Effect of chitosan on the enamel demineralization process *in vitro*: an enamel solubility test

To cite this article: I Surija et al 2018 *J. Phys.: Conf. Ser.* **1073** 052005

View the article online for updates and enhancements.
Effect of chitosan on the enamel demineralization process in vitro: an enamel solubility test

I Suriya, H A Gunawan* and L R Amir

Department of Oral Biology, Faculty of Dentistry, Universitas Indonesia, Jakarta, 10430, Indonesia

*E-mail: atjiek@ui.ac.id

Abstract. Dental caries and erosion are caused by demineralization, which occurs as a result of imbalanced tooth remineralization. Chitosan contains highly acid-reactive hydroxyl and amino groups and thus may affect the solubility of tooth enamel and mechanisms associated with increased environmental pH in the oral cavity. In this study, we examined the effects of chitosan on 11 extracted incisive and pre-molar teeth. The teeth were categorized into baseline group, negative control, acid plus chitosan, and chitosan treatment groups. Compared to the negative control, the teeth subjected to acid plus chitosan treatment led to an increased pH and decreased calcium concentration. It can be concluded that chitosan inhibits the tooth enamel demineralization process in vitro.

1. Introduction
In Indonesia, issues surrounding oral health problems, particularly dental caries, require attention and effective solutions. A family health survey (Survey Kesehatan Rumah Tangga) conducted in 2004 by the Indonesian Health Department reported a dental caries prevalence of 85–99%. Additionally, the prevalence of early dental caries and the Decayed Missing and Filled Teeth (DMF-T) index were found to increase by 70% each decade [1-3].

Dental caries is a pathologic manifestation of damaged tooth tissue and is caused by organic acid, a by-product of carbohydrate fermentation by bacteria in dental plaque. This organic acid diffuses throughout pores in the enamel and dentine and triggers mineral dissolution or demineralization. In a normal dental cycle, this demineralization process is followed by remineralization and is fueled continuously by the existence of saliva, carbohydrate, and bacteria in the mouth. However, if the demineralization and remineralization cycle remains imbalanced over time or if demineralization takes place without remineralization, dental caries will eventually occur [4].

The loss of minerals in the enamel is triggered not only by dental caries, but also by dental erosion, a process in which hard tissue on the surfaces of teeth is lost because of chemical demineralization in the absence of bacterial involvement. In contrast to the slow process of subsurface demineralization, surface structure losses mediated by erosion occur rapidly. Both intrinsic acids, or those produced within the body via processes such as eructation, chronic vomiting, and regurgitation, and extrinsic acids, such as those found in acidic foods, beverages, and drugs, can cause surface enamel erosion.

Various methods have been developed to prevent enamel demineralization, an early form of dental caries. Both topical and systemic fluoridation are proven methods for increasing the durability of enamel against demineralization. However, fluorine can be toxic when applied excessively. Systemic
fluoridation methods are no longer used because it is difficult to measure the dosage and duration of usage, which can lead to over-consumption or fluorosis of the teeth and bones [3]. Consequently, the most effective prevention methods involve reducing the acidic environment inside the mouth. Tactics such as the application of chlorhexidine as an antibacterial agent, promotion of oral hygiene, and consumption of a low-acid and/or low-carbohydrate diet can reduce the bacterial production of organic acids and, consequently, the demineralization process [5,6]. Alternative methods for preventing enamel demineralization have yet to be determined.

Recent studies in dentistry have focused on the use of natural ingredients, such as chitosan. This linear polysaccharide derivative of chitin is synthesized by various organisms and is a characteristic component of arthropod exoskeletons and fungal cell walls. Chitosan is a non-toxic, highly biocompatible, and biodegradable compound with antimicrobial and polymerization activities and thus could be used broadly in biomedical applications, foods, cosmetics, and pharmaceutical agents [5-9]. In terms of dentistry, chitosan could be used to maintain tooth structure by reducing the velocity of hydroxyapatite solubility under acidic conditions. Specifically, chitosan maintains the pH of dental plaque above the critical value required for enamel demineralization. This activity is mediated by chitosan amino groups (-NH₂) that are reactive to acid (H⁺) and can thus rapidly increase the pH in the mouth by absorbing this organic anion [5].

A few previous studies have addressed the ability of chitosan to inhibit enamel demineralization inhibition. Arnaud, Visveswaraiah, and Schlueter have all reported the ability of chitosan to protect against dental enamel demineralization upon exposure to various types of acid, using evaluations of enamel phosphor release, chitosan penetration, and surface microhardness [5,7,10]. However, these studies have not proven the mechanism underlying the ability of chitosan to inhibit enamel demineralization. Therefore, the present study aims to confirm the ability of chitosan to protect against acid-mediated enamel demineralization by increasing the pH inside the mouth, and to compare calcium solubility in enamel samples exposed to an acidic environment in the presence and absence of chitosan.

2. Methods

2.1. Tools and materials
This in vitro experimental study used the following tools: masks, gloves, silicon carbide paper (1000- and 1500-grit), metal wire, micromotor, duct tape, measuring cup, pipette, digital milligram scale, dark-colored glass bottle, spoon, electronic pH meter, reaction tube, round diamond burr, carborundum disc, beaker glass, magnetic stirrer, and atomic absorption spectrometer (AAS). The following materials were used: 11 post-extraction teeth, nail polish (water-proof and acid-proof nitrocellulose), saline solution, aquadem 10% phosphoric acid solution, 1% acetic acid solution, and chitosan powder. The post-extraction sample teeth were obtained from several clinics and public health centers and were clinically caries-free, abrasion-free, and erosion-free. The tested material, chitosan, was manufactured by Biotech Surindo Cirebon (degrees of deacetylation >90%).

2.2. Study design
This study was performed in two phases. (i) pH testing in the presence of a demineralization solution with and without chitosan, and (ii) enamel solubility testing in the presence of a demineralization solution with and without chitosan. During the first phase, the pH values of demineralization and chitosan solutions were measured. The demineralization solution was 10% phosphoric acid. The chitosan solution had a concentration of 2.5 mg/ml and was made by dissolving chitosan powder in 1% acetic acid followed by magnetic stirring for 24 h. The pH values of Aquadem, a 10% phosphoric acid, 2.5 mg/ml chitosan solution, 10% phosphoric acid with aquadem (1:1 volume:volume ratio), and 10% phosphoric acid with chitosan (1:1) were measured.

During the second phase, enamel specimens were prepared by grinding the tooth surfaces with 1000- and 1500-grit silicon carbide paper to achieve a matte surface. The surface was then coated with nail polish, leaving a 5-mm × 5-mm window on the buccal surface. A small hole was made in the tooth apex for wire insertion. Each specimen was then immersed for 6 h in one of four different solutions:
20 ml aquadem (baseline group), 10 ml of 10% phosphoric acid + 10 ml of aquadem (negative control group), 10 ml of 10% phosphoric acid + 10 ml of chitosan solution (treatment group I), and 10 ml of chitosan solution + 10 ml of aquadem (treatment group II). During tooth immersion, the window on the buccal surface was fully immersed. After the 6-h immersion period, all specimens were removed and all solutions were subjected to pH analysis.

Next, the calcium concentration in each treatment solution was measured to determine enamel solubility using a AAS set at a specific wavelength of 422.7 nm [11]. In the AAS measurement process, combustion energy atomizes the sample to free atoms at a temperature of 2300°C, using an oxidant gas mixture of acetylene-air [11,12,13].

Prior to testing, the AAS was calibrated using five standard calcium solutions with concentrations of 2, 4, 6, 8, and 10 ppm. The absorption values of the standard solutions were noted and used to calculate a calibration curve, which was subsequently applied to determine the concentrations of sample solutions.

Measurement data of standard calcium solution concentration that was obtained from this study are presented as numeric values. A parametric test was used to identify differences between groups at a significance level of 2.5% ($p < 0.025$).

3. Results

3.1. Changes in pH according to demineralization solution

Changes in pH following exposure to the treatment solutions were determined using an electronic pH meter. Tooth samples in all four groups (baseline, negative control, treatment I, and treatment II) were subjected to pH measurement before and after enamel immersion for 6 h.

| Treatment group                      | Without Immersion | After Immersion |
|--------------------------------------|-------------------|-----------------|
| Baseline (Aquadem)                   | 6.261             | 6.385           |
| Negative control (10% Phosphoric Acid)| 0.751             | 1.136           |
| Treatment I (Phosphoric Acid + Chitosan)| 0.909             | 1.181           |
| Treatment II (Chitosan)              | 3.321             | 3.574           |

As shown in Table 1, treatment solution I had a slightly higher pH value than the negative control solution both before and after enamel immersion, and the values in all four solutions increased value after enamel immersion relative to before immersion.

3.2. Solubility of enamel calcium

AAS was used to determine the elemental Ca concentration in the solutions after enamel immersion. The results are presented in ppm (parts per million) of Ca, and a higher concentration indicates a higher enamel solubility.

As shown in Figure 1, no calcium was detected in the baseline sample. By far, the highest concentration of 2,029.72 88.23 ppm was recorded in the control negative group. The concentration in treatment group I was 857.78 ± 609.85 ppm, while that of treatment group II was 69 times lower at 12.40 ± 2.39 ppm.

A statistical analysis revealed normally distributed measurements of calcium concentration in each group, and an analysis of variance (ANOVA) revealed significant differences in this parameter between the negative control, treatment I, and treatment II groups ($p < 0.025$). Additionally, a post hoc test also identified a $p < 0.025$ in comparisons among the groups. In summary, significant differences were observed between all treatment groups.
Figure 1. AAS comparison of calcium concentrations in the sample solutions

Table 2. Results of an ANOVA of the AAS measurements

| Treatment Group       | One-way ANOVA p value | Results (p < 0.025) |
|-----------------------|-----------------------|---------------------|
| Negative Control      | 0.0001                | H0 denied           |
| Treatment I           | 0.0001                | H0 denied           |
| Treatment II          | 0.0001                | H0 denied           |

H0, Hypothesis 0, no significant difference (p < 0.025) in the measured calcium concentrations between the negative control, treatment I, and treatment II:
Hypothesis 1, a significant difference in measured calcium concentrations between the negative control, treatment I, and treatment II

4. Discussion

4.1. Shifting of pH values in treatment solutions

Our findings demonstrate that the addition of chitosan to the demineralization solution led to an increase in pH, as demonstrated by the difference between the negative control and treatment I groups. Notably, chitosan increased the pH of an acid solution not only before but also after a 6-h period of enamel immersion. The higher pH values of the enamel-immersed solutions are attributed to the ability of hydroxyl groups and enamel phosphate to bind H⁺ ions. Delvar reported that an increasing pH value in a demineralization solution over time could be used as a measure of high enamel hydroxyapatite solubility [15]. Notably, we observed increases in pH from before to after immersion in all four solutions, which was attributed to the ability of aquadem to decrease the surface tension of a material. Accordingly, the reduced surface tension led to enlargement of the enamel pores and allowed H⁺ ions to penetrate the pores between enamel crystals. If pH measurement was performed on aquadem, decreasing of pH value would occur.

The differences in the pH shift between the negative control group and treatment I demonstrated the differing abilities of aquadem and chitosan to increase pH in a phosphoric acid solution. Specifically, the acid-reactive amino groups (–NH₂) and hydroxyl (–OH) groups in chitosan could more strongly increase the pH value by absorbing free H⁺ ions from the solution, which would otherwise bind to phosphate and hydroxyl groups in enamel and thus trigger the dissolution of hydroxyapatite. Therefore, chitosan leads to competitive binding with enamel for the H⁺ ions in an acid solution.
[5,8,16], and the mechanism by which chitosan inhibits enamel demineralization could thus be explained by the increase in pH in the demineralization environment.

We note that we did not subject the changes in treatment solution pH values to statistical testing. We consider this appropriate in terms of the study aims, namely the intent to determine whether chitosan could reduce enamel demineralization by increasing the pH value of an acid solution. Our findings prove our hypothesis without requiring us to determine the significance of this difference.

4.2. AAS measurement of enamel solubility
As noted, we used AAS to evaluate the inhibition of enamel demineralization underlying the decreased enamel solubility observed in the first phase of the study. In mature enamel, the proportion of inorganic calcium ranges from 33.6 to 39.4% by weight, while the proportion of phosphate groups may be as high as 16.1–18.0% by weight [16]. During the process of enamel dissolution, the reaction of OH$^-$ groups with H$^+$ ions weakens the enamel structure and triggers phosphate group and calcium leached out of apatite cell unit, followed by dissolution in the demineralization solution. Consequently, the measurement of soluble elemental calcium with an AAS could be used as a proxy of hydroxyapatite demineralization [3]. and would be easier to measure than phosphate groups, which contain both phosphate and oxygen atoms and are thus difficult to determine in AAS absorbance readings and hydroxyapatite demineralization measurements. Accordingly, we decided to measure the calcium concentration in demineralization solutions.

As shown in Figure 2, the highest calcium concentration was detected in the negative control solution, indicating a great amount of damage to the enamel. However, this value was reduced by roughly half in the treatment I solution, suggesting that chitosan protected the enamel against acid-induced mineral demineralization. However, we note that calcium was also found in the treatment II solution, which would not be expected if chitosan protected against enamel demineralization. However, we note that the calcium concentration in the treatment II group was far lower than that in the group with demineralization solution. We could attribute this event to the use of a 1% acetic acid solution to dissolve chitosan and yield a solution with a pH of 3.321, as chitosan is a strongly alkaline substance that can only be dissolved in an acid solution (pH < 6). At such low pH values, the amino groups of chitosan are protonized and produce polyelectrolytic cations [5]. The presence of H$^+$ ions from acetic acid may have increased the solubility of enamel in the absence of the demineralization solution. This led to the unfortunate finding that the chitosan solution itself could demineralize enamel, although not to the same extent as the 10% phosphoric acid solution (pH = 0.51).

The results obtained with our enamel solubility test, which were used to evaluate the effects of acid and chitosan on enamel specimens, were consistent with those of a study by Gunawan in which the calcium concentrations of 10% phosphate acid solutions were evaluated after the immersion of enamel and anchovy specimens [3]. Two factors might explain the mechanism by which chitosan decreases the solubility of enamel in an acidic environment. First, chitosan increases the pH value of a demineralization solution. Second, chitosan binds to the enamel surface in the acidic environment, following the interactions of the -NH$_2$ groups in chitosan with H$^+$ ions in solution to form -NH$_3^+$ and render the molecule positively charged. This positively charged molecule could then interact with negatively charged substances, such as the enamel surface [5,7].

5. Conclusions
In conclusion, this study proved that chitosan could reduce enamel demineralization in a 10% phosphoric acid in vitro. However, the potential risk that an acidic chitosan solution might itself dissolve enamel must be considered in future studies. However, enamel solubility test suggests that chitosan can inhibit the enamel demineralization process in vitro by increasing the environmental pH and thus reducing enamel solubility.

References
[1] Nurhidayat O, Tunggul E and Wahyono B 2012 Perbandingan media power point dengan flip chart dalam meningkatkan pengetahuan kesehatan gigi dan mulut. Unnes J. Public Health. 1 31.
[2] Indonesia 2013 Badan Penelitian dan Pengembangan Kesehatan Kemenkes RI. Riset Kesehatan Dasar. Jakarta.

[3] Gunawan H A 2006 Pengaruh perubahan kristal apatit, tingkat retensi, dan intrusi fluor terhadap kelarutan email setelah perlakuan larutan ikan teri jengki (S insularis) [Disertasi]. Depok: Universitas Indonesia.

[4] Featherstone J D B 2008 Dental caries: A dynamic disease process. Aust. Dent. J. 53 286.

[5] Visveswariah P M, Prasad D and Johnson S 2014 Chitosan A novel way to intervene in email demineralization - An in vitro study. Int. J. Curr. Microbiol. Appl. Sci. 3 617.

[6] McIntyre J M 2005 Preventive management of dental caries Preservation and restoration of tooth structure eds Mount G J and Hume W R (Queensland: Knowledge Books and Software) 2nd ed. pp. 35.

[7] Arnaud T M S, De Barros Neto B and Diniz F B 2010 Chitosan effect on dental email de-remineralization: An in vitro evaluation. J. Dent. 38 848.

[8] Lee H S, Tsai S, Kuo C C, Bassani A W, Pepe-Mooney B, Miksa D, Masters J, Sullivan R and Composto R J 2012 Chitosan adsorption on hydroxyapatite and its role in preventing acid erosion. J. Colloid Interface Sci. 385 235.

[9] Rinaudo M 2006 Chitin and chitosan: Properties and applications. Prog. Polym. Sci. 31 603.

[10] Schluerer N, Klimek J, Ganss C 2014 Effect of a chitosan additive to a Sn2+-containing toothpaste on its anti-erosive/anti-abrasive efficacy- a controlled randomised in situ trial. Clin. Oral Investig. 18 107.

[11] Bishop M L, Fody E P and Schoeff L E 2010 Clinical chemistry: techniques, principles, correlations (Philadelphia, Pa.: Wolters Kluwer Health/Lippincott Williams & Wilkins).

[12] Noriyanti T 2012 Analisis kalsium, kadmium, dan timbal pada susu sapi secara spektrofotometri serapan atom [Skripsi]. Depok: Universitas Indonesia.

[13] Delvar A, Lindh L, Arnebrant T and Sotres J 2015 Interaction of polyelectrolytes with salivary pellicles on hydroxyapatite surfaces under erosive acidic conditions. ACS Appl. Mater. Interfaces. 7 21610.

[14] Robinson J W, Frame E M S and Frame G M 2005 Undergraduate instrumental analysis (6th ed) (New York: M. Dekker) p. 1079.

[15] Barbour M E, Shellis R P, Parker D M, Allen G C and Addy M 2005 An investigation of some food-approved polymers as agents to inhibit hydroxyapatite dissolution. Eur. J. Oral Sci. 113 457.

[16] Williams R A D and Elliott J C 1989 Basic and applied dental biochemistry (2nd ed) (Edinburgh: Churchill Livingstone) p 342.