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■ RESEARCH ARTICLE

Development of Dextranase for Toothpaste Supplement for Efficient Removal of Dental Biofilm

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ABSTRACT: The accumulation of plaque is produced by oral bacterial pathogens, *Streptococcus mutans* (*S. mutans*), which causes dental cavities. Dextranase can prevent dental caries by degrading dextran (the main component of plaque biofilm). However, dextranase is unstable in dental care products such as toothpaste. Therefore, this project aims to increase the stability and specificity of dextranase in toothpaste. We hypothesized that agarose encapsulation would increase dextranase stability and encapsulated hydroxyapatite nanoparticle (HA)-immobilized dextranase in toothpaste would increase biofilm (produced by *S. mutans)* degradation specificity. We tested the immobilization yield with a DNSA assay. The results indicated that 5 % HA was the most optimized immobilization condition. To increase the stability of the immobilized HA-dextranase by preventing dextranase degradation, agarose, a natural non-toxic polysaccharide, was used to encapsulate HA-dextranase into uniform agarose beads. To test the stability of dextranase, 1% Dextranase, 5% HA-Dextranase, and Encapsulated HA-dextranase were mixed in sodium acetate, 1 % toothpaste, and 100 % toothpaste conditions at 25 ˚C and 50 ˚C for 48 hours. The result showed that encapsulated HA-dextranase had the highest relative stability in 100% toothpaste at 50 ˚C. Biofilm formation of *S. mutans* was quantified to test the specificity of dextranase in degrading biofilm. The results showed the greatest amount of *S. mutans* degradation occurred with encapsulated HA-dextranase. Overall, immobilized HA-dextranase removed biofilm most effectively and increased the specificity of dextranase from agarose encapsulation. This research can be applied in formulating toothpaste with dextranase to increase dental plaque biofilm removal efficiency.

KEYWORDS: Molecular Biology; Biochemistry; *Streptococcus mutans*; Dextranase; Hydroxyapatite.

■ Introduction

Most toothpaste contains fluoride to help prevent dental caries.[1] However, there are limitations to fluoride in removing dental cavities. Fluoride, since it is a chemical, in large amounts can cause several health issues.[2] Excess fluoride exposure can cause skeletal fluorosis, a bone disease that impairs joint mobility, causing stiffening and fractures in bones and joints.[3] Exposure to large fluoride concentrations during childhood can result in mild dental fluorosis, where white specks appear on tooth enamel.[4] Abrasives in toothpaste is another limitation with current too. If there is a more significant number of abrasives than is safe, relative dentin abrasion (RDA) levels amount above 250 can lead to microabrasions, harming tooth enamel.

Dextranase is a substance that can be applied to toothpaste to overcome the current limitations.[5] Dextranase degrades dextrans preventing dental caries. When applying it to toothpaste, the most important problems with dextranase paste are low stability (easily degraded in toothpaste) and low specificity (randomly distributed in the mouth).[6] Therefore, in this research, dextranase is engineered to increase its stability and specificity for the efficient removal of dental biofilm. First, dextranase is encapsulated with hydrophilic agarose gel to increase stability; then, dextranase is immobilized with hydroxyapatite (HA) nanoparticles (<200nm) to enhance the specificity of degrading biofilm.

Dextranase is a hydrolase (an enzyme that uses water to break chemical bonds) that prevents tooth decay.[5] Dextranase prevents dental caries by breaking down or cleaving dextran (a complex branched glucan that is a component of plaque biofilm and occurs naturally in sucrose) and eliminating dental plaque. Due to its properties, dextranase has several industrial applications as a potential oral wash ingredient (to prevent dental caries), clearing contaminants such as dextran during cane sugar manufacturing, and synthesizing oligosaccharides.[7]

As mentioned above, dextran is a complex branched glucan, or a group of glucose polymers made by a specific bacterium, *Streptococcus mutans (S. mutans)*, and sucrose.[8] Dextran is used as plasma volume expanders and anticoagulants. Although dextran is biodegradable, it can also be cleaved by dextranase (an enzyme). In biological experiments and industry, it serves various purposes. Additionally, dextran is found naturally in sucrose, confectionery, jams, and syrups.[9]

S. mutans is a bacterium that causes the formation of dental caries and inhabits the human oral cavity.[10] It causes tooth decay when inhabiting dental plaque, a biofilm that forms on human teeth. *S. mutans* use sugars such as sucrose derived from food to build a capsule on the tooth's hard surface. Inside the formed capsule, the bacteria use more sugar to fuel metabolism and lactic acid, attacking the tooth's enamel. Moreover, *S. mutans* have specific properties that allow them to form dental caries.[11] They can colonize the tooth, which damages the tooth enamel by synthesizing sucrose and glucan, metabolizing various carbohydrates into organic acids tolerating low pH environments.

Overall, the purpose of this experiment was to engineer dextranase with two processes: 1) immobilize dextranase with hydroxyapatite nanoparticles (<200nm), 2) encapsulating the

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immobilized dextranase with hydrophilic agarose gel. Then, the stability of dextranase and specificity of degrading biofilm produced from *S. mutans* was tested. The results demonstrated increased stability and specificity of dextranase, supporting the hypothesis.

Methods

Chemicals stock solution preparation:

205.07 mg of sodium acetate (Sigma) was weighed on a weighing boat on a densimeter. The sodium acetate was added into 50 mL of water and measured and added into a 50 mL tube (BD). 80 mL of 0.5M Sodium hydroxide was measured from the previously made solution. 2.18 g of 3,5-Dinitrosalicylic acid (DNSA) (Daejung) was weighed on a densimeter. On a heating plate, at 70 ˚C, it was stirred until the DNSA powder was completely dissolved. After 30 g of Sodium potassium tartrate (Sigma) was weighed on a densimeter, it was added to the mixture. Inside the bottle, water was added up to 100 mL. 10 mL of 50 mM sodium acetate was measured into a 15 mL tube using a pipetting aid. 0.3 g of Dextran 70 (Tokyo Chemical Industry) was weighed on a weighing boat on a densimeter then added into the tube with sodium acetate. 100 mL of water was measured. 2 g of Sodium Hydroxide (Duksan) was measured using a weighing boat on a densimeter. Both 100 mL of water and 2 g of Sodium Hydroxide were added to a 250 mL glass bottle (Pyrex). To prepare 1% dextranase 1 µL of Dextranase (Sigma) and 99 µL of sodium acetate were taken with pipettes to a tube for a total of 100 µL. To prepare 1% toothpaste solution, 0.05 g of toothpaste (Perioe) was measured directly inside a 15 mL tube on a densimeter. 5 mL of 50mM sodium acetate was added into the same 15 mL tube. The solution was mixed well with a vortexer. 0.9% Sodium chloride was made with 500 mL water measured inside a beaker. 4.5 g of Sodium Chloride (Bio Basic Inc.) was weighed and added to the glass bottle. 50 mL of Paraffin liquid (Samchun) was measured and added to a glass bottle. 1.5 mL of Tween #80 (Samchun) was measured and added into a 50 mL tube (BD) to make 3 % Tween #80 in Paraffin oil. The Tween #80 liquid is an emulsifier in this solution. 2 g of agarose powder was weighed and added to the glass bottle with 50 mL of 0.9% sodium chloride to make a 4% agarose solution. The solution was microwaved so the agarose dissolved in the solution at a temperature between 80-90˚C.

Dextranase activity assay:

The DNS/DNSA method was used for the dextranase activity assay. 1 µL of the dextranase solution was put inside a 1.5 mL tube. 49 µL of sodium acetate was added to the same 1.5 mL tube to make a diluted dextranase solution. 50 µL of each (dextranase) enzyme solution and 150 µL of 3 % dextran was pipetted into 1.5 mL tubes. The contents were mixed well on a vortexer. The tubes were placed on a 60˚C heating block for 15 minutes. 200 µL of DNSA reagent was added to stop the reaction. The samples were boiled for 5 minutes on a heating plate. The samples were pipetted into 15 mL tubes containing 3 mL of distilled water added with a pipetting aid to dilute the concentration solutions and then mixed well using a pipette. In order to determine dextranase activity, the samples were pipetted into a microplate to measure the absorbance of the mixture at 540 nm using a spectrometer. The same assay was repeated for HA-dextranase Immobilization.

HA-Dextranse immobilization:

100 µL of dextranase and 9900 µL of sodium acetate were pipetted into a 15 mL tube to make 10 mL of 1 % dextranase solution. Eight 1.5 mL tubes were prepared for each Hydroxyapatite nanoparticle (HA) (Aldrich) amount. With a volume to weight ratio of 1 mL: 1 g = 100 %, the HA (g) percentage was determined. In this experiment, 0 % (0 g), 0.1 %, (0.001 g), 0.5 % (0.005 g), 1 % (0.01 g), 5 % (0.05 g), and 10 % (0.1 g) were prepared. The HA powder was measured on weighing boats inside a densimeter. The HA beads were crushed by the spoon, and powder was left on the weighing boat. The samples were incubated on a shaker for 10-12 minutes. The tubes were centrifuged at 13,800 g for 5 minutes to settle the solution from the sides of the tubes to be compacted. 1 µL of each HA-dextranase solution sample was pipetted into other tubes with 49 µL of sodium acetate. Dextranase activity assay was used to determine HA-dextranase immobilization yield by measuring the dextranase that did not bind with the HA nanobeads.

Calculation of immobilized dextranase yield:

In order to measure the activity of free dextranase and remaining dextranase activity, a DNSA assay was performed after HA-dextranase immobilization. Immobilized dextranase yield (in percent) is calculated by defining the control variable or free dextranase (C) and the supernatant variable or remaining dextranase without HA (S). Then the S value is divided by the C value and subtracted from 1. The final value is multiplied by 100 to calculate the yield in a percentage, as shown in equation (1). Immobilized dextranse yield (%) =

$$1 - \left(\frac{S}{C}\right) \times 100 \qquad (1)$$

Encapsulation of HA-dextranase:

Three buffers were prepared in agarose bead preparation for dextranase encapsulation. First, the 0.9% Sodium chloride solution was made with 500 mL of water and 4.5 g of Sodium Chloride. A 3 % Tween 80 in Paraffin oil was made with 50 mL Paraffin liquid and 1.5 Tween #80. The last buffer is a 4 % agarose solution made with 50 mL of 0.9% NaCl water and 2 g of agarose. The solution was microwaved, so the agarose dissolved in the solution at a temperature between 80-90 ˚C. The temperature of the 4 % agarose solution was measured and left until it cooled down to 65 ˚C. Immobilized HA-dextranase (5 %) was added to the 4 % agarose solution at 65˚C and then mixed well with a magnetic stirrer. The 3 % Tween 80 in Paraffin liquid solution was separated in half into 2 15 mL tubes (BD). One 15 mL tube was heated at 60-70˚C, and the other 15 mL tube was frozen at -20˚C. The heated (60-70 ˚C) paraffin liquid solution was poured into the 5 % HA-dextranase agarose solution mixture. After adding the 60-70˚C Paraffin liquid solution, the mixture was at 50˚C. With a magnetic stirrer, the solution was mixed for 10 minutes. After 10 minutes, the cold (-20 ˚C) Paraffin liquid solution was put into the mixture. When all the

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solutions were mixed well with a magnetic stirrer, the beads were observed under a microscope.

Streptococcus mutants biofilm formation and quantification:

The quantification of *Streptococcus mutans* and biofilm cells was performed in 96-well-plates. The bacteria were inoculated in LB broth. They were incubated for 24 hours at 37°C. After incubation, the supernatant from each well was removed. The biofilm remaining in each well was washed three times with sterile distilled water, followed by staining with 1% crystal violet (Biorad) solution for 15 minutes. The cells were then washed three times with sterile distilled water and air-dried for an hour. Stained biofilm cells were de-stained using 95% ethanol. After washing with sterile distilled water, 10% SDS was added to each well to lysis the biofilm-forming cells. The absorbance was measured at 600 nm.

Results and Discussion

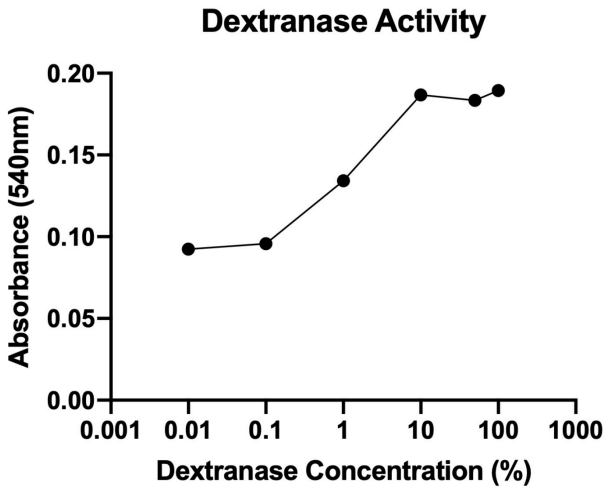

Figure 1: Effect of dextranase concentration on dextranase activity measured by DNSA assay. Line graph showing the 540 nm absorbance (n=1). Six different dextranase concentrations were prepared: 0.01, 0.1, 1, 10, 50, 100 %. For the DNSA assay, each dextranase enzyme solution was mixed with 3 % dextran. Then the samples were placed at 60°C for 15 minutes.

This experiment aims to determine a linear range of dextranase activity. The linear detection range is the region in which the amount of dextranase concentration is proportional to the absorbance (540 nm), representing dextranase activity.

The dextranase activity was measured through DNSA (3,5-dinitrosalicylic acid) assay. DNSA (3,5-dinitrosalicylic acid) assay method determined dextranase activity. The DNSA method is used in biochemistry to determine the total amount of reducing sugars. During the reaction, when the reducing sugars previously treated with DNSA are placed inside boiling water (at 100°C), the 3,5-dinitrosalicylic acid (DNSA) is reduced to 3-Amino-5-Nitrosalicylic acid (ANSA). Depending on the concentration of reducing sugar in the solution, the color of the solution turns darker, ranging from an orange to a red color. We placed the samples into a microplate inside the spectrometer to measure dextranase activity precisely using absorbance at 540 nm.

Since the optimized dextranase concentration is critical to measure the activity change of dextranase, we tested six different dextranase concentrations (in percent): 0.01, 0.1, 1, 10, 50, 100 % dextranase. At dextranase concentrations ranging from 10 to 100 %, the 540 nm absorbance is nearly constant because the dextranase activity was nearly saturated (Figure 1). Therefore, 1%, which is in the middle of the linear range of dextranase activity, was chosen to be an optimized concentration to test the change in the activity of dextranase in downstream experiments.

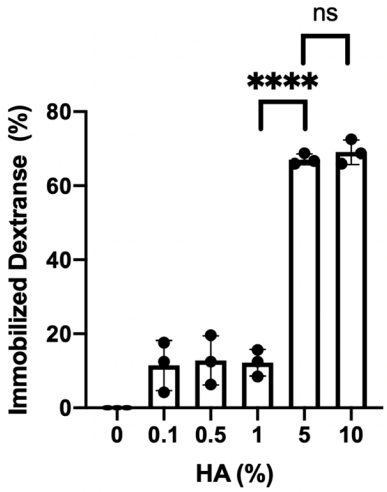

Figure 2: Effect of immobilized HA-dextranase formation yield by increasing HA concentration. Bar graph showing mean ± SD percentage of immobilized dextranase (n=3). Dextranase was immobilized by incubating dextranase with HA powder for 10 minutes· Five different HA concentrations (0 %, 0.1 %, 0.5 %, 1 %, 5 %, and 10 %) were tested. Unpaired t-test, $^{****}p < 0.0001$.

When dextranase is in the mouth, dextranase specificity decreases due to being randomly distributed throughout the mouth instead of precisely targeting dental caries and dental plaque. Hydroxyapatite nanoparticle (HA) is an inorganic, non-toxic nanomaterial used to immobilize proteins. By immobilizing dextranase with the HA bead, the dextranase will specifically sink deeper between the teeth in areas with dental plaque biofilm to degrade dextran with an increased dextranase concentration in targeted areas; therefore, dextranase specificity increases.

The purpose of determining HA-dextranase immobilization yield is to measure the most efficient yield of dextranase immobilization for a corresponding amount of HA. The optimized condition of HA-dextranase is applicable for downstream experiments, specifically in HA-dextranase encapsulation. In total, five distinct HA concentrations were tested: 0 % (0 g), 0.1 % (0.001 g), 0.5 % (0.005 g), 1 % (0.01 g), 5 % (0.05 g), and 10 % (0.1 g).

The results demonstrated that at 1 % HA, the immobilized dextranase yield is very low (~10%). However, at 5 % HA, the results showed an about 50 % increase in the immobilization yield compared to 1% HA (Figure 2). At 10 % HA, although the percentage of HA was two times the HA amount in 5 % HA, there was no significant increase in the immobilization yield. The final optimized HA-dextranase immobilization yield condition was 5 % HA.

Agarose is a natural polysaccharide obtained from red seaweed. It is edible as agarose is a natural substance without toxicity.[12] It has a high melting temperature and solidifies to a hydrogel at room temperature. Furthermore, using the

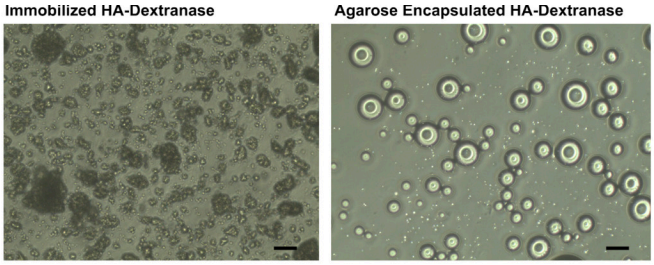

Figure 3: Different sizes of immobilized HA-Dextranase and agarose encapsulated HA-Dextranase particles were observed in the microscopic images. The difference between immobilized HA-Dextranase and agarose encapsulated HA-Dextranase is shown. Agarose encapsulated HA-Dextranase is larger with a visible coating layer. Microscopic images of naked immobilized HA-dextranase and agarose encapsulated HA-dextranase were taken. Scale bar = 200 µm.

emulsification technique, agarose encapsulation results in uniform beads.

Previous research indicated that immobilizing dextranase to an HA-dextranase complex increases the specificity of dextranase.[13] However, HA-dextranase lacks stability since dextranase is diluted throughout the entire mouth, resulting in protein degradation. Therefore, in this experiment, immobilized HA-dextranase was encapsulated by agarose beads with the emulsification technique. By encapsulating the HA-dextranase complex, we hypothesized that dextranase stability inside the toothpaste would be enhanced due to agarose encapsulation. Overall, the purpose of this experiment aimed to determine whether encapsulation of HA-dextranase was successfully conducted.

We used the same method of emulsification to prepare agarose beads for encapsulation as the previous paper described. However, in this experiment, specifically, HA-dextranase was encapsulated. From Figure 2, 5 % HA was used as the optimized condition of HA-dextranase immobilization yield. Additionally, the agarose was dissolved with a 4 % agarose solution of 50 mL NaCl and 2 g agarose.

This sample was taken from the top of the encapsulated solution to a separate tube; then, the sinking precipitate was carefully pipetted for observation under a microscope. The left image displays the HA-dextranase complex observed in a microscope in both separated and aggregated conditions (Figure 3). The small speckles on the right image are naked immobilized HA-dextranase. The encapsulated HA-dextranase is larger and circular with a visible coating layer since the agarose bead encapsulates the HA-dextranase complex (Figure 3).

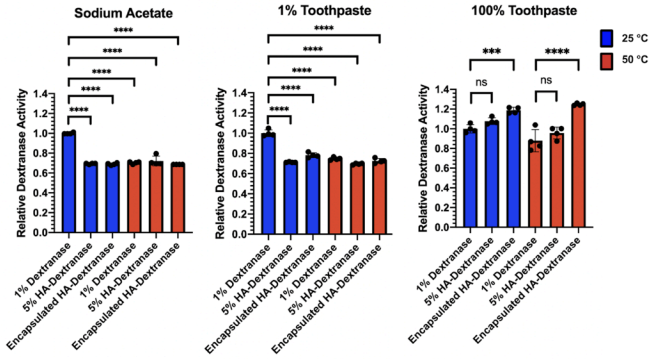

Figure 4: At both 25 °C and 50 °C, encapsulated HA-Dextranase showed the highest relative dextranase activity, indicating that agarose encapsulation provided temperature-sensitive protection. The blue bar represents 25 °C and the red bar represents 50 °C showings mean ± SD relative dextranase activity ($n=4$). Analysis of 1 % Dextranase, 5 % HA-Dextranse, and Encapsulated HA-dextranase stability in sodium acetate, 1% toothpaste, and 100% toothpaste at 25 °C and 50 °C. Two-way ANOVA and Tukey test, nsp > 0.01, ***p < 0.001, ****p < 0.0001.

Three types of dextranases (1 % Dextranase, 5 % immobilized HA-dextranase, and encapsulated immobilized dextranase) from previous experiments were tested for their stability. The stability of each dextranase type was tested under three different conditions: 100 % toothpaste, 1 % toothpaste, and sodium acetate (no toothpaste). We added both sodium acetate and toothpaste (100 % and 1 %) to each sample. To test the thermostability, each set of dextranase samples was placed at two temperature conditions: 25 ˚C and 50 ˚C, and incubated for 48 h.

Since the white translucent substances from toothpaste increased the absorbance by 540 nm, the background absorbance measurement increased. Therefore, the relative value of each condition was calculated and presented as a graph. Therefore, we used the relative value of each condition to calculate and present the data. Relative value was used to organize the data to easily compare the ratio differences of dextranase stability within each condition. We calculated the relative dextranase value by setting the average of four 1 % dextranase measurements at 25˚C as the relative value of 1.0. Every value in each condition was divided by each respective average value. The mean and standard deviation of four measurements in each condition is represented by a bar graph, while every value is also plotted as a dot (Figure 4).

In the sodium acetate condition at 25 ˚C, the stability of 5% HA-dextranase and encapsulated HA-dextranase was decreased compared to 1% dextranase. This result suggested that both HA immobilization and encapsulation were insufficient to protect dextranase from degradation in sodium acetate solution. All three samples incubated at 50 ˚C decreased the stability of all samples compared to 1% dextranase at 25 ˚C. This result showed that dextranase was not a thermostable protein (Figure 4).

In 1 % toothpaste, at both 25 ˚C and 50 ˚C, relative dextranase activity in all conditions was decreased compared to 1% dextranase at 25 ˚C. However, 1% dextranase activity at 50 ˚C was significantly decreased compared to 1% dextranase activity at 25 ˚C. This result shows that dextranase is not a thermostable protein since enzyme activity decreased in a condition with a higher temperature. 5 % HA-dextranase at 25 ˚C and 50 ˚C displayed the lowest activity compared to 1 % dextranase and encapsulated HA- dextranase in its respective temperatures (Figure 4).

At 100 % toothpaste, 1 % dextranase, immobilized HA-dextranase, and encapsulated HA-dextranase showed increased relative dextranase values. At both temperatures, immobilized HA-dextranase showed no change in dextranase activity compared to 1 % dextranase (Figure 4). However, encapsulated, immobilized HA-dextranase showed significantly higher activity than 1% dextranase at 25 ˚C and

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50 °C (Figure 4). This result demonstrates that encapsulating dextranase provides temperature-sensitive protection.

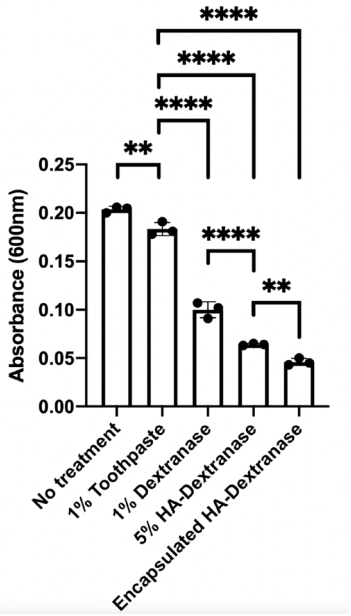

Figure 5: Encapsulated HA-Dextranase efficiently removed the biofilm produced from *S. mutans*. Bar graph showing means ± SD absorbance measurement for biofilm quantification (n=3). Quantification of biofilm produced from *S. mutants* after treatment of 1 % Dextranase, 5 % HA-Dextranse, and Encapsulated HA-dextranase in 1% toothpaste solution for 24 hours. Mean ± S.D. was plotted with statistical significance (two-way ANOVA and Tukey test): $p < 0.05$ (*) $p < 0.01$ (**).

We measured 600 nm absorbance to quantify the biofilm formation to analyze the effect of different dextranase on biofilm formation from *S. mutans* in both sodium 1% toothpaste solutions. Between the no treatment and 1 % toothpaste treated *S. mutans*, the no toothpaste showed a higher biofilm absorbance, indicating that toothpaste is effective, to an extent, in biofilm removal. However, 1 % dextranase significantly decreased the absorbance compared to no toothpaste, showing the effectiveness of dextranase in removing biofilm. Furthermore, 5 % immobilized dextranase to an HA bead to form an HA-dextranase complex showed increased efficiency of biofilm removal with an absorbance reading of 0.06. Yet, encapsulated HA-dextranase showed the most effective biofilm removal showing the lowest absorbance reading at 0.04.

■ Conclusion

We hypothesized that the stability of dextranase would increase with agarose encapsulation. This hypothesis was supported by comparing the dextranase activity determined by the DNSA assay with the increased stability of encapsulated HA-dextranase. Encapsulated HA-dextranase showed the highest relative dextranase activity in 100% toothpaste at 25 °C and 50 °C, indicating that encapsulated dextranase was most stable in 100% toothpaste. However, we only tested the stability of dextranase only after seven days of incubation with 100% toothpaste. Therefore, we should test the long-term storage of dextranase in toothpaste in the future.

A previous study indicated that dextranase specificity in removing biofilm produced by bacteria is limited in toothpaste solution.[6] Therefore, we hypothesized that the specificity of degrading biofilm produced by *S. mutans* would increase with encapsulated HA-dextranase. We quantified biofilm formation after the dextranase samples were treated in 1% toothpaste to test the specificity of three different dextranase. Figure 5 showed that encapsulated HA-dextranase had the most effective biofilm removal. However, we only tested one type of biofilm produced by *S. mutans*. Also, we only tested the effect of dextranase on biofilm removal in a 1% toothpaste condition. Therefore, further tests with various toothpaste concentrations are needed.

There are some acknowledged limitations in this study. First, some minor errors occurred while scraping HA powder from the weighing boat into the 1.5 mL tubes. This error occurred due to some HA powder sticking to the plastic weighing boat. This may cause inaccurate final concentrations of HA. Secondly, we only performed *in vitro* experiments analyzing the stability and specificity of engineered dextranase. Therefore, *in vivo* experiments such as animal experiments should be performed to verify the effect of encapsulated HA-dextranase on removing biofilm and dental cavities. Overall, this research can be applied in formulating toothpaste with dextranase to increase dental plaque biofilm removal efficiency.

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