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Research Report

The ability of IgY to recognize surface proteins of *Streptococcus mutans*

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ABSTRACT

Background: *Streptococcus mutans are gram positive bacteria classified into viridians group, and have a role in pathogenesis of dental caries. It's adhesion to the tooth surface is mediated by cell surface proteins, which interact with specific receptor located in tooth pellicle. Glucan binding protein, Glukosyltransferase, and antigen I/II are basic proteins of S. mutans, which have a role in initiating the interaction. A previous study showed that chicken's IgY can interfere the interaction.* ***Purpose****: The objective of this study was to assess the ability of IgY in recognizing the surface molecule of Streptococcus mutans expressed by various serotypes (c, d, e, f) and a strain derived from IPB, Bogor.* ***Method****: Western blot was used as a method to determine such capability.* ***Result****: The result showed that IgY has a potency to recognize antigen I/II, but not the other proteins on the cell surface of all bacteria tested.* ***Conclusion:*** *The ability of IgY to bind the surface protein, antigen I/II, indicates that this avian antibody could be used as a candidate for anti-adhesion in preventing dental caries.*

Key words: *IgY, Streptococcus mutans, adhesion, surface proteins, and dental caries*

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INTRODUCTION

Streptococcus mutans (*S. mutans*) are bacteria that have an important role in the pathogenesis of dental caries. Among bacterium species in oral cavity, *S. mutans* are known as one of bacteria that have recently been discussed because of its ability in forming extracellular polysaccaride and plaque. These bacteria were isolated from dental plaque for the first time by Clarke in 1924.[1] These bacteria are classified into the *Monera* kingdom, *Firmicutes* division, *Bacilli* class, *Lactobacillus* ordo, *Streptococcaceae* family, *Streptococcus* genus, and *Streptococcus mutant* species.[2] In 1890, Miller formulated chemo-parasitic theory of dental caries, known as hypothesis of non-specific plaque describing a process from decalcification process of enamel to the forming of dental caries as the cumulative effect of acid produced by bacteria of dental plaque.[3]

Molecularly, the surface proteins of *S. mutans* that are commonly involved in the process of dental caries are *Glucan binding protein* (Gbp) and Antigen I/II (Ag I/II).[4,5] Besides that, *S. mutans* also express molecules as enzyme in the process of carbohydrate fermentation that has a role in the activity of *S. mutans*, such as *Glucosyltransferase* (Gtf), Dextranase (Dex), and Fruktosiltranferase (Ftf). Each of those three enzymes then breaks sucrose in order to form glukan, dextran, and fruktan. Actually, there are other proteins that have a role in supplying energy reverse of *S. mutans* in order to be able in oral cavity, such as Dextranase A (DexA), Dextranase B (DexB), Fruktanase, and Dlt1-4 (protein intracellular cells of *S. mutans*).[6,7]

Furthermore, there are two approaches for preventing caries, which are *host* aspects like diet control, clean control of oral cavity, fluorides, and agent aspects obtained from both active and passive immunizations. The use of mammalian Immunoglobulin G (IgG) in active and passive immunizations can prevent the adhesion of *S. mutans* on dental pellicle by recognizing cell surface proteins of *S. mutans*, especially Gbp, Gtf, and AgI/II, so the antibody

can be used for preventing dental caries.[8] Besides mammalian antibody like IgG, Immunoglobulin Y (IgY) can also be used as immune-prophylaxis matter for preventing dental caries through its use as complement materials of tooth paste used for decreasing the percentage of *S. mutans* in human saliva.[9] Tests for the IgG in passive immunization actually has been done, but the producing process of IgG has many technique limitations, is not economics, and can hurt animal that produce it during blood taking process. Based on the phenomenon, passive immunization with antibody (IgY) specifically located in egg yolk was used to substitute the role of IgG in preventing the interaction of adhesive molecule of *S. mutans* on dental surface.[10,11]

Chicken's egg is the source of IgY that potentially can be used as immunotherapy materials since it is more resistant to temperature (60–65° C) and pH alteration, and does not cause cross-reaction with structural component of tissue and protein in mammalians compared with IgG. These characters indicate that IgY can be applied to diagnose and prevent disease.[12] Based on some aspects in dentistry, IgY anti *S. mutans* actually can be used to decrease the frequency of caries in the experiments on caries.[13] For instance, based on the result of the experiment in which rats are used as testing animal, it was known that IgY has a potency in preventing the activity of Gtf and GbpB, so it can present colonization of *S. mutans* in dental pellicle, but the serotype is not known.[14,15] Besides that, the reference about the ability of IgY relating with antigen I/II of *S. mutans* with many serotypes is still not known since protein has an important role in the adhesion of *S. mutans* to dental pellicle.[3]

Therefore, this research was aimed to analyze the potency of IgY anti *S. mutans* in recognizing cell surface proteins of *S. mutans* with serotype c, d, e, and f. The significance of this experiment is to inform both the potency of IgY located in egg yolk of chicken as anti-*S. mutans*, especially for the researchers and pharmacy companies, and the possibility of IgY as passive immunization materials.

MATERIAL AND METHOD

This research was an experimental laboratory conducted in Molecular Laboratory in Faculty of Dentistry-UI and Faculty of Veterinary-IPB. Specific IgY anti *S. mutans* produced by Faculty of Veterinary, Pertanian Bogor Institute (FKH-IPB) was used as detector of bacteria from 4 serotypes, *S. mutans* serotype c (*mutans* Xc), *S. mutans* serotype d (*sobrinus* OMZ176), *S. mutans* serotype e (*mutans* LM7), and *S. mutans* serotype f (*mutans* OMZ175). Those four serotypes of *S. mutan* were laboratory strain, obtained from Professor Yamashita, Department of Preventive Dentistry, Faculty of Dentistry, Kyushu University, Japan. *S. mutans* strain derived from IPB was used as positive control.

In order to obtain antigen, each serotype of *S. mutans* was cultured in the selective solid media of Trypticase Soy with Sucrose and Bacitracin (TYS20B) and incubated for 12–72 hours at the temperature of 37° C. Next, one colony was taken usingh *oase* after being cultivated in the water media of *Trypticase Soy Broth* (TSB), and incubated for 24–72 hours at the temperature of 37° C in microaerophilic setting. Afterwards, *whole cell S. mutans* were centrifuged at 3000 rpm for 10 minutes. Sediment obtained was added with 200 μl lisozim, and incubated at ice temperature for about 5 minutes before centrifuged again at 3000 rpm for 5 minutes. Next, the sediment, as *S. mutans* antigen, was re-suspended in 500 μl Phosphate Buffer Saline (PBS) and added with 12.5% Sodium Dodecyl Sulphate (SDS) for about 65 μl.

Before the profile of cell proteins of *S. mutans* with serotype c, d, e, f, and a strain derived from IPB with western blot method was detected, the level of protein was determined by using Bradford (Bio-Rad) method in order to make the number of cell proteins of *S. mutans* that was analyzed have the same level of protein for each. *S. mutans* cells that have already been prepared with lisozim then were put into *Elisa plate* wells for about 160 μl (10 μl sample + 150 μl PBS). Next, other same *Elisa plate* wells were given with *Bovine Serum Antibody* (BSA) as standard proteins about 160 μl (10 μl BSA + 150 μl PBS). Either sample or BSA was added with 40 μl protein assay (Bradford), and then re-suspended by using *multi-chanel pipet* and incubated at the room temperature for 1 hour. The concentration of proteins was measured by using Elisa reader based on Optical Density with 655 nm wave length.

Western blot technique was used in order to detect the reactivity of IgY. Based on the method conducted by Yera *et al*.,[16] the method will be as the following; SDS PAGE**.** The prepared collector gel was put into SDS tank that has already been filled with reservoir running buffer stock (Bio-Rad). Next, separator gel was put into 20 μl sample and 5 μl standard protein (Invitrogen) in different wells as the mass indicator of the sample protein molecule. Then, protein was separated through 2 phases. First, the sample and marker proteins in the wells were collected near separator gel by setting up the electricity vertically at 100 mA, 100 volt, and 16 watt for 30 minutes. Second, the proteins in separator gel were separated by setting up the electricity at 100 mA, 150 voltage, 25 watt, for 80 minutes.

The next step was electrotransfer of antigen protein. First, gel containing antigen protein was put onto blotting nitrocellulose paper, and then was immersed in buffer transfer blot for about 2 minutes inside glass container. At the same time, transferring cassette that has already been halved was prepared. Second, each of them was put in foam pad (sponge), and then filter papers of mini trans-blot were put on them. Third, gel was put on one of them with blotting nitrocellulose on it. Afterward, the surface part was leveled up in order not to cause any bubble. Then, the transferring cassette was girded again and put into electrophoresis tank. Finally, electro-transfer was used with 100 Voltage of electricity for 1 hour.

Detection of Antigen Protein with IgYwas done. First, Blotting *nitrocellulose* (Bio-Rad) paper containing antigen was incubated in 20 ml 5% milk on sheker for about 1 hour at the temperature of 30° C. Next, it was washed 3 times with PBS (Sigma) for about 5 minutes. IgY in 10 ml 5% non-fat milk with the concentration 1:2000 was poured into the surface of blotting papers that have already been washed and incubated on shaker for about 1 hour at 30° C. Then, they were washed 4 times with PBS for 5 minutes. In order to visualize the interaction between IgY and targeted antigen protein, those blotting papers must be added with anti IgY antibody labeling peroxides (Horse rabbit peroxide anti-Chicken/Turkey IgG) with 1:2000 concentration that have already diluted with 10 ml 5% milk. Next, they were incubated on shaker for 1 hour at the temperature of 30° C. After that, those blotting papers were washed 4 times with PBS for 5 minutes. To visualize the binding between IgY and targeted antigen, finally, developing reagent was used.

RESULT

In this research, the series of working procedures for determining the profile and reactivity of IgY towards cell proteins of *S. mutans* with serotype c, d, e, and f, were conducted by using Western Blot technique. *Streptococus mutans* that have already been extrated with lisozim was analyzed by SDS PAGE method and colored with *Commasie blue* in order to analyze the profile of *S. mutans* proteins visualised in Figure 1. Paralelly, blotting of IgY anti *S. mutans* was conducted by using Western Blot technique in order to analyze cell proteins of *S. mutans* binding with IgY (Figure 2).

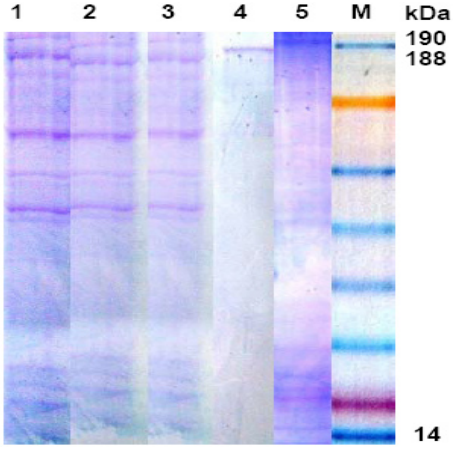

Figure 1. The protein profile of *S. mutans* colored with Commassie blue using SDS-PAGE method *S. mutans* in line 1, 2, and 3 showed similar protein ribbon profile, while, *S. mutans* in line 4 and 5 shows the different protein ribbon from the previous one.

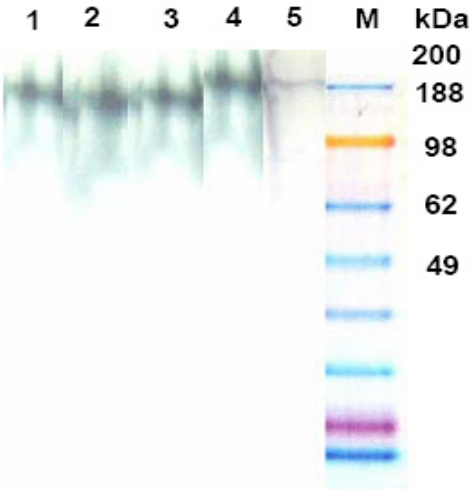

Figure 2. The surface proteins of *S. mutans* recognized by IgY anti *S. mutans*, using Western Blot method (Figure 2), showed similar protein ribbon with Molecule Mass (MM) about 188 kDa for all of those five isolates of *S. mutans*. Number 1–5 show that the well with 20 μl antigen extracted with lisozim from *whole cels* of *S. mutans*. Line 1 (*S. mutans* Isolate FKH IPB), Line 2–4, each of which shows *S. mutans* with serotype f, serotype e, serotype d, and serotype c. M = prestaining protein marker, Kda = kilo Dalton, and the number under it showed the molecule mass of proteins.

DISCUSSION

Chicken's egg yolk is considered as the food component which also contain specific antibody towards antigen inducing the respond of the chicken's immune system. The production of IgY from chicken's egg actually can give more advantages than the production of imunoglobulin from other animals, like mammals.[14] IgY, moreover, can be used to detect some viral brands through Elisa test, immunodiffusion, and immunofluorescence since the score of its isoelectrics is lower than human's IgG. [17] IgY can also be used to detect immunoglobulin in animal serum.[14]

The technology for producing IgY as an alternative antibody substituting antibody produced by mammalian animals, moreover, is considered as one of reasons for using it in immunotherapy and immunopropilaxis.[18] in dentistry, IgY can actually be used as antibody of anti *S. mutans*.[15,19] Thus, IgY, as anti adhesion, is expected to have potency to recognize the surface proteins causing the dental caries. In this case, the surface proteins of *S. mutans* commonly reported as the initiator of the adhesion of bacteria to dental pellicle are Gbp, Gtf, and Ag I/II.[20]

The choosing of whole cell *S. mutans* as the vaccine materials used for producing chicken's antibody was aimed to expose many kinds of surface epitope used for producing many kinds of paratop with many binding sites in hypervariabel regio, thus, IgY produced can interact with epitop of the surface proteins of *S. mutans* with high aviditas.[21] It means that the binding of the surface proteins of *S. mutans* by IgY antibody can decrease the character of

S. mutans pathogens since they can restrain the adhesion of those bacteria in the *host* dental surface, so it can possibly restrain the process of caries.[22]

The result from this research can give important information about the potency of IgY as anti surface proteins of *S. mutans.* This research may oven answer whether IgY produced by FKH IPB can interact with high avidities towards the surface proteins of *S. mutans*. Therefore, in order to answer the question, Western Blot technique then was used in this research.

Based on the analysis result of protein profile with commasie blue and the use of IgY anti *S. mutans* with Western Blot technique (Figure 1 and 2), the surface proteins of *S. mutans* that can be recognized by IgY were proteins with Molecule Mass (BM) about 188 kDa, while other proteins was not detected by Western Blot technique. Idone[7] and Matos-Graner[23] report that proteins, about 180-190 kDa, are considered as Ag I/II proteins of *S. mutans*, which means that IgY used in this research can specifically recognize antigens I/II considered as the surface proteins expressed by those serotypes of *S. mutans*, meanwhile Gtf and Gbp can not be detected. The reason of this IgY potency is because fragment antigen binding (FAB) of IgY can recognize the surface proteins of *S. mutans*.[24]

Thus, this result supports the assumption that chicken immunized with whole cell *S. mutans* can produce specific IgY towards Ag I/II expressed by those four serotypes of *S. mutans*. The potency of IgY is possibly related with the higher concentration of antibody in egg yolk.[25] However, this hypothesis still needs further researches. One of them conducted by Nikki,[26] shows that besides Ag I/II has 190 kDa Molecule Mass, Ag I/II also locates in the position that is relatively higher than the cell surface of *S. mutans* compared with Gtf and Gbp. Therefore, Ag I/II become the surface proteins that are more dominant as the initiator of adhering to dental pellicle. By using of IgY with water soluble fraction (WSF) and immune-blotting technique, Wibawan[14] and Smith[24] reports that IgY WSF can detect 59 kDa proteins considered as the surface proteins of *S. mutans* (GbpA), nevertheless, IgY only shows the weak reactivation towards Pac proteins classified into antigen I/II family.[10]

Method used in this research, therefore, supports and is relevant with the aim of IgY anti *S. mutans* in recognizing both the surface proteins of *S. mutans* with serotype c, d, e, and f, and affinity of its interaction. Thus, the potency of IgY in recognizing Ag I/II specifically shows that IgY used in this research is specific and can be used to restrain Ag I/II of *S. mutans* expressed by all those serotypes. Petersen[27] reports that Ag I/II has a good role as adhesin of *S. mutans,* strong tendency to bind saliva component, and a good role as the main initiator in the adhesion and colonization of *S. mutans* in dental pellicle.

The potency of IgY in restraining the surface proteins of *S. mutans* in *in-vitro way*, thus, can be assumed that IgY can prevent the synthesis of glucan from sucrose caused by *S. mutans,* and can decrease the colonization and carcinogenicity of *S. mutans* in the dental surface. Furthermore, Kruger[28] reports that the use of IgY through passive immunization is very effective in protecting caries in rats as the testing animals, which means that IgY can restrain the colonization of *S. mutans* and *S. sobrinus* in *in vivo* experiment. Not only in *S. mutans,* the use of IgY anti *Salmonella enteritis* can also show the good reactivation through ELISA Test,[29] Similarly, Rawendra[11] reports that IgY can interact well with *Enterophatogenic Escherichia coli* and restrain the colonization of bacteria in the surface of intestine.

Moreover, the research conducted by Smith [6] showed that the use of sIgA antibody of *S. mutans* with serotype c, showed that the reactivation of sIgA in the caries free group was higher than that in the caries sensitive group. It means that the antibody can prevent the adhesion of *S. mutans* to hydroxiapatite layers. In other words, the content of sIgA in saliva can prevent the process of caries by restraining the adhesion of *S. mutans*.[30] Like sIgA, the result of this *in-vitro* research shows that IgY could be able to recognize Ag I/II considered as the surface proteins of *S. mutans*.

Chismirina[31] in her research also reports that the use of Polymerase chain reactions (PCR) method successfully determines the serotype of *S. mutans* strain derived from IPB with serotype d (*S. mutans* used as the positive control of antigen produced by IgY). Thus, it may be concluded that IgY anti *S. mutans* with serotype d can recognize proteins of Ag I/II cell surface of *S. mutans* with serotypes c, d, e, and f similar with *S. mutans* strain derived from IPB used as the positive control in this research. However, in other to strengthen the result of this research, it needs the further researches about clinical isolate *S. mutans* strain, especially concerning with the allergic test and cross reaction with some bacteria and fungi of oral cavity, both of which facilitate the adhesion of *S. mutans* to dental pellicle. Therefore, IgY is expected to be used as biology material in the prevention program of dental caries through passive immunization.

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