chosen as a marker in a dental caries detection kit and its association with daily consumption of glucose, lactose, soy protein and iron.

In conclusion, *S. mutans* biofilm induced by 5% glucose has one protein band candidate, 5% lactose has four protein bands, while soy protein contains seven protein bands. All protein bands from each inducer possess different densities which can be used in the further test to make a biomarker for dental caries detection kits.

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