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Research Report

Characterization of *Streptococcus sanguis* molecular receptors for *Streptococcus mutans* binding molecules

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ABSTRACT

Background: *Dental caries is a major problem in oral cavity. If dental caries causes cavity, the structure of dental hard tissue will not be reversible because of damage in the structure of the hard tissue. The early pathogenesis mechanism of dental caries is an adhesion interaction between cariogenic Streptococcus mutans microorganisms and tooth surface pellicles. The attachment involves a specific molecular component interaction between the bacterial complement molecules and the surface of the host. Streptococcus sanguis as a dominant ecology at the beginning of bacterial plaque aggregation will colonize the tooth surface earlier than S. mutans. The surface of bacterial cells can express some adesin. The bacteria also can express receptors for adhesins of other bacteria. Specific receptors for adhesions of S. Mutans bacteria are not only found in the pellicles, but also present in pioneer bacteria, such as S. sanguis. Adhesion between those bacteria is called as coagregation.* ***Purpose:*** *This study aimed to analyze the characterization of Streptococcus sanguis molecular receptors for Streptococcus mutans binding molecules.* ***Method:*** *This study used a sonication method for protein isolation of S. mutans and S. sanguis bacterial biofilms, as well as electrophoresis method using 12 % SDS-PAGE gel and Western Blot analysis.* ***Result:*** *Results of the protein profile analysis of S. mutans biofilms using 12% SDS-PAGE showed that there were 17 bands, each of which molecular weights was 212, 140, 81, 65, 61, 48, 45, 44, 40, 39, 33 , 25, 23, 19, 17, 12, and 11 kDa. On the other hand, results of the protein profile analysis of S. sanguis biofilms using 12% SDS-PAGE showed that there were 15 bands, each of which molecular weight was 130, 85, 65, 61, 48, 46, 40, 37, 29, 25, 23, 21, 17, 15, and 12 kDa. And, results of the analysis of S. sanguis receptor molecules using Western blot showed that there were three bands, each of which molecular weight was 130, 85, and 40 kDa.* ***Conclusion:*** *S. sanguis bacteria have specific receptor molecules for S. mutans bacteria with the molecular weight of 130, 85, and 40 kDa.*

Keywords: *receptor; adherence; coagregation; biofilms*

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INTRODUCTION

Dental caries is a multifactorial phenomenon. This disease is a major problem in oral cavity. If dental caries cause cavity, the structure of dental hard tissue will not be reversible because of damage in the structure of the hard tissue.[1] According to Riskesdas 2013, the prevalence of active caries in the population of Indonesia increased compared to the prevalence of active caries in 2007, ie from 43.4% (2007) to 53.2% (2013). To decrease the caries index as referenced by WHO guidelines, prevention of all aspects triggering dental caries is necessary.[2]

Streptococcus mutans (*S. mutans)* is a normal flora of the mouth. However, *S. mutans* can frequently become pathogenic in acidic environment. Cariogenic potential of these bacteria is manifested by its ability to ferment various carbohydrates, produce large amounts of acid, and to participate in the formation of tooth plaque.[1]

S. mutans as the main oral cariogenic bacteria, moreover, excrete glucosyltransferase (GTF) enzyme, which is useful to synthesize extracellular polysaccharides (glucans) from sucrose. Glucan is an important virulence factor since it helps the attachment of bacteria to the pellicles of teeth, and also contributes to the integrity of the structure.[3,4] Biofilm

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is a community of microbes attached to a solid surface, embedded in a matrix of extracellular polymeric substance produced by microorganisms.[5,6] Biofilm community is a complex and dynamic structure that has accumulated over several oral bacterial colonizations.[7]

The early mechanism of pathogenesis of dental caries is preceded by an adhesion interaction between cariogenic microorganisms and salivary glycoprotein components (pellicles) of the tooth surface. The attachment then involves a specific molecular component interaction between bacterial complement molecules and the surface of the host, then the surface of bacterial cells express some adhesins. The bacteria also express receptors for other bacterial adhesins.[8]

In the initial stage of dental plaque formation, furthermore, tooth surface is coated with pellicles, followed with adhesion of several bacterial species (pioneer colonizers), such as *Streptococcus sanguis (S. sanguis)*.[6,9] *S. sanguis* will provide a place of attachment for other bacteria (secondary colonizers) to stick to the pellicles and form biofilms.[6] Similarly, a research conducted by Okahashi explains that Pil B and Pil C proteins on the fimbriae of *S. sanguis* are capable of binding salivary α-amylase to the tooth surface. This indicates a specific attachment by the pili of *S. sanguis* facilitating other organisms to adapt well in the oral cavity.[12]

Alternative attempts to suppress the incidence of dental caries are by diagnosing the risk of dental caries early and using inhibitor compounds for proteins that play a role in the formation of biofilm. Proteomic study involving determination of protein profiles composing biofilms and *S. mutants* adhesin molecules is a way to identify biomarker candidates of dental caries risk in humans. Therefore, this research aimed to examine the molecular components of *S. sanguis* bacterial receptors pioneer bacteria involved in the initial attachment of *S. mutans* biofilm formation, triggering dental caries.

MATERIAL AND METHOD

This research was a laboratory exploratory observational research. The bacteria used in this research were *S. mutans* from the Central Health Laboratory Surabaya, and isolates of *S. sanguis* from the Laboratory of Microbiology, Faculty of Medicine, Universitas Brawijaya, Malang. Experimental animals used in this research, moreover, were New Zealand rabbits aged 3 months and weighed 1.5-2 kg. Meanwhile, tools required for this research were micropipette, incubators, digital scales analytical balance (OHAUS), shaker incubator (Tungtec instruments, laminar flow cabinets Kottermann 8580), electrophoresis tool (Bio Rad, USA) and Western blot device (Biorad, USA).

This research was conducted in several stages, namely identification of bacteria, a tests on *S. mutans* and *S. sanguis* bacterial biofilm formations, isolation of bacterial protein biofilms, treatment in experimental animals, manufacture of *S. mutans* anti-biofilms, electrophoresis stage, and Western Blotting stage. Identification of each bacteria was performed using tripticase yeast cysteine (TYC) media, then incubated at a temperature of 37^0 C for 24 hours. A test of bacterial biofilm formation was conducted using Brain Heart Infusion Broth (BHIB) media, then incubated at 37^0 C for 24 hours in a candle jar. BHIB as the bacterial culture media were put into vacutainer tubes, and then centrifuged for 15 minutes to separate the bacterial pellets from the medium. The supernatant was discarded. Part of the *S. mutans* bacterial sediment was added with 2 ml of 0.05% NOG, and then centrifuged at a speed of 12,000 rpm for 30 minutes. Isolation of protein biofilms in each group was carried out using sonication method with a power of 7 x 30 sec at a frequency of 40Hz in the TEM buffer (10 mM of Tris-HCl [pH 6.8], 1 mM of EDTA, 5 mg of MgSO4).[13,15] They then were stored at a temperature of -20^0. As a result, they were ready to be used as samples for analysis of crude protein of *S. mutans* and *S. sanguis* biofilms using 12% sodium dodecyl sulfate poly acrilamida gel electrophoresis (SDS-PAGE). The analysis of the crude protein of *S. mutans* and *S. sanguis* biofilms was performed using 12% SDS-PAGE (2.5 ml of Acrylamide, 1.2 ml of Tris HCl (pH 8.8), 1.2 ml of 0.5% SDS, 1.1 mL of distilled water, 50 mL of TEMED, and 30 mL of 10% APS). Staining then was conducted using silver stain and standard molecules of sigma low range marker.

In the next stage, *S. mutans* anti-biofilms was made of protein derived from *S. mutans* biofilms mixed with adjuvant materials at a ratio 1 : 1. After that, they were vortexed to be homogeneous for 30 minutes, and then injected in the sub-cutaneous area of those rabbits to facilitate the absorption of the antigens. Adjuvant materials used for the initial vaccination were Complete Freund's adjuvant, while Incomplete Freund's *adjuvant* was used as booster. Booster was administrated until the 35^{th} day of vaccination, and then the polyclonal antibodies were harvested.[15]

S. sanguis receptor molecules were analyzed by running the *S. sanguis* protein biofilms, and then transferring proteins in the Nc membrane. They were incubated together with *S. mutans* biofilm suspensions and *S. mutans* polyclonal antibodies, and anti rabbit Ig G as secondary antibodies. Blotting process then was performed to generate bands, converted with bands on Broad Marker Proteins from Bio-Rad.

RESULTS

Analysis results of the protein profiles of *S. mutans* biofilms revealed seventeen bands, each of which molecular weight was 212, 140, 81, 65, 61, 48, 45, 44, 40, 39, 33, 25, 23, 19, 17, 12, and 11 kDa. On the other hand, analysis results of the protein profiles of *S. sanguis* biofilms indicated fifteen bands, each of which molecular weight

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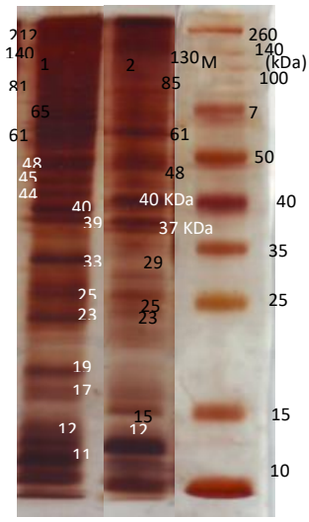

Figure 1. BM protein profiles of *S. mutans* (1) and *S. sanguis* (2). M as a protein marker of 12% SDS-PAGE.

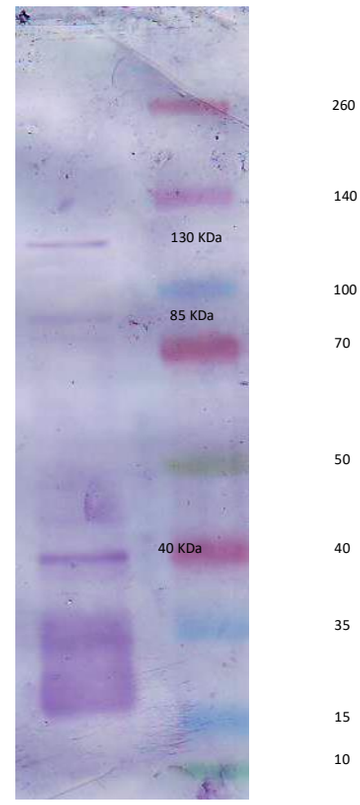

Figure 2. BM receptor molecules of *S. sanguis* (2). M as a Western Blot marker of Biorad.

was 130, 85, 65, 61, 48, 46, 40, 37, 29, 25, 23, 21, 17, 15, and 12 kDa.

Analysis results of *S. sanguis* receptor molecules using Western blot showed three bands, each of which molecular weight was 130, 85, and 40 kDa.

DISCUSSION

In the stage of the isolation of bacterial protein biofilms using the sonication method, TEM buffer (10 mM of Tris-HCl [pH 6.8], 1 mM of EDTA, and 5mm of MgSO4) was added.[13,14] This additional buffer aims to protect proteins from denaturation due to heat produced by ultrasonic sound vibrations.[21]

The analysis of the crude protein of *S. mutans* and *S sanguis* biofilms was performed to determine the protein profiles of *S. mutans* and *S sanguis* biofilms based on their molecular weight. The protein profiles of their biofilms depicted all the constituent proteins of *S. mutans* and *S. sanguis* biofilms. The protein profiles observed were molecular weight, existed protein bands, thick and thin protein bands, and total protein formed from the samples. The presence or absence of the bands at a certain migration distance indicates the presence or absence of migrated proteins that stop at such distances during electrophoresis process. The thickness of the bands can basically be divided into two, namely thick and thin bands. Thick bands indicate high total protein or large protein concentration, while thin bands demonstrate low total protein.[16,17] Thick bands can be distinguished from the thin bands due to the number of molecules migrated. Thick bands are formed from ixation of several bands. Bands that have a greater ionic strength will migrate farther than the bands with small ionic strength.[19]

Moreover, results of the analysis of the protein profile of *S. mutans* biofilms using 12% SDS-PAGE showed that there were 17 bands, each of which molecular weight was 212, 140, 81, 65.61, 48, 45, 44, 40, 39, 33, 25 , 23, 19, 17, 12, and 11 kDa. According to Kyle, there are 185,000 surface proteins that can be expressed by *S. mutans* serotype c. Previous researches have established some *S. mutans* surface protein molecules that play a role in initial attachment process to other organisms on the tooth surface, which have multiple names or designations, namely PAC molecules, Antigen I/ II, P1, SR and MS. Antigen I/ II in the cell walls of S. mutans has a molecular weight of 150 kDa - 215 kDa.[22] The molecular weights of other *S. mutans* protein molecules in this research were 81, 65, 61, 48, 45, 44, 40, 39, 33 , 25, 23, 19, 17, 12, and 11 kDa. Those protein molecules could be considered as fraction components of the fimbriae, as a degradation of other adhesins with greater molecular weights, or as expressions of other genes that have not known the role of proteins encoded. However, the protein molecular weights are not always in line with the protein molecular weights referenced since it can be affected by several factors, such as concentration of the gel, flow of supplied electricity, and effects of buffer, for instance, pH will affect the density of protein charge, consequently, affecting the level and direction of the movement.[24]

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The addition of sodium dodecyl sulfate (SDS) in the electrophoresis process, furthermore, serves as a electrophoresis buffer to make proteins become denatured, causing dissolved hydrophobic molecules, resulting in a negative charge on the entire structure of a protein by binding to the hydrophobic residues of each amino acid. As a result, protein molecules are separated based on their molecular weight only.[18] On the other hand, Western Blot technique aims to determine the character of *S. sanguis* receptor molecules in recognizing *S. mutans*. This technique will detect a protein with a particular molecular weight as gene expression results on the nitrocellulose membrane by using a labeled antibody.

In addition, the analysis results of the *S. sanguis* receptor molecules using Western blot demonstrated that there were three bands, each of which molecular weight was 130, 85, and 40 KDa. Adhesin proteins in *S. Sanguis,* according to some previous researches, are known as SrtA, Fim A, Abp A, Abp B, Pil B, and Pil C. ABP B in this research had a molecular weight of 85 kDa. Similarly, Nikitkova argues that ABP B has a molecular weight of 82 kDa - 87 kDa.[23] Pil B and Pil C, moreover, in this research had a molecular weight of 40 kDa. Like this research, Pil B and Pil C as proteins/ receptors/ adhesins in *S. sanguis* bacteria, according to the previous research, also have a molecular weight of 38 kDa - 45 kDa.[23] Meanwhile, SrtA in this research had a molecular weight of 130 kDa. Similarly, in a previous research conducted by Yamaguchi, SrtA molecules as *S. sanguis* surface antigens have molecular weights of 100 kDa, 130 kDa and 170 kDa. Yamaguchi states that Srta involves in adhesion process to the tooth surface, dental restorative materials, and oral cavity epithelial cells.[11] Therefore, it can be said that the Western Blot results obtained in this research are in line with the previous researches.

It can be concluded that *S. mutans* biofilms have an ability to recognize specific epitopes of the constituent proteins in *S. sanguis* biofilms. *S. sanguis* as pioneer bacteria on tooth surfaces have specific receptors against cariogenic *S. mutans* bacteria to facilitate *S. mutans* attach to the tooth surface receptors.

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